Recent Advances in Microalgal Biotechnology

OMICS eBooks Group
www.esciencecentral.org/ebooks

Edited by
Dr. Jin Liu
Dr. Zheng Sun
Dr. Henri Gerken

Recent Advances in Microalgal Biotechnology

Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

ISBN: 978-1-63278-066-9

DOI: http://dx.doi.org/ 10.4172/978-1-63278-066-9-67

Published Date: January, 2016

Printed Version: January, 2016

Published by OMICS Group eBooks

731 Gull Ave, Foster City, CA 94404, USA.

A free online edition of this book is available at www.esciencecentral.org/ebooks

Additional hard copies can be obtained from orders @ www.esciencecentral.org/ebooks

Preface

Microalgae are sunlight driven single-cell factories for protein, lipids, carbohydrates, pigments, vitamins and minerals, etc. Microalgae have long been used as health food and additives for human consumption, as well as animal feed in aquaculture. Microalgae also prove to be beneficial to environmental cleanup such as bioremediation of industrial flue gases and waste water. Recently, owing to the demand of renewable energy, microalgal biofuels, biodiesel in particular, have attracted unprecedentedly interest. Also, microalgae emerge as promising hosts for the expression of recombinant proteins.Nevertheless, there are still tremendous challenges involved in the algae production pipeline such as strain improvement, mass cultivation, harvest and drying, biomass disruption, and recycling of water and nutrients, which have been impeding commercial application of microalgae in many different ways. The great opportunities lying ahead will be the innovations and breakthroughs occurred in microalgal biotechnology. This book brings together recent advances in microalgal biotechnology, dedicated to both the understanding of the fundamentals and development of industry-oriented technologies.

About the Editors

Dr. Jin Liu is an assistant professor at Peking University and a National Youth Thousand Talents Program Fellow, China. Dr. Liu received a BSc in biology and an MSc in biochemistry and molecular biology from Sun Yat-sen University, and a PhD in algal biotechnology from The University of Hong Kong. He worked in world top laboratories on plants and microalgae over ten years. He is broadly trained and highly experienced molecular biologist and phycologist with interdisciplinary background. Particular areas of expertise include molecular biology, genetic engineering, biotechnology, transcriptomics, mass cultivation, algal physiology, algal biofuels and high-value products. He has published over 30 peer-reviewed research articles on top journals of algae fields and wrote 7 book chapters. He also serves as editorial board members for several peer-reviewed journals.

Thank you

Signature

Dr. Zheng Sun is an Associate Professor in the College of Fisheries and Life Science at Shanghai Ocean University, China. Zheng received his M.Res. in 2006 from the University of Bath, England and Ph.D. in 2011 from the University of Hong Kong. His doctoral research was focused on algal biotechnology under the supervision of Prof. Steven Feng Chen. During 2011-13, Zheng worked as a postdoctoral researcher at Hong Kong Baptist University and City University of Hong Kong. In 2013, Dr. Sun joined Shanghai Ocean University as an Associate Professor via the fast-track promotion. Dr. Sun's research interests include algal biochemistry, algae-derived metabolites and biorefinery of algal biomass. He has published over 20 peer-reviewed articles in top journals in the field of food, agricultural and natural resource sciences. He is also a recipient of the 'Shanghai Pu-Jiang Talent Programme' Award in 2013 and 'Shanghai Young Eastern Scholar' Award in 2015.

SUN ZHENG

Thank you

Signature

Dr. Henri Gerken is a Senior Research Scientist at the Arizona Center for Algal Technology and Innovation (AzCATI) at Arizona State University, USA. Dr. Gerken received his PhD from Arizona State University in Microbiology in 2009 and completed Post Doctoral fellowships at the National Renewable Energy Laboratory in Golden Colorado and AzCATI. Research interests include cell wall compositional analysis and enzymatic digestion of industrially relevant microalgae as well as genetic engineering of microalgae for fuels and fine products.

Thank you

Signature

Introduction to the eBook

Microalgae represent a highly diverse group of photosynthetic microorganisms with simple life cycle. Microalgae can be found in a wide range of environmental conditions, including water, land, and even unusual environments such as snow and desert soils. It is estimated that there are more than 50,000 species around the world, among which only a small portion have been studied and analyzed. Through efficient photosynthesis, microalgae grow fast requiring only sunlight, carbon dioxide, water and a small amount of nutrients. Because of its ease of growth and being synchronized and having similar metabolic pathways to higher plants, the green alga *Chlorella* has long been used as a model organism to investigate the photosynthesis and pathways of carbon dioxide assimilation (Calvin-Benson cycle). The potential of using microalgae for multiple uses triggers the marriage of microalgae with biotechnology, which has taken phycology research to new heights.

An early report "Alga Culture – From Laboratory to Pilot Plant" edited by J.S. Burlew summarized the pioneering studies on outdoor mass cultivation of microalgae, which were launched in the late 1940s in United States, Germany and Japan, and represented the initial phase of microalgal biotechnology. Many professional societies were established at that time, including Phycological Society of America (1947), British Phycological Society (1952), Japanese Society of Phycology (1952), International Phycological Society (1960), Australian Phycological Society (1966), etc. Later, the report "Feeding the Expanding World Population: International Action to Avert the Impending Protein Crisis" by United Nations Advisory Committee on the Application of Science and Technology to Development in 1968 initiated the idea of using microalgae as a food source for human being, which served as a strong impetus to the development of microalgal biotechnology and the first international meeting of applied phycology held in 1978 in Israel. From then on, there was a steadily growing interest in this field, and in the beginning of this century an unprecedented boom occurred due to the concept of utilizing microalgae for biofuel production.

Nevertheless, the commercial success in microalgae has been restricted to aquaculture and high-value products. The Chapter 4 of this book gives an overview of current situation and prospective of microalgae for aquaculture. There are a wide range of value-added products from microalgae, such as carotenoids including astaxanthin (Chapter 5 and Chapter 7) and ω-3 polyunsaturated fatty acids (Chapter 6). Astaxanthin possesses a broad range of applications in food, feed, nutraceutical, and pharmaceutical industries, and natural astaxanthin sourced from microalgae (e.g., *Haematococcus pluvialis*) costs over $7,000 per kg. Polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are widely used in food, nutritional supplements, and health and beauty products, with an estimated global market of $13 billion in 2015. Although the microalgal value-added products are in high demand by the market, their production capacity is limited. Challenges in the mass production remain to be addressed to fulfil the ever-increasing human needs for these natural products.

Chapters 8-12 deal with topics devoted to microalgal biofuels. Microalgae are capable of producing many types of biofuels, including crude oil, biodiesel, hydrocarbon, hydrogen, and ethanol. The utilization of microalgae has presently become a major topic in both scientific and commercial meetings of phycology, which take place several times each year worldwide hosted by different organizations. There is a debate in the feasibility of using microalgae for commercial biofuel production. At least, based on the current technologies, the microalgal biofuel production is far away from being cost-effective. The great opportunities lying ahead will be the development of next generation culture systems (Chapter 13 and Chapter 14) and technologies in downstream processes (Chapter 16,

microalgae harvest). Harvest contributes substantially to the whole cost of microalgae production. Another R&D priority should be given to the development of a stable and robust genetic transformation system for production-promising strains so that they can be manipulated for improvements (Chapter 15). Also, the integrated production of biofuels coupled with the environmentally beneficial applications such as flue gas biomitigation and wastewater treatment (Chapters 1-3) represents a promising direction toward production cost reduction. To this end, 'Omics' analysis for microalga is underway (Chapter 17), which may benefit the future genetic engineering of microalge. The breakthroughs and innovations occurred in the field of microalgal biotechnology may have the potential to drive the microalgae market from high-value products to low-cost commodities.

Acknowledgements

Seventeen chapters included in this e-book are authorized by over thirty scientists worldwide and consist of the major works in their respective aspects of microalgal biotechnology. The e-book serves a platform bringing together the updated knowledge and ideas in the field of mciroalgal biotechnology and should contribute to advance this novel science and to unveil its great potential. All merits this book deserves are due to the professional competence, distinguished experience, and great vision of its contributors, to whom the editors extend their deepest gratitude.

Jin Liu wishes to thank his wife, Jiang Guo, not only for her thorough understanding and infinite support during his long-time working on this book but also for her great patience and everlasting love all over the years while he has been pursuing his career in science worldwide, from Hong Kong to Arizona to Maryland to Beijing. He is grateful to his lovely son, Zeno Liu, for bringing endless happiness and luck. Also, he would like to express thanks to his parents and young brother's family for their infinite physical and emotional support and encouragement.

Zheng Sun and Henri Gerken wish to thank their respective family for the patience and support on the editorial work on this book.

Potential Applications of Microalgae in Wastewater Treatments

Wenguang Zhou*

Center for Biorefining, Bioproducts and Biosystems Engineering Department, University of Minnesota, USA

***Corresponding authors:** Wenguang Zhou, Center for Biorefining, Bioproducts and Biosystems Engineering Department, University of Minnesota, 1390 Eckles Avenue, Saint Paul, MN 55108, USA, Tel: 612-625-7733; E-mail: zhouw@umn.edu

Introduction

Algae are oxygen-evolving photosynthetic microorganisms and commonly grow in various aquatic environments, such as fresh and marine water, wastewater steams from a variety of wastewater sources (e.g., agricultural run-off, concentrated animal feed operations, and industrial and municipal wastewaters). Some species can grow on rocks, soils, plants, etc. as long as there are adequate amounts of C (organic or inorganic carbon), N (ammonium, nitrate, urea, yeast extract, etc.), and P as well as other essential trace elements presented. Wastewaters are unique in their chemical profile and physical properties as compared with fresh and marine waters. The research of cultivation of algae on waste streams for wastewater treatment was conducted as early as the 1950s, and the symbiotic algal-bacterial relationship in waste stabilization pond was first proposed in which algae were used as tiny aeration devices to provide large amount of Oxygen (O_2) through photosynthesis for aerobic bacteria to oxidize and degrade the organic compounds in wastewaters while heterotrophic bacteria concomitantly release CO_2 and the nutrients needed by microalgae during photosynthesis [1]. While the initial purpose of this study is to treat the secondary effluent for N and P removal before discharged to water bodies [2]. Recent researches indicated the great potential of mass production of algal biomass on waste streams for simultaneous wastewater bioremediation and biofuel and other applications [3-6]. Wastewaters provide not only water medium but also most of the necessary nutrients suitable for cultivation of microalgae evidenced by high algae growth rate and productivity and nutrient removal efficiency. The significant savings of water and fertilizers costs are expected to tip the economic equation in favor of wastewater based algae-to-fuel approach. Therefore, coupling wastewater treatment with algae cultivation may offer an economically viable and environmentally friendly way for sustainable renewable algae-based biofuel and bio-based chemicals production as well as credits for wastewater treatment since large quantities of freshwater and nutrients required for algae growth could be saved and the associated life cycle burdens could be reduced significantly [4,6-8]. They can also couple carbon-neutral fuel production with CO_2 sequestration from power industries and other sources such as municipal wastewater treatment plants, in turn generating carbon credits. Therefore, growing algae on waste streams offers many advantages over traditional algae farms. (Figure 1),

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

shows the simplified process diagram for wastewater-based algae cultivation system with multiple benefits of water and nutrient reuse, biofuels and bio-based chemicals production, wastewater remediation and CO_2 fixation.

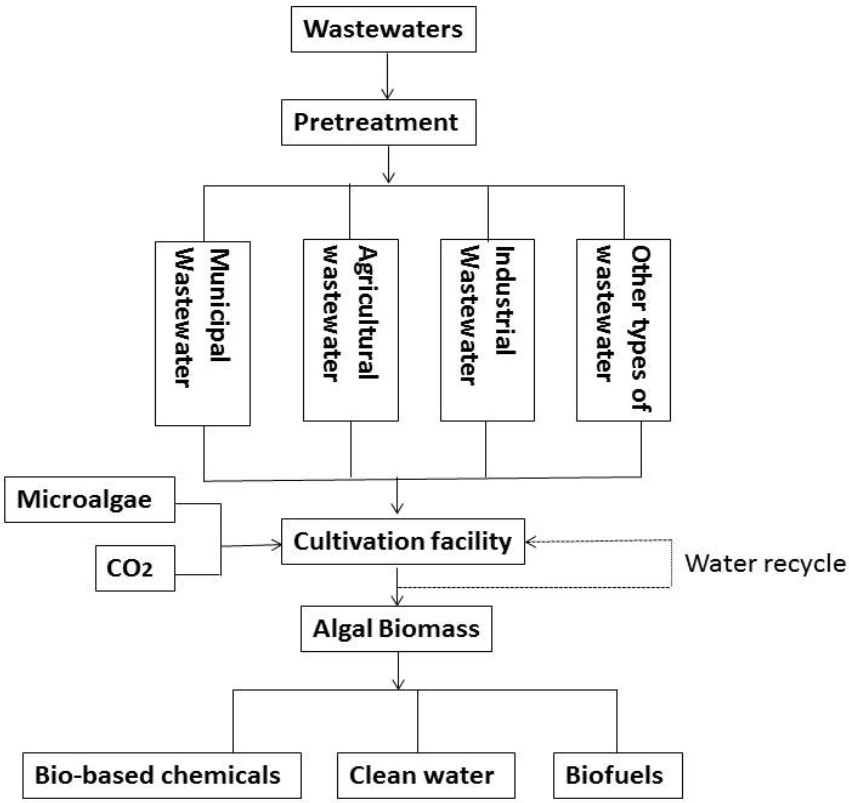

Figure 1: Process diagram of integrated wastewater-based algae cultivation system for production of biofuels, bio-based chemicals and effective nutrients removal.

Principles of Microalgae-Based Wastewater Treatment

The main pollutants in a variety of wastewater sources are Nitrogen (N) and Phosphorus (P) in different forms, which contribute to eutrophication of bodies of water in lakes, rivers or oceans worldwide if discharged improperly. On the other hand, these pollutants are ideal nutrients to stimulate fast algae growth. The typical metabolic processes in microalgae include mechanisms of light harvesting, carbon acquisition through photosynthesis, and N and P assimilation as well as formation of unique secondary metabolites (e.g., DHA and EPA, unique pigments and active compounds, etc.) for different applications [9-11]. It is worth noting that although most microalgae in nature are obligate photoautotrophs and their growth strictly depends on photosynthetically derived energy, there are some species that can grow in a Heterotrophic Mode (HM) in the presence of organic carbon under complete darkness or in a Mixotrophic Culture Mode (MM) when supplied with both organic and inorganic carbon under light/dark conditions [4,12-15]. Thus the main mechanisms for nutrients removal in wastewater by microalgae include typical photosynthetic assimilation and/or chemosynthetic assimilation by heterotrophic/mixotrophic metabolic pathway as well as abiotic factors such as ammonia volatilization and phosphorus precipitation through elevated pH.

Nitrogen Metabolism

Nitrogen is the most important nutrient contributing to the algal biomass produced and is a key constituent of many algal cellular components such as alkaloids, amides, amino acids, proteins, DNA, RNA, enzymes, vitamins, and hormone, accounting for 1 to 10% of total algal biomass (dry weight), depending on the supply and availability [10,16-18]. Most of microalgae species are capable of utilizing a variety of nitrogen sources including organic nitrogen (e.g., urea, glycine, glutamine, peptone, yeast extract, etc.) and inorganic nitrogen (e.g., ammonia, nitrate, and nitrite, etc.) [10,18,19]. Waste streams from agricultural run-off, concentrated animal feed operations, and industrial and municipal activities are ideal nitrogen sources. It is commonly accepted that nitrogen metabolism is linked to carbon metabolism in algae because they share organic carbon and energy supplied directly from photosynthetic electron transport and CO_2 fixation as well as from the metabolic pathway of organic carbon through heterotrophic culture [10,20]. For example, uptake and assimilation of inorganic nitrogen (e.g., ammonium) by microalgae to form amino acids requires carbon skeletons in the form of keto-acids (2-oxalogutarate and oxaloacetate) and energy in the form of ATP and NADPH to synthesize the amino acids glutamate, glutamine, and aspartate with energy provided by typical TCA cycle under both autotrophic and heterotrophic culture mode [20-22]. The metabolic pathways involved in nitrogen assimilation are depicted in (Figure 2).Among the above nitrogen forms, ammonium is the most preferred form of nitrogen source for microalgae in part because its uptake and utilization by microalgae is most energy-efficient [10,23,24]. In general, ammonium is transported across the membranes by a group of proteins belonging to the Ammonium Transporter family (AMT). Some ammonium transporters belonging to the AMT family have been found and identified in *Diatoms* and *Chlamydomonas* [22,25]. The Glutamine Synthetase (GS) and Glutamate Synthase (GOGAT) were reported to catalyze assimilation of ammonium under both Autotrophic Culture mode (AC) and HC, and at same time producing glutamine, glutamate and a-ketoglutarate, respectively [26-30] (Figure 2). Sometimes ammonium is incorporated into glutamate by the reversible reductive amination of a-ketoglutarate, which is catalyzed by Glutamate Dehydrogenase (GDH) [31]. In general, the GS/GOGAT pathway is regarded as the main pathway for ammonium assimilation, while the GDH pathway plays a minor part in the formation of glutamate. However, GDH plays an important role as a catabolic shunt in regulating nitrogen metabolism and mitochondrial function [21]. Additionally, the activity of GDH is closely related to conditions of stress [29]. GS has high affinity for ammonia and ability to incorporate ammonia efficiently into amino acids, and is regarded as an important enzyme in photosynthetic microalgae species, even under heterotrophic metabolism [32]. Nitrogen is distributed to different amino acids after ammonium is incorporated into glutamate via the GS/GOGAT cycle or GDH, much of it through transamination with oxaloacetate by aspartate aminotransferase to yield aspartate [10]. The organic nitrogen compounds, such as amino acids, nucleotides, chlorophylls, polyamines, and alkaloids were composed of glutamate, aspartate, and asparagines [31,33].

With regard to nitrate and nitrite metabolism, studies showed that only two enzymes, namely Nitrate Reductase (NR) and Nitrite Reductase (NiR), help catalyze nitrate to ammonium [22,26]. Firstly, NR catalyzes the reduction of nitrate to nitrite, using reduced pyridine nucleotides as physiological electron donors [34,35]. Subsequently, NiR catalyzes nitrite to ammonium. The whole reaction uses ferredoxin as the electron donor [36] (Figure 2). It is worth noting that, for most microalgae, NR is actively expressed in cells when supplied with nitrate as the sole nitrogen source while it is fully repressed in cells when culture media contains excess ammonium or both of ammonium and nitrate [34,37-39]. This further explains the preference of microalgae species like *Chlorella* for ammonium.

For catabolic metabolic pathway of organic nitrogen assimilation, the urease, Urea Carboxylase (UAlase) and allophanate lyase are considered to be the key enzymes to metabolize these organic nitrogens. For example, urea could be metabolized to ammonia

and bicarbonate by above key enzymes [23]. However, utilization of organic nitrogen such as urea is far less important in the growth cycle of microalgae than ammonium and nitrate. It is apparent from above discussion that the preference for microalgae nitrogen uptake and assimilation by most microalgae species is: ammonium > nitrate > nitrite > urea [10].

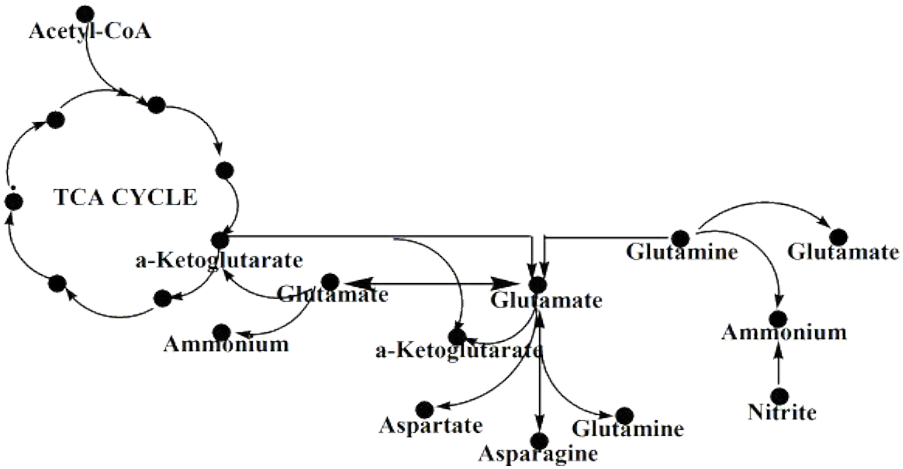

Figure 2: Typical Nitrogen metabolic pathway in microalgae.

Phosphorus Removal Metabolism

Phosphorous, representing around 1%-3% of microalgae (dry weight), is another major macronutrient that plays an important role in cellular metabolic processes such as energy transfer, biosynthesis of nucleic acids, DNA, and forms many structural and functional components required for growth and development [18]. Orthophosphate is incorporated into organic components through various types of phosphorylation processes. Microalgae grown in phosphorus-rich wastewaters could exhibit the capability of luxury uptake of phosphorus stored in polyphosphate bodies which is influenced by a variety of factors such as phosphate concentration in wastewater, light intensity and temperature. However, the mechanisms of how these factors affect luxury uptake are not fully understood [18,40]. The ratio of N/P also plays an important part in N and P removal in algae-based wastewater treatment system as it not only determines the potential productivity but also is important in maintaining the dominance of candidate species in culture [18]. The inorganic N/P ratio varies with different types of wastewaters. A range of approximately 6.8-10 is considered as the optimal N/P ratio for algae growth. A ratio of N/P much higher than the optimal ratio indicates a high probability of phosphorus limitation while N/P ratio much lower than the optimal ratio indicates a high probability of nitrogen limitation [41].

Abiotic Removal Metabolism

Apart from photosynthetic and chemosynthetic assimilation by microalgae, N and P removal from wastewaters are also affected by abiotic factors significantly. Numerous researches demonstrated that high pH contributed greatly to N and P removal through ammonia volatilization and chemical precipitation (e.g., calcium phosphate) in algae-based wastewater treatment system [3,4,12,42]. For example, at high pH, ammonia could be easily removed through stripping and phosphorous could be coagulated with metal ions that naturally existed in wastewaters and removed through precipitation [7,12,43,44]. Therefore, the N and P removal in wastewater-based algae cultivation system were attributed to assimilation by microalgae as well as volatilization and precipitation caused by abiotic factors.

Municipal Wastewater

The research of growing algae in municipal wastewater-based stabilization pond was conducted as early as the 1950s while the initial purpose of this algae-based pond process was to further treat the secondary effluent and avoid eutrophication before discharge to water bodies [1]. In contrast to industrial wastewater, the nutrient profiles of municipal wastewater are less variable and can be easily treated by algae-based cultivation system, which is one of reasons for active research for this type of wastewater during the past decades [45]. In general, there are three different types of municipal wastewaters which include: (1) primary wastewater, which referred to wastewater before primary settling; (2) secondary effluent, which is wastewater after activated sludge tank; (3) centrate wastewater, which is generated after sludge centrifuge [6] (Table 1). The influence of nutrient profiles of different types of municipal wastewaters above on algae growth is significant. For example, when grown on nutrient-poor secondary effluent (TN: 15.5 mgL^{-1}; TP: 0.5 mgL^{-1}; COD: 24 mgL^{-1}) using *Scenedesmus sp.*, 9.2 $mgL^{-1}d^{-1}$ of algal biomass productivity was reached while more than 20 times higher algal biomass productivity (193.8 $mgL^{-1}d^{-1}$) was achieved when the strain grown on nutrient-rich centrate wastewater (TN: 134 mgL^{-1}, TP: 212 mgL^{-1}, COD: 2324 mgL^{-1}) [3,24], which may offer a new option for enhanced algal biomass accumulation and efficient nutrient removal by growing algae on nutrient-rich municipal wastewater.

Agricultural Wastewater

Agricultural wastewaters especially animal manure wastewaters are widely available all over the world and can cause severe eutrophication issue if not properly managed. The unique properties of high turbidity, high nutrient concentration, and large insoluble organic compound prevent their application in algae-based wastewater treatment (Table 1). The commonly adopted strategy to tackle these challenges is to dilute concentrated animal manure with freshwater to obtain proper dilution to satisfy the basic nutrient requirement for algae growth [9]. Other methods include screening robust microalgal strains tolerant high concentrated animal manure wastewaters [46], including suspended and benthic algae [8,9], use of concentrated algal biomass at initial inoculums and pretreatment of animal manure to release more Volatile Fatty Acids (VFAs) for some facultative heterotrophic strains to be utilized for fast growth and effective nutrient removal [47,48].

Parameters (mg/L)	Dairy Wastewater[a]	Dairy Wastewater[b]	Swine Manure wastewater[a]	Swine Manure wastewater[b]	Wastewater before primary settling	Primary settled sewage	Secondary treated wastewater	Centrate
NH_4^+-N	1782	2232	2820.3	3630.1	33.4	32.2-35.5	2.5-27.4	71.8
NO_3^--N	ND	ND	ND	ND	ND	0.4	< 1	ND
NO_2-N	ND	ND	ND	ND	ND	ND	< 1	ND
TP	266	249.7	48.2	38.9	5.66	4.29-6.86	0.5-11.8	201.5
TN	3305	3456	3272.1	4317.0	40.65	38.95-48.4	15.5-ND	131.5
COD	38230	23760	14707	8933	231.0	157.5-224	24-ND	2250.0
Inorganic N/P	6.7	8.9	58.5	93.3	5.9	4.7-8.3	2.3-5	0.36
Reference	Wang et al., [6]	Wang et al., [6]	Zhou et al., [4]	Zhou et al., [4]	Wang et al., [6]	Wang et al., [6]	Hodaifa et al., [49]	Wang et al., [6]

Table 1: Characteristics of animal wastewaters and municipal wastewaters derived from different treatment stages of WWTP.

Note: ND: Not Detected a: Represents fresh manure; b: Represents anaerobic digested manure

Industrial Wastewater

Unlike municipal and agriculture wastewaters, there are no universal treatment methodologies for industrial wastewaters due to their unique properties of complex constituents and high toxic compounds including toxic metals, which is specific to

each industry. Microalgae are very sensitive to metal toxicity and also can remove these metal pollutants through absorption and adsorption. For some industrial wastewaters, in which the heavy metals are at very low concentrations, these heavy metals can easily enter microalgal cells through the help of micronutrient transporters and are attached to peptides or proteins and finally are transported to specific cellular compartments for detoxification. Additional heavy metals can be further removed by microalgae adsorption through their unique extracellular ultrastructure [18]. Therefore, algae-based technology is mainly focused on remediation and removal of heavy metals such as copper, nickel, zinc, cadmium, chromium, lead, and mercury, etc. and other chemical toxins (e.g., hydrocarbons, biocides, and surfactants) rather than algal biomass accumulation [18]. Although the fact that industrial wastewater are not suitable for algae growth is proved by many research groups, there are a few studies proved the feasibility of microalgae grown on some specific industrial wastewaters such as carpet mill effluent and wastewater derived from olive-oil extraction for algal biomass production [5,49].

Cultivation Facility

Reactors used for mass cultivation of phototrophic microorganisms (including microbial, algal or plant cells) in a photobiological reaction are termed Photobioreactors (PBR) [18,50]. Depending on whether or not exposure to the air, PBR for microalgae cultivation can be simply classified as open systems or closed systems. The commonly used open systems in wastewater treatment plants include lagoon, high stabilization pond, aerated ponds, raceway pond, multi-layer pond-like bioreactor, etc. The most prominent features of open-pond system include simple construction, low cost and simple operation. However, the disadvantages of such system are also obvious, such as large occupation area, not stable culture system, difficult to control, easy to get polluted, water evaporation loss issue and the fact that light intensity decays rapidly with medium depth. Nonethelessly, from the perspective of economic viability, advanced raceway pond with paddle-wheel is considered as the most promising culture system for low-cost treatment of different types of wastewaters generated from sewage, industry and agriculture as well as biofuels feedstock accumulation [51,52].

Summary

Coupling algal biofuel production with wastewater treatment is considered as the most viable solution to the traditional wastewater treatment processes due to the advantages of technological feasibility, economic viability, environmental sustainability, and credits for wastewater treatment. The success of algae-based cultivation system for wastewater treatment relies on the ability of the algal cells to effectively assimilate both organic (through mixotrophic/heterotrophic growth) and inorganic carbon (e.g. CO_2 from flue gas) and other nutrients such as N, P from wastewater for algal biomass and lipid production. Therefore, a comprehensive characterization of wastewaters from different sources and a better understanding of the mechanisms of N and P uptake and utilization by microalgae are essential not only to the fundamental study of photosynthesis and chemosynthesis, but also to the applied research in wastewater treatment. In addition, microalgae-based wastewater treatment has other advantages such as reduced sludge formation and the fact that the resulting sludge with algal biomass is energy rich and can be further processed to produce biofuel or other valuable products such as fertilizers compared with traditional wastewater treatment process. Moreover, for effective organic-rich wastewater treatment, screening facultative heterotrophic microalgae strains which could adapt well in various wastewater environments, developing effective pretreatment for enhanced VFA profile in wastewaters, and exploiting efficient cultivation system seem to be the most promising for highly efficient wastewater remediation and maximal algal biomass production. Overall, the high cost of algae-biofuel arises from enormous amounts of water and nutrients for algal biomass production, intensive energy for algae harvesting and downstream processing for

traditional algal farm could also be reduced significantly by using advanced wastewater-based cultivation system and process integration.

References

1. Oswald WJ, Gotaas HB, Golueke CG, Kellen WR (1957) Algae in waste treatment. Sewage Ind Wastes 29: 437-455.
2. Oswald WJ, Golueke CG (1960) Biological transformation of solar energy. Adv Appl Microbiol 2: 223-262.
3. Zhou W, Li Y, Min M, Hu B, Chen P, et al. (2011) Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresour Technol 102: 6909-6919.
4. Zhou WG, Li Y, Min M, Hu B, Zhang H, et al. (2012) Growing wastewater-born microalga *auxenochlorella protothecoides* UMN280 on concentrated municipal wastewater for simultaneous nutrient removal and energy feedstock production. Applied Energy 98: 433-440.
5. Chinnasamy S, Bhatnagar A, Claxton R, Das K (2010) Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium. Bioresource Technol 101: 6751-6760.
6. Wang L, Min M, Li Y, Chen P, Chen Y, et al. (2010) Cultivation of green algae *Chlorella sp.* in different wastewaters from municipal wastewater treatment plant. Appl Biochem Biotechnol 162: 1174-1186.
7. Tam NF, Wong YS (1990) The comparison of growth and nutrient removal efficiency of *Chlorella pyrenoidosa* in settled and activated sewage. Environ Pollut 65: 93-108.
8. Mulbry WW, Wilkie AC (2001) Growth of benthic freshwater algae on dairy manures. J Appl Phycol 13: 301-306.
9. Zhou W, Hu B, Li Y, Min M, Mohr M, et al. (2012) Mass cultivation of microalgae on animal wastewater: a sequential two-stage cultivation process for energy crop and omega-3-rich animal feed production. Appl Biochem Biotechnol 168: 348-363.
10. Perez-Garcia O, Escalante FM, de-Bashan LE, Bashan Y (2011) Heterotrophic cultures of microalgae: metabolism and potential products. Water Res 45: 11-36.
11. Wang Z, Ma X, Zhou W, Min M, Cheng Y, et al. (2013) Oil crop biomass residue-based media for enhanced algal lipid production. Appl Biochem Biotechnol 171: 689-703.
12. Zhou WG, Min M, Li Y, Hu B, Ma X, et al. (2012) A hetero-photoautotrophic two-stage cultivation process to improve wastewater nutrient removal and enhance algal lipid accumulation. Bioresour Technol. 110: 448-455.
13. Li Y, Zhou W, Hu B, Min M, Chen P, et al. (2012) Effect of light intensity on algal biomass accumulation and biodiesel production for mixotrophic strains *Chlorella kessleri* and *Chlorella protothecoide* cultivated in highly concentrated municipal wastewater. Biotechnol Bioeng 109: 2222-2229.
14. Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. Bioresour Technol 97: 841-846.
15. Das P, Aziz SS, Obbard JP (2011) Two phase microalgae growth in the open system for enhanced lipid productivity. Renew energy 36: 2524-2528.
16. Becker EW (1994) Microalgae: Biotechnology and microbiology. PP: 293. Cambridge University Press. United Kingdom.
17. Rhee GY (1978) Effects of N: P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. Limnology and Oceanography 23: 10-15.
18. Richmond A (2004) Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwel science Ltd. Oxford, UK.
19. Cheng Y, Zhou WG, Gao CF, Gao Y, Wu QY (2008) Biodiesel production from Jerusalem artichoke (*Helianthus Tuberosus L.*) tuber by heterotrophic microalgae *Chlorella protothecoides*. J Chem Technol Biotechnol 84: 777-778.
20. Huppe HC, Turpin DH (1994) Integration of carbon and nitrogen metabolism in plant and algal cells. Ann Rev Plant Physiol Plant Mol Biol 45: 577-607.
21. Lea PJ, Miflin BJ (2003) Glutamate synthase and the synthesis of glutamate in plants. Plant Physiol Biochem 41: 555-564.
22. Fernandez E, Galvan A (2007) Inorganic nitrogen assimilation in *Chlamydomonas*. J Exp Bot 58: 2279-2287.
23. Morris I (1974) Nitrogen assimilation and protein synthesis. In: Stewart WDP (ed.). Algal Physiology and Biochemistry. PP: 513-613. Blackwell Scientific Publications, Oxford, UK.
24. Li X, Hu HY, Yang J (2010) Lipid accumulation and nutrient removal properties of a newly isolated freshwater microalga, *Scenedesmus sp.* LX1, growing in secondary effluent. N Biotechnol. 27: 59-63.
25. Allen AE, Ward BB, Song B (2005) Characterization of diatom (*Bacillariophyceae*) nitrate reductase genes and their detection in marine phytoplankton communities. J Phycol 41: 95-104.
26. Ahmad I, Hellebust JA (1990) Regulation of Chloroplast Development by Nitrogen Source and Growth Conditions in a *Chlorella protothecoides* Strain. Plant Physiol 94: 944-949.

27. Tischner R (1984) Evidence for the participation of NADP glutamate dehydrogenase in the ammonium assimilation of *Chlorella sorokiniana*. Plant Sci Lett 34: 73-80.

28. Kaplan D, Richmond AE, Dubinsky Z, Aaronson S (1986) Algal nutrition. In: Richmond A (ed.). Handbook for Microalgal Mass Culture. PP: 147-198. CRC Press, Boca Raton, FL, USA.

29. Lu B, Yuan Y, Zhang C, Ou J, Zhou W, et al. (2005) Modulation of key enzymes involved in ammonium assimilation and carbon metabolism by low temperature in rice (*Oryza sativa L.*) roots. Plant Sci 169: 295-302.

30. Vanoni MA, Curti B (2005) Structure--function studies on the iron-sulfur flavoenzyme glutamate synthase: an unexpectedly complex self-regulated enzyme. Arch Biochem Biophys 433: 193-211.

31. Inokuchi R, Kuma KI, Miyata T, Okada M (2002) Nitrogen-assimilating enzymes in land plants and algae: phylogenic and physiological perspectives. Physiol Plant 116: 1-11.

32. Miflin BJ, Habash DZ (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J Exp Bot 53: 979-987.

33. Coruzzi GM, Primary N-assimilation into Amino Acids in Arabidopsis (2003) The Arabidopsis Book. American Society of Plant Biologist. Maryland, USA.

34. Gewitz HS, Piefke J, Vennesland B (1981) Purification and characterization of demolybdo nitrate reductase (NADH-cytochrome c oxidoreductase) of Chlorella vulgaris. J Biol Chem 256: 11527-11531.

35. Nakamura Y, Ikawa T (1993) Purification and properties of NADH: nitrate reductase from red alga Porphyra yezoensis. Plant Cell Physiol 34: 1239-1249.

36. López-Ruiz A, Verbelen JP, Bocanegra JA, Diez J (1991) Immunocytochemical localization of nitrite reductase in green algae. Plant Physiol 96: 699-704.

37. Di Martino Rigano V, Vona V, Fuggi V, Di Martino C, Rigano C (1982) Regulation of nitrate reductase in *Chlorella* nitrate requirement for the appearance of nitrate reductase activity. Plant Sci Lett 28: 265-272.

38. Sherman TD, Funkhouser EA (1989) Induction and synthesis of nitrate reductase in *Chlorella vulgaris*. Arch Biochem Biophys 274: 525-531.

39. Cannons AC, Pendleton LC (1994) Possible role for mRNA stability in the ammonium-controlled regulation of nitrate reductase expression. Biochem J 297 : 561-565.

40. Powell N, Shilton A, Chisti Y, Pratt S (2009) Towards a luxury uptake process via microalgae--defining the polyphosphate dynamics. Water Res 43: 4207-4213.

41. Olguín EJ (2012) Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. Biotechnol Adv 30: 1031-1046.

42. Zhou W, Cheng Y, Li Y, Wan Y, Liu Y, et al. (2012) Novel fungal pelletization-assisted technology for algae harvesting and wastewater treatment. Appl Biochem Biotechnol 167: 214-228.

43. Li Y, Chen YF, Chen P, Min M, Zhou WG, et al. (2011) Characterization of a microalgae *Chlorella sp.* well adapted to highly concentrated municipal wastewater in nutrient removal and biodiesel production. Bioresource Technology 102: 5138-5144.

44. Tam NF, Wong YS (1989) Wastewater nutrient removal by *Chlorella pyrenoidosa* and *Scenedesmus sp.* Environ Pollut 58: 19-34.

45. Pittman JK, Dean AP, Osundeko O (2011) The potential of sustainable algal biofuel production using wastewater resources. Bioresour Technol 102: 17-25.

46. Martin C, Delanoue J, Picard G (1985) Intensive cultivation of fresh-water microalgae on aerated pig manure. Biomass 7: 245-259.

47. Hu B, Min M, Zhou W, Liu Y, Mohr M, et al. (2012) Enhanced mixotrophic growth of microalga *Chlorella sp.* on swine manure for simultaneous biofuel feedstock production and nutrient removal. Bioresour Technol 126: 71-79.

48. Hu B, Zhou W, Min M, Du Z, Chen P, et al. (2013) Development of an effective acidogenically digested swine manure-based algal system for improved wastewater treatment and biofuel & feed production. Appl Energy 107: 255-263.

49. Hodaifa G, Martínez MA, Sánchez S (2008) Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*. Bioresour Technol 99: 1111-1117.

50. Pulz O (2001) Photobioreactors: production systems for phototrophic microorganisms. Appl Microbiol Biotechnol 57: 287-293.

51. Park JB, Craggs RJ, Shilton AN (2011) Wastewater treatment high rate algal ponds for biofuel production. Bioresour Technol 102: 35-42.

52. Zhou WG, Ruan R (2014) Biological mitigation of carbon dioxide via microalgae: recent development and future direction. Scientia Sinca Chimica 44: 63-78.

Adsorption and Absorption of Heavy Metals by Microalgae

Li Li*, Dachun Sun and Hongxin Tan

College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China 201306

***Corresponding authors:** Li Li, College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China 201306; E-mail: l_li@shou.edu.cn

Abstract

Heavy metal contamination of natural water due to mining and industrial activities has posed one of the biggest environmental problems for today. Biosorption using algae is one of the new developed and promising metal removal methods. The state of the art in the field of biosorption of metals by algae is discussed in this article. Classification of major algae groups used in biosorption is illustrated. Since the function groups on the cell wall of algae are found to be responsible for metal uptake, cell wall constituents of different groups of algae are compared. Mechanisms for metal adsorption and absorption are introduced. The mostly used adsorption isotherm and kinetic models in recent literatures are briefly discussed. Several factors could affect the efficiency of biosorption of algae and these factors are studied to provide the optimum environment to gain the best biosorption efficiency in the future. Current state of use of algae as the biosorbent of heavy metals and future perspectives of the technology are also provided to provide insights into this research frontier.

Keywords

Absorption; Adsorption; Algae; Bio sorption; Heavy Metals; Wastewater Treatment

Introduction

The industrial revolution happened in the middle of 18th century propelled development of machine tools, brought changes in chemical manufacturing and iron production processes, and improved many technologies in manufacturing. Since then, more liquid effluents with high levels of heavy metals have been discharged into the environment, which not only contaminated the soil and natural water system, but also threatened the health of human being. More than 40 heavy metals have been widely found in wastewater and the most common ones include Cu, Zn, Mn, Ni, Al, Hg, Cd, Pb, Cr, As, Mo, Co and Sn etc.

Methods for removal of heavy metals from aqueous environment including chemical precipitation, ion exchange, adsorption, membrane filtration, coagulation, flocculation, flotation, and electrochemical treatment have been widely studied by researchers [1]. However, these methods have several disadvantages. For example, chemical precipitation

is the most widely used process for removal of heavy metals from solution. The process is relatively simple and cost is low. However, the process produces large amount of sludge requiring treatment, which, in turn, may contain toxic compounds and may be difficult to treat [2]. Ion exchange resins are effective means of heavy metal removal from wastewater. The high cost makes the method not appropriate for smaller processing plants. Apart from the cost, other disadvantages included resin vulnerable to oxidation by chemicals, and prone to fouling by precipitates and organics [3]. The advantages and disadvantages of main metal removal technologies are summarized in Table 1.

Method	Disadvantages	Advantages
Chemical precipitation	pH dependence Difficult separation Adverse effect by completing agent Resulting sludge's Chemicals required	Simple and low cost
Ion exchange	Sensitive to particles High operational cost Oxidation by chemicals of resin Prone to fouling of resin by precipitates and organics	No sludge generation Pure effluent metal recovery possible
Membrane	No selectivity to alkaline metals Metallic fouling Limited life of membrane Expensive High pressure	Pure effluent
Flocculation and Coagulation	Chemicals required (electrolytes) Depend on basin design	Generate very fine particles of precipitates
Flotation	Less selective for heavy metals	Cheap
Electrodialysis	Takes time Large electrode surface area required Fouling Expensive	Metal Selective

Table 1: Advantages and disadvantages metal remotechnolog**[4]**.

Compared with methods mentioned above, the biosorption method of adsorbing, accumulating, eliminating, and purifying the heavy metals in wastewater with the help of algae, bacterial, fungal or plants is a process gained more and more research interest in recent years and is considered as one of the most effective, economic and promising metal removal methods [4,5,6-13]. In this chapter we will discuss the use of algae as a biosorbent for heavy metal removal in the following aspects. We will start the topic with a brief introduction of the classification of algae. Mechanisms and process of metal uptake will be described. Factors affecting the efficiency of metal biosorption by algae including pH, T, contact time etc. will be discussed. The last topic would be future perspectives of use of algae as biosorbent of heavy metals.

Classification of Algae

Various groups of algae was used as biosorbents of heavy metals and classifications of algae are based on pigments, cell walls chemistry, stored food materials, reproductive structures, life history patterns, etc. Seven major algae groups are classified: Chrysophyta, Euglenophyta, Pyrrhophyta,Charophyta, Chlorophyta, Phaeophyta and Rhodophyta [3]. The Phaeophyta or brown algae have been proved to be most effective biosobent for metal removal, based on statistical review among those algae tested in bio sorption [14]. All the brown algae are almost exclusively marine. They produce a variety of reserve substances, some polymeric and some as free monomers. This group contains many large, complex sea-weeds such as kelp. The Chlorophyta or green algae is also been widely used in heavy metsorption [15,16]. This group of algae produces starch in a form very similar to that of higher plants to reserve carbon. There are more than seventeen thousand species of green

algae abounded in both freshwater and marine. Some species of green algae live in snow, or in symbiotic associations as lichens, or with sponges or other aquatic animals[17]. There are also examples of using the Rhodophyta or red algae to treat heavy metal contaminated wastewater [18]. Like the brown algae, almost all the red algae are marine. [2] Algae could be simply classified into two categories i.e. microalgae and macroalgae. Microalgae are unicellular species which exist individually, or in chains or groups. Depending on the species, their sizes can range from a few micrometers (μm) to a few hundreds of micrometers. Unlike macroalgae, microalgae do not have roots, stems and leaves. Some groups of algae are all microalgae such as Chrysophyta, while other groups have both microalgae and microalgae such as Rhodophyta.

Components of cell wall of various groups of algae will be described since it is closely related to the metal binding capacity of algae. Structure and chemistry of cell walls of different groups of algae varied. In many cases the cell wall is a network of polysaccharides such as pectin, xylans, mannans, alginic acids or fucinic acid. In some algae, calcium carbonate is deposited in the wall to strengthen the structure. Sometimes chitin, a polymer of N-acetylglucosamine, is also present in the cell wall [3]. There is no cell wall present in the group Euglenophyta . Diatom contains a special kind of siliceous component responsible for the rigidity of the cell. All of the three major groups of algae (green, brown, and red algae) of research interest contain cellulose; however, the structure of the cell wall of the three groups differed. The cell walls of brown algae (Phaeophyta) generally contain three components: cellulose, alginic acid, and sulphated polysaccharides (fucoidan matrix). Red algae (Rhodophyta) also contain cellulose, but their interest in connection with biosorption lies in the presence of sulphated polysaccharides made of galactanes (agar and carragenates). Green algae (Chlorophyta) are mainly cellulose, and a high percentage of the cell wall is proteins bonded to polysaccharides to form glycoproteins [3].

Mechanisms of Metal Uptake

Binding Sites on Algae

Cell wall of algae contains a variety of functional groups (amino, carboxyl, sulphate and hydroxyl etc.), which works as the binding site of metals [4,5] . Table 2 listed some major functional groups and classes of organic compounds in algae possibly involved in the biosorption process. The cell wall component difference in different species of algae resulted in different functional groups. Carboxyl is proved to be the most abundant acidic functional group in the cell wall of brown algae [6]. The carboxyl, hydroxyl, and amino and sulphonate groups on cell walls of marine brown macro algae *Cystoseira barbata* are responsible for the binding of metal ions [7] . The presence of amino, carboxylic and sulfonate groups on the cell wall of green microalgae *Chlamydomonas reinhardtii* are responsible for adsorption of the metal ions [8].

Functional group	Class of compounds	Species examples
Hydroxyl	Alcohols, carbohydrates	*Chlamydomonas reinhardtii*
Carboxyl	Fatty acids, proteins, organic acids	*Cladophora fascicularis, Phaeophyta Spp., Oedogonium Spp.*
Amino	Proteins, nucleic acids	*Caulerpa lentillifera, Cladophora fascicularis Chlamydomonas reinhardtii,* Oedogonium Spp.
Ester	Lipids	
Sulfhydryl	Cysteine (amino acid), proteins	*Caulerpa lentil lifera, Phaeophyta Spp.*
Carbonyl, terminal end	Aldehydes, polysaccharides	*Chlamydomonas reinhardtii*
Carbonil, internal	Ketones, polysaccharides	
Phosphate	DNA, RNA, ATP	*Cladophora fascicularis*

Table 2 : Functional groups and classes of organic compounds in algae biomass.

Technology involved in characterizing the active sites involved in metal uptake including: Titration, Infrared And Raman Spectroscopy (XPS), electron microscopy (scanning and/or transmission), Nuclear Magnetic Resonance (NMR), X-Ray Diffraction Analysis (XRD), X-Ray Adsorption Fine Structure Spectroscopy (XAFS), FT-IR Spectra etc [2,8,9].

Process of Metal Uptake

Adsorption is the adhesion of atoms, ions or molecules from a gas, liquid, or dissolved solid to a surface. Adsorption is carried out by extracellular cell-associated materials. It is a non-metabolic and rapid process, which occurs in both living and non-living cells. Absorption is a physical or chemical phenomenon or a process in which atoms, molecules, or ions enter some bulk phase-gas, liquid, or solid material . It is an intracellular accumulation process. This is a different process from adsorption, since molecules undergoing absorption are taken up by the volume, not by the surface (as in the case for adsorption). In the case of algae, the biosorption process took place in two stages: a very rapid surface adsorption and a slow intracellular diffusion or absorption [5]. The quality of metals removed by absorption is usually much less than the quantity of surface adsorbed metals [10]. For example, a large fraction of Pb and Cd (85 % Pb and 79 % Cd) was surface adsorbed by living cells of *Nostoc muscorum*, and only small amount was taken up through the intracellular metal transport system [11].

Mechanisms of adsorption and Absorption n algae

Bio sorption of heavy metals by algae is a complex process involving many physical, chemical and biological reactions. The adsorption is a passive event, which is independent of cell metabolism, and is based on physicochemical interactions between metal and functional groups on the cell wall. Conversely, the absorption depends on cell metabolism and takes place when metal ions are incorporated intracellular [12]. Mechanism of biosorption by algae is either the monolayer chemisorption, in which the functional groups such as O-H bond, N-H bond, C-N bond and C-H bond are responsible for binding the heavy metal atoms, or physical adsorption accompanied by the ion exchange [13]. A good example of ion exchange is that in the cell walls of brown algae, where the sulfated fucans and carboxylate alginic acids act as cation-exchangers. These cations such as Ca^{2+}, K^{+}, Na^{+} are substituted by heavy metal ions [14]. Besides ion-exchange, the precise binding mechanism in the biosorption assays may range from physical (i.e., electrostatic or London–van der Waals forces) to chemical binding (i.e.,ionic and covalent) [6] . Also, in many biosorption processes, several metal binding mechanisms usually take place simultaneously and it is very difficult to distinguish between the mechanisms [8].

Adsorption Isotherms

Empirical models of simple mathematical relations between equilibrium metal concentration in solid phase and equilibrium concentration in liquid phase of the different metals present in solution are used for representing equilibrium biosorption data of single- and multi-metallic systems. The Langmuir and Freundlich are most widely accepted models used to evaluate metal uptake capacity and to compare the difference in metal uptake between various species in single-metallic systems. Since the two models are frequently found in literatures studying metal uptake by algae, principles of the two isotherms will be discussed.

Langmuir Adsorption Isotherm

Langmuir adsorption isotherm is a widely-used model for describing metal adsorption to a sorbent. Langmuir equation quantifies the amount of adsorbate adsorbed on an adsorbent, as a function of adsorbate concentration in solution contacting the adsorbent surface. This isotherm is based on the following assumptions, namely (1) adsorption is

limited to monolayer coverage, (2) maximum adsorption equal to a complete monomolecular layer on all reactive adsorbent surfaces, (3) ability of molecule to be adsorbed on a given site is independent of its neighboring sites occupied, and (4) Equilibrium could be reached [8].

The Langmuir equation is

$$q_e = \frac{q_{max} b C_e}{1 + b C_e} \quad (1.1)$$

Where q_e is weight of adsorbate per unit weight of adsorbent, q_{max} is maximum amount of adsorbate that can be adsorbed on monomolecular layer, *b* is a constant related to binding strength, and C_e is the concentration of adsorbate in the solution. The Langmuir isotherm is a non linear model, whose parameters can be found by using non linear regression. Alternatively, the isotherm could be linearised in four different ways. For example, the above equation also could be written in linear form as:

$$\frac{C_e}{q_e} = \frac{1}{q_{max} b} + \frac{C_e}{q_{max}} \quad (1.2)$$

If $\frac{C_e}{q_e}$ is plotted against C_e, a straight line could be generated. The q_{max} and constant *b* can be calculated from the intercepts and slopes of the plot. This form of linearization is called Langmuir linearization. Other forms of the linearization include Lineweaver-Burk linearization, Eadie-Hoffsie linearization, and Scatchard linearization. Langmuir parameters obtained from tfour linearization can differ among eOther due to an improper weight associated to the linearised variables in linear regression [17].

Freundlich Adsorption Isotherm

Freundlich adsorption isotherm is an alternative of Langmuir developed for heterogeneous surfaces. Unlike Langmuir, there is no adsorption maximum for Freundlich.

The Freundlich equation is

$$q_e = K_F C_e^{1/n} \quad (1.3)$$

Where q_e is weight of adsorbate per unit weight of adsorbent, K_F is the adsorption coefficient, which is a measure of adsorption capacity or fundamental effectiveness of the adsorbent. Empirical constant *n* is a measure of the adsorption intensity, and C_e is the concentration of adsorbate (mg L^{-1}).

The equation (1.3) also could be written in linear form as

$$\text{Log}\,(q_e) = \left(\frac{1}{n}\right) \log C_e + \log K_F \quad (1.4)$$

If the dataset fits the model, the line is straight when log (q_e) is plotted against $\log C_e$ and the value of K_F and *n* could be determined. High K_F and high *n* values indicate high adsorption throughout the concentration range. Low K_F and high *n* values mean low adsorption throughout the studied concentration range. A low *n* value is indicative of high adsorption at strong solute concentration [11].

Many studies have been successfully using the Langmuir and Freundlich models to represent biosorption behavior of a variety of algae species [7,8,11,15-18]. Adsorption behavior of Pb and Cd by cyanobacterium *N. muscorum* was able to be explained by both Langmuir and Freundlich isotherm models [11]. However, the adsorption of Pb (II) and Cu (II) by *Spirogyra* and *Cladophora* filamentous macroalgae showed a better fit with the Langmuir isotherm model than the Freundlich isotherm model [15].

Besides Langmuir and Freundlich models, in the literature, a wide number of models including Redlich-Peterson, Temkin, Sips, Toth, and Dubinin-Radushkevich etc. were applied to represent biosorption of single metal systems onto biomasses

Adsorption Kinetics

The pseudo-first order and pseudo-second order models are often applied to estimate adsorption kinetic process of metal uptake. Principles of the two models will be briefly illustrated.

Pseudo -First-Order Model

The pseudo-first order model also called Lagergren's first-order model. The model described the adsorption rate based on the adsorption capacity.

$$\frac{dq}{dt} = k_1(q_e - q) \quad (1.5)$$

Where q_e is the equilibrium metal concentration in solid phase, q is the amounts of absorbed metal ions at time t, and k_1 is the rate constant of pseudo-first-order kinetics equation.

Integration of Eq. 1.5 (boundary conditions: t = 0, q = 0 and t = t, q = q) gives the linear form of the equation.

$$\ln (q_e - q) = \ln q_e - k_1 t \quad (1.6)$$

-By linear regression using plots ln (q_e − q) versus t, both q_e and K_1 could be calculated.

Pseudo -Second- Order Model

Pseudo-second-order model is derived assuming a second order dependence of the sorption rate on available sites.

$$\frac{dq}{dt} = k_2(q_e - q)^2 \quad (1.7)$$

Where q_e is the equilibrium metal concentration in solid phase, q is the metal concentration in solid phase at time t, and k_2 is the rate of pseudo-second order rate constant. Integration of Eq. 1.7 (boundary conditions: t = 0, q = 0 and t = t, q = q) gives the linear form of the equation.

$$\frac{t}{q} = \frac{t}{q_e} + \frac{1}{K_2 q_e^{\,2}} \quad (1.8)$$

By plotting t/q against t, the pseudo-second-order parameters (q_e and k_2) could be determined.

Both q_e, K_1, K_2 and the corresponding correlation coefficients (R^2) are usually very important parameters evaluating the efficiency of the models. The two models have been widely applied to many biosorption kinetic studies. However, in some of the studies, the pseudo-second-order model is found to be priority to the pseudo-first-order model. In a study investigating the bio sorption of Pb(II), Cd(II), and Co(II) from aqueous solution on green algae waste biomass, the pseudo-second-order kinetic equation could better describe the biosorption kinetics of heavy metals than the pseudo-first-order model [18] . The adsorption of Pb and Cd by cyanobacterium *N. muscorum* followed pseudo-second-order kinetics [11].The pseudo-second-order kinetics provided the best description of data with R^2 in the range of 0.992–0.999 in a kinetics study of biosorption of selenium from aqueous solution by green algae (*Cladophora hutchinsiae*) biomass [16].

Factors Affecting the Efficiency of Metal Bio sorption by Algae

Factors affecting the bio sorption capacity of metals by algae are widely studied by researchers to determine the optimum environment for biosorption efficiency. A brief discussion of several critical factors is as follows.

pH

pH is one of the most important parameters affecting the biosorption process in two aspects.

First, pH could affect the physicochemistry and hydrolysis of the metals in solution. In natural water, only a small amount of metals exist as free, ionic form and many exist as soluble hydrolysis products such as $Al(OH)_2^+$, $Al(OH)_4^-$, etc., ion pairs such as $CuCO_3^\circ$ and $ZnCl_2^\circ$, etc., and coordination compounds formed through chelation of positively charged metal ions by dissolved organic matter [19]. Solubility of most metals in water decreases as pH increases. Concentration of free, ionic form of manganese, copper, zinc, cobalt, and aluminum are particularly low in waters with pH above 5 or 6 [19]. The $Al(OH)_3$ dissolves in water as the follows: $Al(OH)_3 + 3H^+ = Al^{3+} + 3H_2O \quad K=10^9$

The solubility of $Al(OH)_3$ decreased as pH increases and there is little aluminum in solution unless pH is below 5.

Second, the pH levels affect the total negative charges on the surface of the cell walls and the chemistry of the walls [5]. At extremely low pH levels, binding sites on the cell wall of algae will take up more H^+, consequently not only reducing metal ions binding sites on the sorbent surface but also resulting in higher repulsion between bio sorbents and metallic ions [5,20]. With increase in pH, the sorbent surface takes more negative charges, thus attracting greater metal ions. However, concentration of free metal ion will decrease with increasing pH.

Optimum pH for metal biosorption varies for different algae species and metal types. The optimum pH for bio sorption of nickel (II) from aqueous solution by brown algae was 6 [5]. The optimum pH was 6 for the recovery of Cd, Ni and Zn, and less than 5 for Cu and Pb when six different algae were used to remove heavy metals in a laboratory system [21]. The biosorption of heavy metal ions by brown seaweeds (*Hizikia fusiformis*, *Laminaria japonica* and *Undaria pinnatifida*) showed a minimum absorption at pH 2 and a maximum biosorption at pH 4 for Pb^{2+}, Cd^{2+}, Mn^{2+}, and $Cr_2O_7^{2-}$ or pH 6 for Cu^{2+} [22].

Temperature

Temperature can influence the physiological metabolic activity of biosorbents, as well as thermal dynamics and thermal capacity of binding sites on the surface of biosorbents to affect the biosorption of metallic ions [11].The temperature range of 30-40°C is most suitable for the metal binding as well as intracellular uptake of heavy metals by cyanobacterium *Nostoc muscorum* [11]. Adsorption of nickel (II) ion by brown algae was controlled by an endothermic process and the rate of removal increased with increasing temperature up to 40°C [9]. In the case of red macro alga *Hypnea valentiae*, Rathinam et al.,[23] reported an increase in surface availability as well as the increased diffusion rate of adsorbate, both of which are main reasons for increase of cadmium biosorption with the increase of temperature from 30 – 60°C.

Contact Time

Initially, metal uptakes increase as contact time increases. However, equilibrium usually could be attained in a short time. Biosorption of nickel (II) from aqueous solution by brown algae increased with increasing contact time and a large part of nickel was removed by dried algal biomass in the first 20min of contact time [5]. Liu et al., [17] demonstrated that 2h is enough to reach adsorption equilibrium of Cd^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} from aqueous solutions by pretreated biomass of brown algae. All in all, biosorption of metals by algae is a fast process. High removal rates are obtained initially due to abundant adsorbent sites available and high solute concentration [17]. As the adsorption process proceeds, saturation state reached and then the sorbed solute tends to desorbs back into the solution. Eventually, the rates of adsorption and desorption will be equal at equilibrium with no further net

adsorption occurs. In industry, the time at which adsorption equilibrium occurs was very important for process optimization [24].

Initial Metal Concentration

As a rule, increasing the initial metal concentration results in an increase in the biosorption capacity because it provides a driving force to overcome mass transfer resistance between the biosorbent and biosorption medium. The algae *Spirogyra* sp. sorbed each heavy metal ions – Mn^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} – from their aqueous solution proportionally to the amount of the metal ions in solution [25] Higher sorption capacities were obtained at higher initial concentrations of nickel (II) by brown algae [5] .

Co-Exist Ions

The positive ions in effluents are likely to compete for the biosorption sites on algae cell wall resulting in less binding position available for the target heavy metals [20]. Kratochvil et al., [26] reported with the increase of Fe^{2+} in the effluent, the amount of Cu^{2+} removed from wastewater decreased. The negative ions could form ion pairs with metals in water like $CuCO_3^0$, $FeCl_2$, and $CdSO^0$, thus reducing the free metal concentration in water. Addition of NaCl influence cadmium adsorption onto *H. valentiae* seaweed due to formation of chloro-complex with cadmium and the competitive effect of Na^+ cations on metal binding with cadmium [23]. Also, increased amount of electrolyte such as NaCl can swamp the sorbent surface, decreasing metal ion access to the surface for sorption and therefore the metal sorbed may be significantly decreased [23]

Biomass Concentration

More metal ions are tended to be taken up at lower than higher cell densities [27]. Loss of biosorbent effectiveness at high concentrations was observed because high biomass concentrations can exert a shell effect to protect the active sites from being occupied by metal [21]. The other reason is that the electrostatic interaction between cells at high densities may reduce the availability of binding sites per unit mass. A trend of decreasing in biosorption capacity with an increase in biosorbent dosage has been reported by Rathinam et al., [23] for the adsorption of cadmium from simulated wastewaters by red macro algae.

Use of Algae as Bio Sorbent of Heavy Metals and Future Perspectives

Studies have been carried out to determine metal biosorption capacities of diverse types of algae[5,7,8,10,12,15-18,21,23,28-32]. Lesmana et al.,[33] summarized applications of species of marine green macroalgae, fresh water green microalgae, marine red macroalgae, and marine brown macroalgae for the separation of heavy metals from water and wastewater. Initially, researches in the field of bio sorption have mostly concerned itself with brown algae, and then the green algae and red algae were used in heavy metal removal study [21]. Recently, using cyanobacteria for bioremediation has also received research attention [4,11,28] .

The use of living cells for the biosorption of heavy metals has the disadvantage in nutrient requirements, metal toxicity, and cell death system failure [4]. Earlier studies have mainly focus on the efficiency of metal removal by dead algae biomass , due to potential toxicity of high metal levels to living biomass and recently potential of metal removal using living algae have also been explored [10]. Ji et al., [30] proved the possibility of heavy metal removal from wastewater using living freshwater green alga Cladophora fracta. Mane et al., [31] also demonstrated the living Algae Spirogyra sp. and Spirullina could remove several metals from aqueous solution. Moreover, the algal biomass is often pretreated to gain better sorption uptakes, better metal yields or higher mechanical stability. A wide variety of reagents was used for pretreatment of biomass:acids, alkalis, salts, organic compounds, enzymes, metal chelates (EDTA) and distilled water [2] . Some of these methods could provide desirable results

while others cannot and there is still no generalized criterion that can be used whether on the biomass or the right type of treatment to be employed.immobilizationimmobilized [39].

Though various studies have proved the efficiency of using algae for heavy metal removal from wastewater, as a novel biosorbent, studies on biosorption with algae are still few compared to other biomass (mainly fungi and bacteria) [3] . What's more, most of these researches are in experimental scale. Further investigation in the mechanisms of metal uptake in multi-metallic systems, modeling, effective methods for algae pretreatment, and technology commercialization are required.

Acknowledgements:

This study was funded by the Shanghai Young College Teachers Support Program and the PhD Start-up Fund of Shanghai Ocean University (Grant No. A1-0209-13-0105386).

References

1. Fu FL, Wang Q (2011) Removal of heavy metal ions from wastewaters: A review. Journal of Environmental Management 92: 407-418.
2. Tchobanoglous G, Burtonm FL (1991) Wastewater engineering: In Metcarf and Eddy Inc.(Eds) [3rd edn] Wakefield, MA, 756-759.
3. Kuyucak N, Volesky B (1997) Biosorption by algal biomass. In: Volesky B (ed.)_Bio sorption of heavy metals CRC, Boca Raton, FL, 173-198
4. Wang LK, Tay JH, Tay STL, Hung YT (2010) Heavy Metal Removal by Microbial Bio sorbents. Environmental Bioengineering, Humana Press, USA.
5. Brinza L, Dring MJ, Gavrilescu M (2007) Marine micro algal species as bio sorbents for heavy metals. Environ Eng Manag J. 6: 237-251.
6. Ji L, Xie SL, Feng J, Li YH, Chen L (2012) Heavy metal uptake capacities by the common freshwater green alga *Cladophora fracta*. J ApplPhycol 24: 979-983.
7. Wang J, Chen C (2009) Biosorbents for heavy metals removal and their future. Biotechnol Adv. 27: 195-226.
8. Çelekli A, Yavuzatmaca M, Bozkurt H (2010) An eco-friendly process: Predictive modelling of copper adsorption from aqueous solution on *Spirulina platensis*. J. Hazard Mater. 173: 123-129.
9. Pahlavanzadeha H, Keshtkarb AR, Safdarib J, Abadia Z (2010) Biosorption of nickel(II) from aqueous solution by brown algae: Equilibrium, dynamic and thermodynamic studies J. Hazard Mater. 175: 304-310.
10. Davis TA, Volesky B, Mucci A (2003) A review of the biochemistry of heavy metal biosorption by brown algae. Water Res. 37: 4311-4330.
11. Yalçın S, Sezer S, Apak R (2012) Characterization and lead (II), cadmium (II), nickel (II) biosorption of dried marine brown macro algae Cystoseira barbata Environ Sci Pollut Res 19: 3118-3125.
12. Kondo K, Shigehisa H, Matsumoto M (2012) Adsorption of Heavy Metal Ions onto Green Microalga *Chlamydomonas reinhardtii*. Chemical Engineering Transactions 29: 1303-1308.
13. Li XS, Liu SL, Na ZY, Lu DN, Liu Z (2013) Adsorption, concentration, and recovery of aqueous heavy metal ions with the root powder of *Eichhornia crassipes*. Ecological Engineering 60: 160– 166.
14. Romera E, González F, Ballester A, Blázquez ML, Muñoz JA (2006) Biosorption with algae: a statistical review. Crit. Rev. Biotechnol. 26(4): 223-235.
15. Zhou GJ, Peng FQ, Zhang LJ, Ying GG (2012) Biosorption of zinc and copper from aqueous solutions by two freshwater green microalgae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. Environ Sci Pollut Res 19: 2918-2929.
16. Tuzen M, Sarı A (2010) Biosorption of selenium from aqueous solution by green algae (*Cladophora hutchinsiae*) biomass: Equilibrium, thermodynamic and kinetic studies. Chemical Engineering Journal 158: 200-206.
17. Kotrba P, Mackova M, Macek T (2011) Microbial Biosorption of Metals. Springer Dordrecht Heidelberg London New York
18. Ibrahim WM (2011) Biosorption of heavy metal ions from aqueous solution by red macroalgae. J. Hazard Mater. 192: 1827-1835
19. Dixit S, Singh DP (2013) An evaluation of phycoremediation potential of *cyanobacterium Nostoc muscorum:* characterization of heavy metal removal efficiency J Appl Phycol 1-12.

20. Monteiro CM, Castro PML, Malcata FX (2009) Use of the microalga *Scenedesmus obliquus* to remove cadmium cations from aqueous solutions. World J Microbiol Biotechnol 25: 1573-1578.

21. Yu Q, Matheickal JT, Yin P, Kaewsarn P (1999) Heavy metal uptake capacities of common marine macro algal biomass. Water Res. 33 (6): 1534-1537.

22. Lee YC, Chang SP (2011). The bio sorption of heavy metals from aqueous solution by *Spirogyra and Cladophora* filamentous microalgae. Bioresour Technol. 102: 5297–5304.

23. Liu YH, Cao QL, Luo F, Chen J (2009) Biosorption of Cd^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} ions from aqueous solutions by pretreated biomass of brown algae. J. Hazard Mater. 163: 931-938.

24. Bulgariu D, Bulgariu L (2012) Equilibrium and kinetics studies of heavy metal ions biosorption on green algae waste biomass. Bioresour. Technol. 103: 489-493.

25. Quintelas C, Rocha Z, Silva B, Fonseca B, Figueiredo H, Tavares T (2009) Biosorptive performance of an *Escherichia coli* biofilm supported on zeolite NaY for the removal of Cr(VI), Cd(II), Fe(III) and Ni(II). Chem Eng Sci 152: 110-115.

26. Vaghetti JCP, Lima EC, Royer B, da Cunha BM, Cardoso NF, Brasil JL, Dias SLP (2009) Pecan nutshell as bio sorbent to remove Cu(II), Mn(II) and Pb(II) from aqueous solutions. J Hazard Mater 162: 270-280.

27. Dang VBH, Doan HD, Dang-Vu T, Lohi A (2009) Equilibrium and kinetics of biosorption of cadmium(II) and copper(II) ions by wheat straw. Biores. Technol. 100:211-219.

28. Boyd CE (2000) Water quality: an introduction. Kluwer Academic Publishers, Boston, Massachusetts, USA.

29. Cai ZP, Duan SS (2008) Microalgal biosorption on the heavy metals in polluted waters. Ecological Science 27(6): 499-505.

30. Romera E, González F, Ballester A, Blázquez ML, Muñoz JA (2007) Comparative study of bio sorption of heavy metals using different types of algae. Bioresour. Technol. 98: 3344-3353.

31. Lee SH, Park CH (2012) Biosorption of Heavy Metal Ions by Brown Seaweeds from Southern Coast of Korea. Biotechnology and Bioprocess Engineering 17: 853-861.

32. Rathinam A, Maharshi B, Janardhanan SK, Jonnalagadda RR, Nair BU (2010) Bio sorption of cadmium metal ion from simulated wastewaters using *Hypnea valentiae* biomass: A kinetic and thermodynamic study. Bioresour. Technol. 101: 1466-1470.

33. Montazer-Rahmatia MM, Rabbania P, Abdolalia A, Keshtkarb AR (2011) Kinetics and equilibrium studies on biosorption of cadmium, lead, and nickel ions from aqueous solutions by intact and chemically modified brown algae. J. Hazard Mater. 185: 401-407.

34. Rajfur M, Kłos A, Wacławek M (2010) Sorption properties of *algae Spirogyra* sp. and their use for determination of heavy metal ions concentrations in surface water. Bioelectrochemistry 80: 81-86.

35. Kratochvil D, Volesky B (1998) Biosorption of Cu from ferruginous wastewater by algal biomass. Water Research 32(9): 2760-2768.

36. Mehta SK, Gaur JP (2001) Characterization and optimization of Ni and Cu sorption from aqueous solution by *Chlorella vulgaris*. Ecol. Eng. 18: 1-13.

37. Ribeiro R, Magalhães S, Barbosa F, Nascentes C, Campos I, et al. (2010) Evaluation of the potential of microalgae *Microcystis novacekii* in the removal of Pb^{2+} from an aqueous medium. J Hazard Mater 179: 947-953.

38. Mane PC, Bhosle AB (2012) Bioremoval of Some Metals by Living Algae *Spirogyra* sp. and *Spirullina* sp. from aqueous solution. Int. J. Environ. Res. 6(2): 571-576.

39. Kumar SD, Santhanam P, Jayalakshmi T, Nandakumar R, Ananth S. et al. (2013) Optimization of pH and retention time on the removal of nutrients and heavy metal (Zinc) using immobilized marine microalga *Chlorella marina*. Pakistan Journal of Biological Science 1-6.

40. Lesmanaa SO, Febrianaa N, Soetaredjoa FE, Sunarsob J, Ismadjia S (2009) Studies on potential applications of biomass for the separation of heavy metals from water and wastewater. Biochemical Engineering Journal 44: 19-41.

41. Ajjabi LC, Chouba L (2009) Biosorption of Cu^{2+} and Zn^{2+} from aqueous solutions by dried marine green *macroalga Chaetomorpha*

42. linum. J Environ Manage 90: 34853489

43. Mata YN, Torres E, Blázquez ML, Ballester A, González F, Muñoz JA (2009) Gold(III) biosorption and bioreduction with the brown alga Fucus vesiculo Hazard.

CO2 Sequestration by Microalgae: Advances and Perspectives

T Chakrabarti*, K Krishnamurthi*, S Saravana Devi and Abhay B Fulke

Environmental Health Division, CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nagpur, India

***Corresponding authors:** T Chakrabarti, Environmental Health Division, CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nagpur, India, Tel: +91-712-2249885-889; Fax: +91-712-2249961; E-mail: tapan1249@gmail.com

K Krishnamurthi, Environmental Health Division, CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nagpur, India, Tel: +91-7122249885-889; Fax: +91-7122249961; E-mail: k_krishnamurthi@neeri.res.in

Abstract

Carbon dioxide, a Greenhouse Gas (GHG), is the one of the principle pollutant, warming earth. In the past 150 years, anthropogenic activities have pumped enough carbon dioxide into the atmosphere to raise its levels to 400 ppm, higher than they have been for hundreds of thousands of years. In the global effort to combat the climate change, several CO_2 capture and storage technologies are deliberated. Because of photosynthetic ability of microalgae, the potential microalgae such as *Chlorella vulgaris*, *Scenedesmus* sp., *Chroococcus* sp. and *Chlamydomonas* sp. have been actively used globally in closed and open photobioreactor for CO_2 mitigation. Algae as feed stocks for bio-energy refer to a diverse group of organisms that include microalgae, macroalgae (seaweed) and cyanobacteria (formerly called "blue-green algae"). Algae occur in a variety of natural aqueous and terrestrial habitats in environment. Under certain conditions, some microalgae have the potential to accumulate lipids. Algal biofuel generation imbibes both carbon sequestration and energy production. Further, special emphasis is required on regulation of biosynthetic genes and its expression in microalgae under various CO_2 stress conditions for sequestration.

Introduction

Reducing the rate of global climate change which in turn linked to increasing levels of Greenhouse Gases (GHGs) in the atmosphere is recognized as the world's biggest environmental challenge [1-3]. In recent years, researchers are focusing on biotechnological routes which might help to reduce CO_2 emissions in the air vis-a-vis sequester more carbon from the air into the ground and oceans. Now, amply clear that no single technology holds the answer to achieving fuel related GHG emission reductions. Instead, only full use of all available alternatives such as switching to renewable energy sources, enhancing energy efficiency, curbing emissions from agriculture, forestry and deploying Carbon Sequestration and Storage (CSS) paradigm will solve the energy and climate challenge. A rational scientific policy and networking with pockets of excellence conducting their climate change related

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

research in equivalent, elsewhere in the world, must exists. A strategy for low-carbon escalation and renewable energy systems, especially in the transport sector must exist in India. The country has three alternatives to high-carbon systems: carbon sequestration, renewable energy and nuclear power, despite high costs. According to Mr. Sam Pitroda, Chairman of the Knowledge Commission, "India has to invent, sometimes reinvent, model and not to depend on western nations for simulations as western model of consumption, generally, is unsustainable." Biomass from plants, bagasse, cow dung, agricultural residues etc which could either be anaerobically digested to produce biogas or could be transformed into gas. A related study was reported by Prajapati et al. [4] and Prajapati et al.[5]. This biomass can be used to generate steam or power or used as a fuel. Various examples of gasified power plants in India showed that power is generated using rice husk in Andhra Pradesh State, while several bagasse based plants are present elsewhere in the country. Potential use of biomass as first, second, third and fourth generations of biofuels are summarized in brief as under:

- **First generation biofuels:** This utilizes food-based feedstocks for example sugar cane, corn or soybean as raw materials, and utilize processing technologies like fermentation (for ethanol) and trans-esterification for biodiesel [6].
- **Second generation biofuels:** These exploit non-food feedstocks, for example lingo-cellulosic plant biomass and non-edible oilseeds through fermentation or thermo-chemical routes. However, the technology has yet not been commercialized.
- **Third generation biofuels:** This purposely utilizes transgenic bioenergy crops to improve biofuel yield. An example is the development of lignin deficient plants, improve ethanol production, or corn with implanted enzyme cellulase. However, the use of transgenic plants are still experimented on laboratory scale and their future use in developing countries is questionable because of lack of monitoring and control strategies to manage transgenic plants under open field conditions.
- **Fourth generation biofuels:** This involves algae (microalgae/macroalgae) production with carbon sequestration and more carbon neutral to the other generation biofuels [7]. Thus, symbolize "Bioenergy with Carbon Storage (BECS)".

Literature review revealed that growth of algae is negatively influenced by increasing CO_2 [1,8]. Strains that grow well at CO_2 concentrations of 5-10% show severe reduction in their growth rate [3,9]. Efficient carbon dioxide capture from industrial flue-gas is an important development to permit carbon management [1,3,10-14]. Algae have been successfully used to clear outgases from power-plant [15].Various CO_2 mitigation strategies such as chemical absorption, membrane separation, cryogenic fractionation and CO_2 adsorption using molecular sieves have been investigated though their techno-economic feasibility is not so encouraging [16,17]. The key focus for Government should, therefore, be to support the demonstration projects involving microalgae biomass based biofuel production thereby enabling the entrepreneurs to go ahead with commercial scale production enabling such cleaner technologies facilitating prosperous future for everyone.

The key bio-molecules that can be extracted from algal biomass are;

a) Lipids
b) Carbohydrates
c) Proteins

Lipids and carbohydrates can be used as fuel precursors for example, gasoline and biodiesel, while proteins can be used as animal/fish feeds [18].

In this context, present chapter discusses about the scope of reduction in carbon dioxide emission using microalgae.

Bio-Mitigation of CO_2 Emissions with Microalgae

One of the largely considered methods for CO_2 mitigation is the use of microalgae in biomass conversion in photo bioreactor [3]. Marine and freshwater microalgae are

microscopic photosynthetic organisms. Microalgae namely, Cyanobacterial (Cyanophyceae) and eukaryotic microalgae, green algae (Chlorophyta) and diatoms (Bacillariophyta) can be used to capture CO_2 from three different sources: atmospheric CO_2, CO_2 emission from power plants and industrial processes and soluble carbonate [1,19]. In this context, possibly the transfer of CO_2 from the atmosphere to the microalgae through photosynthesis is fundamental route for CO_2 capture [3]. However, 360ppm CO_2 concentration in atmospheric air makes what economically non-feasible [20]. In contrast, CO_2 capture from flue gas emissions from fossil fuel based power plants achieves better recovery because of, "The higher concentration is in exhaust stream, raceway pond systems for microalgae production, and not in photobioreactor" [21]. However, only a small number of algae are tolerant to the high levels of Sox and NOx present in flue gases. Further, the gases need to be cooled prior to injection into the growth medium. Some microalgae species can assimilate CO_2 from soluble carbonates such as Na_2CO_3 and $NaHCO_3$ [19] leading to high pH of the medium because of conversion of carbonate/bicarbonate alkalinity to hydroxyl alkalinity. Such a condition tends to control invasive species since only a very small number of algae can grow in such extreme conditions [19]. The high cost of process technology and price competitiveness of biodiesel extraction from microalgae can be offset by bio-mitigation of CO_2 emissions which may be simultaneously exploited to reduce cost [19,22]. The harvested biomass methods, include sun drying, low-pressure shelf drying, drum drying, spray drying [23,24], fluidized bed drying [25], freeze drying [26] and refractance Window™ technology drying[27]. Intracellular oils are extracted more easily from dried biomass [26,28].

Fulke et al., [3] evaluated the photosynthetic ability of different microalgae leading to higher CO_2 fixation and calcite formation vis-a-vis their ability to synthesize biodiesel precursors. Further, Fulke et al.[3]observed the presence of Fatty Acid Methyl Esters (FAME) such as docosapentaenoic acid (C22:5), palmitic acid (C16:0) and docosahexaenoic acid (C22:6) and superior quality calcite production with simultaneous CO_2 mitigation using *Chlorella* species. Demirbas, [29] reported that microalgae could covert CO_2 into chemical energy via photosynthesis, subsequently leading to fuels biosynthesis. Thus, the photosynthetic potential of microalgae can be integrated with advance CO_2 sequestration and biodiesel production which is a new area of research interest [1,3].

Biofuel from CO_2 Sequestered Microalgae Biomass

Microalgae have a higher CO_2 fixation ability compared to plants [30] and produce value-added products [1,3,31,32]. Microalgae oil production was extensively reviewed by Brennan and Owende [7]. Wastewater effluent from the agri-food industry can be used for microalgae cultivation [33]. However, care should be taken to prevent introduction of wastewater algal predators such as protozoa and other species.

Three different algae production mechanisms showed photoautotrophic (requires inorganic carbon and light), heterotrophic (requires organic carbon and light) and mixotrophic (autotrophic photosynthesis and heterotrophic assimilation) production. Algae can be commercially cultivated in open raceway ponds or closed photo-bioreactors [34]. According to Brennan and Owende [7] raceway pond consist of a closed loop, oval shaped recirculation channels as shown in (Figure: 1). Photo-bioreactors, on the other hand, consist of an arrangement of straight glass or plastic tubes as made known in (Figure: 2) by Ugwu et al. [35]. The tubular array captures sunlight, minimize contamination and favor constant cell growth [12,36]. Ketheesan and Nirmalakhandan [37] studied an airlift-driven raceway reactor for microalgal cultivation with the highest CO_2 consumption. Cheng et al. [38] had showed that increasing retention time of CO_2 in photo bioreactor could significantly enhance the CO_2 fixation efficiency. Similar result was also reported by Fulke et al. [3] in which *Chlorella* sp. could clearage CO_2 gas up to 15% concentration with air lift photobioreactor. Many researchers have studied microalgal CO_2 fixation with closed photo-bioreactors [3,38-40]. A variety of microalgae species have been applied to CO_2 fixation, including *Chlorella*

vulgaris, Scenedesmus obliquus, Chlamydomonas reinhardtii, Spirullina sp. And *Botrycoccus braunii* [1,38,39,41,42]. Thus, algal based CO_2 sequestration is promising technology for carbon dioxide sequestrations vis-a vis biofuel production [1,3,14,43,44].

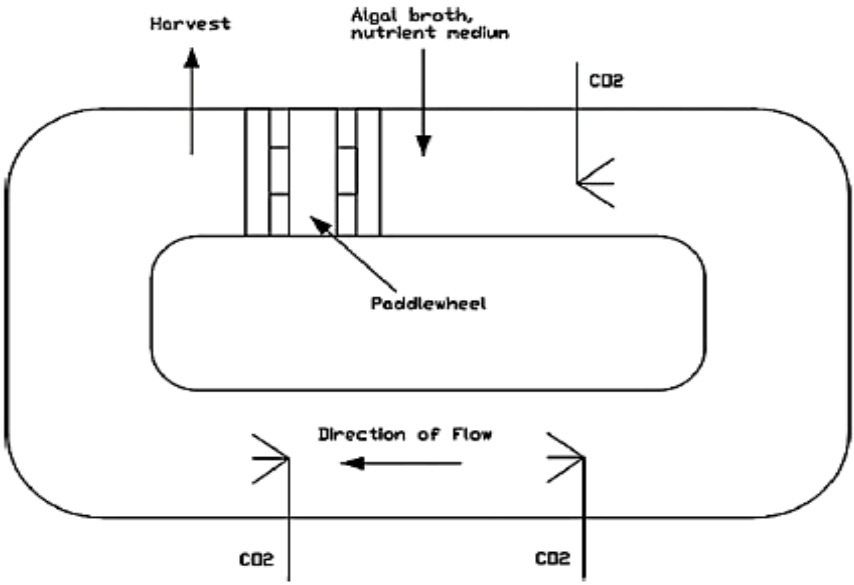

Figure 1: Plan view of a raceway pond. Algae broth is introduced after the paddlewheel, and completes a cycle while being mechanically aerated with CO_2. It is harvested before the paddlewheel to start the cycle again (adapted from [7,10]).

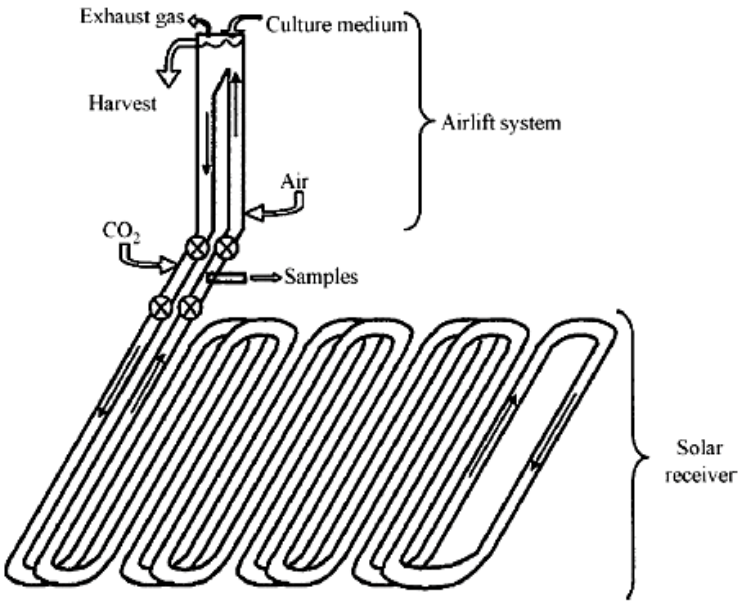

Figure 2: Basic design of a horizontal tubular photobioreactor (adapted from [7,84]) Two main sections: airlift system and solar receiver; the airlift systems allow for the transfer of O_2 out of the systems and transfer of CO_2 into the system as well as providing a means to harvest the biomass. The solar receiver provides a platform for the algae to grow by giving a high surface area to volume ratio.

In heterotrophic production [45-47], microalgae used not only glucose as organic carbon substrates. Leading to low cost harvesting systems with higher biomass growth was discussed by Chen and Chen [48]. Li et al. and Miao and Wu [47,49] investigated the heterotrophic cultivation of *Chlorella protothecoides* for high lipid content, used for biodiesel production. Chojnacka and Noworyta [50] compared *Spirulina* sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. Akkerman et al.,[51] studied importance of Photosynthetic Efficiency (PE) during photoautrophic growth. Microalgae, therefore, appear to be the most efficient biomass resource, used for biofuel production due to its lipid content and specific growth rate [52]. Sheehan et al., [11] reported the site specific variation is the choice for industrial microalgae production. Yoo et al., [32] evaluated three microalgae species, *Chlorella vulgaris, Botryococcus braunii,* and *Scenedesmus* sp. under high level CO_2 growth condition for biodiesel production, authors observed that *B. braunii* is the most suitable one for biodiesel production while, *Scenedesmus* sp. is suitable for CO_2 mitigation. Chiu et al., and Tang et al., [40,53] revealed the high concentration of CO_2 supplement, stimulate microalgae to grow faster comparison to atmospheric air. Furthermore, authors found microalga; *Haematococcus pluvialis* can tolerate 34% of CO_2 and could fixed CO_2 with fixation rate of 0.14 $g^{-1}L^{-1}$ d^{-1} [12,54,55]. Overall summary on recent studies on microalgae CO_2 sequestration is presented in (Table 1).

S.No.	Microalgae	CO_2concentration tolerance (%)	CO_2 fixation rate ($g^{-1}L^{-1}d^{-1}$)	References
1.	*Scenedesmus dimorphus*	0.03%	1.27	Fulke et al. [1]
2.	*Scenedesmus incrassatulus*		1.50	
3.	*Chroococcus cohaerens*		0.78	
4.	*Chlorella* sp.		1.62	
5.	*Chlorella* sp.	15	0.46	Jin et al. [56]
6.	*Chlorella* sp.	5	0.7	Ryu et al. [57]
7.	*Chlorella* sp.	--	1.38	Zhao et al. [58]
8.	*Chlorella* sp.	15	--	Fulke et al. [3]
9.	*Chlorella vulgaris*	10	0.25	Sydney et al. [59]
10.	*Chlorella vulgaris*	2	0.43	Yeh and Chang [60]
11.	*Scenedesmus* sp.	15	0.61	Jin et al. [56]
12.	*Spirulina platensis*	15	0.92	Kumar et al. [61]
13.	*Scenedesmus obliquus*	10	0.55	Ho et al. [62]
14.	*Scenedesmus obliquus*	10	0.29	Tang et al. [53]
15.	*Dunaliella* sp.	3	0.31	Kishimoto et al. [63]
16.	*Scenedesmus obliquus*	2.5	1.19	Ho et al. [64]
17.	*Chlorella kessleri*	18	--	de Morais and Costa [39]
	Chlorella vulgaris			
	Scenedesmus obliquus			
	Spirulina sp.			
18.	*Dunaliella tertiolecta*	10	0.27	Sydney et al. [59]
19.	*Haematococcus pluvialis*	34	0.14	Huntley and Redalje [35]
20.	*Chlorella kessleri*	18	0.16	de Morais and Costa [65]

Table 1: Recent studies on microalgal CO_2 sequestration.

From the table, *Haematococcus pluvialis, Spirulina* sp., *Chlorella* sp., *Chlorella vulgaris, Scenedesmus incrassatulus, Scenedesmus obliquus, Scenedesmus dimorphus* and *Scenedesmus* sp. have been recognized as promising microalgae strains, capable to assimilate much of carbon dioxide, provide a biological tool for reducing GHG emissions from coal-fired power plants and other carbon severe industrial processes. Whereas, numerous

microalgae strains have high lipid content, it is possible to increase the concentration by optimizing nitrogen level in the feed [66,67], light intensity [68], temperature, salinity [67], CO_2 percentage [39,69] and harvesting procedure [66,69]. However, increases in lipid accumulation will not outcome in lipid productivity as biomass productivity and lipid accumulation ratios diverges in microalgae.

The algae can be processed into biofuel instead of first extracting oils then processing. This result in reduced costs associated with the interim process from biomass to biofuel. A degradable alternative liquid called bio-oil, an advantage to enter directly in refinery stream with some hydro-treating and hydro-cracking, produce a suitable diesel feedstock. "Flash pyrolysis" technology has high efficiency, where feedstock is quickly heated to 350 - 500˚C for 2 seconds. Since an alga exists fundamentally in small units and has no fiber tissue to deal with, algae have a major advantage over other biomass sources. A significant roadblock in using pyrolysis for algae conversion is moisture content, and significant dehydration must be performed upstream for the process to work efficiently. The gasification of the microalgal biomass to liquid fuels during Fischer-Tropsch Synthesis (FTS) or mixed alcohol synthesis results in syngas [70-72]. FTS tends to require production at a very large scale to make the process efficient overall. However, most significance problem with FTS is the generation of tars during the gasification process, causes coking of the synthesis catalyst and any other catalysts used in the syngas cleanup process and must be removed. Liquefaction with the direct hydrothermal (defined as water held in a liquid state above 100°C by applying pressure) can be employed to convert wet algal biomass to liquid fuels [73]. A maximum oil yield of 64% using *Botryococcus braunii* as a feedstock has also been reported that algal biomass was processed by liquefaction [74]. Prajapati et al. [30] studied the novel integration of phycoremediation and biogas production from algal biomass. Sialve et al. [75] evaluated that microalgae biomass contains considerable amount of lipids, carbohydrates and proteins. This makes it a favorable substrate to convert into biodiesel, biogas and other biofuel production along with their integration with CO_2 sequestration.

Value Added Products from Algal Biomass

Borowitzka [76] examined that some of Chlorophyceae group algal species accumulate high concentrations of carotenoids. Hirata et al., [77] suggested that phycobiliproteins, phycocyanin and phycoerythrin from algae can be usedincosmetics, pharmacy and nutrition. Francisco et al., [78] investigated *Chlorella vulgaris* as for biodiesel production with lipid content of 27.0%. Muller-Feugaet al., [79] reported bactericides production using Ulvophyceae, Charophyceae and Spirogyra. Lorenz and Cysewski [80] produced astaxanthin from *H.Pluvialis*. The schematic diagram of the overall process of CO_2 conversion into algal biomass to biofuel and other Value-Added Products (VAPs) is shown by authors in (Figure 3). In wastewater stabilization ponds, the algae produce oxygen through photosynthesis [1,81]. Hejazi and Wijffels [82] highlighted the microalgal milking for commercialization of microalgal products.

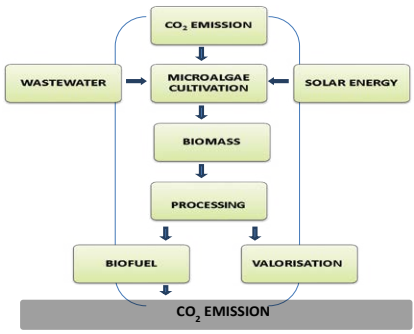

Figure 3: The overall process of CO_2 conversion into algal biomass which can be used for production of biofuel and other value added products.

CO_2 Sequestration by Microalgae: Recent Advances and Perspectives

Crucial need is integrated CO_2 bio-fixation, bio-fuel production and value addition of algal biomass as an expectant substitute to current CO_2 mitigation strategies. However, the option for most frequently quoted microalgae for competent and economical combination of CO_2 biofixation, wastewater treatment and lipid synthesis toward biofuel production should be explored.

Various studies reported CO_2 capture technologies have the adequate capability to reduce greenhouse gas emissions and are able to address the prospects and challenges based on cost and engineering finances of CO_2 capture and sequestration [1,3,83].

Special emphasis is required on regulation of biosynthetic genes and its expression in microalgae under various stress conditions for CO_2 sequestration. To increase the photosynthetic efficiency of microalgae, significant genetic modifications in Rubisco gene and its functions should be investigated.

References

1. Fulke AB, Chambhare K, Giripunje MD, Sangolkar L, Krishnamurthi K, et al. (2013) Potential of wastewater grown algae for biodiesel production and CO_2 sequestration. African Journal of Biotechnology 12:2939-2948.
2. Khan SA, Rashmi, Hussain MZ, Prasad S, Banerjee UC (2009) Prospects of biodiesel production from microalgae in India. Renewable and Sustainable Energy Reviews 13: 2361-2372.
3. Fulke AB, Mudliar SN, Yadav R, Shekh A, Srinivasan N, et al. (2010) Bio-mitigation of CO_2, calcite formation and simultaneous biodiesel precursors production using Chlorella sp. BioresourTechnol 101: 8473-8476.
4. Prajapati SK, Malik A, Vijay VK (2014) Comparative evaluation of biomass production and bioenergy generation potential of *Chlorella* spp. through anaerobic digestion. Applied Energy 114:790-797.
5. Prajapati SK, Kaushik P, Malik A, Vijay VK (2013) Phycoremediation and biogas potential of native algal isolates from soil and wastewater. BioresourTechnol 135: 232-238.
6. IEA (2006) World energy outlook 2006. International Energy Agency, Paris.
7. Brennan L, Owende LP (2010) Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews 14: 557-577.
8. Lee JS, Lee JP, (2003) Review of advances in biological CO_2 mitigation technology. Biotechnol Bioprocess Eng8:354-359.
9. Watanabe Y, Ohmura N, Saiki H (1992) Isolation and determination of cultural characteristics of microalgae which functions under CO_2 enriched atmosphere. Energy Convers Manage 33: 545-552.
10. Chisti Y (2007) Biodiesel from microalgae. BiotechnolAdv 25: 294-306.
11. Sheehan J, Dunahay T, Benemann J, Roessler P (1998) A look back at the U.S. Department of Energy's Aquatic Species Program:Biodiesel from algae. National Renewable Energy Laboratory, Colorado.
12. Huntley M, Redalje D (2007) CO_2 Mitigation and Renewable Oil from Photosynthetic Microbes: A New Appraisal. Mitigation and Adaptation Strategies for Global Change 12: 573-608.
13. Yadav RR, Mudliar SN, Shekh AY, Fulke AB, Sivanesan SD, et al. (2012) Immobilization of carbonic anhydrase in alginate and its influence on transformation of CO_2 to calcite. Process Biochemistry 47: 585-590.
14. Shekh AY, Kannan K, Yadav RR, Sivanesan SD, Chakrabarti T, et al.(2013) Biotechnological Applications of Microalgae: Biodiesel and Value Added Products. Taylor & Francis CRC Press, Florida.
15. Zeiler KG, Heacox DA, Toon ST, Kadam KL, Brown LM (1995)The use of microalgae for assimilation and utilization of carbon dioxide from fossil fuel-fired power plant flue gas. Energy Conversion and Management 36:707-712.
16. Huston MA, Marland G (2003) Carbon management and biodiversity. J Environ Manage 67: 77-86.
17. Stewart C, Hessami MA (2005) A study of methods of carbon dioxide capture and sequestration-the sustainability of a photosynthetic bioreactor approach. Energy Conversion and Management 46:403-420.
18. Ferrel J, Sarisky-Read V U.S. DOE (2010) National Algal Biofuels Technology Roadmap. US Department of Energy, Office of Energy Efficiency and Renewable Energy, Biomass Program, Maryland.
19. Wang B, Li Y, Wu N, Lan CQ (2008) CO_2 bio-mitigation using microalgae. ApplMicrobiolBiotechnol 79: 707-718.
20. Stepan DJ, Shockey RE, Moe TA, Dorn R(2002) Carbon dioxide sequestering using microalgae systems.US Department of Energy, Pittsburgh, PA.

21. Bilanovic D, Andargatchew A, Kroeger T, Shelef G (2009) Freshwater and marine microalgae sequestering of CO_2 at different C and N concentrations-Response surface methodology analysis. Energy Conversion and Management 50: 262-267.

22. Gudin C, Therpenier C (1986) Bioconversion of solar energy into organic chemicals by microalgae. Advances in Biotechnological Processes 6:73-110.

23. Prakash J, Pushparaj B, Carlozzi P, Torzillo G, Montaini E, et al. (1997) Microalgal Biomass Drying by a Simple Solar Device. International Journal of Solar Energy 18: 303-311.

24. Desmorieux H, Decaen N (2005)Convective drying of Spirulina in thin layer. Journal of Food Engineering 66: 497-503.

25. Leach G, Oliveira G, Morais R (1998) Spray-drying of *Dunaliellasalina* to produce a β-carotene rich powder. Journal of Industrial Microbiology and Biotechnology 20: 82-85.

26. Molina Grima E, Medina A, Gime´nez A, Sa´nchez Pe´rez J, Camacho F, et al. (1994) Comparison between extraction of lipids and fatty acids from microalgal biomass. Journal of the American Oil Chemists' Society 71: 955-959.

27. Nindo CI, Tang J (2007) Refractance Window Dehydration Technology: A Novel Contact Drying Method. Drying Technology 25: 37-48.

28. Molina Grima E, Belarbi EH, Acién Fernández FG, Robles Medina A, Chisti Y (2003) Recovery of microalgal biomass and metabolites: process options and economics. BiotechnolAdv 20: 491-515.

29. Demirbas A (2004) Current Technologies for the Thermo-Conversion of Biomass into Fuels and Chemicals. Energy Sources 26: 715-730.

30. Prajapati SK, Kaushik P, Malik A, Vijay VK (2013) Phycoremediation coupled production of algal biomass, harvesting and anaerobic digestion: possibilities and challenges. BiotechnolAdv 31: 1408-1425.

31. Vunjak-Novakovic G, Kim Y, Wu X, Berzin I, Merchuk J (2005) Air-Lift Bioreactors for Algal Growth on Flue Gas: Mathematical Modeling and Pilot-Plant Studies. IndEngChem Res 44: 6154-6163.

32. Yoo C, Jun SY, Lee JY, Ahn CY, Oh HM (2010) Selection of microalgae for lipid production under high levels carbon dioxide. BioresourTechnol 101: 71-74.

33. Cantrell KB, Ducey T, Ro KS, Hunt PG (2008) Livestock waste-to-bioenergy generation opportunities. BioresourTechnol 99: 7941-7953.

34. Jime´nez C, Cossi´o BR, Labella D, Xavier Niell F (2003) The feasibility of industrial production of *Spirulina (Arthrospira)* in southern Spain. Aquaculture 217: 179-190.

35. Ugwu CU, Aoyagi H, Uchiyama H (2008) Photobioreactors for mass cultivation of algae. BioresourTechnol 99: 4021-4028.

36. Rodolfi L, ChiniZittelli G, Bassi N, Padovani G, Biondi N, et al. (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. BiotechnolBioeng 102: 100-112.

37. Ketheesan B, Nirmalakhandan N (2012) Feasibility of microalgal cultivation in a pilot-scale airlift-driven raceway reactor. BioresourTechnol 108: 196-202.

38. Cheng L, Zhang L, Chen H, Gao C (2006) Carbon dioxide removal from air by microalgae cultured in a membrane-photobioreactor. Sep PurifTechnol 50: 324-329.

39. de Morais MG, Costa JAV, (2007) Carbon dioxide fixation by Chlorella kessleri, C. vulgaris, Scenedesmusobliquus and Spirulina sp. cultivated in flasks and vertical tubular photobioreactors. Biotechnology Letters 29: 1349-1352.

40. Chiu SY, Kao CY, Chen CH, Kuan TC, Ong SC, et al. (2008) Reduction of CO2 by a high-density culture of Chlorella sp. in a semicontinuousphotobioreactor. BioresourTechnol 99: 3389-3396.

41. Giripunje MD, Fulke AB, Meshram PU (2013) A Study of Functional Genomics for Unknown Proteins in Chlamydomonasreinhardtii. International Journal of Computer Applications 81: 1-6.

42. Packer M (2009) Algal capture of carbon dioxide; biomass generation as a tool for greenhouse gas mitigation with reference to New Zealand energy strategy and policy. Energy Policy 37: 3428-3437.

43. Ramanan R, Kannan K, Deshkar A, Yadav R, Chakrabarti T (2010) Enhanced algal CO_2 sequestration through calcite deposition by Chlorella sp. and Spirulinaplatensis in a mini-raceway pond. BioresourTechnol 101: 2616-2622.

44. Ramanan R, Vinayagamoorthy N, Sivanesan SD, Kannan K, Chakrabarti T (2012) Influence of CO_2 concentration on carbon concentrating mechanisms in cyanobacteria and green algae: a proteomic approach. Algae 27: 295-301.

45. Chen F, Zhang Y, Guo S (1996) Growth and phycocyanin formation of Spirulinaplatensis in photoheterotrophic culture. Biotechnology Letters 18: 603-608.

46. Eriksen NT (2008) Production of phycocyanin--a pigment with applications in biology, biotechnology, foods and medicine. ApplMicrobiolBiotechnol 80: 1-14.

47. Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. BioresourTechnol 97: 841-846.

48. Chen GQ, Chen F (2006) Growing phototrophic cells without light. BiotechnolLett 28: 607-616.

49. Li X, Xu H, Wu Q (2007) Large-scale biodiesel production from microalga Chlorella protothecoides through heterotrophic cultivation in bioreactors. BiotechnolBioeng 98: 764-771.

50. Chojnacka K, Noworyta A (2004) Evaluation of *Spirulina* sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. Enzyme and Microbial Technology 34: 461-465.

51. Akkerman I, Janssen M, Rocha J, Wijffels RH (2002) Photobiological hydrogen production: photochemical efficiency and bioreactor design. International Journal of Hydrogen Energy 27: 1195-1208.

52. Minowa T, Yokoyama S-y, Kishimoto M, Okakura T (1995) Oil production from algal cells of *Dunaliella tertiolecta*by direct thermo chemical liquefaction. Fuel 74: 1735-1738.

53. Tang D, Han W, Li P, Miao X, Zhong J (2011) CO_2 biofixation and fatty acid composition of Scenedesmusobliquus and Chlorella pyrenoidosa in response to different CO2 levels. BioresourTechnol 102: 3071-3076.

54. Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol 26: 126-131.

55. Pokoo-Aikins G, Nadim A, El-Halwagi MM, Mahalec V (2010) Design and analysis of biodiesel production from algae grown through carbon sequestration. Clean Technol Environ Policy 12: 239-254.

56. Jin HF, Lim BR, Lee K (2006) Influence of nitrate feeding on carbon dioxide fixation by microalgae. J Environ Sci Health A Tox Hazard Subst Environ Eng 41: 2813-2824.

57. Ryu HJ, Oh KK, Kim YS (2009) Optimization of the influential factors for the improvement of CO_2 utilization efficiency and CO2 mass transfer rate. Journal of Industrial and Engineering Chemistry 15: 471-475.

58. Zhao B, Zhang Y, Xiong K, Zhang Z, Hao X, et al. (2011) Effect of cultivation mode on microalgal growth and CO2 fixation. Chemical Engineering Research and Design 89: 1758-1762.

59. Sydney EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, et al. (2010) Potential carbon dioxide fixation by industrially important microalgae. BioresourTechnol 101: 5892-5896.

60. Yeh KL, Chang JS (2011) Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga Chlorella vulgaris ESP-31: implications for biofuels. Biotechnol J 6: 1358-1366.

61. Kumar A, Yuan X, Sahu AK, Erga SJ, Langenhove V, et al. (2010) A hollow fiber membrane photo-bioreactor for CO_2 sequestration from combustion gas coupled with wastewater treatment: a process engineering approach. Journal of Chemical Technology and Biotechnology 85: 387-394.

62. Ho SH, Chen CY, Yeh KL, Chen WM, Lin CY, et al. (2010) Characterization of photosynthetic carbon dioxide fixation ability of indigenous *Scenedesmus obliquus* isolates. Biochemical Engineering Journal 53: 57-62.

63. Kishimoto M, Okakura T, Nagashima H, Minowa T, Yokoyama SY, et al. (1994) CO_2 fixation and oil production using microalgae. J Ferment Bioeng 78: 479-482.

64. Ho SH, Li PJ, Liu CC, Chang JS (2013) Bioprocess development on microalgae-based CO2 fixation and bioethanol production using Scenedesmusobliquus CNW-N. BioresourTechnol 145: 142-149.

65. deMorais MG, Costa JAV (2007) Isolation and selection of microalgae from coal fired thermoelectric power plant for biofixation of carbon dioxide. Energy Convers Manag 48: 2169-2173.

66. Widjaja A, Chien CC, Ju YH (2009) Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris.* Journal of the Taiwan Institute of Chemical Engineers 40: 13-20.

67. Wu WT, Hsieh CH (2008) Cultivation of microalgae for optimal oil production. Journal of Biotechnology 136: 1521-1521.

68. Weldy CS, Huesemann M (2007) Lipid Production by Dunaliellasalina in Batch Culture: Effects of Nitrogen Limitation and Light Intensity. US Department of Energy Journal of Undergraduate Research 7: 115-122.

69. Chiu SY, Kao CY, Tsai MT, Ong SC, Chen CH, et al. (2009) Lipid accumulation and CO_2 utilization of Nannochloropsisoculata in response to CO_2 aeration. BioresourTechnol 100: 833-838.

70. Okabe K, Murata K, Nakanishi M, Ogi T, Nurunnabi M, et al. (2009) Fischer–Tropsch Synthesis over Ru Catalysts by Using Syngas Derived from Woody Biomass. Catalysis Letters 128: 171-176.

71. Srinivas S, Malik RK, Mahajani Sanjay MFT (2007) Fischer-Tropsch synthesis using bio-syngas and CO_2.Energy for Sustainable Development 11: 66-71.

72. Balat M (2006) Sustainable transportation fuels from biomass materials. Energy, Education, Science and Technology. 17: 83-103.

73. Patil V, Tran KQ, Giselrød HR (2008) Towards sustainable production of biofuels from microalgae. Int J MolSci 9: 1188-1195.

74. Sawayama S, Inoue S, Dote Y, Yokoyama SY (1995) CO_2 fixation and oil production through microalga. Energy Conversion and Management 36: 729-731.

75. Sialve B, Bernet N, Bernard O (2009) Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. BiotechnolAdv 27: 409-416.

76. Borowitzka MA (1998) Company news. J ApplPhycol 10: 417.

77. Hirata T, Tanaka M, Ooike M, Tsunomura T, Sakaguchi M (2000) Antioxidant activities of phycocyanobilin prepared from *Spirulina platensis*. J ApplPhycol 12: 435-439.

78. Francisco EC, Neves DB, Jacob-Lopes E, Franco TT (2010) Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. J ChemTechnolBiotechnol 85: 395-403.

79. Muller-Feuga A, Moal J, Kaas R (2003) The microalgae for aquaculture. In: Stottrup JG, McEvoy LA (eds) Life feeds in marine aquaculture. Blackwell, Oxford.

80. Lorenz RT, Cysewski GR (2000) Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. Trends Biotechnol 18: 160-167.

81. Oswald WJ (2003) My sixty years in applied algology. J ApplPhycol 15: 99-106.

82. Hejazi MA, Wijffels RH (2004) Milking of microalgae. Trends Biotechnol 22: 189-194.

83. Biggs S, Herzog H, Reilly J, Jacoby H (2001) Economic modelling of CO2 capture and sequestration. Proceedings of the Fifth International Conference on Greenhouse Gas Control Technologies.

84. Becker EW (1994) Microalgae. Cambridge: Cambridge University Press.

Microalgae as the Production Platform for Carotenoids

Zheng Sun[1], Jin Liu[2], Yan-hui Bi[1], Zhi-gang Zhou[1*]

[1]College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, People's Republic of China

[2]Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science, Baltimore, MD

***Corresponding author:** Zhi-gang Zhou, Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science, Baltimore, MD 21202, USA, Tel: +86-21-61900424; E-mail: zgzhou@shou.edu.cn

Abstract

Microalgae represent a sustainable source of natural products. Over 15,000 novel compounds have been identified in microalgae. This chapter focuses on algae-derived carotenoids, a group of high-value products. Carotenoids are a group of structurally diverse terpenoid pigments with broad applications in feed, food, nutraceutical and pharmaceutical industries. Production of carotenoids has been one of the most successful activities in microalgal biotechnology achieving great commercial success. This chapter aims to give a mini review of biology and industrial potential of using microalgae as a production platform for carotenoids. The path forward for further expansion of the microalgae carotenoid production with respect to both challenges and opportunities is also discussed.

Introduction

Carotenoids represent a group of structurally diverse terpenoids and are responsible for a broad variety of colors such as brilliant yellow, orange and red of fruits, leaves and aquatic animals [1]. The reason is because carotenoids have a conjugated polyene chain that acts as a chromophore giving rise to distinct colors with different numbers of conjugated double bonds. The demand for carotenoids in pigmentation industry is huge. They have been widely used as feed supplements for aquaculture, such as salmonids and crustacean, because they cannot be synthesized by themselves and the reddish-orange color characteristic is regarded by consumers as one of the key quality attributes. They are also used in the tropical marine ornamental industry and poultry, aiming to pigment ornamental fish or egg yolk. In addition to pigmentation, carotenoids are essential components of human nutrients (e.g. β-carotene acts as the precursors for vitamin A). Besides, they exert significant beneficial effects on human health, contributing to the enhancement of immune system and the attenuation of the risk of several diseases. Effects of carotenoids on human health are, in general, associated with their antioxidant activities [2]. These important properties make carotenoids valuable chemicals with extensive applications in feed, food, nutraceutical, and pharmaceutical industries. Beta-carotene, astaxanthin, lutein, and canthaxanthin are

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

the major carotenoids with commercial interest. The global market value of carotenoids in 2010 was nearly $1.2 billion, and according to report from financial organizations, it is estimated to reach $1.4 billion by 2018 with a compound annual growth rate of 2.3%. To be more specific, the market of β-carotene is expected to grow to $334 million by 2018 with a compound annual growth rate of 3.1%, whilst the market of lutein is expected to grow to $309 million by 2018 with a compound annual growth rate of 3.6 %

(http://www.companiesandmarkets.com/Market/Food-and-Drink/Market-Research/The-Global-Market-for-Carotenoids/RPT988273).

Microalgae represent an outstanding natural source of carotenoids. Some species are able to accumulate high contents of carotenoids as a part of their biomass. A number of algal strains have been intensively studied regarding their carotenoid-producing activities, such as β-carotene from *Dunaliella salina* [3], zeaxanthin from *Synechocystis* sp. [4], lutein from *Chlorella protothecoides* [5], and astaxanthin from *Haematococcus pluvialis* and *Chlorella zofingiensis* [6,7]. Production of carotenoids has been one of the most successful activities in microalgal biotechnology with great commercial success achieved. This article intends to provide a mini review of the recent advances in microalgae-based carotenoid production.

Chemistry of Carotenoids

Carotenoids are a group of tetraterpenes (C_{40}) composed of 8 isoprene (C_5) units joining together to form a common $C_{40}H_{56}$ hydrocarbon skeleton phytoene [8]. The most typical feature of carotenoids is a long polyene chain, which may be terminated by cyclic groups (rings) or complemented with oxygen elements. Elements other than carbon, hydrogen and oxygen are not directly attached to the carbon skeleton in naturally occurring carotenoids [9]. Chemical structures of some typical carotenoids are shown in (Figure 1). The hydrocarbon carotenoids are known as carotenes (e.g. α-carotene, β-carotene and lycopene), whilst the oxygenated derivatives are named xanthophylls. In the latter, oxygen may be present as OH groups (e.g. lutein), as oxo-groups (e.g. canthaxanthin) or in a combination of both (e.g. astaxanthin). The carbon-carbon double bond in the polyene chain of carotenoids may exhibit two configurations, i.e. geometric isomers *cis* or *trans*. It has been found that most carotenoids in nature are *trans* isomers and they are thermodynamically more stable than *cis* isomers [10].

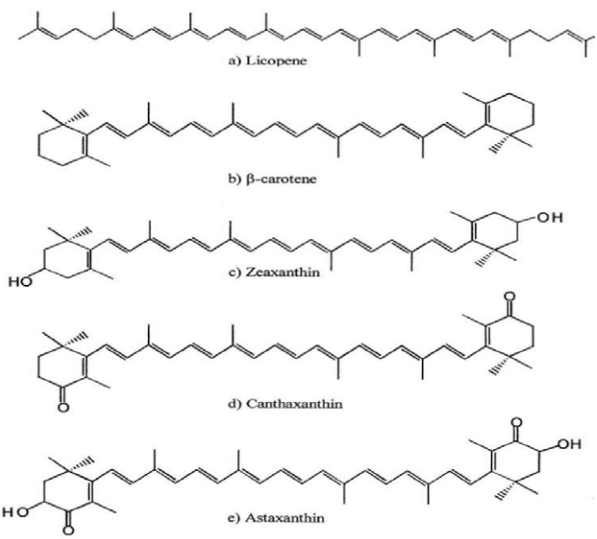

Figure 1: Chemical structures of some typical carotenoids.

So far, over 700 carotenoids have been identified. The biosynthetic pathway is shown in (Figure 2). Phytoene is converted to lycopene through a series of desaturation reactions. All cyclic carotenoids are subsequently derived from lycopene through either one or more processes including hydrogenation, dehydrogenation, cyclization and oxidation. In diatom *Phaeodactylum tricornutum*, a single isomerization of one of the allenic double bonds in neoxanthin yields diadinoxanthin. Meanwhile, fucoxanthin may also be generated from neoxanthin through two reactions: hydroxylation at C8 in combination with a keto-enol tautomerization and acetylation of the 3'-HO group [11]. In general, carotenoids that have important applications in health and nutrition have rather simple structures, such as beta-carotene, lycopene, lutein and astaxanthin [9].

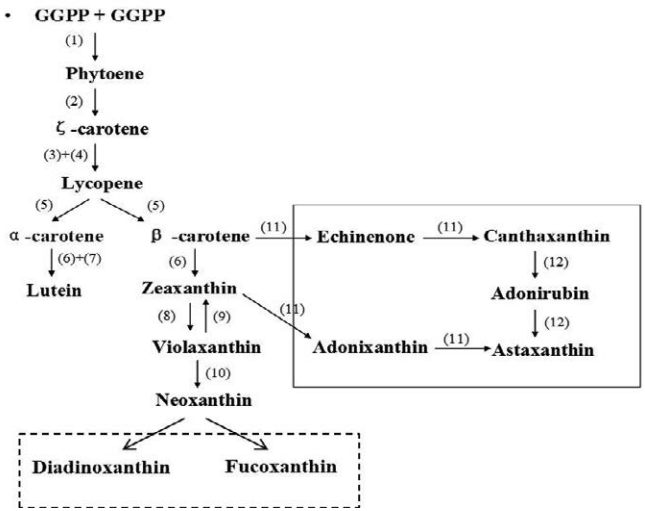

Figure 2: Biosynthetic pathways of carotenoids.

GGPP: Geranylgeranyl diphosphate. Enzymes: (1) phytoene synthase, (2) phytoene desaturase, (3) ζ-carotene desaturase, (4) carotene isomerase, (5) lycopene α- or β-cyclase, (6) β-ring hydroxylase, (7) ε-ring hydroxylase, (8) violaxanthin de-epoxidase, (9) zeaxanthin epoxidase, (10) neoxanthin synthase, (11) β-carotene ketolase, (12) β-carotene 3′3-hydroxylase. Box in solid line: the main pathway for astaxanthin production in typical astaxanthin-producing microalgal species; box in dotted line: the proposed pathway of diadinoxanthin and fucoxanthin in diatom *Phaeodactylum tricornutum*.

Antioxidant Activities

Most carotenoids have potent antioxidant activities. For example, lutein and zeaxanthin are the essential component of the pigment present in *macula lutea* (or yellow spot) in the eye retina and lens. These organs are susceptible to oxidant damage, so lutein and zeaxanthin exert antioxidant action to protect eyes. Another carotenoid, astaxanthin, is described as a 'super vitamin E' because its antioxidant effect is as high as 150 times more than anthocyanin and 1000 times more than vitamin E [12]. Carotenoids can scavenge harmful radicals through 3 ways: electron transfer (CAR + ROO·→CAR·$^+$ + ROO$^-$), hydrogen abstraction (CAR + ROO·→CAR·$^+$ +ROOH) and addition to a radical species (CAR + ROO·→ROOCAR·). Rodrigues et al., (2012) conducted a membrane-mimicking system to evaluate the scavenging activity of carotenoids against various reactive species based on the fluorescence loss of a fluorescent lipid (C11-BODIPY$^{581/591}$). As shown in Table 1, astaxanthin exhibited the strongest activity among all carotenoids. Its overall antioxidant effect was stronger than quercetin, a well-known bioactive flavone [13]. Such potent anti-

oxidative capacity is associated to a great extent with astaxanthin's molecular structure: 1) it has a long chain of thirteen conjugated double bonds to provide greater reducing potential than β-carotene's eleven, and 2) it has a Hydroxyl (OH) and a keto (C=O) moieties on each ionone ring, making the molecule highly polar with enhanced membrane function activity [14].

Carotenoids	Scavenging Capacity [a]			
	ROO•	HOCl	ONOO⁻	HO•
β-carotene	0.14	0.71	NA[b]	1.02
zeaxanthin	0.56	1.41	3.87	0.77
lutein	0.6	0.97	4.81	0.78
lycopene	0.08	0.35	0.4	0.31
fucoxanthin	0.43	1.18	6.26	NA
canthaxanthin	0.04	0.28	0.1	NA
astaxanthin	0.64	1.66	9.4	0.73
α-tocopherol	0.48	1.77	NA	0.37
quercetin	0.84	1.42	5.63	0.97
trolox	1.00	1.00	NA	NA
ascorbic acid	NA	NA	0.41	1.00
cysteine	0.04	NA	1.00	0.02

Table 1: Scavenging capacity of carotenoids [13].

Peroxyl Radical (ROO•), Hydroxyl Radical (HO•), Hypochlorous Acid (HOCl) and Peroxynitrite Anion ($ONOO^-$)

[a] The scavenging capacity was calculated considering as reference: trolox for ROO• and HO•, cysteine for HOCl and ascorbic acid for $ONOO^-$.

[b] NA: No activity was found within the tested concentrations.

Bioactivity and Disease Prevention

The powerful anti-oxidative properties make carotenoids a class of important nutrients in health promotion. There has been ever increasing evidence supporting their protective effects in preventing or delaying chronic disease (Table 2). Taking astaxanthin, the strongest antioxidant agent as an example, it plays important roles in following human health conditions:

Carotenoids	Therapeutic indications	Model
β-carotene	Liver fibrosis [16], Crohn's disease [17], renal damage [18] and CVD [19]	LPS-stimulated RAW264.7 cell, primary macrophages and LPS-administrated mice [20]
Lutein	AMD [21,22], atherosclerosis [23,24], retinal neural damage [25,26] and ultraviolet radiation [27,28]	EIU in Lewis rats [29] CNV in C57BL/6J mice induced by laser photocoagulation and b-End3, RAW264.7 and ARPE-19 cell lines [21]
Fucoxanthin	Inflammation	LPS-stimulated RAW264.7 cells [30]
Astaxanthin	Colitis and colitis-associated colon carcinogenesis [31], cardiac function [32], diabetes [33,34] and EIU [35]	CNV in C57BL/6J mice induced by laser photocoagulation [36] LPS-stimulated RAW264.7 cells and primary macrophages [37]
Violaxanthin	Inflammation	LPS-stimulated mouse RAW264.7 cells [38]

Table 2: Benefits of carotenoids to health [15].

LPS: Lipopolysaccharide; CNV: Choroidal Neovascularization; EIU: Endotoxin-Induced Uveitis; AMD: Age-Related Macular Degeneration.

1) Given the tight association between oxidation processes and DNA damage, the positive roles of astaxanthin in DNA damage control have been highlighted by a number of studies [39,40];
2) In animal studies, the attenuated gastric inflammation and bacterial load were

observed after the administration of an algal extract containing astaxanthin. Meanwhile, such modifications were accompanied by a shift from Th1-response to a mixed Th1/Th2-response [41];

3) Through the interaction with membranes, astaxanthin may help decrease the amount of lipid peroxidation and preserve the structure of membranes [42]. Astaxanthin is also effective in the maintenance of mitochondrial redox state [43];
4) Astaxanthin has shown promising effects on preventing or slowing the progression of cancer. In a cell-based study, it demonstrated to inhibit the proliferation of murine mammary tumor cells in a dose-dependent manner [44]. Similarly, the viability of human colon cancer cells was significantly decreased after the treatment with astaxanthin [45]. Positive effects were also observed in animal studies [46].
5) Benefits of astaxanthin in vascular diseases have long been proposed. In animal studies, the administration of astaxanthin significantly reduced the blood pressure through the suppressing of nitric oxide expression [47]. The pre-treatment with astaxanthin may attenuate the damage of heart attacks and reduce the area of infarction, indicating the cardio-protective effects of astaxanthin [48]. Yoshida et al. (2010) reported that the administration of astaxanthin may improve blood lipid profiles through modifying LDL- and HDL-cholesterol, and lowering triglyceride levels [49].
6) Since astaxanthin is able to cross the blood-brain barrier, it can attenuate the cell damage caused by oxidative stress in the brain and neurological dysfunction, therefore providing neuroprotective effects [47].
7) The UV radiation from sunlight may act as a huge environmental risk factor that can result in eye and skin diseases. Results of *in vitro* studies showed that the treatment with an algal extract containing astaxanthin can effectively protect cell lines from UV-mediated photo-oxidation, suggesting the benefits of astaxanthin for eye and skin health [50].
8) Astaxanthin may help improve the immune system by heightening the production of antibody and increasing the total number of T cells [51].

Recently, our studies have revealed a novel physiological function of astaxanthin: the anti-glycation effect. Glycation, also known as Maillard reaction, is a process in which reducing sugars react spontaneously with amino acids and amino groups of proteins. This reaction is believed to be a key factor contributing to the pathogenesis of diabetic complications [52]. We evaluated over 20 microalgae and found that the extract of *Chlorella* exhibited potent anti-glycation effect. At the concentration of 500 ppm, the ethyl acetate extract significantly inhibited the formation of Advanced Glycation Endproducts (AGEs) in a BSA-glucose system. The inhibitory rate reached up to 80.64%, comparable to the effect of aminoguadine, a well-known synthetic anti-glycation agent [53]. We subsequently identified astaxanthin as a major effective ingredient [54]. In cultured human-derived retinal pigment epithelial ARPE-19 cells, astaxanthin exhibited dual functions against the intracellular glycoxidative process: it blocked the formation of endogenous AGEs through the suppression of intracellular oxidative stress such as nitric oxide, reactive oxygen species and lipid peroxidation; meanwhile, it prevented the deleterious effects induced by exogenous AGEs such as cell proliferation and mRNA up-regulation of Vascular Endothelial Growth Factor (VEGF) and Matrix Metalloproteinase (MMP)-2. Taken together, these results clearly suggested the significant potential of astaxanthin in the management of diabetic retinopathy [55].

Microalgae as the Source of Carotenoids

Compared with higher plants, another main source of naturally occurring carotenoids, algae can be cultivated in bioreactors so that a continuous and reliable source can be guaranteed. Besides, the growth of algae in photobioreactors is independent of season or weather, so that a homogeneous biomass which is ready for processing without the labor-

intensive separation can be obtained [56]. Carotenoids accumulated in microalgae can be classified into primary and secondary ones. The primary carotenoids are essential for survival, acting as the functional components of cellular photosynthetic apparatus. Lutein and β-carotene belong to primary carotenoids. They are located within the thylakoidal membrane to quench free radicals in chlorophylls [57,58]. Different from the primary carotenoids, secondary carotenoids are only accumulated after exposure to specific environmental stimuli via carotenogenesis [59]. If the amount of primary carotenoids is not enough to deal with the excessive energy, secondary carotenoids, e.g. astaxanthin, canthaxanthin and adonixanthin will accumulate out of thylakoid membrane to protect cells against oxidative damage. They are usually found in lipid bodies [58,60]. A couple of ketocarotenoids, such as astaxanthin and canthaxanthin are abundantly present in algae, whereas they rarely accumulate in higher plants. Some species in *Adonis* are the only known higher plants that produce ketocarotenoids [61]. Alloxanthin and diadinoxanthin, which have acetylenic bonds, are also only found in algae [62]. Allene (C=C=C) is a unique structure of natural products, and fucoxanthin is the first reported allenic carotenoid, which was obtained from brown algae [63]. Epoxy carotenoids are also abundant in algae, such as antheraxanthin, violaxanthin and fucoxanthin [15]. Distribution of principle carotenoids in some representative algae is summarized in Table 3.

Carotenoids	Microalgae	Quantity [a] (mg g^{-1})
α-Carotene	*Dunaliella salina*	2.7±0.5 [64]
β-Carotene	*Dunaliella salina*	138.3±10.0 [64]
Lutein	*Chlorella protothecoides*	4.6 [65], 5.4 [5]
	Dunaliella salina	6.6±0.9 [27]
	Scenedesmus almeriensis	5.3 [66]
	Galdieria sulphuraria	0.4±0.1 [67]
Astaxanthin	*Haematococcus pluvialis*	22.7 [68], 40.0 [69] 98.0 [70]
	Chlorella zofingiensis	0.9 [71], 1.0 [72,73], 2.2 [74], 6.8 [68]
	Galdieria sulphuraria	0.6±0.1 [67]
Zeaxanthin	*Dunaliella salina*	11.3±1.6 [64]

Table 3: Distribution of principle carotenoids in various microalgae [15].

[a]Different quantities of carotenoids are obtained from different cultivation methods for the same microalgae.

Beta-carotene from *Dunaliella*

Dunaliella is a genus of unicellular and motile green algae commonly found in marine water. *D. salina* is a type species of this genus well known for its ability of accumulating high levels of β-carotene. Under nutrient-stressed, high light and high salt conditions, *D. salina* is capable of producing β-carotene up to 10% of its dry weight [75], the highest level achieved in nature. Unlike the chemically synthesized β-carotene which has a stereo geometry of all-trans, most β-carotene in *D. salina* (~80%) is composed of two stereoisomers, all-trans and 9-cis in approximately equal amount [76,77]. As a consequence, the algal β-carotene has superiority in bioavailability and physiological properties [2]. The mass culture of *D. salina* for commercial production of β-carotene is indeed one of the most successful examples of halophile biotechnology throughout the whole world. Details are given in section 6. There are many studies attempting to maximize β-carotene production at industrial scale, such as improvement in extraction/isolation techniques [78,79] and optimization of cultivation conditions [80]. A couple of genetic engineering projects have also been conducted. Yan et al., (2005) isolated the complete gene of phytoene synthase (*Psy*) from *D. salina*, which catalyzes the dimerization of two molecules of Geranylgeranyl Pyrophosphate (GGPP) to phytoene. The *Psy* gene of total length of 2982 bp was found to consist of five exons and four introns [81]. The Phytoene desaturase (*Pds*) was also cloned from *D. salina*. The phylogenetic analysis showed that *D. salina Pds* was closer to higher plants and cyanobacteria than bacterial and fungi [82]. Feng et al., (2009) compared three

transformation methods, namely glass beads, bombardment particle and electroporation to screen a high-efficiency transformation way for gene engineering of *D. salina*. Results showed that transformation efficiency of the glass beads was the highest, approximately 10^2 transformants μg^{-1} DNA [83].

Astaxanthin from *Haematococcus*

The genus *Haematococcus* is a group of biflagellate and unicellular green algae. The species *H. pluvialis*, also known as *H. lacustris* or *Sphaerella lacustris*, is the best natural astaxanthin producer: its astaxanthin content is up to 2-3% of the dry biomass [84]. Since double bonds in the long-chain are susceptible to oxidation, astaxanthin in *H. pluvialis* needs to be conjugated with proteins or esterified with fatty acids to stabilize molecules. For example, the esterified astaxanthin (mainly monoester) reaches up to 95% of total astaxanthin [1]. The accumulation of astaxanthin in *H. pluvialis* can be affected by both environmental and nutritional stresses. Light is the best inductive carotenogenic factor, and it varies with its intensity and direction of illumination and Effective Culture Ratio (ECR, volume of culture medium/volume of flask). Tripathi et al., (2002) observed a 6-fold increase in astaxanthin production (37 mg L^{-1}) under the high light intensity (5.1468×10^7 erg m^{-2} s^{-1}) [85]. Kobayashi et al., (1992) found that continuous illumination was beneficial for the formation of astaxanthin and blue light was more effective than red light [86]. In terms of temperature, it was found that when the alga was cultivated at 30°C, the production of astaxanthin was 3-fold more than at 20°C [87]. Moreover, with acetate supplement to 30°C culture, the production was 2-fold more than without addition. Fabregas et al., (1998) observed that N-deficiency had a greater effect than light intensity on astaxanthin synthesis [88].

Natural astaxanthin in *H. pluvialis* is predominantly present in the form of 3S, 3'S, which is very different from synthetic astaxanthin from petrochemical sources that contains a mixture of all enantiomers where only ~25% is 3S, 3'S. Indeed, the 3S, 3'S form of astaxanthin has been reported to give a stronger pigmentation to rainbow trout than other isomers and therefore is a preferred feed additive for fish farming [89]. The isomeric structure is almost the same as the krill, the main food for salmons. Furthermore, 3S, 3'S is believed to be the major form that contributes to human health benefits of astaxanthin, whilst other enantiomers, although may not be harmful, had no significant biological effects [90] (Figure 3). As a major source of natural astaxanthin, *H. pluvialis* has been approved as a color additive in salmon feeds and a dietary-supplement ingredient for human consumption in USA by FDA, Japan as well as several European countries. Numerous studies have been conducted to expand the production capacity and economics of *H. pluvialis* astaxanthin, including the development of advanced extraction techniques [91], genetic engineering for increasing the cellular astaxanthin level [92], and the exploration of next-generation culture systems [6,93].

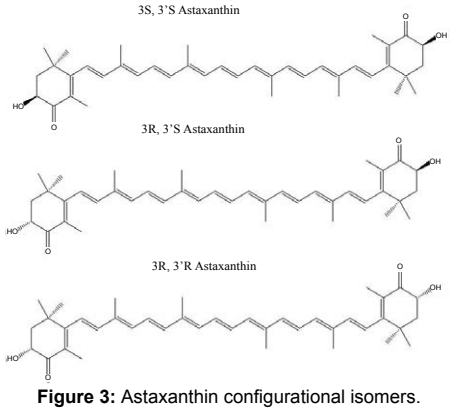

Figure 3: Astaxanthin configurational isomers.

Lutein from *chloreophycean* microalgae

Similar to other members of carotenoids, lutein also contains a series of conjugated double bonds and has strong antioxidant abilities. Lutein is an important nutraceutical ingredient and a daily intake of 6 mg is recommended [95]. It may contribute to the enhancement of immune function as well as the prevention of various human diseases. There are various products of lutein supplement available in the market, containing 6 to 25mg of lutein per capsule. Pure lutein is also available especially for patients at a high risk of Age-related Macular Degeneration (AMD), and it is usually extracted from marigold flowers, the most abundant natural source of commercial lutein. A large amount of lutein present in marigold flowers is esterified with half of the weight corresponding to fatty acids [2], and therefore chemical saponification is necessary in the manufacture of lutein products. In this regard, microalgae are regarded as a promising alternative to the conventional plant sources as the algae-derived lutein is in the free non-esterified form. The most commonly-studied algae include *Dunaliella bardawil* [94], *Chlamydomonas reinhardtii* [95], *Chlorella protothecoides* [5], *Chlorella zofingensis* [96], *Muriellopsis* sp. [2,96], *Scenedesmus almeriensis* [2], *Chlorococcum citriforme* and *Neospongiococcus gelatinosum* [96]. They are potential lutein producers because they are capable of accumulating a much higher lutein content (0.5%-1.2% dry weight) than marigold petals, and their growth is independent of season or weather, producing a homogeneous biomass which is ready for processing without labor-intensive separation required. Moreover, these algae synthesize valuable by-products which can be processed to protein hydrolysates, pigments and valuable lipids depending on the strain, hence increasing the profitability of the manufacturing process [56].

Mass Cultivation for Carotenoid Production

There are two major cultivation modes for microalgae: photoautotrophic and heterotrophic cultures (if a strain is able to grow heterotrophically, a mixotrophic mode may also be developed). The idea of photoautotrophic cultivation was first proposed in Germany in the early 1940s, in which CO_2 and light energy are used as the carbon and energy sources, respectively. This mode of culture has been commonly employed in the culture of *Dunaliella* for β-carotene production. As mentioned earlier, *D. salina* can survive under extreme conditions such as high salinity and high temperature, hence its cultivation is easy to be maintained. Australian researchers proposed the method of 'extensive cultivation', consisting of large open ponds without CO_2 addition and with minimal control [76]. Facilities are usually located in places where solar irradiance is maximal, cloudiness is minimal, and climate is warm [75]. The 'extensive cultivation' approach is easy to operate and economical. To reduce the contamination of plankton, the hypersaline water is used; on the other hand, algae grow slowly under such high-salinity condition, and the concentration of β-carotene is less than 0.1 g m^{-3} [97]. Another approach, 'intensive cultivation' has also been employed for the large-scale production of *Dunaliella* β-carotene. It is a two-step system conducted at high cell densities under carefully controlled conditions. The aim of stage 1 is to optimize biomass production of cells with a low β-carotene-to-chlorophyll ratio; and in stage 2, the culture is diluted to about one third to increase the light availability to cells, and carotenogenesis is further enhanced by nitrogen deficiency [98]. García-González et al., (2003) also reported the intensive cultivation under semi-continuous regime in open tanks. By optimizing culture depth, cell density and dilution cycles, an average annual productivity of 1.65g m^{-2} d^{-1} was obtained for the algal biomass, being that for β-carotene of 0.1g m^{-2} d^{-1} [99].

Although photoautotrophic cultivation is easy to be maintained and has been commonly employed in microalgal industries, it is difficult to achieve a high growth rate due to the hostile or unsteady environment. Besides, in photoautotrophic cultures, the cells are often limited by light because of the mutual shading of cells (especially in denser cultures),

which results in a poor cell yield as well as a low content of algae-derived metabolisms (including carotenoids) [100]. To solve the problems, another culture system, heterotrophic cultivation has been developed. Its most remarkable feature is the utilization of organic carbon substances as the sole carbon and energy source. Since the requirement for light is eliminated, a significant increase of cell density is possible, which may lead to a much higher productivity than the photoautotrophic cultivation [101].

To date, only a small number of microalgal species can survive in complete darkness and, hence, be cultured heterotrophically. For most obligate photoautotrophic microalgae, they may lack the efficient uptake of essential substrates (especially sugars) into cells, or the inside Tricarboxylic Acid (TCA) cycle is incomplete due to the absence of key enzymes such as α-ketoglutarate dehydrogenase [102]. Microalgae are able to utilize a wide range of carbon sources including sugars, hydrolyzed carbohydrates, waste molasses, acetate and glycerol as well as organic carbons from wastewater [103]. Glucose is the most superior carbon source. It has been found that the ATP generated from heterotrophic mode using glucose as the energy supplier exceeds more than 600% over the photoautotrophic mode in which energy is supplied by light [104]. In contrast to light conditions where Embden-Meyerhof-Parnas (EMP) pathway acts as the predominant glycolysis, under darkness, glucose is mainly metabolized via the Pentose Phosphate (PP) pathway [105]. For example, in alga *Chlorella pyrenoidosa*, PP pathway accounts for over 90% of glucose metabolic flux distribution [104]. So far, the maximum biomass content in heterotrophic cultures achieves up to 150 g L^{-1} of cell dry weight, much higher than photoautotrophic cultures (< 40 g L^{-1}) [106]. There are a number of factors that can significantly influence cell growth and synthesis of metabolites in heterotrophic culture. The major medium nutrients include carbon source, nitrogen source and phosphorus. The major environmental factors include temperature, salinity, pH and dissolved oxygen level. The heterotrophic cultivation of microalgae for carotenoid production is shown in Table 4. The high cell density can be achieved by the employment of fed-batch, continuous and perfusion culture strategies that are commonly used in the fermentation of bacteria or yeasts. Fed-batch culture is an effective way to minimize the substrate inhibition as a high initial concentration of substrates (e.g. sugars) may lead to growth inhibition. For instance, the optimal sugar concentration for growing *Chlorella zofingiensis* was reported below 20 g L^{-1} [107]. On the other hand, the fed-batch culture cannot overcome the inhibition caused by the toxic metabolites generated during the algal culture. They may accumulate as the cells build up and prevent further enhancement of cell density. Such metabolite-driven inhibition can be eliminated by continuous or semi-continuous cultivation systems, in which the fresh medium is continuously added to a well-mixed culture whilst cells or products are simultaneously removed to keep a constant volume. Liu et al., (2013) conducted a semi-continuous *Chlorella zofingiensis* culture coupled with feeding with waste molasses at a low concentration [7]. It was found that the diluted raw molasses provided better productivities of biomass and astaxanthin than glucose. However, from the view of cost-effectiveness, more energy input is required to harvest the diluted cells. Besides, cell density as well as substrate utilization efficiency is decreased in continuous cultivation. Perfusion culture is a technique combining the advantages of fed-batch and continuous culture systems. Cells are physically retained by a retention device whereas the spent medium was removed. Meanwhile, fresh medium was fed into the bioreactor to maintain sufficient nutrient supply. Park et al., (2014) established a two-stage mixotrophic culture system for *Haematococcus pluvialis* combining with stepwise increased light irradiance [110]. By perfusion process, the biomass density achieved was 3.09 and 1.67 times higher than batch and fed-batch processes, respectively. Under light irradiance, 12.3g L^{-1} of biomass and 602mg L-1 astaxanthin were obtained. Zhang et al., (2014) established an 'attached cultivation' using the immobilized biofilm. Under the optimized condition, the maximum astaxanthin productivity reached 160mg $m^{-2}d^{-1}$ [111].

Carotenoids	Strain	Device	Culture mode	Cell concentration (g L^{-1})	Productivity (mg $L^{-1}d^{-1}$)	Yield (mg L^{-1})	Ref
Astaxanthin	*Chlorella zofingiensis*	Flask	Batch	10.2	0.7	10.3	[72]
	Chlorella zofingiensis	Flask	Batch	7.5	1.3	11.8	[107]
	Chlorella zofingiensis	Flask	Batch	7.3	1.4	12.6	[108]
	Haematococcus pluvialis	Flask	Batch	NA	0.8	9.0	[109]
Lutein	*Chlorella protothecoides*	Fermentor	Batch	19.6	11.3	83.8	[65]
	Chlorella protothecoides	Fermentor	Fed-batch	46.9	22.7	225.3	[5]

Table 4 : Heterotrophic cultivation of microalgae for carotenoid production [106].

Conclusion and Future Perspectives

Microalgae represent a promising source of natural carotenoids that can potentially replace the chemically synthesized ones. To date, microalgae-based carotenoid production has achieved some commercial success. But there are still substantial challenges to be addressed, which restrict the production capacity and economics of microalgal carotenoids. Future exploration of using microalgae for carotenoid production may lie in better understanding of carotenoid biosynthesis and regulation, genetic engineering for enhanced accumulation of carotenoids, development of next generation culture systems for sustainable and cost-effective production of carotenoid-rich biomass, and exploration of state-of-art biorefinery-based integrated production strategies. Advances occurring in these areas will greatly expand the production capacity and lower the production cost, allowing microalgae *to* be a competitive source of natural carotenoids.

To this end, there are more than ten microalgae strains with complete genome sequences available, and 'omics' analyses (genomics, transcriptomics, and proteomics) of several production strains such as *H. pluvialis* and *C. zofingiensis* are underway, which will benefit the understanding of carotenoid biosynthesis and development of a sophisticated molecular toolbox for more efficient manipulation of the production strain to meet the ever increasing need in natural carotenoids.

Acknowledgements

Authors acknowledge financial support from the Shanghai Pujiang Program (Grant No. 13PJ1403500), the Shanghai Early Career Faculty Program (Grant No. ZZHY13005) and the Doctoral Fund of Ministry of Education of China (Grant No. 20133104120004).

References

1. Lorenz RT, Cysewski GR (2000) Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends Biotechnol 18: 160-167.

2. Del Campo JA, García-González M, Guerrero MG (2007) Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. Appl Microbiol Biotechnol 74: 1163-1174.

3. Tafreshi A, Shariati M (2006) Pilot culture of three strains of *Dunaliella salina* for ß-carotene production in open ponds in the central region of Iran. World J Microb Biot 22: 1003-1006.

4. Lagarde D, Beuf L, Vermaas W (2000) Increased production of zeaxanthin and other pigments by application of genetic engineering techniques to *Synechocystis sp.* strain PCC 6803. Appl Environ Microbiol 66: 64-72.

5. Shi XM, Jiang Y, Chen F (2002) High-yield production of lutein by the green microalga *Chlorella protothecoides* in heterotrophic fed-batch culture. Biotechnol Prog 18: 723-727.

6. Zhang BY, Geng YH, Li ZK, Hu HJ, Li YG (2009) Production of astaxanthin from *Haematococcus* in open pond by two-stage growth one-step process. Aquaculture 295: 275-281.

7. Liu J, Sun Z, Zhong Y, Gerken H, Huang J, et al. (2013) Utilization of cane molasses towards cost-saving astaxanthin production by a *Chlorella zofingiensis* mutant. J Appl Phycol 25: 1447-1456.

8. Croteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites). In: BB Buchanan, W Gruissem, RL Jones (eds.). Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, MD, USA. pp: 1250-1317.

9. Liaaen-Jensen S (2004) Basic carotenoid chemistry. In: ST Mayne, NI Krinsky, H Sies (eds.). Carotenoids in health and disease. Marcel Dekker Press, New York, USA. pp: 1-30.

10. Britton G (1995) Structure and properties of carotenoids in relation to function. FASEB J 9: 1551-1558.

11. Dambek M, Eilers U, Breitenbach J, Steiger S, Büchel C, et al. (2012) Biosynthesis of fucoxanthin and diadinoxanthin and function of initial pathway genes in *Phaeodactylum tricornutum*. J Exp Bot 63: 5607-5612.

12. Miki W (1991) Biological functions and activities of animal carotenoids. Pure Appl Chem 63: 141-146.

13. Rodrigues E, Mariutti LR, Mercadante AZ (2012) Scavenging capacity of marine carotenoids against reactive oxygen and nitrogen species in a membrane-mimicking system. Mar Drugs 10: 1784-1798.

14. Hussein G, Sankawa U, Goto H, Matsumoto K, Watanabe H (2006) Astaxanthin, a carotenoid with potential in human health and nutrition. J Nat Prod 69: 443-449.

15. Zhang J, Sun Z, Sun P, Chen T, Chen F (2014) Microalgal carotenoids: beneficial effects and potential in human health. Food Funct 5: 413-425.

16. Seifert WF, Bosma A, Hendriks HF, van Leeuwen RE, van Thiel-de Ruiter GC, et al. (1995) Beta-carotene (provitamin A) decreases the severity of CCl4-induced hepatic inflammation and fibrosis in rats. Liver 15: 1-8.

17. Lavy A, Naveh Y, Coleman R, Mokady S, Werman MJ (2003) Dietary *Dunaliella bardawil*, a beta-carotene-rich alga, protects against acetic acid-induced small bowel inflammation in rats. Inflamm Bowel Dis 9: 372-379.

18. Kaliappan G, Nagarajan P, Moorthy R, Kalai Gana Selvi S, Avinash Raj T, et al. (2013) Ang II induce kidney damage by recruiting inflammatory cells and up regulates PPAR gamma and Renin 1 gene: effect of β carotene on chronic renal damage. J Thromb Thrombolysis 36: 277-285.

19. Helmersson J, Arnlöv J, Larsson A, Basu S (2009) Low dietary intake of beta-carotene, alpha-tocopherol and ascorbic acid is associated with increased inflammatory and oxidative stress status in a Swedish cohort. Br J Nutr 101: 1775-1782.

20. Bai SK, Lee SJ, Na HJ, Ha KS, Han JA, et al. (2005) beta-Carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated macrophages by suppressing redox-based NF-κB activation. Exp Mol Med 37: 323-334.

21. Izumi-Nagai K, Nagai N, Ohgami K, Satofuka S, Ozawa Y, et al. (2007) Macular pigment lutein is antiinflammatory in preventing choroidal neovascularization. Arterioscler Thromb Vasc Biol 27: 2555-2562.

22. Bian Q, Gao S, Zhou J, Qin J, Taylor A, et al. (2012) Lutein and zeaxanthin supplementation reduces photooxidative damage and modulates the expression of inflammation-related genes in retinal pigment epithelial cells. Free Radic Biol Med 53: 1298-1307.

23. Xu XR, Zou ZY, Xiao X, Huang YM, Wang X, et al. (2013) Effects of lutein supplement on serum inflammatory cytokines, ApoE and lipid profiles in early atherosclerosis population. J Atheroscler Thromb 20: 170-177.

24. Kim JE, Leite JO, DeOgburn R, Smyth JA, Clark RM, et al. (2011) A lutein-enriched diet prevents cholesterol accumulation and decreases oxidized LDL and inflammatory cytokines in the aorta of guinea pigs. J Nutr 141: 1458-1463.

25. Sasaki M, Ozawa Y, Kurihara T, Noda K, Imamura Y, et al. (2009) Neuroprotective effect of an antioxidant, lutein, during retinal inflammation. Invest Ophthalmol Vis Sci 50: 1433-1439.

26. Li SY, Fung FK, Fu ZJ, Wong D, Chan HH, et al. (2012) Anti-inflammatory effects of lutein in retinal ischemic/hypoxic injury: in vivo and in vitro studies. Invest Ophthalmol Vis Sci 53: 5976-5984.

27. González S, Astner S, An W, Goukassian D, Pathak MA (2003) Dietary lutein/zeaxanthin decreases ultraviolet B-induced epidermal hyperproliferation and acute inflammation in hairless mice. J Invest Dermatol 121: 399-405.

28. Lee EH, Faulhaber D, Hanson KM, Ding W, Peters S, et al. (2004) Dietary lutein reduces ultraviolet radiation-induced inflammation and immunosuppression. J Invest Dermatol 122: 510-517.

29. Jin XH, Ohgami K, Shiratori K, Suzuki Y, Hirano T, et al. (2006) Inhibitory effects of lutein on endotoxin-induced uveitis in Lewis rats. Invest Ophthalmol Vis Sci 47: 2562-2568.

30. Kim KN, Heo SJ, Yoon WJ, Kang SM, Ahn G, et al. (2010) Fucoxanthin inhibits the inflammatory response by suppressing the activation of NF-κB and MAPKs in lipopolysaccharide-induced RAW 264.7 macrophages. Eur J Pharmacol 649: 369-375.

31. Yasui Y, Hosokawa M, Mikami N, Miyashita K, Tanaka T (2011) Dietary astaxanthin inhibits colitis and colitis-associated colon carcinogenesis in mice via modulation of the inflammatory cytokines. Chem Biol Interact 193: 79-87.

32. Nakao R, Nelson OL, Park JS, Mathison BD, Thompson PA, et al. (2010) Effect of astaxanthin supplementation on inflammation and cardiac function in BALB/c mice. Anticancer Res 30: 2721-2725.

33. Chan KC, Pen PJ, Yin MC (2012) Anticoagulatory and antiinflammatory effects of astaxanthin in diabetic rats. J Food Sci 77: H76-80.

34. Kim YJ, Kim YA, Yokozawa T (2009) Protection against oxidative stress, inflammation, and apoptosis of high-glucose-exposed proximal tubular epithelial cells by astaxanthin. J Agric Food Chem 57: 8793-8797.

35. Ohgami K, Shiratori K, Kotake S, Nishida T, Mizuki N, et al. (2003) Effects of astaxanthin on lipopolysaccharide-induced inflammation in vitro and in vivo. Invest Ophthalmol Vis Sci 44: 2694-2701.

36. Izumi-Nagai K, Nagai N, Ohgami K, Satofuka S, Ozawa Y, et al. (2008) Inhibition of choroidal neovascularization with an anti-inflammatory carotenoid astaxanthin. Invest Ophthalmol Vis Sci 49: 1679-1685.

37. Lee SJ, Bai SK, Lee KS, Namkoong S, Na HJ, et al. (2003) Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I(kappa)B kinase-dependent NF-kappaB activation. Mol Cells 16: 97-105.

38. Soontornchaiboon W, Joo SS, Kim SM (2012) Anti-inflammatory effects of violaxanthin isolated from microalga *Chlorella ellipsoidea* in RAW 264.7 macrophages. Biol Pharm Bull 35: 1137-1144.

39. Santocono M, Zurria M, Berettini M, Fedeli D, Falcioni G (2007) Lutein, zeaxanthin and astaxanthin protect against DNA damage in SK-N-SH human neuroblastoma cells induced by reactive nitrogen species. J Photochem Photobiol B 88: 1-10.

40. Tripathi DN, Jena GB (2009) Intervention of astaxanthin against cyclophosphamide-induced oxidative stress and DNA damage: a study in mice. Chem Biol Interact 180: 398-406.

41. Bennedsen M, Wang X, Willén R, Wadström T, Andersen LP (2000) Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol Lett 70: 185-189.

42. McNulty HP, Byun J, Lockwood SF, Jacob RF, Mason RP (2007) Differential effects of carotenoids on lipid peroxidation due to membrane interactions: X-ray diffraction analysis. Biochim Biophys Acta 1768: 167-174.

43. Wolf AM, Asoh S, Hiranuma H, Ohsawa I, Iio K, et al. (2010) Astaxanthin protects mitochondrial redox state and functional integrity against oxidative stress. J Nutr Biochem 21: 381-389.

44. Kim H, Park J, Chew B (2001) Beta-carotene and astaxanthin inhibit mammary tumor cell growth and induce apoptosis in mice in vivo. J FASEB 15: 298.

45. Onogi N, Okuno M, Matsushima-Nishiwaki R, Fukutomi Y, Moriwaki H, et al. (1998) Antiproliferative effect of carotenoids on human colon cancer cells without conversion to retinoic acid. Nutr Cancer 32: 20-24.

46. Lockwood SF, Penn MS, Hazen SL, Bikádi Z, Zsila F (2006) The effects of oral Cardax (disodium disuccinate astaxanthin) on multiple independent oxidative stress markers in a mouse peritoneal inflammation model: influence on 5-lipoxygenase in vitro and in vivo. Life Sci 79: 162-174.

47. Hussein G, Nakamura M, Zhao Q, Iguchi T, Goto H, et al. (2005) Antihypertensive and neuroprotective effects of astaxanthin in experimental animals. Biol Pharm Bull 28: 47-52.

48. Gross GJ, Lockwood SF (2004) Cardioprotection and myocardial salvage by a disodium disuccinate astaxanthin derivative (Cardax). Life Sci 75: 215-224.

49. Yoshida H, Yanai H, Ito K, Tomono Y, Koikeda T, et al. (2010) Administration of natural astaxanthin increases serum HDL-cholesterol and adiponectin in subjects with mild hyperlipidemia. Atherosclerosis 209: 520-523.

50. Lyons NM, O'Brien NM (2002) Modulatory effects of an algal extract containing astaxanthin on UVA-irradiated cells in culture. J Dermatol Sci 30: 73-84.

51. Chew BP, Park JS (2004) Carotenoid action on the immune response. J Nutr 134: 257-261.

52. Ahmed N (2005) Advanced Glycation Endproducts--role in pathology of diabetic complications. Diabetes Res Clin Pract 67: 3-21.

53. Sun Z, Peng XF, Liu J, Fan KW, et al. (2010) Inhibitory effects of microalgal extracts on the formation of Advanced Glycation Endproducts (AGEs). Food Chem 120: 261-267.

54. Sun Z, Liu J, Zeng XH, Huangfu JQ, et al. (2011) Astaxanthin is responsible for anti-glycoxidative properties of microalga *Chlorella zofingiensis*. Food Chem 126: 1629-1635.

55. Sun Z, Liu J, Zeng X, Huangfu J, Jiang Y, et al. (2011) Protective actions of microalgae against endogenous and exogenous Advanced Glycation Endproducts (AGEs) in human retinal pigment epithelial cells. Food Funct 2: 251-258.

56. Fernández-Sevilla JM, Acién Fernández FG, Molina Grima E (2010) Biotechnological production of lutein and its applications. Appl Microbiol Biotechnol 86: 27-40.

57. Siefermann-Harms D (1987) The light-harvesting protective functions of carotenoids in photosynthetic membranes. Physiol Plantarum 69: 561-568.

58. Rise M, Cohen E, Vishkautsan M, Cojocaru M, Gittlieb HE, et al. (1994) Accumulation of secondary carotenoids in *Chlorella zofingiensis*. J Plant Physiol 144: 287-292.

59. Guedes AC, Amaro HM, Malcata FX (2011) Microalgae as sources of carotenoids. Mar Drugs 9: 625-644.

60. Bar E, Rise M, Vishkautsan M, Arad S (1995) Pigments and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. J Plant Physiol 146: 527-534.

61. Cunningham FX Jr, Gantt E (2011) Elucidation of the pathway to astaxanthin in the flowers of Adonis aestivalis. Plant Cell 23: 3055-3069.

62. Takaichi S (2011) Carotenoids in algae: distributions, biosyntheses and functions. Mar Drugs 9: 1101-1118.

63. Willstätter R, Page HJ (1914) Chlorophyll. XXIV. The pigments of the brown algae. Eur J Org Chem 404: 237-271.

64. Hu CC, Lin JT, Lu FJ, Chou FP, Yang DJ (2008) Determination of carotenoids in *Dunaliella salina* cultivated in Taiwan and antioxidant capacity of the algal carotenoid extract. Food Chem 109: 439-446.

65. Shi X, Zhang X, Chen F (2000) Heterotrophic production of biomass and lutein by Chlorella protothecoides on various nitrogen sources. Enzyme Microb Technol 27: 312-318.

66. S´anchez JF, Fern´andez JM, Aci´en FG, Rueda A, P´erez-Parra J, et al. (2008) Influence of culture conditions on the productivity and lutein content of the new strain *Scenedesmus almeriensis*. Process Biochem 43: 398-405.

67. Graziani G, Schiavo S, Nicolai MA, Buono S, Fogliano V, et al. (2013) Microalgae as human food: chemical and nutritional characteristics of the thermo-acidophilic microalga *Galdieria sulphuraria*. Food Funct 4: 144-152.

68. Orosa M, Valero JF, Herrero C, Abalde J (2001) Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other green microalgae under N-starvation and high light conditions. Biotechnol Lett 23: 1079-1085.

69. Boussiba S, Bing W, Yuan JP, Zarka A, Chen F (1999) Changes in pigments profile in the green alga *Haeamtococcus pluvialis* exposed to environmental stresses. Biotechnol Lett 21: 601-604.

70. Domínguez-Bocanegra AR, Guerrero Legarreta I, Martinez Jeronimo F, Tomasini Campocosio A (2004) Influence of environmental and nutritional factors in the production of astaxanthin from *Haematococcus pluvialis*. Bioresour Technol 92: 209-214.

71. Chen T, Wang Y (2013) Optimized astaxanthin production in *Chlorella zofingiensis* under dark condition by response surface methodology. Food Sci Biotechnol 22: 1343-1350.

72. Ip PF, Chen F (2005) Production of astaxanthin by the green microalga *Chlorella zofingiensis* in the dark. Process Biochem 40: 733-738.

73. Sun N, Wang Y, Li YT, Huang JC, Chen F (2008) Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). Process Biochem 43: 1288-1292.

74. Wang Y, Liu Z, Qin S (2013) Effects of iron on fatty acid and astaxanthin accumulation in mixotrophic *Chromochloris zofingiensis*. Biotechnol Lett 35: 351-357.

75. Ben-Amotz (1993) Production of β-carotene and vitamins by the halotolerant alga *Dunaliella*. In: Attaway DH, Zaborsky OR (eds.). Marine biotechnology volume 1: pharmaceutical and bioactive natural products. Plenum Press, New York, pp: 411-417.

76. Borowitzka M, Borowitzka L (1988) *Dunaliella*. In: M Borowitzka, L Borowitzka (eds.). Micro-algal Biotechnology. Cambridge University Press, Cambridge, pp: 27-58.

77. Ben-Amotz A, Avron M (1989) The wavelength dependence of massive carotene synthesis in *Dunaliella bardawil* (Chlorophyceae). J Phycol 25: 175-178.

78. Macías-Sánchez MD, Mantell C, Rodríguez M, Martínez de la Ossa E, Lubián LM, et al. (2009) Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from *Dunaliella salina*. Talanta 77: 948-952.

79. Macías-Sánchez MD, Mantell Serrano C, Rodríguez M, Martínez de la Ossa E, Lubián LM, et al. (2008) Extraction of carotenoids and chlorophyll from microalgae with supercritical carbon dioxide and ethanol as cosolvent. J Sep Sci 31: 1352-1362.

80. Kleinegris DM, Janssen M, Brandenburg WA, Wijffels RH (2011) Continuous production of carotenoids from *Dunaliella salina*. Enzyme Microb Technol 48: 253-259.

81. Yan Y, Zhu YH, Jiang JG, Song DL (2005) Cloning and sequence analysis of the phytoene synthase gene from a unicellular chlorophyte, *Dunaliella salina*. J Agric Food Chem 53: 1466-1469.

82. Zhu YH, Jiang JG, Yan Y, Chen XW (2005) Isolation and characterization of phytoene desaturase cDNA involved in the beta-carotene biosynthetic pathway in *Dunaliella salina*. J Agric Food Chem 53: 5593-5597.

83. Feng S, Xue L, Liu H, Lu P (2009) Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. Mol Biol Rep 36: 1433-1439.

84. Rao AR, Sarada R, Ravishankar A (2007) Stabilization of astaxanthin in edible oils and its use as an antioxidant. J Sci Food Agr 87: 957-965.

85. Tripathi U, Sarada R, Ravishankar GA (2002) Effects of culture conditions on growth of green alga - *Haematococcus pluvialis* and astaxanthin production. Acta Physiol Plant 24: 323-329.

86. Kobayashi M, Kakizono T, Nishio N, Nagai S (1992) Effects of light intensity, light quality and illumination cycle on astaxanthin formation in a green alga, *Haematococcus pluvialis*. J Ferment Bioeng 74: 61-63.

87. Tjahjono AE, Hayama Y, Kakizono T, Terada Y, Nishio N, et al. (1994) Hyperaccumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. Biotechnol Lett 16: 133-138.

88. Fábregas J, Domínguez A, Álvarez DG, Lamela T, Otero A (1998) Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in *Haematococcus pluvialis*. Biotechnol Lett 20: 623-626.

89. Osterlie M, Bjerkeng B, Liaaen-Jensen S (1999) Accumulation of astaxanthin all-E, 9Z and 13Z geometrical isomers and 3 and 3' RS optical isomers in rainbow trout (*Oncorhynchus mykiss*) is selective. J Nutr 129: 391-398.

90. Guerin M, Huntley ME, Olaizola M (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. Trends Biotechnol 21: 210-216.

91. Plaza M, Herrero M, Cifuentes A, Ibáñez E (2009) Innovative natural functional ingredients from microalgae. J Agric Food Chem 57: 7159-7170.

92. Steinbrenner J, Sandmann G (2006) Transformation of the green alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. Appl Environ Microbiol 72: 7477-7484.

93. Fábregas J, Otero A, Maseda A, Domínguez A (2001) Two-stage cultures for the production of astaxanthin from *Haematococcus pluvialis*. J Biotechnol 89: 65-71.

94. Salguero A, León R, Mariotti A, de la Morena B, Vega JM, et al. (2005) UV-A mediated induction of carotenoid accumulation in *Dunaliella bardawil* with retention of cell viability. Appl Microbiol Biotechnol 66: 506-511.

95. Francis GW, Strand LP, Lien T, Knutsen G (1975) Variations in the carotenoid content of *Chlamydomonas reinhardii* throughout the cell cycle. Arch Microbiol 104: 249-254.

96. Del Campo JA, Moreno J, Rodríguez H, Vargas MA, Rivas J, et al. (2000) Carotenoid content of chlorophycean microalgae: factors determining lutein accumulation in *Muriellopsis sp. (Chlorophyta)*. J Biotechnol 76: 51-59.

97. Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechnol 70: 313-321.

98. Ben-Amotz A (1995) New mode of *Dunaliella* biotechnology: two-phase growth for β-carotene production. J Appl Phycol 7: 65-68.

99. García-González M, Moreno J, Cañavate JP, Anguis V, Prieto A, et al. (2003) Conditions for open-air outdoor culture of *Dunaliella salina* in Southern Spain. J Appl Phycol 15:177-184.

100. Chen GQ, Chen F (2006) Growing phototrophic cells without light. Biotechnol Lett 28: 607-616.

101. Chen F (1996) High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol 14: 421-426.

102. Benedict CR (1978) Nature of obligate photoautotrophy. Annu Rev Plant Physiol 29: 67-93.

103. Liu J, Sun Z, Chen F (2013) Heterotrophic production of microalgal oils. In: A Pandey, DJ Lee, Y Chisti, CR Soccol (eds.). Biofuels from algae. Elsevier, Amsterdam, Netherlands, pp: 111-142.

104. Yang C, Hua Q, Shimizu K (2000) Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochem Eng J 6: 87-102.

105. Perez-Garcia O, Escalante FM, de-Bashan LE, Bashan Y (2011) Heterotrophic cultures of microalgae: metabolism and potential products. Water Res 45: 11-36.

106. De Swaaf ME, Sijtsma L, Pronk JT (2003) High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*. Biotechnol Bioeng 81: 666-672.

107. Ip PF, Chen F (2005) Peroxynitrite and nitryl chloride enhance astaxanthin production by the green microalga *Chlorella zofingiensis* in heterotrophic culture. Proc Biochem 40: 3595-3599.

108. Ip PF, Chen F (2005) Employment of reactive oxygen species to enhance astaxanthin formation in *Chlorella zofingiensis* in heterotrophic culture. Proc Biochem 40: 3491-3496.

109. Kobayashi M, Kurimura Y, Tsuji Y (1997) Light-independent, astaxanthin production by the green microalga *Haematococcus pluvialis* under salt stress. Biotechnol Lett 19: 507-509.

110. Park JC, Choi SP, Hong ME, Sim SJ (2014) Enhanced astaxanthin production from microalga, *Haematococcus pluvialis* by two-stage perfusion culture with stepwise light irradiation. Bioprocess Biosyst Eng DOI: 10.1007/s00449-014-1180-y.

111. Zhang W, Wang J, Wang J, Liu T (2014) Attached cultivation of *Haematococcus pluvialis* for astaxanthin production. Bioresour Technol 158: 329-335.

Omega-3 Polyunsaturated Fatty Acids from Algae

Mala Khan[1], Md. Moshiur Rahman[1], S Zaman[2], Turjo Arif Jahangir[2], M H Razu[1]

[1] Designated Reference Institute for Chemical Measurements, BCSIR, Qudrat-i-Khuda Road, Dhaka-1205

[2] Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205

***Corresponding author:** Mamudul Hasan Razu, Designated Reference Institute for Chemical Measurements (DRiCM), Bangladesh Council of Scientific and Industrial Research (BCSIR), Qudrat-i-Khuda Road, Dhaka-1205, Bangladesh; Tel: +88-0172-1218185; E-mail: razu_ss86@yahoo.com

Abstract

Currently, public awareness of healthcare importance increasing. Polyunsaturated fatty acid is an essential nutrition for human, such docosahexaenoic acid and eicosapentaenoic acid derivatives of omega-3. The need of omega-3 polyunsaturated fatty acid generally derived from fish oil, but fish oil has a high risk in chemical contamination. Algae are single cell microorganism, which have relatively high content of omega-3. Commercial production of omega-3 polyunsaturated fatty acid is very promising for both foods and feeds, because the availability of abundant raw materials and suitable to develop in the tropics. This chapter aims to give a mini review of biology and human health benefits of using algae as a production platform for omega-3. In addition, this literature also reviews about the stimulation of omega-3 production and biotechnological approach in algae with respect to both challenges and opportunities.

Introduction

Omega-3 is unsaturated essential fatty acids which cannot be synthesized by human due to lack of desaturase enzyme acts to insert double bonds at ω3 position [1]. There are three types of omega-3 fatty acids involved in human physiology are α-linolenic acid (ALA), Eicosapentaenoic Acid (EPA), And Docosahexaenoic Acid (DHA).ALA Is The Shortest Chain Of Omega-3 Polyunsaturated Fatty Acids and mainly found in vegetables oil and nuts; other two important derivatives EPA and DHA found in fish and many other microorganisms, such as microalgae and bacteria [2-5]. Even, ALA can be converted into EPA and DHA in the body, but the conversion is very limited and inefficient [3], therefore omega-3 must be provided in the form of dietary supplement.These long chains Polyunsaturated Fatty Acid (PUFA) in algae have profound benefits and functions in dietetics and therapeutic uses [6-8]. They are believed to have positive effects for the treatment of hypertension, premenstrual tension, hypertonia, cancer, hyperlipidemia and number of other cases [7-11]. In addition, ALA is the precursors of prostaglandins (E2 and F2) which possess potent vasodilators, antiflammatory and antiaggrgatory properties as well as may be useful to correct defects occur in metabolism of essential fatty acid and imbalance of EPA [12]. EPA plays an important role in mammals as an agent to prevent blood platelet aggregation [13-

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

15] whereas DHA is significant for the reception and transmission of impulses between brain cells [16]. Furthermore, dietary omega-3 (T3) PUFAs especially EPAand DHA are plays a major role in modulating the biosynthesis of eicosanoids and in controlling the levels blood lipids and lipoproteins [17]. Currently, some government agencies and nutritional organizations recommended daily intake level of DHA and EPA ranging from 0.2-0.3 g/day for the general population [18-19] up to 1.0- 4.0 g/day for patient with coronary heart disease [18].These important properties make omega-3 (T3) PUFAs valuable chemicals with extensive applications in dietary supplements, pet nutrition, beveragesand pharmaceutical industries. Worldwidemarket analysis in consumption of PUFAs estimated at 123.8 thousand metric tons worth US$2.3 billion in 2013, is forecast to be 134.7 thousand metric tons valued at US$2.5 billion in 2014. By 2020, it is projected that demand for Omega-3 PUFAs globally would reach 241 thousand metric tons with a value of US$4.96 billion, thereby posting a volume Compound Annual Growth Rate (CAGR) of almost 10% and a value CAGR of 11.6% between 2013 and 2020 [20].

It is known that global fish stocks are in danger, so, fish production may decrease in the future. In addition to this, some fishes, especially marine fishes like salmon, sardine, tuna, anchovy, mackerel or hake, are sometimes contaminated with heavy metals as copper or mercury, and organic pollutants as PCBs or dioxins, which have a toxic effect for human health [21]. For this reason, currently algaecame forwardas the major alternative source for production of omega-3 PUFAs. There are various strains of microalgae with a wide range of components, such as proteins, carbohydrates, fats, and nucleic acids, though in varying proportions. Typically, fatty acids make up to 40 percent of microalgae mass, and some of the fatty acids are unsaturated. The proportion of PUFA differs greatly depending on the species. For example, *Crypthecodinium cohnii* microalgae species possesses only DHA and no other PUFAs; *Chaetoceros* sp., *Skeletonema costatum* and *Nannochloropsis* sp. contain predominantly EPA, whereas *Porphyridium cruentum* contains predominantly ARA.

In addition, omega-3 biosynthesis can be stimulated by sudden change of growth condition and metabolic engineering. There are several methods for extraction, purification and quantification is existing for omega-3 productions from algae through lipid extraction. Therefore, on the basis of commercial importance of omega-3 PUFAs production from algae, microalgal biotechnology have emerged with current developments in genomics, bioinformatics analyses, genetic and metabolic engineering. This article intends to provide a mini review of the recent advances in algae-based omega-3 production.

Chemistry of Omega-3

Fatty acids are hydrocarbon-chains with a methyl group in one end of the molecule (termed omega = ω or n) and an acid group (often called carboxylic acid) in the other end. There are two major nomenclatures of fatty acids. In the most commonly used nomenclature the carbon atom next to the carboxyl group is called the alpha-carbon (α-carbon) with the consecutive carbons named β-carbon etc. The letter n is often used instead of the Greek ω to describe the methyl end. Furthermore, it is quite common to use the systematic nomenclature for fatty acids where the locations of double bonds are indicated with reference to the carboxyl group. In PUFAs the first double bond may be between the 3rd and the 4th carbon atom from the ω carbon, and these are called omega-3 fatty acids. Some physical and chemical property of omega-3 has been given below [22]:

sMolecular Weight	909.36888 g/mol
Molecular Formula	$C_{60}H_{92}O_6$
Hydrogen Bond Donor Count	3

Hydrogen Bond Acceptor Count	6
Rotatable Bond Count	40
Exact Mass	908.689391 g/mol
Monoisotopic Mass	08.689391 g/mol
Topological Polar Surface Area	112 A^2
Heavy Atom Count	66

Table 1: Chemical and physical properties of Omega-3.

There are three most important Omega-3 fatty acids are α-linolenic acid (18:3, *n*-3; ALA), eicosapentaenoic acid (20:5, *n*-3; EPA), and docosahexaenoic acid (22:6, *n*-3; DHA) (Figure 1) [23].These three polyunsaturates have either 3, 5, or 6 double bonds in a carbon chain of 18, 20, or 22 carbon atoms, respectively. As with most naturally-produced fatty acids, all double bonds are in the *cis*-configuration, in other words, the two hydrogen atoms are on the same side of the double bond; and the double bonds are interrupted by methylene bridges (-CH 2-), so that there are two single bonds between each pair of adjacent double bonds.

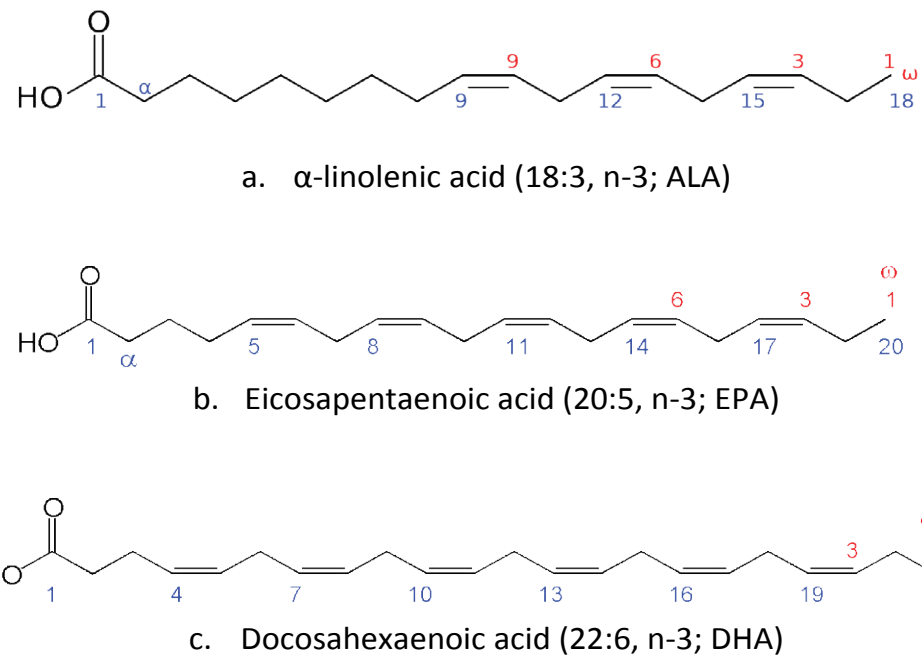

Figure 1: Chemical structures of ALA, EPA and DHA.

The biosynthesis of omega-3 fatty acids begins with de novo synthesis of short chain fattyacids, typically oleic acid, from acetate [23]. Most organisms are capable of de novo fatty acid synthesis. Next, the oleic acid is subjected to a series of desaturation and elongation reactions to form longer chain fatty acids as can be seen in Figure 2 [23].

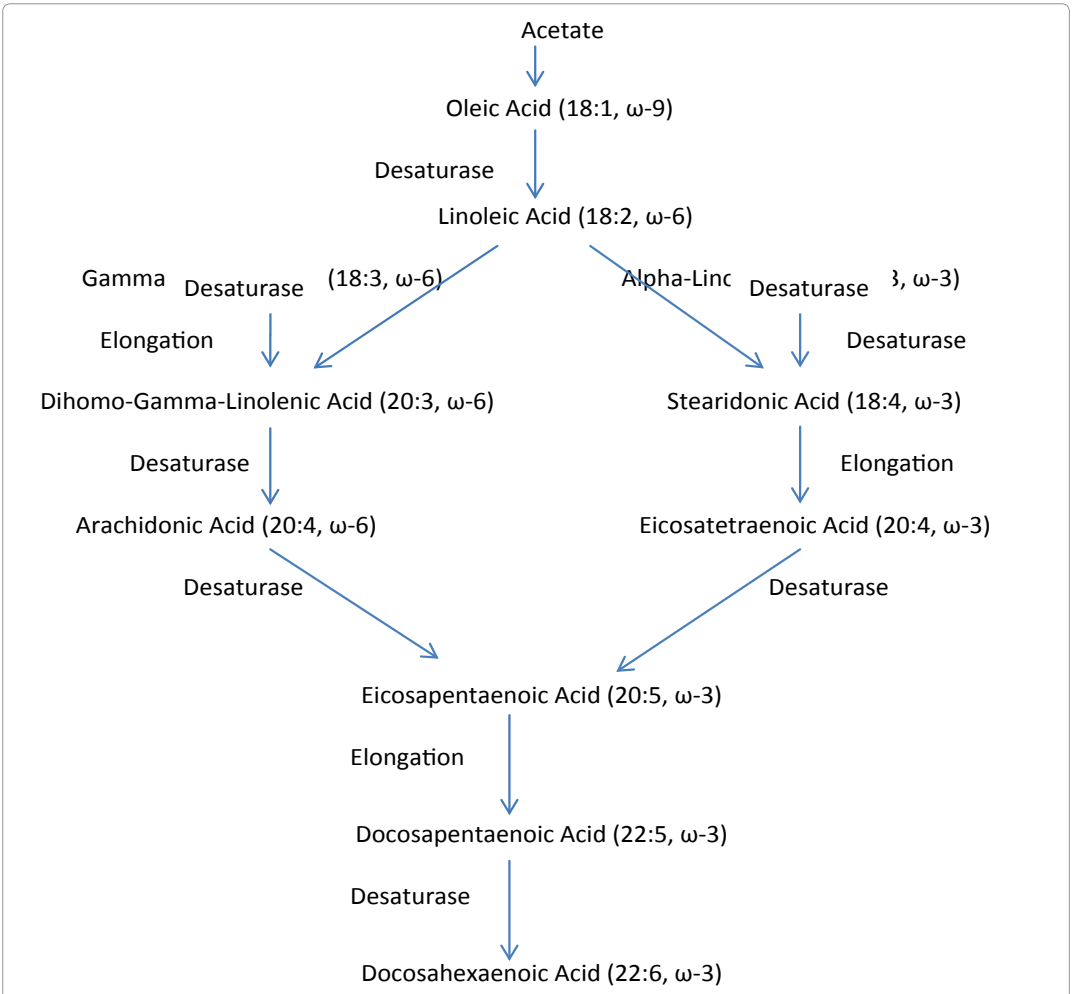

Figure 2: The biosynthetic pathways for omega-3 polyunsaturated fatty acids.

Importance of Omega-3 PUFA

Development and Growth

The combination of several months of omega-3 fatty acid deficiency and small stores of essential omega-3 fatty acids in the adiopse tissue, prepares the ground for clinical signs of omega-3 fatty acid deficiency such as scaly and haemorrhagic dermatitis, haemorrhagic folliculitis of the scalp, impaired wound healing, reduced growth in children, reduced visual acuity and neuropathy [24-31]. During foetal development the essential fatty acids are transferred from the mother via placenta to the developing foetus [32]. There are special proteins in placenta preferentially transporting DHA and AA [33-35]. Some studies suggest that supplementation with long-chain PUFA during pregnancy promotes a small increase in pregnancy duration, birth weight and a reduced risk of early preterm delivery in high risk pregnancies [36-38]. Visual functions are incompletely developed at birth, but there is a rapid development during the first year of life. DHA is a major structural lipid of retinal photoreceptor outer segment membranes. Biophysical and biochemical properties of DHA may affect photoreceptor membrane function by altering permeability, fluidity, thickness,

and lipid phase properties. Tissue DHA status affects retinal cell signaling mechanisms involved in photo transduction and DHA insufficiency is associated with alterations in retinal function [39].

Biological Effect

Eicosanoids

Eicosanoids are important signal molecules including leukotrienes, prostaglandins, thromboxanes, prostacyclins, lipoxins and hydroxy-fatty acids. In addition, two new families of lipid mediators have been discovered, resolvins (resolution phase interaction products) and protectins, both derived from omega-3 PUFA [40]. These have potent anti-inflammatory, neuro protective and pro-resolving properties.

Membrane Fluidity

When large amounts of very long-chain omega-3 fatty acids are ingested, there is high incorporation of EPA and DHA into membrane phospholipids, which may alter the physical characteristics of cell membranes [41]. Altered fluidity may lead to changes of membrane protein functions.

Lipid Peroxidation

Lipid peroxidation products may act as biological signals in certain cells [42]. One of the major concerns with intake of omega-3 fatty acids has been the high degree of unsaturation and thereby the possibility of promoting peroxidation. Modified Low-Density Lipoprotein (LDL) might be endocytosed by macrophages and initiate development of atherosclerosis. Oxidatively modified LDL has been observed in atherosclerotic lesions [43], and LDL rich in oleic acid has been found to be more resistant to oxidative modification than LDL enriched with omega-6 fatty acids in rabbits [44].

Acylation of Proteins

Acylation of proteins is important for anchoring certain proteins in membranes or folding of the proteins, and it seems to be crucial for the function of these proteins [45-46]. Although saturated fatty acids are most commonly linked to proteins, omega-3 fatty acids may also acylate proteins [47]. It has been demonstrated that PUFAs (AA and EPA) may inhibit palmitoylation and alter membrane localization of a protein kinase (Fyn) [48], whereas C14:2 may be acylated to a protein kinase (Fyn), thereby altering its raft localization and promote reduced T cell signal transduction and inflammation [49].

Gene Interactions

Fatty acids or their derivatives (acyl-CoA or eicosanoids) may interact with nuclear receptor proteins that bind to certain regions of DNA and thereby alter transcription of these genes. The receptor protein, often interacting with another nuclear receptor, may, in combination with a fatty acid, function as a transcription factor.The omega-3 and omega-6 PUFAs are feed forward activators of Peroxisome Proliferator-Activated Receptors (PPARs), whereas these same fatty acids are feed-back inhibitors of liver X receptorS (LXRs) and Sterol regulatory element-binding proteins (SREBPs). Saturated fatty acyl coenzyme A thioesters activate HNF-4α, whereas coenzyme A thioesters of PUFAs antagonize HNF-4α action. It has also been shown that PUFAs including DHA are ligands for another transcription factor, retinoid X receptor (RXR), which is important for expression of several genes regulated by different nutrients [50-51].

VLDL and Chylomicrons

Omega-3 fatty acids promote a striking reduction of Triacylglycerols (TAGs) in VLDL and chylomicron particles [52-54]. With daily intake of 2-4 g/day of very long-chain omega-3

fatty acids, the plasma concentration of TAGs is reduced by 20-30% [55], and the hepatic synthesis of triaglycerols is decreased, probably because omega-3 fatty acids inhibit esterification of other fatty acids in addition to being poor substrates for TAG-synthesising enzymes [56]. Very long-chain omega-3 fatty acids may also reduce TAG production by increasing fatty acid oxidation via peroxisomal β-oxidation [57].

High Density Lipoprotein (HDL)

In most studies of human lipoprotein metabolism, gram quantities of very long-chain omega-3 fatty acids cause a small increase (1-3%) in the plasma concentration of HDL cholesterol [52, 54, 58]. Very often this increase in HDL occurs simultaneously with a fall in plasma VLDL concentration. The increased concentration of HDL cholesterol may be explained by the reduced concentration of free fatty acids in plasma [57, 59-60] causing reduced net flux of cholesteryl esters from HDL to LDL and VLDL via reduced activity of the cholesteryl ester transfer protein [61-62].

Vascular Changes

It has been suggested that blood platelets, viscosity, coagulation and fibrinolysis might be influenced in advantageous ways by dietary intake of omega-3 fatty acids.Provision of very long-chain omega-3 fatty acids to healthy volunteers as well as to subjects with increased risk of cardiovascular disease, reduced the generation of platelet activating factor (PAF; important for platelet aggregation) [63-64], which may be of importance for suppression of rolling and adherence of monocytes on activated endothelial cells [65]. Supplementation with omega-3 fatty acids may reduce blood pressure, in particular among hypertensives [66-68]. From a meta-analysis of clinically controlled trials it has been estimated that systolic and diastolic blood pressures are decreased by 0.66 and 0.35 mmHg, respectively, per gram of very long-chain omega-3 fatty acids supplied through the diet [67]. It is also possible that EPA and DHA might improve endothelial function [69] via eicosanoids or working as ligands for transcription factors.

Diseases

Cardiovascular Diseases

Cardiovascular diseases are mainly caused by atherosclerosis, and give rise to development of myocardial infarction, cerebral infarction, cognitive decline, and gangrene and loss of function in the extremities.It has been shown that dietary factors such as saturated and trans fatty acids, cholesterol, some coffee lipids and sodium, in addition to lack of omega-3 fatty acids may promote development of atherosclerosis [70-74].

Atherosclerosis

The effects of very long-chain omega-3 fatty acids on some parameters of importance for the development of atherosclerosis are outlined. Some of these effects of omega-3 fatty acids have been demonstrated in controlled clinical trials, whereas other findings refer to studies in animals or *in vitro* [42, 55, 75-77]. Among the positive effects of omega-3 fatty acids are reduced activity of blood platelets via thromboxanes (TXA2), reduced Platelet Activating Factor (PAF), reduced PDGF, reduced chemotactic effect via Leukotrienes (LTB4) And Relaxation Of Smooth Muscle Cells Via EDRF Which Promotes Increased Blood Flow and reduced blood pressure.

Arrhythmias

In cells and animal studies it has been shown that incorporation of marine omega-3 fatty acids reduces the risk of arrhythmias, probably due to inhibition of the fast voltage-dependent sodium-channel [78-79]. Arrhythmias causing sudden cardiac death often arise from ischemia-induced electrical instability in the heart muscle. Ischemia may promote

depolarization of cardiac membranes by reducing the activity of sodium/potassium ATPase, which enhances interstitial potassium concentration, making the resting membrane potential more positive. This may make myocytes more likely to depolarize due to small stimuli and thereby initiate an arrhythmia [80].

Cancer

In spite of the fact that animal fat is associated with many of the carcinogenic effects of dietary fat [81], there is some scientific evidence that omega-3 fatty acids may protect against development of certain types of cancers [82-83]. Narayanan et al., [84] observed altered expression of genes important for apoptosis and regulators of colon cancer cell proliferation, explaining how DHA might protect against colonic cancer development. Another explanation for the inhibitory effect of omega-3 fatty acids on colorectal cancer may be due to cytotoxic peroxidation products generated during lipid peroxidation of EPA and cyclooxygenase (COX) activity [85]. Feeding experiments with rodents showed that omega-3 fatty acids decreased colon carcinogenesis at both the initiation and promotion stages [86-88]. A large number of studies in animals and in cultured cells demonstrate that omega-3 fatty acids may inhibit initiation, growth and metastasis of malignant cells [65].

Inflammatory Diseases

A significant number of reports indicate that supplementation with a few grams daily of very long-chain omega-3 fatty acids provide health benefits in relation to some inflammatory diseases [76,79]. The mechanism behind these effects may be related to altered eicosanoid formation or any of the other mechanisms described. For the eicosanoids, it has been shown that LTB5 derived from EPA may replace some of the more potent inflammatory agent LTB4 derived from AA. Omega-3 fatty acids may inhibit the adhesion of monocytes and endothelial cells possibly due to generation of platelet activating factor [65].

Rhematoid Arthritis / Joints

Several studies have shown that dietary supplementation with very long-chain omega-3 fatty acids (>3 g/day) reduces the clinical symptoms of rheumatoid arthritis as evaluated by morning stiffness and number of swollen joints, in meta-analyses [86-90].

Asthma and Allergy

In a randomized controlled study among asthmatic children, the results suggest that dietary supplementation with long-chain omega-3 fatty acids was beneficial in a strictly controlled environment in terms of inhalant allergens and diet [91].

Diabetes

In a meta-analysis it was concluded that intake of omega-3 fatty acids had no significant effect on glycemic control or fasting insulin [92].

Effect on Central Nervous System

Several reports have suggested that supplementation with marine omega-3 fatty acids are important for treatment of schizophrenia, depressions or borderline personality disorder [93-97].

Schizophrenia

Data indicate that the level of omega-3 fatty acids is low in red blood cells as well as in some cortical areas of the brain in schizophrenic patients [98-99], although the data are based on very small numbers of cases and are somewhat controversial [100].

Alzheimer's and Parkinson Disease

The omega-3 fatty acids in fish might explain part of this beneficial effect. It is clearly possible to influence the content of DHA in the brain by dietary intake [101]. In two prospective studies it was observed that lower plasma DHA levels increased the risk of developing AD later in life [102-103]. There are too few data on Parkinson disease in relation to dietary intake of omega-3 fatty acids to conclude on the potential effects of supplementation.

Natural Source of Omega -3 Fatty Acid

Microalgae offer a promising non-polluted resource for biotechnology and bioengineering of LC-PUFA production, as an alternative to fish oils [104-105]. Microalgae can also be grown on low to no-cost nutrients, which make them an economically viable source of omega-3 fatty acids [106]. Compared to traditional source, microalgae present a few advantages for n-3 LC-PUFA production, such as commonly occurring genes for the biosynthesis of these nutrients, simpler fatty acid profiles and higher growth rates.

Various auto- and heterotrophic marine species from different classes produce EPA and DHA, whereas AA is generally found in scarce amounts [107-108]. According to recent reviews of total lipid extracts, *Bacillariophyceae* (diatoms) and *Chrysophyceae* species may be rich sources of EPA and DHA; *Cryptophyceae*, *Prasinophyceae*, *Rhodophyceae*, *Xanthophyceae*, *Glaucophyceae* and *Eustigmatophyceae* can represent interesting EPA sources, whereas DHA is found in significant amounts mostly in *Dinophyceae*, *Prymnesiophyceae*, and *Euglenophyceae* [109-110]. Moreover, some important microalge has been given in following table 2 with their fatty acid profile [111].

	Chroococcus sp	***Phaeodactylum tricornutum***	***Isochrysis galbana***	***Pavlova sp.***	***Oocystis sp.***	***Tetraselmis sp.***	***Rhodomonas baltica***	***Nannochloropsis oceanica***
Saturated								
C12:0	2.1	–	–	–	–	–	2.0	1.2
C14:0	0.1	8.8	8.9	7.5	0.2	0.5	4.1	16.9
C16:0	21.3	16.6	11.5	13.4	3.8	6.3	6.0	17.2
C18:0	0.3	0.6	-	0.4	–	1.2	0.8	1.8
C20:0	0.2	–	-	–	–	–	0.1	–
C24:0	–	1.6	-	–	0.1	–	4.0	–
Sum	24.0	27.6	20.4	21.3	4.1	8.0	17	37.1
Mono saturated								
C 16:1	1.1	26.0	3.3	12.8	1.5	1.3	0.4	18.2
C 18:1^{c}	0.4	1.8	13.1	2.9	3.9	10.7	3.4	4.1
C 20:1^{c}	–	–	–	-	–	0.9	0.1	0.5
C 22:1^{c}	0.1	0.3	0.6	0.8	–	–	0.1	-
Sum Polyunsaturated	1.6	28.1	17.0	16.5	5.4	12.9	4.0	22.8
C 18:2^{b}	10.7	1.5	7.0	2.1	6.4	2.5	11.7	9.7
C 18:3^{a}	1.0	0.3	3.8	1.8	8.1	6.4	12.0	0.5
C 18:4^{a}	–	3.3	12.5	4.3	0.7	4.1	5.1	-
C 20:2^{b}	0.1	–	–		–	–	0.1	0.5
C 20:4^{b}	0.1	2.2	–	0.4	0.5	0.6	0.2	3.7
C 20:5^{a}	–	28.4	0.8	18.0	1.1	4.8	4.4	23.4
C22:5^{b}	–	1.3	–		–	–	0.2	–
C22:6^{a}	–	0.2	15.8	13.2	–	0.2	–	–
Sum	11.9	37.2	39.9	39.8	16.8	18.6	33.7	37.8

Table 2: Fatty acid profile of some microalgae. Data are given as mg g^{-1} of dry weight.

Dashes indicate FA not detected, ax-3 fatty acids, bx-6 fatty acids, cx-9 fatty acids

Photoautotrophic Microalgae

It is generally thought that photosynthetic microalgae tend to produce higher levels of EPA than heterotrophs. *Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Nitzschia laevis* and *Porphyridium cruentum* can present elevated levels of EPA in total fatty acids, although relatively low cell lipid contents tend to result in small EPA amounts in the biomass (Table 3) [112].

Species	EPA content (%TFA)[1]	EPA content (DW)[2]
Nannochloropsis sp.	38–39	2–3
Phaeodactylum tricornutum	31	5
Nitzschia laevis	25-33	3-4
Porphyridium cruentum	25	3
Odontella aurita	26	-
Pavlova lutheri	22-29	-
Cyclotella cryptica	17-23	1
Cylindrotheca sp.	24-25	-

Table 3. Examples of marine microalgae species characterized by EPA production.

[1]% TFA, % of total fatty acids; [2] % DW, % of biomass dry weight

Heterotrophic Microalgae

Several marine heterotroph microalgae are considered as the most preeminent alternative industrial sources of oils rich in DHA (Table 4) [113], with approved use in human foods, especially for application in infant formulas, since they are considered to be non-pathogenic and nontoxigenic.

Species	DHA content (% TFA) [1]	DHA content (% DW) [2]
Schizochytrium mangrovei	31-41	12-21
Schizochytrium limacinum	25-35	5-15
Thraustochytrium aureum	32-37	6-7
Thraustochytrium striatum	37	2
Ulkenia sp.	10-23	5
Aurantiochytrium sp.	40	18
Crypthecodinium cohnii	53-57	5-6

Table 4: Examples of marine microalgae species characterized by DHA production.

[1]% TFA, % of total fatty acids; [2] % DW, % of biomass dry weight

Stimulation of Omega-3 in Microalgae

Environmental Stress

Omega-3 fatty acid biosynthesis can be prompted by the applying of growth environmental stresses, such as low temperature and change of salinity or UV radiation. For example, *Pavlova lutheri* increased its relative EPA content from 20.3 to 30.3 % when the culture temperature was reduced to 15°C [114]. Similarly, *Phaeodactylum tricornutum* had a higher EPA content when the temperature was shifted from 25°C to 10°C for 12 h [115]. An increase in PUFAs is expected as these fatty acids have good flow properties and would be predominately used in the cell membrane to maintain fluidity during low temperatures. Salinity may also regulate PUFA biosynthesis, although not in a consistent manner. For example, *Crythecodinium cohnii* ATCC 30556 increased its total DHA content up to 56.9% of total fatty acids when cultured in 9 g/L NaCl. Other treatments that cause the generation of reactive oxygen species and lipid peroxidation also result in higher PUFA contents. For example, *Phaeodactylum tricornutum* increased its EPA content up to 19.84% when stressed with UV light [116].

Metabolic Engineering

Apart for external stresses, metabolic engineering is another promising approach to increase the production of omega-3 in microalgae. Synthesis of ω-3 fatty acids occurs via the elongation and desaturation of long chain fatty acids. Work has been performed to create recombinant sources of ω-3 fatty acids in a variety of systems with some success [117-118]. Canola (*Brassica napus*) seeds have been produced which overexpress the *B. napus* Δ15 desaturase, as well as the Δ6 and Δ12 desaturases from the commercially grown fungus *Mortierella alpine* to synthesize the ω-3 fatty acid Stearidonic Acid (SDA) [119]. Moreover, a promising cisgenic approach for microalgae maybe to increase EPA or DHA production by overexpressing at least some of their native elongases and desaturases. It may be necessary to use promoters inducible by external stimuli rather than constitutive promoters that may interfere with normal cell function and growth. Another, yet unexplored option may lie in the inhibition of PUFA degradation. β-oxidation of fatty acids occurs in the peroxisomes but before PUFAs can be metabolized, saturases are required to fill in the double bonds. Mutations in one or several saturases may result in less efficient β-oxidation of PUFA and a higher percentage of these fatty acids. However, at present the mechanism behind the selection and storage of fatty acids for triacylglycerol production remains unclear.

Procedure of Omega-3 Production

Extraction of omega-3 depends on strain and physical properties of algae. A flow chart is given below for the production of omega-3 (Figure 3),

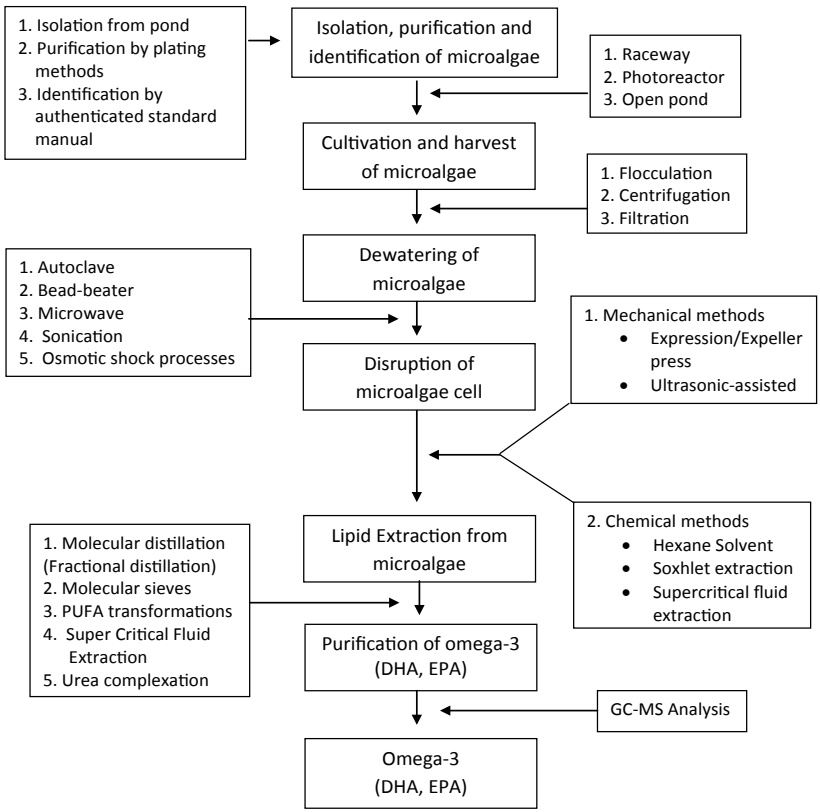

Figure 3: Flow chart for omega-3 production.

Conclusion and Future Perspectives

Natural limitations favor a novel approach for the production of omega-3 fatty acids. A series of PUFAs including Eicosapentanoic Acid (EPA) and Docosahexanoic Acid (DHA) have widespread nutritional and pharmaceutical values. Microalgae cells have the potential to rapidly accumulate lipids, such as triacylglycerides that contain fatty acids important for high value fatty acids (e.g., EPA and DHA) production. However, lipid extraction methods for microalgae cells are not well established, and there is currently no standard extraction method for the determination of the fatty acid content of microalgae. Production yields are also highly dependent on microalgal cultivation conditions, biomass processing, cell disruption and strain selection in addition to solvent polarity and extraction processing.

From a commercial perspective, a techno-economic assessment is needed and should ideally be carried out for large scale extraction where costs are likely to be very different compared to the presents laboratory-based study. It is necessary to study deeper about the extraction and purification of omega-3 PUFA which is more economic with good quality and quantity along with biotechnological approach.

Acknowledgements

The authors are thankful to the authorities of Designated Reference Institute for Chemical Measurements (DRiCM), Bangladesh Council of Scientific and Industrial Research (BCSIR) and Algasol Bangladesh Limited for providing required facilitates and financial support.

References

1. Van West Dirk, Maes, Michael (2003) Polyunsaturated fatty acids in depression. CtaNeuropsychiatrica 15 (1): 15-21.
2. Chew YL, Lim YY, Omar M, Khoo KS (2008) Antioxidant activity of three edible seaweeds from two areas in South East Asia. LWT-Food Sci. Tech. 41: 1067-1072.
3. Kalogeropoulos N, Chiou A, Gavala E, Christea M, Andrilkopoulos NK, et al. (2010) Nutritional evaluation and bioactive micro constituents (carotenoids, tocopherols, sterols and squalene) of raw and roasted chicken fed on DHA-rich microalgae. Food Research International 43: 2006–2013.
4. Guedesb AC, Amaro HM, Barbosa CR, Pereira RD, Malcata FX (2011) Fatty Acid Composition of Several Wild Microalgae and Cyanobacteria, with a focus on Eicosapentaenoic, Docosahexaenoic and Linolenic Acids for Eventual Dietary Uses. Food Research International 2: 12-21.
5. Ward OP, Singh A (2005) Omega-3/6 fatty acids: Alternative sources of production. Process Biochemistry 40: 3627–3652.
6. Innis SM1 (1991) Essential fatty acids in growth and development. See comment in PubMed Commons below Prog Lipid Res 30: 39-103.
7. Carlson SE, Salem N (1991) Essential of T3 fatty acids in growth and development of infants. In: Health effects of T3 polyunsaturated fatty acids in seafood. Ed. Simopoulus AP, Kifer RR and Barlow SM. World Rev. Nutr., Karger, Basel, Switzerland, pp: 74-86.
8. Ogata J, Hagiwara Y, Hagiwara H, Shibamoto T (1996) Inhibition of malonaldehyde formation by antioxidants from T3 polyunsaturated fatty acids. JAOCS 73: 654-656.
9. OtleÅŸ S, Pire R (2001) Fatty acid composition of Chlorella and Spirulina microalgae species. See comment in PubMed Commons below J AOAC Int 84: 1708-1714.
10. Roughan GP (1989). Spirulina: A source of dietarygamma-linolenic acid. J. Sci. Food Agric 47: 85-93.
11. Saito T, Saito T, Oka N (1992) Scientific reports on Chlorella in Japan, Japan Treatment Association, Silpaque Publishing, Kyoto, Japan.
12. Quoc KP, Pascaud M (1996) Effects of dietary gamma-linolenic acid on the tissue phospholipid fatty acid composition and the synthesis of eicosanoids in rats. See comment in PubMed Commons below Ann Nutr Metab 40: 99-108.

13. Croft KD, Beilin LJ, Legge FM, Vandongen R (1987) Effects of diets enriched ineicospentaenoic or docosahexaenoic acid onprostaglandin metabolism in rats. Lipids 22: 647-650.

14. Cohen Z, Vonshak A, Boussiba S, Richmond A (1988) The effect of temperature and cellconcentration on the fatty acid composition of outdoor cultures of porphyridium cruentum. In: Algal Biotechnology: Ed. Stadler T, Mollion J, Verdus MC, Karamanos Y, Christiaen D. ElsevierAppl. Sci. London, pp: 412-419.

15. Veloso V, Reis L, Gouveia L, Fernandes HL, Novias JM (1991) Lipid production by Phaeodactylum tricornum. Bioresou. Technol. 38:115-119.

16. Hoffman DR, Birch EE, Birch DG, Uauy RD (1993) Effects of supplementation with omega 3 long-chain polyunsaturated fatty acids on retinal and cortical development in premature infants. See comment in PubMed Commons below Am J Clin Nutr 57: 807S-812S.

17. Mokady S, Sukenik AS (1995) A marine unicellular alga in diets of pregnant and lactating asa source of T3 fatty acids for the developing brain oftheir progeny. J. Sci. Food Agric. 68: 133-139.

18. Kalogeropoulos N, Chiou A, Gavala E, Christea M, Andrilkopoulos NK, et al. (2010) Nutritional evaluation and bioactive micro constituents (carotenoids, tocopherols, sterols and squalene) of raw and roasted chicken fed on DHA-rich microalgae. Food Research International 43: 2006–2013.

19. Ruxton CHS, Reed SC, Simpson MJA, Millington KJ (2004) The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J Hum Nutr Dietet 70: 449-459.

20. Matthan NR, Ooi EM, Van Horn L, Neuhouser ML, Woodman R, et al. (2014) Plasma phospholipid fatty acid biomarkers of dietary fat quality and endogenous metabolism predict coronary heart disease risk: a nested case-control study within the Women's Health Initiative observational study. See comment in PubMed Commons below J Am Heart Assoc 3.

21. Domingo JL, Bocio A, Falcó G, Llobet JM (2007) Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants. See comment in PubMed Commons below Toxicology 230: 219-226.

22. http://pubchem.ncbi.nlm.nih.gov/compound/56842239#section=Chemical-and-Physical-Properties

23. Wen ZY, Chen F (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. See comment in PubMed Commons below Biotechnol Adv 21: 273-294.

24. Bjerve KS (1989) n-3 fatty acid deficiency in man. See comment in PubMed Commons below J Intern Med Suppl 731: 171-175.

25. Bjerve KS, Fischer S, Wammer F, Egeland T (1989) a-Linolenic acid and long-chain omega-3 fatty acid supplementation in three patients with omega-3 fatty acid deficiency: effect on lymphocyte function, plasma and red cell lipids, and prostanoid formation. Am J Clin Nutr 49:290-300.

26. Bjerve KS, Thoresen L, Børsting S (1988) Linseed and cod liver oil induce rapid growth in a 7-year-old girl with N-3- fatty acid deficiency. See comment in PubMed Commons below JPEN J Parenter Enteral Nutr 12: 521-525.

27. Bjerve KS, Mostad IL, Thoresen L (1987) Alpha-linolenic acid deficiency in patients on long-term gastric-tube feeding: estimation of linolenic acid and long-chain unsaturated n-3 fatty acid requirement in man. See comment in PubMed Commons below Am J Clin Nutr 45: 66-77.

28. Carlson SE, Salem N Jr (1991) Essentiality of omega 3 fatty acids in growth and development of infants. See comment in PubMed Commons below World Rev Nutr Diet 66: 74-86.

29. Carlson SE, Werkman SH (1996) A randomized trial of visual attention of preterm infants fed docosahexaenoic acid until two months. See comment in PubMed Commons below Lipids 31: 85-90.

30. Uauy R, Peirano P, Hoffman D, Mena P, Birch D, et al. (1996) Role of essential fatty acids in the function of the developing nervous system. See comment in PubMed Commons below Lipids 31 Suppl: S167-176.

31. Crawford MA (1993) The role of essential fatty acids in neural development: implications for perinatal nutrition. See comment in PubMed Commons below Am J Clin Nutr 57: 703S-709S.

32. Al MD, Hornstra G, van der Schouw YT, Bulstra-Ramakers MT, Huisjes HJ, et al. (1990) Biochemical EFA status of mothers and their neonates after normal pregnancy. See comment in PubMed Commons below Early Hum Dev 24: 239-248.

33. Innis SM (2005) Essential fatty acid transfer and fetal development. See comment in PubMed Commons below Placenta 26 Suppl A: S70-75.

34. Duttaroy AK (2004) Fetal growth and development: roles of fatty acid transport proteins and nuclear transcription factors in human placenta. See comment in PubMed Commons below Indian J Exp Biol 42: 747-757.

35. Koletzko B, Larqué E, Demmelmair H (2007) Placental transfer of long-chain polyunsaturated fatty acids (LC-PUFA). See comment in PubMed Commons below J Perinat Med 35 Suppl 1: S5-11.

36. Smuts CM, Huang M, Mundy D, Plasse T, Major S, et al. (2003) A randomized trial of docosahexaenoic acid supplementation during the third trimester of pregnancy. See comment in PubMed Commons below Obstet Gynecol 101: 469-479.

37. Makrides M, Duley L, Olsen SF (2006) Marine oil, and other prostaglandin precursor, supplementation for pregnancy uncomplicated by pre-eclampsia or intrauterine growth restriction. See comment in PubMed Commons below Cochrane Database Syst Rev : CD003402.

38. Szajewska H, Horvath A, Koletzko B (2006) Effect of n-3 long-chain polyunsaturated fatty acid supplementation of women with low-risk pregnancies on pregnancy outcomes and growth measures at birth: a meta-analysis of randomized controlled trials. See comment in PubMed Commons below Am J Clin Nutr 83: 1337-1344.

39. SanGiovanni JP, Chew EY (2005) The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. See comment in PubMed Commons below Prog Retin Eye Res 24: 87-138.

40. Schwab JM, Serhan CN (2006) Lipoxins and new lipid mediators in the resolution of inflammation. See comment in PubMed Commons below Curr Opin Pharmacol 6: 414-420.

41. Salem N (1989) Omega-3 fatty acids: molecular and biochemical aspects, in: Spiller G.A., Scala J. (Eds.), Current Topics in Nutrition and Disease: New Protective Roles for Selected Nutrients, Alan R. Liss, New York 22: 109-228.

42. Drevon CA, Baksaas I, Krokan HE Eds. (1993) Sources, chemistry and biochemistry of dietary lipids. In: Omega-3 fatty acids. Metabolism and biological effects. Birkhäuser, Basel pp 125-386.

43. Ylä-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, et al. (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. See comment in PubMed Commons below J Clin Invest 84: 1086-1095.

44. Parthasarathy S, Khoo JC, Miller E, Barnett J, Witztum JL, Steinberg D (1990) Low density lipoprotein rich in oleic acid is protected against oxidative modification: Implications for dietary prevention of atherosclerosis. Proc Nat l Acad Sci USA 87: 3894-3898.

45. McIlhinney RA (1990) The fats of life: the importance and function of protein acylation. See comment in PubMed Commons below Trends Biochem Sci 15: 387-391.

46. Babu P, Deschenes RJ, Robinson LC (2004) Akr1p-dependent palmitoylation of Yck2p yeast casein kinase 1 is necessary and sufficient for plasma membrane targeting. See comment in PubMed Commons below J Biol Chem 279: 27138-27147.

47. Muszbek L, Laposata M (1993) Covalent modification of proteins by arachidonate and eicosapentaenoate in platelets. See comment in PubMed Commons below J Biol Chem 268: 18243-18248.

48. Webb Y, Hermida-Matsumoto L, Resh MD (2000) Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. See comment in PubMed Commons below J Biol Chem 275: 261-270.

49. Liang X, Nazarian A, Erdjument-Bromage H, Bornmann W, Tempst P, et al. (2001) Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction. See comment in PubMed Commons below J Biol Chem 276: 30987-30994.

50. de Urquiza AM, Liu S, Sjöberg M, Zetterström RH, Griffiths W, et al. (2000) Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. See comment in PubMed Commons below Science 290: 2140-2144.

51. Lengqvist J, De Urquiza AM, Bergman AC, Wilson TM, Sjovall J et al. (2004) Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand binding domain. Mol Cell Proteomics 3: 692-703.

52. Harris WS (1997) n-3 fatty acids and serum lipoproteins: human studies. See comment in PubMed Commons below Am J Clin Nutr 65: 1645S-1654S.

53. de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, et al. (1994) Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. See comment in PubMed Commons below Lancet 343: 1454-1459.

54. Harris WS (1996) n-3 fatty acids and lipoproteins: comparison of results from human and animal studies. See comment in PubMed Commons below Lipids 31: 243-252.

55. Harris WS, Miller M, Tighe AP, Davidson MH, Schaefer EJ, et al.(2008) Omega-3 fatty acids and coronary heart disease risk: clinical and mechanistic perspectives. See comment in PubMed Commons below Atherosclerosis 197: 12-24.

56. Rustan AC, Nossen JO, Christiansen EN, Drevon CA (1988) Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A:1,2-diacylglycerol acyltransferase. See comment in PubMed Commons below J Lipid Res 29: 1417-1426.

57. Rustan AC, Christiansen EN, Drevon CA (1992) Serum lipids, hepatic glycerolipid metabolism and peroxisomal fatty acid oxidation in rats fed omega-3 and omega-6 fatty acids. See comment in PubMed Commons below Biochem J 283 : 333-339.

58. Harris WS (1989) Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. See comment in PubMed Commons below J Lipid Res 30: 785-807.

59. Singer P, Wirth M, Berger I (1990) A possible contribution of decrease in free fatty acids to low serum triglyceride levels after diets supplemented with n-6 and n-3 polyunsaturated fatty acids. See comment in PubMed Commons below Atherosclerosis 83: 167-175.

60. Rustan AC, Hustvedt BE, Drevon CA (1993) Dietary supplementation of very long-chain n-3 fatty acids decreases whole body lipid utilization in the rat. See comment in PubMed Commons below J Lipid Res 34: 1299-1309.

61. Barter PJ (1990) Enzymes involved in lipid and lipoprotein metabolism. Curr Opin Lipidol 1: 518-23.

62. Abbey M, Clifton PM, McMurchie EJ, McIntosh GH, Nestel PJ (1990) Effect of a high fat/cholesterol diet with or without eicosapentaenoic acid on plasma lipids, lipoproteins and lipid transfer protein activity in the marmoset. See comment in PubMed Commons below Atherosclerosis 81: 163-174.

63. Sperling RI (1991) Effects of dietary fish oil on leukocyte leukotriene and PAF generation and on neutrophil chemotaxis. See comment in PubMed Commons below World Rev Nutr Diet 66: 391-400.

64. Mori TA, Beilin LJ, Burke V, Morris J, Ritchie J, et al. (1997) Interactions between dietary fat, fish, and fish oils and their effects on platelet function in men at risk of cardiovascular disease. See comment in PubMed Commons below Arterioscler Thromb Vasc Biol 17: 279-286.

65. Mayer K, Merfels M, Muhly-Reinholz M, Gokorsch S, Rosseau S, et al. (2002) Omega-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. See comment in PubMed Commons below Am J Physiol Heart Circ Physiol 283: H811-818.

66. Bonaa KH, Bjerve KS, Straume B, Gram IT, Thelle D, et al. (1990) Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension. A population-based intervention trial from the Tromso study. N Eng J Med 322: 795-801.

67. Morris MC, Sacks F, Rosner B (1993) Does fish oil lower blood pressure? A meta-analysis of controlled trials. See comment in PubMed Commons below Circulation 88: 523-533.

68. Knapp HR (1996) n-3 fatty acids and human hypertension. See comment in PubMed Commons below Curr Opin Lipidol 7: 30-33.

69. Chin JP, Dart AM (1995) How do fish oils affect vascular function? See comment in PubMed Commons below Clin Exp Pharmacol Physiol 22: 71-81.

70. Keys A (1970) Coronary heart disease in seven countries. Circulation 41:1-211.

71. Selwyn AP, Kinlay S, Libby P, Ganz P (1997) Atherogenic lipids, vascular dysfunction, and clinical signs of ischemic heart disease. See comment in PubMed Commons below Circulation 95: 5-7.

72. Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. See comment in PubMed Commons below Nature 362: 801-809.

73. Johansson L, Drevon CA, Aa Bjørneboe GE (1996) The Norwegian diet during the last hundred years in relation to coronary heart disease. See comment in PubMed Commons below Eur J Clin Nutr 50: 277-283.

74. Harris WS, Miller M, Tighe AP, Davidson MH, Schaefer EJ (2008) Omega-3 fatty acids and coronary heart disease risk: clinical and mechanistic perspectives. See comment in PubMed Commons below Atherosclerosis 197: 12-24.

75. Drevon CA (1992) Marine oils and their effects. See comment in PubMed Commons below Nutr Rev 50: 38-45.

76. Leaf A, Weber PC (1988) Cardiovascular effects of n-3 fatty acids. See comment in PubMed Commons below N Engl J Med 318: 549-557.

77. Endres S, De Caterina R, Schmidt EB, Kristensen SD (1995) n-3 polyunsaturated fatty acids: update 1995. See comment in PubMed Commons below Eur J Clin Invest 25: 629-638.

78. Nair SS, Leitch JW, Falconer J, Garg ML (1997) Prevention of cardiac arrhythmia by dietary (n-3) polyunsaturated fatty acids and their mechanism of action. See comment in PubMed Commons below J Nutr 127: 383-393.

79. Leaf A, Xiao YF, Kang JX, Billman GE (2003) Prevention of sudden cardiac death by n-3 polyunsaturated fatty acids. See comment in PubMed Commons below Pharmacol Ther 98: 355-377.

80. Jenkins DJA, Josse AR, Dorian P, Burr ML, LaBelle Trangmar R, et al. (2008) Heterogeneity in Randomized Controlled Trials of Long Chain (Fish) Ome¬ga-3 Fatty Acids in Restenosis, Secondary Prevention and Ventricular Arrhythmias. J. Am. Coll. Nutr 27: 367-378.

81. Ames BN, Gold LS, Willett WC (1995) The causes and prevention of cancer. See comment in PubMed Commons below Proc Natl Acad Sci U S A 92: 5258-5265.

82. Caygill CP, Charlett A, Hill MJ (1996) Fat, fish, fish oil and cancer. See comment in PubMed Commons below Br J Cancer 74: 159-164.

83. Kaizer L, Boyd NF, Kriukov V, Tritchler D (1989) Fish consumption and breast cancer risk: an ecological study. See comment in PubMed Commons below Nutr Cancer 12: 61-68.

84. Narayanan BA, Narayanan NK, Reddy BS (2001) Docosahexaenoic acid regulated genes and transcription factors inducing apoptosis in human colon cancer cells. See comment in PubMed Commons below Int J Oncol 19: 1255-1262.

85. Dommels YE, Haring MM, Keestra NG, Alink GM, van Bladeren PJ, et al. (2003) The role of cyclooxygenase in n-6 and n-3 polyunsaturated fatty acid mediated effects on cell proliferation, PGE(2) synthesis and cytotoxicity in human colorectal carcinoma cell lines. See comment in PubMed Commons below Carcinogenesis 24: 385-392.

86. Kromhout D (1990) The importance of N-6 and N-3 fatty acids in carcinogenesis. See comment in PubMed Commons below Med Oncol Tumor Pharmacother 7: 173-176.

87. Singh J, Hamid R, Reddy BS (1997) Dietary fat and colon cancer: modulating effect of types and amount of dietary fat on ras-p21 function during promotion and progres¬sion stages of colon cancer. Cancer Res 57: 253-258.

88. Murray NR, Weems C, Chen L, Leon J, Yu W, et al. (2002) Protein kinase C betaII and TGFbetaRII in omega-3 fatty acid-mediated inhibition of colon carcinogenesis. See comment in PubMed Commons below J Cell Biol 157: 915-920.

89. Fortin PR, Lew RA, Liang MH, Wright EA, Beckett LA, et al. (1995) Validation of a meta-analysis: the effects of fish oil in rheumatoid arthritis. See comment in PubMed Commons below J Clin Epidemiol 48: 1379-1390.

90. Goldberg RJ, Katz J (2007) A meta-analysis of the analgesic effects of omega-3 polyunsaturated fatty acid supplementation for inflammatory joint pain. See comment in PubMed Commons below Pain 129: 210-223.

91. Nagakura T, Matsuda S, Shichijyo K, Sugimoto H, Hata K, et al. (2000) Dietary supplementation with fish oil rich in omega-3 polyunsaturated fatty acids in children with bronchial asthma. See comment in PubMed Commons below Eur Respir J 16: 861-865.

92. Hartweg J, Perera R, Montori V, Dinneen S, Neil HA, et al. (2008) Omega-3 polyunsaturated fatty acids (PUFA) for type 2 diabetes mellitus. See comment in PubMed Commons below Cochrane Database Syst Rev : CD003205.

93. Arvindakshan M, Ghate M, Ranjekar PK, Evans DR, Mahadik SP, et al.(2003) Supplementation with a combination of omega-3 fatty acids and antioxidants (vitamins E and C) improves the outcome of schizophrenia. See comment in PubMed Commons below Schizophr Res 62: 195-204.

94. Su KP, Huang SY, Chiu CC, Shen WW (2003) Omega-3 fatty acids in major depressive disorder. A preliminary double-blind, placebo-controlled trial. See comment in PubMed Commons below Eur Neuropsychopharmacol 13: 267-271.

95. Tiemeier H, van Tuijl HR, Hofman A, Kiliaan AJ, Breteler MM, et al.(2003) Plasma fatty acid composition and depression are associated in the elderly: the Rotterdam Study. See comment in PubMed Commons below Am J Clin Nutr 78: 40-46.

96. Saugstad LF (2002) Human nature is unique in the mismatch between the usual diet and the need for “food for the brain” (marine fat, DHA). Adding marine fat is beneficial in schizophrenia and manic-depressive psychosis. This underlines that brain dysfunction in these neurological disorders is associated with deficient intake of marine fat (DHA). Nutr Health 16: 41-44

97. Zanarini MC, Frankenburg FR (2003) omega-3 Fatty acid treatment of women with borderline personality disorder: a double-blind, placebo-controlled pilot study. See comment in PubMed Commons below Am J Psychiatry 160: 167-169.

98. Reddy RD, Keshavan MS, Yao JK (2004) Reduced red blood cell membrane essential polyunsaturated fatty acids in first episode schizophrenia at neuroleptic-naive baseline. See comment in PubMed Commons below Schizophr Bull 30: 901-911.

99. McNamara RK, Jandacek R, Rider T, Tso P, Hahn CG, et al. (2007) Abnormalities in the fatty acid composition of the postmortem orbitofrontal cortex of schizophrenic patients: gender differences and partial normalization with antipsychotic medications. See comment in PubMed Commons below Schizophr Res 91: 37-50.

100. Peet M1 (2008) Omega-3 polyunsaturated fatty acids in the treatment of schizophrenia. See comment in PubMed Commons below Isr J Psychiatry Relat Sci 45: 19-25.

101. Calon F, Cole G (2007) Neuroprotective action of omega-3 polyunsaturated fatty acids against neurodegenerative diseases: evidence from animal studies. See comment in PubMed Commons below Prostaglandins Leukot Essent Fatty Acids 77: 287-293.

102. Heude B, Ducimetière P, Berr C; EVA Study (2003) Cognitive decline and fatty acid composition of erythrocyte membranes--The EVA Study. See comment in PubMed Commons below Am J Clin Nutr 77: 803-808.

103. Schaefer EJ, Bongard V, Beiser AS, Lamon-Fava S, Robins SJ, et al. (2006) Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham heart study, Arch. Neurol 63: 1545–1550.

104. Khozin-Goldberg I, Iskandarov U, Cohen Z (2011) LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. See comment in PubMed Commons below Appl Microbiol Biotechnol 91: 905-915.

105. Apt KE, Behrens PW (1999) Commercial developments in microalgal biotechnology. J Phycol 35: 215–226.

106. Certik M1, Shimizu S (1999) Biosynthesis and regulation of microbial polyunsaturated fatty acid production. See comment in PubMed Commons below J Biosci Bioeng 87: 1-14.

107. Thompson GA Jr1 (1996) Lipids and membrane function in green algae. See comment in PubMed Commons below Biochim Biophys Acta 1302: 17-45.

108. Bigogno C, Khozin-Goldberg I, Boussiba S, Vonshak A, Cohen Z (2002) Lipid and fatty acid composition of the green oleaginous alga Parietochloris incisa, the richest plant source of arachidonic acid. See comment in PubMed Commons below Phytochemistry 60: 497-503.

109. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, et al. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. See comment in PubMed Commons below Plant J 54: 621-639.

110. Lang I, Hodac L, Friedl T, Feussner I (2011) Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. See comment in PubMed Commons below BMC Plant Biol 11: 124.

111. Vishwanath Patil, Torsten Ka¨ llqvist, Elisabeth Olsen, Gjermund Vogt, et al.(2007) Fatty acid composition of 12 microalgae for possible use in aquaculture feed. Aquacult Int 15:1–9.

112. Martins DA, Custódio L, Barreira L, Pereira H, Ben-Hamadou R, et al. (2013) Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. See comment in PubMed Commons below Mar Drugs 11: 2259-2281.

113. Tatsuzawa H, Takizawa E (1995) Changes in lipid and fatty acid composition of Pavlova lutheri. Phytochemistry 40(2): 397–400.

114. Jiang H, Gao K (2004) Effects of lowering temperature during culture on the production of polyunsaturated fatty acids in the marine diatom Phaeodactylum tricornutum (bacillaryophyceae). J Phycol 40(4): 651–654.

115. Liang Y, Beardall J, Heraud P (2006) Effect of UV radiation on growth, chlorophyll fluorescence and fatty acid composition of Phaeodactylum tricornutum and Chaetoceros muelleri (bacillariophyceae). Phycologia 45(6): 605–615.

116. Amiri-Jami M, Griffiths MW (2010) Recombinant production of omega-3 fatty acids in Escherichia coli using a gene cluster isolated from Shewanella baltica MAC1. See comment in PubMed Commons below J Appl Microbiol 109: 1897-1905.

117. Li YT, Li MT, Fu CH, Zhou PP, Liu JM, et al. (2009) Improvement of arachidonic acid and eicosapentaenoic acid production by increasing the copy number of the genes encoding fatty acid desaturase and elongase into Pichia pastoris. See comment in PubMed Commons below Biotechnol Lett 31: 1011-1017.

118. Ursin VM (2003) Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. See comment in PubMed Commons below J Nutr 133: 4271-4274.

119. Catalina Adarme-Vega T, David Lim KY, Matthew Timmins, Felicitas Vernen, Yan Li et al. (2012): Microalgal biofactories: a promising approach toward sustainable omega-3 fatty acid production. Microbial Cell Factories 11: 96.

Microalgae as the Feedstock for Biodiesel Production

Edith Martinez-Guerra* and Veera Gnaneswar Gude

[1]Mississippi State University, Department of Civil and Environmental Engineering

***Corresponding author:** Edith Martinez-Guerra, Mississippi State University, Department of Civil and Environmental Engineering, Fax: 870-200-0423; E-mail: em826@msstate.edu

Abstract

Escalating demands for transportation fuels combined with rapid industrialization and depletion of conventional fossil fuel sources with concomitant environmental emissions have created economic, environmental and political instabilities in many developing and developed countries around the world. The quest to achieve energy self-sufficiency or independence has become the root cause for a strong impetus for research and development of renewable fuel production. Among the various renewable fuel alternatives, algae have been shown to be outstanding and potential renewable fuel contributors due to their higher oil yielding capacity than any other terrestrial plants or crops and their environmental-friendly nature. This book chapter presents an overview of the important stages of algal biofuel production with special emphasis on biodiesel production. Algal biodiesel production essentially involves five important steps such as biomass cultivation, harvesting, extraction and separation of valuable compounds and chemical conversion. Each of these stages is energy, chemical, and cost intensive adding to the final product costs. This implies that the overall process needs some strategic and targeted improvements in all these stages for low cost biodiesel production in the near future. From the recent efforts in this area, it becomes evident that integrated approaches that increase valuable bioproducts recovery from algal biomass could improve the economics of biodiesel production.

Keywords: Algal Biomass, Biodiesel, Energy Footprint, Feedstock, Lipids, Renewable Energy

Introduction

Biodiesel is a biodegradable and renewable fuel which can be produced from a variety of feedstock and is currently being produced in many parts of the world. It is considered the fuel of the future and a major competitor for petroleum diesel. Biodiesel can be used by itself in existing diesel engines without any modification to the engine, or blended at any ratio with petroleum diesel as it is currently practice worldwide [1-2]. Biodiesel production may not completely replace the fossil fuel consumption, rather it can aid but to diminish the dependency on this conventional depleting sources. One of the major drawbacks of

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

biodiesel is the feedstock availability for its production. However, the use of microalgae has demonstrated its capabilities of producing non-toxic, high quality biodiesel. Microalgae accumulate intracellular lipid that can be converted or transesterified to biodiesel via conventional or non-conventional techniques that will be discussed within this chapter. Transesterification is simply the conversion of lipids in the feedstock into biodiesel by adding a short chain alcohol and catalyst. Ethanol and methanol are the most common acyl acceptors used for biodiesel production; while the catalyst can be acidic, basic, or enzymatic. Transesterification enhances biodiesel volatility and lowers the viscosity of the oil [3]. Thus, transesterification produces a high quality and environmental friendly fuel source.

Microalgae for Biodiesel Production

Microalgae are eukaryotic, high photosynthesis efficiency microorganisms that can be found in freshwater, wastewater, and marine water sources. Their characteristics are similar to those of other plants, however, microalgae are more efficient converting solar energy because of their simple cellular structure [4].They use sunlight for reducing CO_2 to biofuels,and they are able to accumulate significant amounts of triglycerides within their cells suitable for biodiesel production through transesterification reaction [5]. The lipid accumulation in microalgal cells ranges from 25 to 75% of its dry weight [6]. Moreover, microalgae have the potential of fixing 1.83 tons of atmospheric CO_2 when producing 1 ton of algae biomass [7]. Also, microalgae are oil producing factories producing nearly 100 times more oil per acre than any other plants [8]. They are the most suitable and viable feedstock for bio-fuel production in the near future.

Other advantages with algae as a potential feedstock for biodiesel production is their ability to grow quickly (1-3 doublings per day), tolerate saline and wastewater compositions, and grow in marginal (arid, dry, semi-arid) lands not suitable for agriculture or any other human needs. Since algae require substantial quantities of water for their cultivation, marine or wastewater sources can be utilized as a growth medium. Algae can utilize organic compounds and nutrients (nitrogen and phosphorous) in the wastewater sources and accumulate lipids which can serve as feedstock for biodiesel production. Wastewater treatment can be successfully integrated with algal growth to result in a synergistic effect where water and energy issues can be dealt with in cooperation. These wastewater sources include agricultural runoff, concentrated animal feed operations, and industrial and municipal wastewaters. Apart from that algal biomass can be used to produce other valuable bioproducts such as proteins, health supplements, pigments, polysaccharides, biopolymers and fertilizers.

Currently, biodiesel production from microalgal biomass is a cost- and energy- intensive process. Energy is required for algae cultivation for biomass production and further separation and harvesting stages [9]. Lipid extraction is also an expensive process that contributes to the high cost of algal biodiesel. The algal oil production costs $10.87-13.32 per gallon whereas the cost of triglyceride production is $3 per gallon [10-11]. This cost makes algal biodiesel non-feasible when compared to petroleum diesel. Moreover, the additional cost from the use and recovery of the solvent for the extraction and the catalyst must be taken into account. However, the improvement or the use of sustainable approaches of feedstock processing and conversion technique can tremendously reduce the biodiesel production cost.

Algal biodiesel production involves five essential steps: 1) algae production, 2) algae harvesting, 3) oil extraction, 4) transesterification, and 5) separation and purification [12-14]. These steps (Figure 1) will be elaborated in the following sections. One of the approaches

to reduce the overall biodiesel production is to omit the oil extraction step by performing in-situ transesterification, which will be discussed within the non-conventional techniques section.

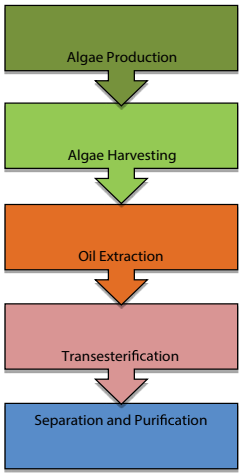

Figure 1: Microalgae biodiesel production step.

Algae Production and Harvesting

Algae have the ability of growing in any types of waters where there are enough nutrients and sunlight or any other type of adequate lightning. They present the advantages of rapid growth, high oil yield per unit area, and being cultivated on land that they are unsuitable for cultivation. However, not all types of algae are suitable for biodiesel production. For example, macroalgae do not store as much lipid as microalgae, and even certain types of microalgae store more lipids than others. They can be cultivated in either a photobioreactor or an open system such as a raceway pond. Photobioreactors are made of thin, transparent materials to ease the penetration of light. After the medium broth has been circulated through a pump to the photobioreactors tube, it is then send back to a reservoir. However, part of the algae is harvested after passing the solar collection tubes [15]. On the other hand, raceway ponds are directly excavated, usually thirty centimeter deep, and made of polypropylene liner covered with a liner of high density polyethylene [16]. In raceway ponds, algae are cultured under similar conditions to their natural environment. Optimal culture medium, light intensity, temperature, water, and CO_2 concentrations required to achieve higher biomass productivity and biomass concentration [2]. Photoautotrophic microalgae are strictly photosynthetic; however, heterotrophic algae species are capable of growing in the dark using organic carbons as energy and carbon sources. Economical way of algal cultivation for oil based fuel depends on the source of water, fertilizer, and organic carbon source [17-18].

Harvesting of microalgal biomass is one of the bottlenecks for biofuel production from microalgae [19]. Harvesting alone, accounts for 20-30% of the total production cost [20]. Moreover, the harvesting process depends on the selected type of algae. Therefore, the challenge is to develop or to modify a process that does not depend on the algal specie, it should reduce energy consumption, and diminish the use of chemicals. Existing harvesting strategies include: centrifugation, gravity sedimentation, filtration, flocculation, electrolytic process, flotation, electrophoresis techniques, and any combination of these [21]. Most of these harvesting methods are mechanical, chemical, biological, and electrical.Biological harvesting are emerging techniques that can lead to further reduction of operational

cost [22]. Each harvesting method has its own advantages and disadvantages, and the efficiency of a method can be increased if combined with another method [20,22]. The integration of sedimentation and flocculation as well as the combination of electro-flotation with dispersed-air flotation are recommended; however, the last mentioned combination has higher performance if the microalgae specie, *Botryococcus braunii*, is used [23]. After all, there is not a universal harvesting technique with the same efficiency to harvest all microalgal strains [22]. There is no single best method for harvesting microalgae. The choice of preferable harvesting technology depends on algae species, growth medium, algae production, end product, and production cost benefit. Algae size is an important factor since low-cost filtration procedures are presently applicable only for harvesting fairly large microalgae. Small microalgae should be flocculated into larger bodies that can be harvested by one of the methods mentioned above. However, the cells' mobility affects the flocculation process, and addition of nonresidual oxidants to stop the mobility should be considered to aid flocculation. The decision between sedimentation or flotation methods depends on the density difference between the algae cell and the growth medium. For oil-laden algae with low cell density, flotation technologies should be considered. Moreover, oxygen release from algae cells and oxygen supersaturation conditions in growth medium support the use of flotation methods. If high-quality algae are to be produced for human consumption, continuous harvesting by solid ejecting or nozzle-type disc centrifuges is recommended. These centrifuges can easily be cleaned and sterilized. They are suitable for all types of microalgae, but their high operating costs should be compared with the benefits from their use. Another basic criterion for selecting the suitable harvesting procedure is the final algae paste concentration required for the next process. Solids requirements up to 30% can be attained by established dewatering processes. For more concentrated solids, drying methods are required. The various systems for algae drying differ both in the extent of capital investment and the energy requirements. Selection of the drying method depends on the scale of operation and the use for which the dried product is intended. [24]. Table 1 shows different algal dewatering processes for algal harvesting.

Dewatering Process	Highest Possible Yield	Reference
Flocculation	> 95% removal of microalgae	[25]
Centrifugation	up to 22% TSS, 80%-90% clarification, >95% cell harvested efficiency, self-cleaning disk stack centrifuge, 12% TSS	[24, 26-27]
Gravity sedimentation	0.5%-1.5% TSS	[24]
Filtration and screening (natural filtration)	1%-6% TSS	[28]
Filtration and screening (pressure filtration)	5%-27% TSS	[28]
Tangential flow filtration	70%-89% microalgal recovery	[29]
Flocculation-flotation	Dissolved air flotation, 1%-6% TSS, dispersed air flotation, 90% microalgae removal	[24, 30]
Electrocoagulation	95% microalgae remocal, 99.5% TSS	[31]
Electroflotation	3%-5% TSS	[24]
Electrolytic flocculation	>90% microalgae removal	[32]

Table1: Different dewatering processes and respective yield [33].

Microalgal harvesting involves the concentration of dilute microalgal suspensions, typically 0.02%–0.06% total suspended solids TSS into slurry or paste with concentrations of 5%–25% TSS or more depending on the target process objective. The desired microalgal concentration can be achieved by a one or two step harvesting process, which involves a primary harvesting step that forms a slurry of 2%–7% TSS followed by a secondary dewatering step that produces an algal paste of 15%–25% TSS. The concentration obtained in the harvesting steps is crucial to the overall process as it influences the subsequent drying process. Microalgae can generally be considered as particles whose stability is due

to surface charge, which is electronegative for *p*H of 2.5–11.5, steric effects due to water molecules bound to the microalgal surface, and adsorbed macromolecules or extracellular organic matter. When compared to other particles in suspension, microalgae have the disadvantage that it is composed of several species with different characteristics such as size, shape, and motility that can influence their behavior toward treatment. This makes it difficult to name one single technique as the best for microalgal recovery [33].

Oil Extraction and Transesterification

Microalgae are a third generation biofuel feedstock, and its lipid content varies species to species (Table 2). They can be used to generate a wide range of energy products; however, conversion of algal oil to biodiesel via transesterification reaction is the most efficient method [20]. The microalgal lipid extraction methods are divided into conventional and non-conventional methods. Among these two methods are several techniques (Table 3), which have been proven to successfully extract the triglyceride from the algal cell. Biodiesel is produced by extracting the oil from the algal cell and through transesterification. These are two separate steps that are still performed separately when using conventional methods; however, the introduction of novel techniques or non-conventional methods has combined these two steps into a single extractive-transesterification step [12, 34]. The strong algal cell boundaries make the oil extraction or cell disruption energy intensive. Microalgae cell wall is composed of different substances such as cellulose, chitin, murein, protein, silica and $CaCO_3$ [35]. Several lipid extractions techniques are available and they are selected based on efficiency, accuracy, cost effectiveness, robustness, and most importantly precision and reproducibility [36].

Transesterification is the conversion the triglycerides esters in oils to mono-alkyl esters (biodiesel) by using a monohydric alcohol and a catalyst [37]. The two major products of transesterification are esters (biodiesel) glycerol. During transesterification triglycerides are sequentially (eq.1) converted to diglycerides, monoglycerides, and finally glycerol (by product), with the alkyl ester being produced at every step [38]. From equation 1: R1, R2, and R3 radicals represent long chain hrydrocarbon known as fatty acids. The major challenge for industrial commercialized algal biodiesel production is the costly recovering of the oil from the microalgae prior to converting it into biodiesel [39]. Continuous problems of fatty acid alkyl esters (FAAE) from microalgae include the high water content in biomass, the necessity of biomass pretreatment, the high free fatty acid content (FFA), low lipids content, and the high complexity of microalgae lipids [40]. However, the transesterification process (Figure 2) reduces significantly the biodiesel production by-products resulting in higher quality and quantity FAAEs by facilitating the conversion of the biomass oil to biodiesel eliminating the solvent extraction step.

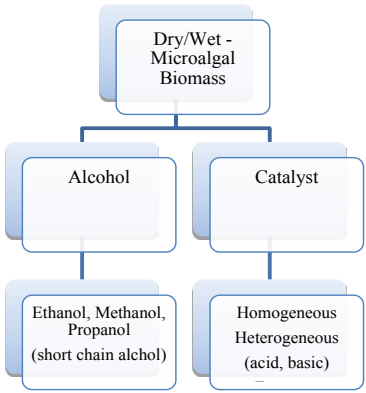

Figure 2: Direct transesterification process.

Freshwater species	(% dry wt)	Marine algae species	(% dry wt)
Botryococcus sp.	25 - 75	*Dunaliella salina*	6-25
Chaetoceros muelleri	34	*Dunaliella primolecta*	23
Chaetoceros calcitrans	15 - 40	*Dunaliella tertiolecta*	18 -71
Chlorella emersonii	25 - 63	*Dunaliella sp.*	18 – 67
Chlorella protothecoides	15 -58	*Isochrysis galbana*	7-40
Chlorella sorokiniana	19 - 22	*Isochrysis sp.*	7-33
Chlorella vulgaris	5-58	*Nannochloris sp.*	20 – 56
Chlorella sp.	10-48	*Nannochloropsis oculata*	23 – 30
Chlorella pyrenoidosa	2	*Nannochloropsis sp.*	12-53
Chlorella sp.	18 - 57	*Neochloris oleoabundans*	29 – 65
Chlorococcum sp.	20	*Pavlova salina*	31
Ellipsoidion sp.	28	*Pavlova lutheri*	36
Haematococcus pluvialis	25	*Phaeodactylum tricornutum*	18 – 57
Scenedesmus obliquus	11-55	*Spirulina platensis*	4-17
Scenedesmus quadricauda	2-19		
Scenedesmus sp.	20 - 21		

Table 2: Microalgae and lipid content [41-46].

Method	Microalgae	Biodiesel Yield (%)	Reaction Time	Reference
Soxhlet	*Nannonchloropsis gaditana*	88.2	20 hours	[47]
Bligh & Dyer Ultrasonication	*Nannonchloropsis gaditana*	43.5		[47]
Bligh & Dyer	*Nannochloropsis gaditana*	37.5		[47]
Direct transesterification (Ultrasonic Bath)	*Nannochloropsis gaditana*	100	90 min	[47]
Subcritical condition (in situ)	*Chlorella vulgaris*	89.71	4hr	[48]
Two-step in situ (pre-esterification process using heterogeneous catalyst followed by the base catalyzed transesterification)	*Chorella sorokiniana*	94.87	10 min	[49]
Conventional heating (base catalyst)	*Chlorella vulgaris*	77.6	75 min	[38]
Conventional heating (acid catalyst)	*Chlorella vulgaris*	96.9	20 hours	[38]
Step-wise methanol with enzymatic catalyst	*Scenedesmus obliquus*	90.81	6 hours	[50]
Two-step esterification (1st step- oxydized pyrite catalyzed esterification of FFAs and 2nd step- alkaline mediated tansesterification of TGs)	*Chlorella sp.*	90	1 hour	[51]
Simultaneous MW and US	*Chlorella vulgaris*	93.07	45 min	[52]
Solvent extraction-conventional heating	*Botryococcus braunii*	80.1	2 hours	[53]
Subcritical methanol: single-step conversion	*Nannochloropsis gaditana*	59.28	90 min	[54]
Microwave Irradiations with hexane as a solvent	*Chlorella sp.*	94.3	6 min	[55]
Microwave Irradiations	*Inoculum Nannochloropsis sp.*	80.13	6 min	[11]
Ultrasound Irradiations	*Chlorella sp.*	95	6 min	[34]

Table 3: Yield percentages for different microalgae biodiesel production.

Conventional Techniques

One of the challenges in utilizing microalgae to make biodiesel is the complexities of extracting the lipids using organic solvents followed by transesterification of the extracts to biodiesel [56]. Conventional techniques convert algal triglycerides to biodiesel by first extracting the lipid using organic solvents such as hexanes, chloroform, and methanol. The triglycerides are then reacted with acid or base and an alcohol to make FAAE [56-58]. In addition, conventional lipid extraction techniques are time consuming and labor intensive making microalgal oil extraction almost non-feasible [23]. Other major disadvantages of

biodiesel production are the high temperature requirements and relative slow reaction rate. Moreover, conventional methods required large volume solvents and is nearly impossible to scale-up [59].

Four of the most commonly used conventional extraction methods are Folch [60], Radin and Hara [61], Chen [62], and Bligh and Dyer [63], which are generally used as bench marks for comparison to non-conventional techniques [34]. Bligh and Dyer is the most common one, and it is a three-step solvent extraction method where the total lipids can be easily determined by the co-solvent mixture of chloroform and methanol. However, this method has adverse effect on the environment due to the use of chloroform. With the same environmental disadvantages as Bligh and Dyer, Folch is one-step solvent extraction commonly used to determine the lipid amount. Folch [60] chloroform-based lipid extraction protocol is efficient for most microalgal lipid analysis. However, chloroform-based lipid extraction is effective for the lab-scale exercises, but an alternative organic solvent such as hexane is easier to handle and more cost effective [64-65]. Similarly to Folch method, Hara and Radin is a one-step solvent extraction method, which uses solvents that are less toxic and cheaper than chloroform and methanol, no interference in the processing by proteolipid protein, extract contain less non-lipids compared to chloroform-methanol extracts of Folch. However, this method is labor-intensive and there is no extraction of gangliosides a minor fraction of total lipids), Chen et al., [62] modified the Folch et al. method using dichloromethane instead of chloroform [66]. Soxhlet extraction is another conventional method considered a baseline for the comparison of lipid extraction methods. During the Soxhlet extraction process, the solvent is vaporized, condense, and percolated through the dried and ground sample repeatedly until the extraction limit has been achieved [67].

Several conventional methods have been modified to improve lipid yield extraction. For example, as previously mentioned, biodiesel production requires heat. Therefore, several conventional heat methods, such as hot plates, have been developed to extract algal oil. Even though, some conventional heating methods are very efficient on increasing the biodiesel yield, they are not apt to achieve an efficient performance other than lab-scale. For that and many more reasons, non-conventional heating methods are being research. Microwave and ultrasound irradiations [68-77], are the most innovative and efficient non-conventional heating method. In conventional heating, heat transfer occurs from the outside to the inside whilst mass transfer occurs from the inside to the outside; however, in microwave assisted solvent extraction occurs from the inside of the extracted material to the bulk solvent [66,78,79]. Microwave and ultrasound irradiation advantages and disadvantages are discussed in the following section. In addition to conventional heating methods, there are jacketed heating, water, and oil bath heating.

Algal cell lipid extraction mechanical pressing (i.e. cell homogenizers, bead mills, autoclave, and spray drying) is a microalgae inefficient process due to the small size of the cells which are able to bypass the press [67]. Among the non-mechanical methods are freezing, the previously discussed organic solvents, osmotic shocks, and catalytic reactions. All conventional methods are capable of microalgae oil extraction, and the method choice depends on the type of microalgal cell grown and thickness of the cell walls impeding liberation of intracellular lipids [80], and more importantly, it depend on the desired conversion scale.

Non-conventional Techniques

Non-conventional technologies for algal biomass processing include microwave and ultrasound irradiations, non-catalytic and solvent free reactions, and sub and supercritical methods. These methods proved to be advantageous over conventional methods of jacketed heating, water and oil bath heating. The vast majority of these methods reduce energy consumption, reaction time, and chemical and volume use. However, supercritical transesterification [80] requires higher volume of solvent/reactant, which is difficult to recover. Also, even though supercritical method has the benefit of no catalyst required; it

may be disadvantageous due to the adverse process economics as well as safety concerns related to the reaction condition [81]. A non-catalytic biodiesel production route with supercritical methanol allows a simple process and high lipid yield because of simultaneous transesterification of triglycerides and methyl esterification of fatty acids [82-83]. A fluid is considered supercritical when its temperature and pressure go above its critical point [84]. Another supercritical method is the supercritical carbon dioxide ($SCCO_2$) extraction, which has low toxicity of the supercritical fluid, tuneable solvating power, favorable mass transfer equilibrium due to intermediate diffusion/viscosity properties of the fluid, and the production of solvent-free extract [85-89]. In addition to conventional methods, pyrolysis decomposes biomass under the condition oxygen deficiency and high temperatures. This process was first used for the production of bio-oils or bio-gases from lignocelluloses. Fast pyrolysis is a new technology, which produces bio-fuel in the absence of air at atmospheric pressure with a relatively low temperature (450-550 °C) and short heating rate (10^3-10^4 $°Cs^{-1}$) as well as short gas residence time to crack into short chain molecules and be cooled to liquid rapidly. However, it has been proven that non-catalytic hrydroyrolysis is an effective process to convert microalgae into liquid fuels with higher quality than fast pyroloysis [90].

Transesterification through non-conventional methods is usually referred to *in-situ* transesterification. The *in-situ* transesterification method has the potential to simplify the conversion process of disrupting the rigid cell wall, reducing the number of unit operations and consequently the overall process costs and consequently the overall process costs and final biodiesel product costs [12,91-92]. In situ transesterification is an efficient way to convert oil-bearing biomass to biodiesel directly, hence, elimination the extraction step which is required in the conventional method [93]. During this process, the alcohol may weaken the cellular and lipid body membranes to facilitate the FAME conversion [91]. Interestingly, in situ transesterification do not degrade the protein in the feedstock meal, while they may degrade components found on the meal. Additionally, in situ transesterification reduces the amount of phospholipids to a level below detection [56,94].

Microwave and ultrasound irradiations are the most commonly used techniques to heat microalgae cells to extract its lipid. They are both non-conventional heating techniques that have demonstrated that they can tremendously enhance microalgae biodiesel production. Microwaves and ultrasound can benefit from shorter extraction and transesterification times and low solvent requirements [95].

Microwave and Ultrasound Irradiation

Microwaves are extremely high frequency radio waves that can cause certain molecules to vibrate around a billion times per second [59]. Microwave irradiation is an unconventional energy source, and it has been proven that transesterification with base homogeneous a catalyst is significantly accelerated under microwave irradiation [14]. This advantageous technology has been reportedly the most simple and most effective method for microalgae lipid extraction among several tested methods [96]. Using microwave irradiation as a selective and instantaneous heating method has many obvious advantages for preparing biodiesel. Microwaves accelerate the disruption of microalgae cells and enable easier lipid release. Microwave irradiation is also a faster and more efficient heating process, which directly contributes to molecular diffusion and mass transfer. In addition, methanol is strong microwave absorption solvent. Thus, microwave irradiation can promote the transesterification reaction. [97].This heating method is able to quickly extract the lipids and simultaneously convert them into FAEE or FAMEs [98]. The microwave effect is two-fold in the extractive-transesterification reaction: first, a thermal effect caused by the microwaves increases the extractive properties of the alcohol to extract the oils from the algal biomass in suspension (diffusive extraction) and next, an extended microwave effect causes the penetration through the cell walls and forces out the oils (from the biological matrix) [12, 34].

Microwave heating is a non-contact heat source, which heats the whole sample volume simultaneously as compared to conductive heating after being reported for extraction of chemicals from environmental matrices. Microwaves transfer the energy into the materials by dipolar polarization, ionic conduction, and interfacial polarization mechanisms and cause localized rapid and superheating of the reaction materials. If a molecule possesses a dipole moment, then, when it is exposed to microwave irradiation, the dipole tries to align with the applied electric field. [99]. The microwave interaction with the reaction compounds (triglycerides and alcohol) results in large reduction of activation energy due to increased dipolar polarization phenomenon [100]. This is achieved due to molecular level interaction of the microwaves in the reaction mixture resulting in dipolar rotation and ionic conduction [101-102]. The amount, by which the activation energy is reduced, is essentially dependent on the medium and reaction mechanism [13, 100].

Ultrasounds can be a valuable tool for the transesterification of fatty acids, aiming to prepare the biodiesel fuel at industrial scale [103]. It is used as as a method for recovery of high purity eicosapentaenoic acid esters from microalgae [104-105]. The association of the cold methods with ultrasound extraction has contributed to the increase the amount of oil extracted from the microalgal biomass. The chemical effect of cavitation is the generation of highly reactive radicals due to dissociation of the entrapped vapor molecules in the cavitation bubble. These form bubbles, filled with solvent and solute vapor and dissolved gases, which grow and recompress. Under proper conditions, ultrasonic cavitation leads to implosive cavitation bubble collapse, producing intense local heating, high pressures and very short lifetimes. The collapse of the cavitation bubbles gives rise to acoustic micro streaming or formation of small eddies that increase the mass and heat transfer in the liquid, and cause velocity gradients that result in shear stresses [106]. Furthermore, the implosion of the cavitation bubbles caused by ultrasound inside and outside an organism may contribute to rupture of cell walls [107-108]. In addition, the formation, growth, and implosive collapse of micro bubbles induced acoustically in the bulk of the liquid phase increase the mass transfer between the two phases by supplying both heating and mixing [109].

Cavitation causes a localized increase in temperature at the phase boundary and provides the mechanical energy for mixing and the required activation energy for initiating the alcoholysis reaction. The collapse of the cavitation bubbles disrupts the phase boundary and causes emulsification by ultrasonic jets that impinge one liquid to another. These effects speed up the alcoholysis reaction rate and shorten its duration, while high final yields of biodiesel are usually achieved [69, 77, 110]. The normal chain alcohols react quite rapidly under ultrasonic irradiation. The velocity of an ultrasonic wave through a material depends on its physical properties. Hence, the ultrasonic velocity decreases with increasing density. Thus, the use of ultrasound enhances the reaction rate and also shifts the equilibrium which results in higher product yields in shorter reaction times. The ultrasound wave frequency seems to affect the reaction rate and the biodiesel yield. The use of ultrasound has an extra advantage as it requires one-third to a half of the energy that is consumed by mechanical agitation [109,111-112]. Transesterification through ultrasonication can be carried out in a water bath (indirect sonication) or using a horn (direct sonication). Direct sonication has been proven to be more efficient resulting in high biodiesel yield [69, 77,110].

Catalytic and non-catalytic Extraction

Catalyst used for transesterification of microalgal oil includes homogeneous and heterogeneous chemical catalyst and enzyme (lipase). Heterogeneous catalysts, either solid acids or solid bases are less corrosive than homogeneous catalyst. However, better results can be obtained if the porosity of the heterogeneous catalyst is higher. Heterogeneous bases proceed by reaction of either the Lewis or the Brønsted basic sites of the catalyst with a monohydric alcohol, and the generated alkoxide mixture interacts with the esters in the oil to yield biodiesel [37).

Sodium hydroxide (NaOH) and potassium hydroxide (KOH) are the most widely used homogenous base catalysts, and they are very efficient on achieving very high conversion rates in short reaction time (2 min); however, the downside of alkali catalyzed process is saponification, the formation of undesirable amounts of soap. Another disadvantage of basic catalysts is they are highly corrosive. The alkali mediated reactions consist of three steps (Figure 3): 1) the base reacts with the alcohol to produce an alkoxide and the protonated catalyst; 2) the nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride generating a tetrahedral intermediate, from which the alkyl ester and the corresponding anion of the diglyceride are formed; and 3) the latter deprotonates the catalyst can react with second molecule of alcohol and start another catalytic cycle [82,84]. Base catalyst is known to react faster than acids; however, acid catalysts are more efficient converting high FFA feedstock into biodiesel [113].

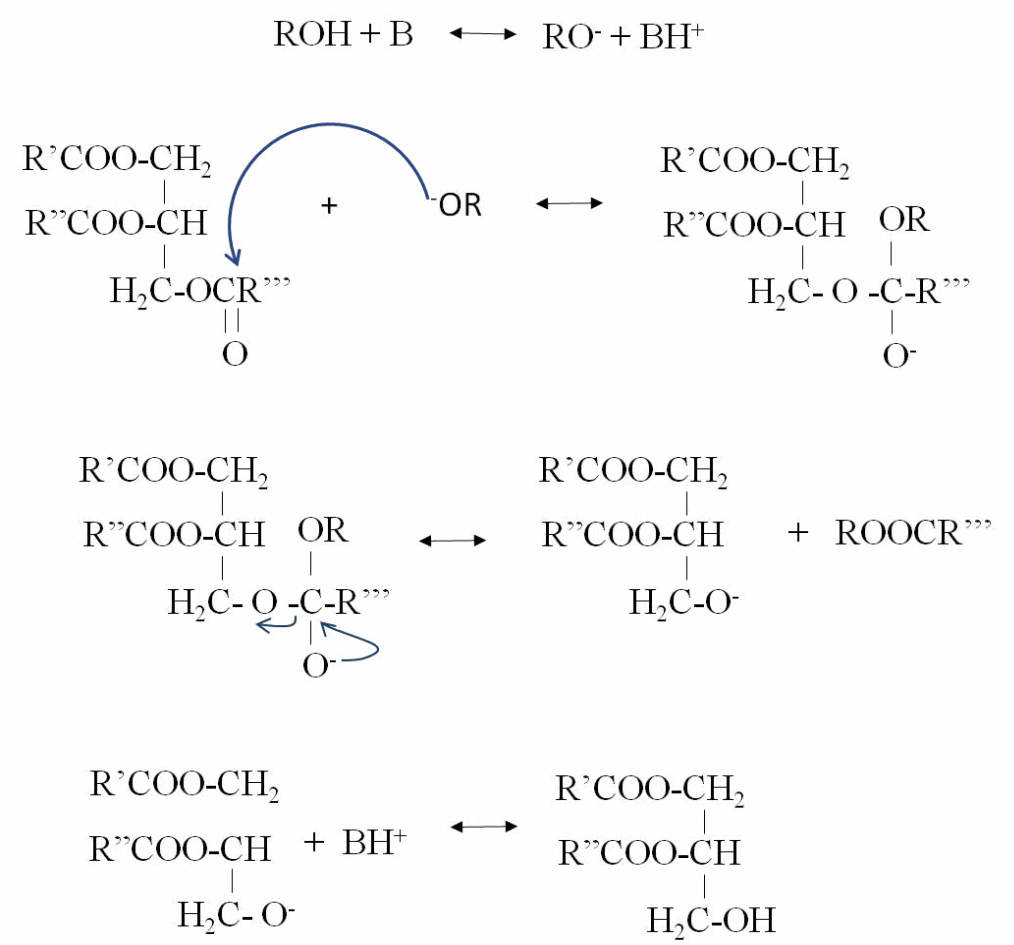

Figure 3: Base catalyst transesterification.

The transesterification process by acids (Figure 4) gives vsery high biodiesel yield and there is not soap formation during the process, but the reactions are slow. Some homogenous acid catalysts are H_2SO_4, HCL, BF_3, H_3PO_4, and some organic sulfonic acids [84]. The addition of the acid catalyst leads to protonation of the carbonyl group of the ester those results in carbocation which after nucleophilic attack of the alcohol produces the

tetrahedral intermediate, which eliminates glycerol to form the new ester and regenerates the catalys H^+ [84]. Nevertheless, acid catalyst required longer reactions time and higher catalyst amounts, which make it more expensive than basic catalysts. The conversion of lipids to biodiesel using an acid catalyst is also known as esterification.

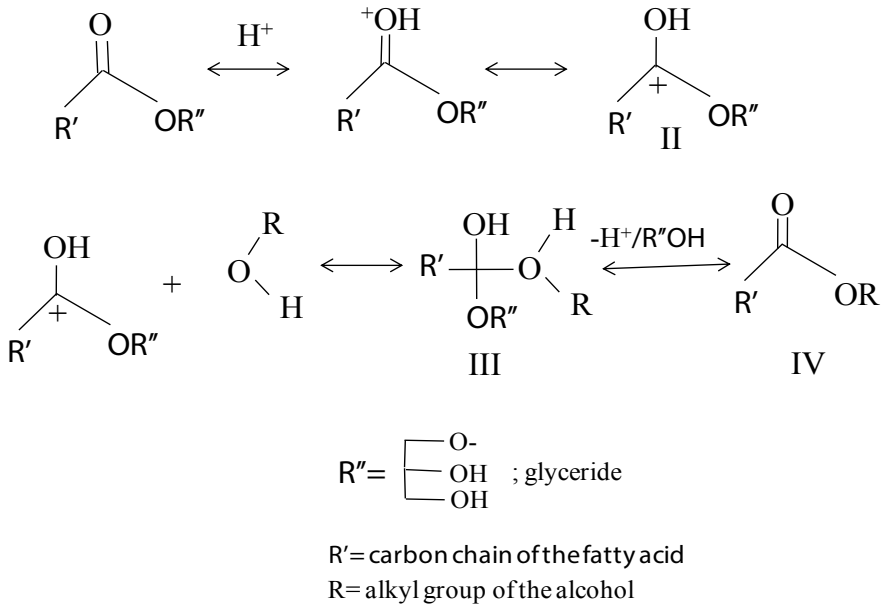

Figure 4: Acid-catalyzed transesterification.

Enzymatic catalysts have also gained attention on biodiesel production. The use of enzymatic catalyst can help to reduce the quantity of methanol, to avoid problems associated to the presence of free fatty acids (FFA), and the circumvent environmental problem caused for the application of chemical catalyst [114-115]. However, lipases has slower reaction rate than chemical catalysts due to its inhibition and mass transfer limitation, and it is also more expensive than other catalysts [116]. Lipase-catalyzed transesterification to biodiesel is a green and sustainable approach because of its environmental-benign nature and low energy requirement [50]. They can esterify FFAs and transesterify TGs [117]. Lipase can be used for conversion of feedstock oil with high FFA [118]. Enzymatic catalyzed transesterification requires mild environment and can tolerate the presence of water and free acids in crude oil [66]. They are divided into three categories: fatty-acid type specific lipases, region- or positional specific lipases, and specific lipases for a certain class of acylglycerols [66].

Solvent/Reactant Role in Biodiesel Production

The key to algal lipid extraction is an effective solvent which can firstly penetrate the solid matrix enclosing the lipid, secondly physically contact the lipid and thirdly solvate the lipid [79]. Solvent extraction methods remove oil from the algal cell via diffusion [119]. A suitable solvent should be insoluble in water, preferentially solubilize the compound of interest, have a low boiling point to facilitate its removal after extraction, and have a considerably different density than water. Also, for process cost-effectiveness, it should be easily sourced, as well as inexpensive and reusable. Therefore, organic solvents, such as benzene, cyclohexane, hexane, acetone and chloroform have shown to be effective when used on microalgae biomass; they degrade microalgal cell walls and extract the oil because oil has a high solubility in organic solvents [120]. Hexane is a popular extraction solvent

for non-polar materials such as lipid, and methane is used to achieve better extraction performance. Solvent extraction entails extracting oil from microalgae by repeated washing or percolation with an organic solvent. However, hexane is the most common solvent of choice for large-scale lipid extractions due to its cost-effectiveness [121]. When extracting lipids from microalgae hexane is less toxic than chloroform, has minimal affinity towards non-lipid contaminants and has higher selectivity towards neutral lipid fractions that can be converted to biodiesel using existing technology [14, 121-124].

In the in situ transesterification process, alcohols perform a vital role, acting as both the solvent, extracting the lipids from the biomass and as the reactant [56]. The energy requirements also depend on the total volume of the reactants as well as the type of the reactants [12]. As previously mentioned, transesterification requires short chain alcohols such as methanol, ethanol, and propanol. Methanol and ethanol produce superior results in transesterification reaction [68]. The physical and chemical properties and the performance of ethyl esters are comparable to those of methyl esters. They both have almost the same heat content; however, the viscosity of the ethyl esters is slightly higher, and the cloud and pour points are slightly lower than those of methyl esters [125]. However, methanol is the most commonly used due to its low cost. One of the disadvantages of methanol is that it is produced from fossil fuel sources and mineral oils [126], while ethanol can be derived from renewable sources. It has been proven that the mixture of methanol and ethanol may benefits from ethanol's better solvent properties and methanol's faster equilibrium conversion kinetics. Therefore, this combination improves the lubricity of the produced esters.

Although there is energy required to recover hexane and alcohols for solvent recycling and lipid separation, the amount of required energy is significantly smaller than that for drying of microalgal biomass, since both hexane and alcohols necessitate much lower heat of evaporation than water. Increasing the polarity of organic solvent by differentiating ratio between a polar solvent (i.e. methanol) and a non-polar solvent (n-hexane) have shown significant effect on lipid recovery. [127]. Unsaturated FAs are preferentially extracted with decreasing polarity of the solvent use [128]. The combination of polar and non-polar solvents enhances the extraction of both polar and non-polar lipids [23].

Separation and Purification

Downstream processes of biodiesel produced from the transesterification process depend on the type and method of catalyst. Three important aspects require specific attention: the purification of the main product, biodiesel, the by-products, glycerol, soap, excess alcohol and trace amounts of water, and the waste products. Effective downstream processing is important to recover energy that can be recouped from the entire process [11]. The efficiency of utilizing the residue left after the lipid extraction has a significant influence on the overall energy balance of the microalgae-to-fuel conversion [129-130]. In addition to the biodiesel and glycerol formation during the transesterification process, significant amounts of solid microalgae biomass residues are produced [131]. This microalgal biomass is protein-rich, and it has the potential of to be used in livestock feeds where they could replace the more commonly used soy and fish meal [131]. The residual alcohol and catalyst can be recycled to the reactor. The by-product glycerol, which is 10% of the overall products [132], is either purified and recycled into the system or sold as crude glycerol. Even without the purification process, the crude glycerol has high potential as a carbon source for microalgal cultivation [133].

The cost removal and recovery of high amounts of unreacted alcohol contribute predominantly to process costs greater than those for oil extraction and transesteirication by contemporary biodiesel synthesis methods. Overall the process is highly energy intensive thus increasing in terms of production cost, the catalyst needs to be removed from the production by purification or separation steps, Biomass harvesting, lipid extraction,

separation and concentration can result in loss of some lipids [23,134-135]. Therefore, more research is necessary to enhance the purification of the bio-crude, and to ease the byproduct separation process.

Conclusion

Algae clearly have the potential to serve as renewable fuel factories in the very near future. This has yet to be realized through important developments in many concerned areas. For example, algal biomass and lipid accumulation yields need to be increased systematically to improve downstream process economics. Developments in selective high value bioproducts recovery schemes should be assigned priority. Concerted efforts in all areas of algal biomass production and utilization should focus on increasing the energy, environmental and economic benefits. Currently, algal biodiesel production is not feasible economically even if reliable supplies of algal biomass can be ensured. Energy-efficient process reaction and separation schemes based on novel techniques such as microwaves and ultrasound should be exploited. Enhancement of process benefits to include production of bulk chemicals, food and feed ingredients and health supplements and pigments should be pursued aggressively. Alternatively, integrated recovery of various algal biofuels such as biodiesel, bioethanol and biogas from the algal carbohydrates, starch, protein and lipids in a systematic manner could improve the energy and environmental footprint of the algal biorefineries.

References

1. Vicente G1, Martínez M, Aracil J (2004) Integrated biodiesel production: a comparison of different homogeneous catalysts systems. See comment in PubMed Commons below Bioresour Technol 92: 297-305.
2. Feng P, Yang K, Xu Z, Wang Z, Fan L, et al.(2014) Growth and lipid accumulation characteristic of Scenedesmus obliquus in semi-continuous cultivation outdoors for biodiesel feedstock production. Bioresource Technology 173, 406-414.
3. Ma F, Hanna MA (1999) Biodiesel production: a review. Bioresource Technology 70, 1-15.
4. Arun N, Singh DP (2012) Microalgae: The future fuel. J. Algal Biomass Utln 3, 46-54.
5. Dai YM, Chen, KT, Chen CC (2014) Study of the microwave lipid extraction from microalgae for biodiesel production. Chemical Engineering Journal 250: 267-273.
6. Boussiba S, Vonshak A, Cohen Z, Avissar Y, Richmond A (1987) Lipid and biomass production by the halotolerant microalga Nannochloropsos salina. Biomass 12, 37-47.
7. Chisti Y (2007) Biodiesel from microalgae. See comment in PubMed Commons below Biotechnol Adv 25: 294-306.
8. Mubarak M, Shaija S, Suchithra T V (2015) A review on the extraction of lipid from microalgae for biodiesel production. Algal Research 7, 117-123.
9. Guldhe A, Singh B, Rawat I, Bux F (2014) Synthesis of biodiesel from Scenedesmus sp. by microwave and ultrasound assisted in situ transesterfication using tungstated zirconia as a solic acid catalyst. Chem. Engr. Research and Design 92, 1503-1511.
10. Sun A, Davis R, Starbuck M, Ben-Amotz A, Pate R, Pienkos PT (2011) Comparative cost analysis of algal oil production for biofuels. Energy 36, 5169–5179.
11. Suali E, Sarbatly R (2012) Conversion of microalgae to biofuel. Renewable and Sustainable Energy Reviews 16, 4613-4342.
12. Martinez-Guerra E, Gude VG, Mondala A, Holmes W, Hernandez R (2014) Extractive-transesterification of algal lipids under microwave irradiation with hexane as solvent. See comment in PubMed Commons below Bioresour Technol 156: 240-247.
13. Patil PD, Gude VG, Mannarswamy A, Cooke P, Nirmalakhandan N, et al.(2012) Comparison of direct transesterification of algal biomass under supercritical methanol and microwave irradiation conditions. Fuel 97, 822-831.
14. Martinez-Guerra E, Gude VG (2014) Synergistic effect of simultaneous microwave and ultrasound irradiations on transesterification of waste vegetable oil. Fuel 137, 100-108.
15. Wen Z, Johnson, M B (2009) Microalgae as a feedstock for biofuel production. Virginia Cooperative Extension, Publication 442-886, 1-7.
16. Collet P, Lardon L, Helias A, Bricout S, Lombaert-Valot I, et al., (2014) Biodiesel from microalgae- Life cycle assessment and recommendations for potential improvements. Renewable Energy 71, 525-533.
17. Yang C, Hua Q, Shimizu K (2000) Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochem. Eng. J. 75, 87-102.

18. Mohan SV, Rohit MV, Chiranjeevi P, Chandra R, Navaneeth B, et al. (2015) Heterotrophic microalgae cultivation to synergize biodiesel production with water remediation: Progress and perspectives. Bioresource Technology 184, 169-178.

19. Bhatt NC, Panwar A, Bisht TS, Tamta S (2014) Coupling of algal biofuel production with wastewater. See comment in PubMed Commons below ScientificWorldJournal 2014: 210504.

20. Pragya N, Pandey KK, Sahoo PK (2013) A review on harvesting, oil extraction and biofuels production technologies from microalgae. Renewable and Sustainable Energy Reviews 24, 159-171.

21. Rawat I, Ranjith Kumar R, Mutanda T, Bux F (2011) Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Appl Energy 88, 3411–24.

22. Barros AI, Goncalvez AL, Simoes M, Pires JCM (2015) Harvesting techniques applied to microalgae: A review. Renewable and Sustainable Energy Reviews 41, 1489-1500.

23. Rawat I, Kumar RR, Mutanda T, Bux F (2013) Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. Applied Energy 103, 444-467.

24. Shelef G, Sukenik A, Green M (1984) Microalgae harvesting and processing: A literature review. A subcontract report for Solar Energy Research Institute. Golden, Colorado. 1-71.

25. Bilanovic D, Shelef G (1988) Flocclulation of microalgae cationic polymers: Effects of medium salinity. Biomass 17, 65-76.

26. Borowitzka MA (1992) Algal biotechnology products and processes: Matching science and economics. J. Appl. Psychol. 4, 267-279.

27. Heasman M, Diemar J, O'Connor W, Sushames T, Foulkes L, et al. (2002) Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve mollusks: A summary. Aquaculture Research 31, 637-659.

28. Semerjian L, Ayoub GM (2003) High-pH-magnesium coagulation-flocculation in wastewater treatment. Adv. Environ. Res. 7, 389-403.

29. Bernhardt H, Clasen J (1994) Investigation into flocculation mechanisms of small algal cells. Aqua J. Water Supply Res Technol 43, 222-232.

30. Sukenik A, Shelef G (1984) Algal autoflocculation--verification and proposed mechanism. See comment in PubMed Commons below Biotechnol Bioeng 26: 142-147.

31. Danquah MK, Ang L, Uduman N, Moheimani N, Forde GM, et al. (2009) Dewatering of microalgal culture for biodiesel production: Exploring polymer flocculation and tangential flow filtration. J. Chem. Technol. Biotechnol. 84, 1078-1083.

32. Edzwald JK (1995) Principles and applications of dissolved air flotation. Water Sci. Technol. 31, 1-23.

33. Uduman N, Qi Y, Danquah MK (2010) Dewatering of microalgal cultures: A major bottleneck to algae fuels. J of Renwable and Sustainable Energy 2, 1-15.

34. Martinez-Guerra E1, Gude VG2, Mondala A3, Holmes W4, Hernandez R4 (2014) Extractive-transesterification of algal lipids under microwave irradiation with hexane as solvent. See comment in PubMed Commons below Bioresour Technol 156: 240-247.

35. Okuda K (2002) Structure and phylogeny of cell coverings. See comment in PubMed Commons below J Plant Res 115: 283-288.

36. Viswanath B, Mutanda T, White S, Bux F (2010) The microalgae – a future source of biodiesel. Dynamnic Biochem, Process Biotechnol Mole Biol 4, 37–47.

37. Galadima A, Muraza O (2014) Biodiesel production from algae by using heterogeneous catalysts: A critical review. Energy 1-12.

38. Velasquez-Orta SB, Lee JGM, Harvey A (2012) Alkaline in situ transesterification of Chlorella vulgaris. Fuel 94, 544–550.

39. Wahidin S, Idris A, Raehanah SRM (2014) Rapid biodiesel production using wet microalgae via microwave irradiation. Energy Conversion and Management 84, 227-233.

40. Huang GH, Chen F, Wei D, Zhang XW, Chen G, et al. (2010) Biodiesel production by microalgal biotechnology. Appl Energ 87, 38–46.

41. Dragone G, Fernandes B, Vicente AA, Teixeira JA (2010) Third generation biofuels from microalgae in Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Mendez-Vilas A (ed.), Formatex, 1355-1366.

42. Chisti Y (2007) Biodiesel from microalgae. See comment in PubMed Commons below Biotechnol Adv 25: 294-306.

43. Sydney EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, et al. (2010) Potential carbon dioxide fixation by industrially important microalgae. See comment in PubMed Commons below Bioresour Technol 101: 5892-5896.

44. Koh LP, Ghazoul J (2008) Biofuels, biodiversity, and people: understanding the conflicts and finding opportunities. Biological Conservation 141, 2450-2460.

45. Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J (2008) Second generation biofuels: high efficiency microalgae for biodiesel production, BioEnergy Research 1, 20-43.

46. Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N, et al.(2008) Biofuels from microalgae. See comment in PubMed Commons below Biotechnol Prog 24: 815-820.

47. Ríos SD, Castañeda J, Torras C, Farriol X, Salvadó J, et al. (2013) Lipid extraction methods from microalgal biomass harvested by two different paths: screening studies toward biodiesel production. See comment in PubMed Commons below Bioresour Technol 133: 378-388.

48. Tsigie YA, Huynh HL, Ismadji S, Engida AM, Ju YH, et al. (2012) In situ biodiesel production from wet Chlorella vulgaris under subcritical condition. Chemical Engineering Journal 213, 104–108.

49. Dong T, Wang J, Miao C, Zheng Y, Chen S (2013) Two-step in situ biodiesel production from microalgae with high free fatty acid content. See comment in PubMed Commons below Bioresour Technol 136: 8-15.

50. Guldhe A, Singh B, Rawat I, Permaul K, Bux F (2015) Biocatalytic conversion of lipids from microalgae Scenedesmus obliquus to biodiesel using Pseudomonas fluorescens lipase. Fuel 147, 117–124.

51. Seo YH, Sung M, Oh YK, Han JI (2015) Lipid extraction and esterification for microalgae-based biodiesel production using pyrite (FeS2). See comment in PubMed Commons below Bioresour Technol 191: 420-425.

52. Ma G, Hu W, Pei H, Jiang L, Song M, et al. (2015) In situ heterogeneous transesterification of microalgae using combined ultrasound and microwave irradiation. Energy Conversion and Management 90, 41-46.

53. Hidalgo P, Ciudad G, Mittelbach M, Navia R (2015) Biodiesel production by direct conversion of Botryococcus braunii lipids: Reaction kinetics modeling and optimization. Fuel 153, 544-551.

54. Sitthithanaboon W, Reddy HK, Muppaneni T, Ponnusamy S, Punsuvon V, et al., (2015) Single-step conversion of wet Nannochloropsis gaditana to biodiesel under subcritical methanol conditions. Fuel 147, 253-259.

55. Patil PD, Gude VG, Mannarswamy A, Deng S, Cooke P (2011) Optimization of direct conversion of wet algae to biodiesel under supercritical methanol conditions. Bioresource Technology 102, 1399-1405.

56. Wahlen BD, Willis RM, Seefeldt LC (2011) Biodiesel production by simultaneous extraction and conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures. See comment in PubMed Commons below Bioresour Technol 102: 2724-2730.

57. Xu H, Miao X, Wu Q (2006) High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters. See comment in PubMed Commons below J Biotechnol 126: 499-507.

58. Cheng Y, Lu Y, Gao C, Wu Q (2009) Alga-based biodiesel production and optimization using sugar cane as the feedstock Energy Fuels 23, 4166–4173.

59. Cui Y, Liang Y (2014) Direct transesterification of wet Cryptococcus curvatus cells to biodiesel through use of microwave irradiation. Applied Energy 119, 438–444.

60. Folch J, Lees M, Sloane Stanley GH (1956) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226-497.

61. Radin A, Radin NS (1978) Lipid extraction of tissues with a low-toxicity solvent. Analytical Biochemistry 90, 420-426.

62. Chen IS, Shen CSJ, Sheppard AJ (1981) Comparison of methylene chloride and chloroform for the extraction of fats from food products. J of the American Oil and Chemistry Society 58, 559-601.

63. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification, Can. J.Biochem. Physiol. 37, 911–917.

64. Halim R, Gladman B, Danquah MK, Webley PA (2011) Oil extraction from microalgae for biodiesel production. See comment in PubMed Commons below Bioresour Technol 102: 178-185.

65. Bai X, Naghdi FG, Ye L, Lant P, Pratt S, et al. (2014) Enhanced lipid extraction from algae using free nitrous acid pretreatment. See comment in PubMed Commons below Bioresour Technol 159: 36-40.

66. Teo CL, Jamaluddin H, Zain NAM, Idris A (2014) Biodiesel production via lipase catalysed transesterification of microalgae lipids from Tetraselmis sp. Renewable Energy 68 (2014) 1-5.

67. De Boer K, Moheimani NR, Borowitzka MA, Bahri PA (2012) Extraction and conversión pathways for microalgae to biodiesel: A review focused on energy consumption. J of Appled Phycology 24, 1681-1698.

68. Martinez-Guerra E, Gude VG (2014) Transesterification of waste vegetable oil under pulse sonication using ethanol, methanol and ethanol-methanol mixtures. See comment in PubMed Commons below Waste Manag 34: 2611-2620.

69. Martinez-Guerra E, Gude VG (2015) Continuous and pulse sonication effects on transesterification of used vegetable oil. Energy Conversion and Management 96, 268-276.

70. Fast SA, Kokabian B, Gude VG (2014) Enhanced coagulation of algal turbid waters- Comparison between rapid mix ultrasound coagulation methods. Chemical Engineering Journal 244, 403-410.

71. Luo Z, Fang Z, Smith RL (2014) Ultrasound-enhanced conversion of biomass to biofuels. Progress in Energy and Combustion Science 41, 56-93.

72. Patil PD, Gude VG, Harvind R, Tapaswy M, Shuguang D.(2012) Biodiesel production from waste cooking oil using sulfuric acid and microwave irradiation processes p:107-113.

73. Adam F, Abert-Vian M, Peltier G, Chemat F (2012) “Solvent-free” ultrasound-assisted extraction of lipids from fresh microalgae cells: a green, clean and scalable process. See comment in PubMed Commons below Bioresour Technol 114: 457-465.

74. Cesaro A, Velten S, Belgiorno V, Kuchta K (2014) Enhanced anaerobic pretreatment of organic residues for energy production. J Clean Prod 74, 119-124.

75. Patil PD, Gude VG, Camacho LM, Deng S (2009) Microwave-assisted catalytic transesterification of Camelina satiba oil. Energy and Fuels 24, 1298-1304.

76. Park JY, Lee K, Choi SA, Jeong MJ, Kim B, et al., (2015) Sonication-assisted homogenization system for improved lipid extraction from Chlorella vulgaris. Renewable Energy 79, 3-8.

77. Martinez-Guerra E, Gude VG (2014) Transesterification of used vegetable oil catalyzed by barium oxide under simultaneous microwave and ultrasound irradiations. Energt Conversion and Management 88, 633-640.

78. Virot M, Tomao V, Colnagui G, Visinoni F, Chemat F, et al., (2007) New microwave-integrated Soxhlet extraction. An advantageous tool for the extraction of lipids from food products. See comment in PubMed Commons below J Chromatogr A 1174: 138-144.

79. Cooney MJ, Young G, Pate R (2011) Bio-oil from photosynthetic microalgae: Case study. Bioresour Technol 11, 166-177.

80. Nan Y, Liu J, Lin R, Tavlarides LL (2015) Production of biodiesel from microalgae oil (Chlorella protothecoides) by non-catalytic transesterification in supercritical methanoland ethanol: Process optimization. J. of Supercritical Fluids 97, 174–182.

81. Takisawa K, Kanemoto K, Miyazaki T, Kitamura Y, et al., (2013) Hydrolysis for direct esterification of lipids from wet microalgae. See comment in PubMed Commons below Bioresour Technol 144: 38-43.

82. Demirbas A (2005) Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods. Prog Energy Comb Sc 31, 466–487.

83. Demirbas A (2002) Diesel fuel from vegetable oil via transesterification and soap pyrolysis. Energy Sources 24, 835-841.

84. Bharathiraja B, Chakravarthy M, Kumar RR, Yuvaraj D, Jayamuthunagai J,et al., (2014) Biodiesel production using chemical and biological methods – A review of process, catalyst, acyl acceptor, source and process variables Renewable and Sustainable Energy Reviews 38, 368–382.

85. Macias-Sanchez MD, Mantell C, Rodriguez M, de la Ossa EM, Lubian LM, Montero O, et al. (2007) Supercritical fluid extraction of carotenoids and chlorophyll a from Synechococcus sp. J of Supercritical Fluids 39, 323–329.

86. Mendes RL, Reis AD, Palabra AF (2006) Supercritical CO2 extraction of gamma-linolenic acid and other lipids from Arthrospira (Spirulina) maxima: Comparison with organic solvent extraction. Food Chemistry 99, 57–63.

87. Pourmortazavi SM, Hajimirsadeghi SS (2007) Supercritical fluid extraction in plant essential and volatile oil analysis. See comment in PubMed Commons below J Chromatogr A 1163: 2-24.

88. Taylor LT (1996) Supercritical Fluid Extraction. John Wiley & Sons Inc, New York.

89. Thana P, Machmudah S, Goto M, Sasaki M, Pavasant P, et al. (2008) Response surface methodology to supercritical carbon dioxide extraction of astaxanthin from Haematococcus pluvialis. See comment in PubMed Commons below Bioresour Technol 99: 3110-3115.

90. Duan P, Bai X, Xu Y, Zhang A, Wang F, et al. (2013) Non-catalytic hydropyrolysis of microalgae to produce liquid biofuels. See comment in PubMed Commons below Bioresour Technol 136: 626-634.

91. Hass MJ, Wagner K (2011) Simplifying biodiesel production: The direct or in situ transesterification algal biomass. European Journal of Lipid Science and Technology 11, 1219-1229.

92. Jazzar S, Olivares-Carrillo P, Pérez de los Ríos A, Marzouki MN, Acién-Fernández FG, et al.(2015) Direct supercritical methanolysis of wet and dry unwashed marine microalgae (Nannochloropsis gaditana) to biodiesel. Applied Energy 148, 210–219.

93. Kim GV1, Choi W2, Kang D3, Lee S1, Lee H4 (2014) Enhancement of biodiesel production from marine alga, Scenedesmus sp. through in situ transesterification process associated with acidic catalyst. See comment in PubMed Commons below Biomed Res Int 2014: 391542.

94. Vicente G, Bautista LF, Rodriguez R, Gutierrez FJ, Sadaba I, Ruiz-Vazquez RM, Torres-Martinez S, Garre V (2009) Biodiesel production from biomass of an oleaginous fungus. Biochemical Engineering Journal 48, 22-27.

95. Gude VG, Grant G, Patil P, Deng S (2013) Biodiesel from low cost renewable feedstock. Open Engineering 3, 595-605.

96. Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM, et al. (2010) Comparison of several methods for effective lipid extraction from microalgae. See comment in PubMed Commons below Bioresour Technol 101 Suppl 1: S75-77.

97. Cheng J, Yu T, Li T, Zhou J, Cen K, et al. (2013) Using wet microalgae for direct biodiesel production via microwave irradiation. See comment in PubMed Commons below Bioresour Technol 131: 531-535.

98. Gude VG, Patil PD, Martinez-Guerra E, Deng S, Nirmalakhandan N, et al. (2013) Microwave energy potential for biodiesel production. Sustainable Chemical Processes 2013, 1-5.

99. Gude PD, Patil PD, Deng S, Nirmalakhandan N (2011) Microwave enhanced methods for biodiesel production and other environmental applications. Green Chemistry for Environ. Remediation, Wiley Publishers, 209-249.

100. L. Perreux A, Loupy (2001) A tentative rationalization of microwave effects in organic synthesis according to the reaction medium, and mechanistic considerations. Tetrahedron, 57, 9199–9223.

101. Lidstrom P, Tierney JP, Wathey B, Westman J (2001) Microwave assisted organic synthesis: A review Tetrahedron 57, 9225–9283.

102. Kanitkar AV (2010) Parameterization of microwave assisted oil extraction and its transesterification to biodiesel. Master's Thesis. Lousiana State University, Baton Rougue, LA.

103. Stavarache C, Vinatoru M, Nishimura R, Maeda Y (2005) Fatty acids methyl esters from vegetable oil by means of ultrasonic energy. See comment in PubMed Commons below Ultrason Sonochem 12: 367-372.

104. Belarbi EH, Molina E, Chisti Y (2000) A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. See comment in PubMed Commons below Enzyme Microb Technol 26: 516-529.

105. Zheng H, Yin J, Gao Z, Huang H, Ji X, et al. (2011) Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. See comment in PubMed Commons below Appl Biochem Biotechnol 164: 1215-1224.

106. Adewuyi YG (2001) Sonochemistry: environmental science and engineering applications.4681-4715.

107. Mason TJ, Paniwnyk L, Lorimer JP (1996) The use of ultrasound in food technology. Ultrason. Sonochem. 3, S253–S256.

108. Araujo GS, Matos LJ, Fernandes JO, Cartaxo SJ, Gonçalves LR, et al. (2013) Extraction of lipids from microalgae by ultrasound application: prospection of the optimal extraction method. See comment in PubMed Commons below Ultrason Sonochem 20: 95-98.

109. Avramovic JM, Stamenkovic OS, Todorovic ZB, Lazic ML, Veljkovic VB, et al. (2010) The optimization of the ultrasound-assisted base-catalyzed sunflower oil methanolysis by a full factorial design. Fuel Processing Technology 91, 1551-1557.

110. Grant GE, Gude VG (2013) Biodiesel from waste cooking oils via direct sonication. Applied Energy 109, 135-144.

111. Ji J, Wang J, Li Y, Yu Y, Xu Z (2006) Preparation of biodiesel with the help of ultrasonic and hydrodynamic cavitation. See comment in PubMed Commons below Ultrasonics 44 Suppl 1: e411-414.

112. Lifka J, Ondruschka B (2004) Influence of mass transfer on the production of biodiesel,Chem. Eng. Technol. 27, 1156–1159.

113. Laurens LML, Quinn M, Van Wychen S, Templeton DW, Wolfrum EJ, et al. (2012) Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification. Analytical and Bioanalytical Chemistry 403, 167-178.

114. Araya K, Ugarte A, Azócar L, Valerio O, Wick LY, et al. (2015) Whole cell three phase bioreactors allow for effective production of fatty acid alkyl esters derived from microalgae lipids. Fuel 144 (2015) 25–32

115. Azócar L, Ciudad G, Heipieper HJ, Muñoz R, Navia R, et al. (2010) Improving fatty acid methyl ester production yield in a lipase-catalyzed process using waste frying oils as feedstock. See comment in PubMed Commons below J Biosci Bioeng 109: 609-614.

116. Fjerbaek L, Christensen KV, Norddahl B (2009) A review of the current state of biodiesel production using enzymatic transesterification. See comment in PubMed Commons below Biotechnol Bioeng 102: 1298-1315.

117. Tahera H, Al-Zuhaira S, Al-Marzouqia AH, Haikb Y, Faridc M, et al. (2014) Enzymatic biodiesel production of microalgae lipids under supercritical carbon dioxide: Process optimization and integration. Biochemical Engineering 90, 103–113.

118. Atadashi IM, Aroua MK, Abdul Aziz AR, Sulaiman NMN (2012) Production of biodieselusing high free fatty acid feedstocks. Renew Sustain Energy Rev2012;16:3275–3285.

119. Ranjan A, Patil, C, Moholkar VS (2010) Mechanistic assessment of microalgal lipid extraction. Ind. Eng. Chem. Res. 49, 2979–2985.

120. Harun R, Singh M, Forde GM, Danquah MK (2010) Bioprocess engineering of microalgae to produce a variety of consumer products. Renew. Sust. Energ. Rev. 14, 1037–1047.

121. Mercer P, Armenta RE (2011) Developments in Oil Extraction from Microalgae. Eur. J. Lipid Sci. Technol., 113, 539–547.

122. Shin HS, Ryu JH, Bae SY, Crofcheck C, Crocker M, et al. (2014) Lipid extraction from Scenedesmus sp. microalgae for biodiesel production using hot compressed hexane. Fuel 130, 66-69.

123. Lee SJ, Yoon BD, Oh HM (1998) Rapid method for the determination of lipid from the green alga Botryococcus braunii. Biotechnol Tech 12, 553–556.

124. Medina AR, Grima EM, Gimenez AG, Gonzalez MJI (1998) Downstream processing of

125. Encinar JM, Gonzalez JF, Rodriguez JJ, Tejedor A (2002) Biodiesel fuels from vegetable oils: transesterification of Cynara cardunculus L. oils with ethanol. Energy Fuels 16, 443–450.

126. Andrade Torres E, Cerqueira GS, Ferrer TM, Quintella CM, Raboni M, et al. (2013) Recovery of Different waste vegetable oils for biodiesel production:A pilot Experience in Bahia State, Brazil.Wate Manage.33,2670-2674.

127. Yoo G, Park WK, Kim CW, Choi YE, Yang JW, et al. (2012) Direct lipid extraction from wet Chlamydomonas reinhardtii biomass using osmotic shock. Bioresource Technology 123, 717–722.

128. Mulbry W, Kondrad S, Buyer J, Luthria D (2009) Optimization of an oil extraction process for algae from the treatment of manure effluent. J. Am. Oil Chem. Soc. 86, 909–915.

129. Xu L, Wim Brilman DW, Withag JA, Brem G, Kersten S (2011) Assessment of a dry and a wet route for the production of biofuels from microalgae: energy balance analysis. See comment in PubMed Commons below Bioresour Technol 102: 5113-5122..

130. Cheng J, Huang R, Yu T, Li T, Zhou J, et al. (2014) Biodiesel production from lipids in wet microalgae with microwave irradiation and bio-crude production from algal residue through hydrothermal liquefaction. Bioresource Technology 151, 415–418.

131. Ehimen EA, Sun ZF, Carrington CG (2010) Variables affecting the in situ transesterification of microalgae lipids. Fuel 89, 677–684

132. Sun F, Chen H (2008) Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw. See comment in PubMed Commons below Bioresour Technol 99: 5474-5479.

133. Liang Y, Sarkany N, Cui Y, Yesuf J, Trushenski J, et al. (2010) Use of sweet sorghum juice for lipid production by Schizochytrium limacinum SR21. See comment in PubMed Commons below Bioresour Technol 101: 3623-3627.

134. Elsey D, Jameson D, Raleigh B, Cooney MJ (2007) Fluorescent measurement of microalgal neutral lipids. See comment in PubMed Commons below J Microbiol Methods 68: 639-642.

135. Viswanath B, Mutanda T, White S, Bux F (2010) The microalgae – a future source of biodiesel. Dynamnic Biochem Process Biotechnol Mole Biol 4, 37–47.

Hydrogen Production by Microalgae

Sufia Hena*

[1]School of Industrial Technology, University Sains Malaysia, Georgetown 11800, Malaysia

***Corresponding author:** Sufia Hena, School of Industrial Technology, University Sains Malaysia, Georgetown 11800, Malaysia, Tel No: 006046532537; Fax No: 006046573678; E-mail: sufiahena@usm.my

Abstract

Biological hydrogen production has been known for over a century, although no intense research was done at that time. Recent environmental issues accelerated the research on renewable sources of energy and thus biological hydrogen production grabbed the attention of researchers. Biological hydrogen production processes offer renewable energy sources which can be utilized for the generation of the cleanest energy carrier for the use of mankind. Hydrogen gas is thought to be the ideal fuel for a world in which air pollution is need to be eased, global warming has to be arrested, and the environment is planned to be protected in an economically sustainable manner. Cyanobacteria and green microalgae split water into molecular hydrogen and oxygen using sunlight under special conditions. This chapter provides an overview of the principles of photobiological hydrogen production in microalgae and various approaches that have been proposed for hydrogen production by microalgae or cyanobacteria are discussed and several critical limiting factors are identified.

Keywords: Direct Biophotolysis; Green Fuel; Indirect Biophotolysis; Microalgae

Introduction

Sharp rise in price due to decrease availability of fossil fuel and increasing concerns about the global climate changes caused by anthropogenic rise of the atmospheric greenhouse gasses concentration, the finding of renewable sources of energy has become more and more important [1,2]. Molecular hydrogen grabbed the attention of researchers is a fuel that has the potential to provide the clean energy required for transport, heating and electricity.

In recent years, microalgae have attracted interest for producing valuable molecules ranging from therapeutic uses to biofuels. Nevertheless, since more than 70 years ago, the pioneering discovery of the German plant Physiologist Hans Gaffron and co-researchers [3,4] about the ability of unicellular green algae to produce H_2 gas upon illumination has been a curiosity in field of sustainable energy. Prior to the finding of Gaffron and co-researchers, Jackson and Ellms (1896) reported that a natural bloom of the cyanobacterium Anabaena, when placed into a glass jar, rapidly started to evolve hydrogen gas [5]. Those days Renewable H_2 production was made less urgent by the excess of fossil fuel but is now again of increasing priority due to global warming and depletion of petroleum resource.

Cyanobacteria and green algae are so far the only known organisms for both hydrogen production and an oxygenic photosynthesis [6]. Recently many intense researches on

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

bio-hydrogen production from microalgae have been done mainly upon a prior anaerobic incubation of the cells in the dark [6-9]. *Chlamydomonas reinhardtii*, a single-celled green microalga is known to produce hydrogen when starved of sulphur under anaerobic conditions [10-13] and causes a specific but reversible decline in the rate of oxygenic photosynthesis [14] however this condition does not affect the rate of mitochondrial respiration [15]. The hydrogenase pathway enables *C. reinhardtii* and other microalgae to dissipate electrons from the photosynthetic electron transport chain in the form of molecular H_2 [16], a volatile and harmless gas for the algae, but an attractive green and clean energy for humans [17].

Mortensen and Gislerød have demonstrated an environmental friendly hydrogen production method where they used flue gases from a power plant as a carbon dioxide (CO_2) source for growing *C. reinhardtii* [18].

Hydrogen gas is seen as the most promising and an ideal future energy in the list of fuel evolution, because of several technical, socio-economic and environmental benefits; such as it is easily converted to electricity in fuel cells, liberates a large amount of energy per unit mass, and generates no air pollutants and thus H_2 fuel is also known as green fuel. Nevertheless hydrogen is one of the most abundant elements on planet earth, but its molecular form exists in environment at extremely low levels which is even less than 1 ppm [19]. Hence a major goal, the production of renewable H_2 fuel at affordable costs has been renewed over the past few years. Presently 88% of total hydrogen produced from non-renewable fossil fuels and costs of fossil fuels are again of concern and 4-6% from electrolysis, while rest is from different source of bio-hydrogen. Bio-hydrogen has gained attention due to its potential as a sustainable alternative to the conventional methods for H_2 production.

Nature has created biological reactions that use sunlight for the oxidation of water (oxygenic photosynthesis), and enzymes (hydrogenase and nitrogenase) that use electrons for the generation of H_2 [20-23] is encoded in the nucleus of the unicellular green algae. Biological hydrogen or bio-hydrogen production is the microbiological conversion of water in sunlight and/or organic substrates into hydrogen by action of enzymes hydrogenase (Hase) or nitrogenase (Nase). However under certain conditions of growth some green algae and cyanobacteria have been dominantly investigated for their capacity to convert captured solar energy to hydrogen. In this process, light energy captured by the photosystem II (PSII) is used to split water, producing oxygen, and generate a high-energy, low-potential reductant capable of reducing protons (H^+) to hydrogen (H_2) via a hydrogenase or nitrogenase enzyme, a process that has been called biophotolysis which is classified into two major groups: direct and indirect biophotolysis. The generated electron from water molecule splitting passes through the membrane-bound electron transport chain that connects the two photosystems (I and II), which subsequently produces ATP and subsequently reduces ferredoxin. Reduced ferredoxin can participate in a number of metabolic reactions (Figure 1).

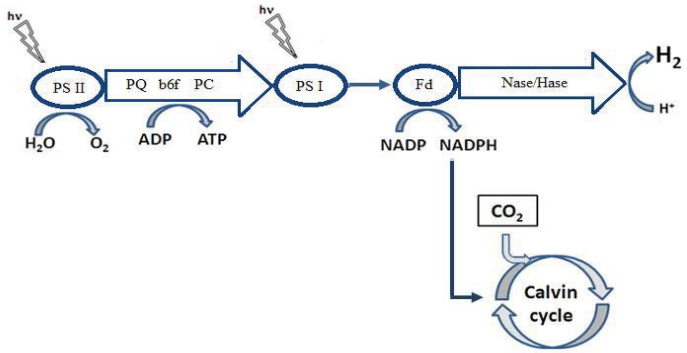

Figure 1: Pathways for possible H_2 production in algae.

Reduced ferredoxin can directly donate electrons to the nitrogenase system which can catalyze the ATP-dependent production of hydrogen. Reduced ferredoxin can either reduce NADP to NADPH which can drive CO_2 fixation by the Calvin cycle.

Direct Biophotolysis

In direct biophotolysis the photoautotrophic microorganisms, either prokaryotic cyanobacteria or eukaryotic green microalgae, possess photosynthetic apparatus chlorophyll a and other pigments to capture sunlight and the recovered energy is used to couple water splitting to the generation of a low-potential reductant or ferredoxin, which can be used directly to reduce a hydrogenase or nitrogenase enzyme without intermediate CO_2 fixation.

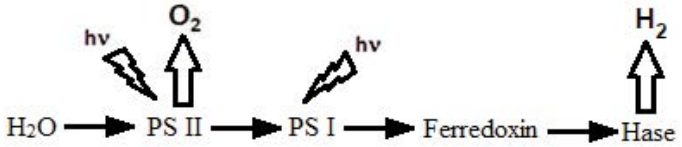

Figure 2: Direct biophotolysis reaction H_2O.

The generated hydrogen ions are converted into hydrogen gas in the medium with electrons donated by reduced ferredoxin to hydrogenase enzyme present in the cells. Light energy absorbed by photosystem II (PSII) generates electrons which are transferred to ferredoxin using light energy absorbed by PSI. A reversible hydrogenase accepts electrons directly from the reduced ferredoxin to generate H_2 in presence of hydrogenase, as shown in Figure 2. The produced reductant, either ferredoxin or NADPH, directly reduces hydrogenase, hence hydrogen production is strictly light dependent in direct biophotolysis. This reaction was first demonstrated with a cell free chloroplast–ferredoxin– hydrogenase system [24], although the existence of such a reaction in green algae had been suggested earlier [25]. Indeed this is an inherently very appealing process since solar energy is used to convert a readily available substrate, water, to produce oxygen and hydrogen, but in practice it is severely limited by other factors. The enzyme hydrogenase is very sensitive to O_2 which is evolved simultaneously during direct biophotolysis process which consequently inhibit the H_2 production. An approach has been investigated to overcome this limitation by removing the O_2 produced, via increasing respiration process using endogenous or exogenous substrates (Figure 3).

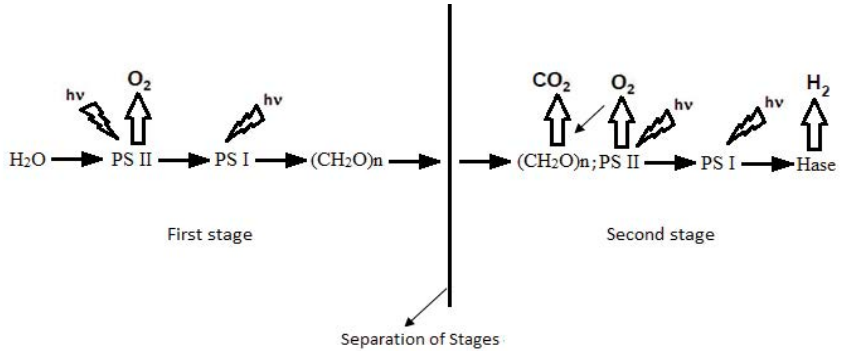

Figure 3: Direct biophotolysis reaction by removing O_2.

Some green algae naturally possess an inducible reversible hydrogenase and in principle could be used in a direct biophotolysis scheme. The well-known H_2-producing green algae, *C.*

reinhardtii, under anaerobic conditions, can either generate H_2 or use H_2 as an electron donor [26]. Hydrogenase activity has also been observed in other green algae like *Scenedesmus obliquus* [26], *Chlorococcum littorale* [27], *Platymonas subcordiformis* [27] and *Chlorella fusca* [26]. Possessing a wide diversity in morphology and physiology, cyanobacteria and green algae are potential microbial species for hydrogen production via direct biophotolysis [28]. Green microalgae such as *C. reinhardtii*, *C. littorale*, *P. subcordiformis* and *S. obliquus* are good example of hydrogen production at certain conditions. Again adaptation is needed for the cells to induce reversible hydrogenase, because of the high sensitivity of hydrogenase to the oxygen generated from water splitting on PSII [26]. In order to overcome the inhibitive effect of oxygen on hydrogenase, various methods have been investigated with limited success.

Methods to Overcome Limitations of Direct Biophotolysis

Since many years, several researches on process of direct biophotolysis has been carried out to improve the efficiency, reaction conditions, stabilizing components and demonstration it in vivo, such as using inert gas sparging, which reduces gas and liquid phase O_2 and H_2 concentrations below 0.1%. Greenbaun [29,30] has demonstrated that the microalgae *C. reinhardtii* simultaneously produces hydrogen and oxygen with 4 photons / H_2, although inert gas sparging is not practical.

The other way is to cover the pond with a simple floating transparent cover to minimize the gas transfer; this technique would cut the cost of sparging inert gas mixing as well. However the fouling and temperature control would still need to be addressed.

Even if a complete stable direct biophotolysis process would achieved; the major issue would then be gas handling, especially the separation of the hydrogen and oxygen and would likely add as much to the cost of the process as the hydrogen production itself. Besides this the greatest obstacle to such a direct biophotolysis process is the safety concerns, production of oxygen-hydrogen mixture is explosive and unstable under even common atmospheric conditions. These limitations headed the research and development and bring it to another level where researchers manage to separate oxygen from hydrogen for three reasons; firstly to reduce the effect of oxygen on hydrogenase, secondly lower the cost of processing to separate hydrogen from oxygen thirdly overcome the safety fears.

In some case the oxygen is removed as it is formed by adding oxygen absorbers such as glucose and glucose oxidase demonstrate by Benemann et al.,(1973) [31]. Rosenkranz and Krasna [32] showed myoglobin and hemoglobin could reduce oxygen concentration effectively to allow a sustainable direct biophotolysis. However, such regeneration would not be practical in full-scale system. Many researchers [33-36] used dithionite; a strong reducing agent that quantitatively reacts with evolved oxygen and leading to anaerobic environment, allowing a sustained direct biophotolysis reaction. Acetate is a good carbon substrate for *C. reinhardtii* cells to maintain a high respiration rate [37] on exogenous acetate and/or endogenous carbohydrates [38]. The objective of these discussed attempts are to lower the photosynthetic oxygen generation rate than the rate of O_2-uptake by respiration, the cells culture gradually becomes anaerobic, which induces the activity of reversible hydrogenase [39]. Kosourov and co researchers [37] had mentioned that it takes almost 40 hrs for the *C. reinhardtii* cells to reach the hydrogen production state that can last up to 60 to 70 hrs. Nevertheless in the presence of CO_2 and under high light intensity production of hydrogen is switch –off as metabolism reverted to normal photosynthesis.

It was observed that in sulfur deprived culture medium *C. reinhardtii* can evolve hydrogen very efficiently in continuous light [39]. This resulted in a decline in photosystem II activity and oxygen evolution rates dropping below the respiration rates, leading to anaerobic condition for hydrogenase and hydrogen evolution, however initially it was presented [37-38,40] as indirect biophotolysis process but further research confirmed it

as direct biophotolysis process. The basic problem of two stage direct process is that the carbohydrate accumulated in the first stage of the reaction Figure 2, contains about the same amount of energy as the hydrogen evolved in the second stage, and this energy is completely wasted in the process of oxygen consumption during high rate of respiration, thus the energy conversion efficiency from light to hydrogen production is quite low [41].

Consequently, despite the great efforts on improvement of direct biophotolysis process, its potential practicality is limited because of the above mentioned reason.

Indirect Biophotolysis

In indirect biophotolysis , light energy captured by the PSII is used to split water, producing oxygen, and generate reduced ferredoxin which are used to fix CO_2 and the resulting reduced carbon compound can be used to drive hydrogen evolution in a separate reaction. Thus, hydrogen production can be separated from oxygen production by time and/or stage, overcoming the problematic production of oxygen at the venue of a highly oxygen-sensitive hydrogenase.

Indirect Single-Stage Biophotolysis

One most common example of indirect biophotolysis is heterocystous cyanobacteria. Heterocystous cyanobacteria are filamentous form of blue-green algae in which vegetative cells are differentiates with specialized cell known as heterocyst, the site of nitrogen fixation by the enzyme nitrogenase and in absence of nitrogen, nitrogenase produces H_2 (Figure 4).

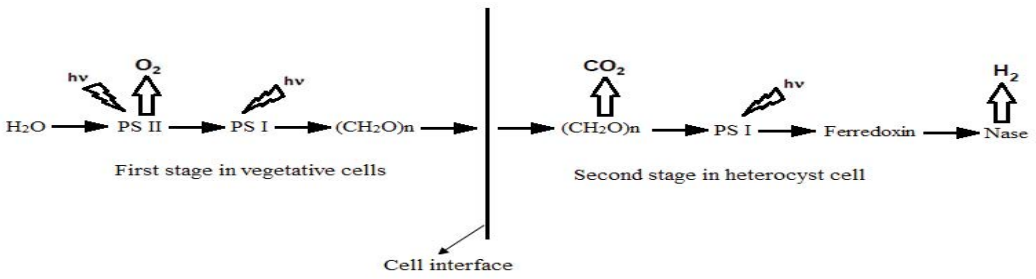

Figure 4: Indirect single-stage biophotolysis in heterocystous cyanobacteria.

In the heterocyst, PSII is completely absent, only PSI is functionally active and generate ATP for nitrogenase activity. The pores, joining heterocysts with vegetative cells, allow the diffusion of carbohydrates from vegetative cells to heterocyst and nitrogenous products in the opposite directions. Heterocystous cyanobacteria are nature made two compartments system at microscopic level for O_2 and H_2 generation reactions. *Anabaena cylindrical* was used for the first time to demonstrate the indirect biophotolysis mechanism that could produce H_2 and O_2 simultaneously using nitrogenase.

Heterocystous nitrogen-fixing, non-nitrogen-fixing cyanobacterial and green algae strains are capable to evolve hydrogen under certain conditions [42,43], using nitrogenase and/or bi-directional hydrogenase enzymes. Heterocystous nitrogen fixing cyanobacteria such as *Anabaena* sp. provides an oxygen-free environment to the oxygen-sensitive nitrogenase that reduces molecular nitrogen into NH_3 as well as protons into H_2 [44]. In nitrogen containing atmosphere, nitrogen fixation is the predominant reaction while hydrogen is a minor byproduct but nitrogen fixing cyanobacteria start producing hydrogen in the absence of molecular nitrogen. The energy required for hydrogen evolution is derived from energy rich carbohydrate molecules present in heterocyst or transferred from vegetative cells [44].

Although the heterocyst system is very appealing for hydrogen production from biological point of view, it has three major limitations: the simultaneous production of O_2 and H_2 which is explosive as well as expensive to separate as discussed earlier, requirement of large closed photo-bioreactor and nitrogenase is a very incompetent enzyme in comparison of hydrogenase and thus it would be necessary to replace nitrogenase by hydrogenase via selecting non-heterocystous or green microalgae over heterocyst cyanobacteria.

Indirect Two-Stage Biophotolysis

Another approach to indirect biophotolysis to overcome the limitation discussed in single-stage biophotolysis is to carry out the two reaction in separate stages, first O_2 production and CO_2 fixation while, second stage is H_2 production with CO_2 release, both the stages are light driven. This process is functionally identical to single-stage heterocyst system, except that O_2 production with CO_2 fixation and H_2 production with CO_2 production are separated with period of time (Figure 5).

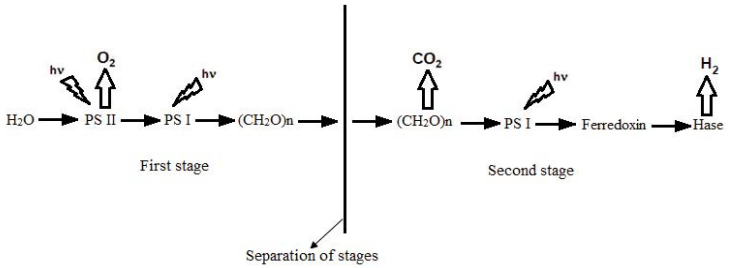

Figure 5: Indirect two-stage biophotolysis with light driven second stage.

Non-heterocystous cyanobacteria *Plectonema boryanum* was used for the first time to demonstrate the two-stage indirect biophotolysis with repeated alternate cycle of aerobic light driven CO_2 fixation and O_2 production with anaerobic light driven H_2 production by Weare and Benemann [45]. The same nitrogen-fixing non-heterocystous cyanobacterium *Plectonema boryanum* was used as a model organism to study hydrogen generation by indirect biophotolysis in nitrogen-limited batch cultures which were continuously illuminated and sparged with argon/CO_2 to maintain anaerobic condition [46].

Practically, this process can be carried out either in two separate reactors, open pond for CO_2 fixation and O_2 production and closed photo-bioreactor for H_2 production or single closed photo-bioreactor for CO_2 fixation with O_2 production and H_2 production in alternate cycle. Benemann (1998) suggested that the algal culture in open pond should be limited for nitrogen to force high accumulation of carbohydrates, which could be used for H_2 production in second stage [47].

The two-stage biophotolysis is modified by replacing light driven second stage of H_2 production with dark fermentation of second stage (Figure 6).

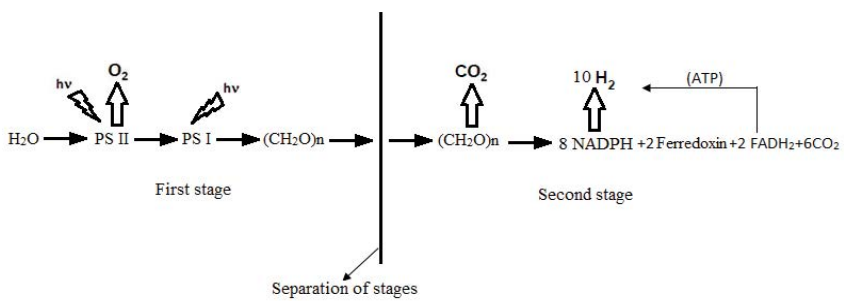

Figure 6: Indirect two-stage biophotolysis with dark fermentation second stage.

This modification in two-stage biophotolysis indirect system might be attained into the day-night cycle, where the dark period allowing development of anaerobic fermentation conditions [48] with H_2 production in the dark followed by CO_2 fixation in day. Light driven H_2 production was also reported in between CO_2 fixation and dark fermentation during sunrise. The main advantage in two-stage dark fermentation process is that the closed photo-bioreactors used in the second stage could be much smaller than for any of the processes discussed earlier, since dark fermentation do not need light exposure. The nitrogen limited open pond culture is concentrated almost about 20-fold and maintained under anaerobic condition in the dark driven by a limited respiration. The noticeable candidate microalgae for this process are those that produce H_2 and that can also be mass cultured with high accumulation of carbohydrates in nitrogen limitation condition. The most common green algae studied are *Chlorella* and blue-green algae *Spirulina* (*Arthrospira*) [28].

The dark anaerobic incubation of algae for H_2 production depends on many other factors such as; duration of anaerobic incubation, nutrients of culture medium, pH of the medium. Intensive research of Guan and co-researchers have revealed that *Platymonas subcordiformis* a marine green algae evolved maximum H_2 in second stage of indirect biophotolysis in sulfur deficient culture medium for optimum time period of 32 h anaerobic condition at pH 8 [49]. However dark anaerobic condition is not required for fresh water green algae *C. reinhardtii*, famous for H_2 production capability, though sulfur deficient medium enhanced the production of H_2 [50].

Comparisons of Hydrogen Production Systems in Algae

The direct biophotolysis system converts solar energy directly into fuel with maximum efficiency of 40.7% at 680 nm wavelength of light using hydrogenase enzyme, while the overall quantum efficiency of 680 nm drop to 20.3% in case of nitrogenase [51,52]. Direct biophotolysis is single stage process and the operation is easy and simpler than indirect photo-biolysis. However the requirement of large hydrogen impermeable photo-bioreactors, possible generation of explosive hydrogen/oxygen mixtures and evolvement of oxygen in the vicinity of oxygen sensitive hydrogenase are limitation of direct biophotolysis system.

In indirect biophotolysis, oxygen and hydrogen evolving phase are separated and required small photo-bioreactors for H_2 producing stage. The maximum energetic efficiency of hydrogen production in indirect biophotolysis system is 27.1%, using hydrogenase at 680 nm wavelength of light, while the energetic efficiency reduces to 16.3%, using nitrogenase as hydrogen production enzyme at 680 nm. The disadvantages of indirect biophotolysis system are a huge consumption of energy in pumping during stage shift and another energy loss happens during production and reuse of stored energy carrier.

Bioreactors for H2 Production

The design and operation of the photo-bioreactors must be suitable for algae culture and capture produced hydrogen. The basic requirements for the most fundamental type of bioreactor are energy supply, either light, carbohydrates or carbon dioxide for H_2 production.

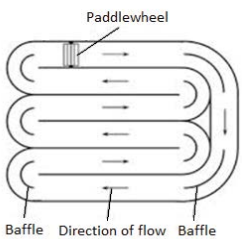

Figure 7: Open pond system.

For biomass accumulation open pond (Figure 7) is the best option. Again open pond is more suitable for robust strain of algae. Mostly the closed photo bioreactor (Figure 8) is used either for high-value microalgae products or for production of H_2.

Figure 8: Commercial scale tubular photobioreactor.

Light driven H_2 production need transparent photobioreactor, collection of generated H_2 and must provide suitable environment for biomass culture [53]. There are several types of closed photobioreactors design for H_2 production such as, covered pond, tubular bioreactors (Figure 8) and flat plate bioreactor. Flat plate bioreactor consists of a rectangular transparent box with a depth of only 1–5 cm. The height and width, can be varied to some extent, but in practice only plates with a height and width both smaller than 1 m have been used. Flat panel reactors show a high photochemical efficiency and biomass yield.

Usually the vertical flat plates are illuminated from one side by direct sunlight or inclined versus the sun. Light/dark cycles are short in these reactors, and this is probably the key factor leading to the high photochemical efficiency. A disadvantage of these systems is that the power consumption of aeration (or mixing with another gas) is high, though mixing is always necessary in any reactor.

Tubular bioreactors are expected to show better efficiencies because of the shorter average light/dark cycles, however practically tubular bioreactors show lesser efficiencies than flat plates, probably due to other factors. Tubular photo bioreactors consist of long transparent tubes with diameters ranging from 3 to 6 cm, and lengths ranging from 10 to 100 m. The culture liquid is pumped through these tubes by means of mechanical or air-lift pumps. The tubes can be positioned on many different ways Other fundamental matter of concern during photobioreactor design is to provide the facility to manage the partial pressure of CO_2/O_2 and H_2. There are two common ways to control the partial pressure: internal gas exchanger and external gas exchanger. In internal gas exchanger the gas is dispersed as fine bubbles through the culture media, while in the external gas exchanger the gas exchange through the degassing chamber, placing outside the photobioreactor. Fortunately overpressure of H_2 is not the root cause of low production of H_2 in any of the biophotolysis process except dark fermentation, where high pressure of H_2 lowers the rate as well as production of H_2. The second stage of H_2 production in direct and indirect biophotolysis process release CO_2, which has to be managed, since presence of excess CO_2 lower the mass transfer capacity because of highly soluble nature and also decrease the pH which are generally not suitable for microalgae culture. Closed photobioreactor faces another problem such as high temperature which can be controlled by water spray.

Despite the current restrictions and challenges in hydrogen production by microalgae, the fundamental advantages would allow further research to get better yield. The most important fundamental advantage for the development of hydrogen production by microalgae is greenhouse gas abatement. Molecular hydrogen (H_2) is a fuel that has the potential to provide the clean energy required for transport, heating and electricity. Hydrogen production by microalgae is a carbon-neutral process that uses unlimited natural resources – sunlight and water.

References

1. Hena S, Fatimah S, Tabassum S (2015) Cultivation of algae consortium in a dairy farm wastewater for biodiesel production. Water Resources and Industry 10: 1-14.
2. Tsygankov AA (2007) Biological Generation of Hydrogen. Russian Journal of General Chemistry 50: 26-33.
3. Gaffron H (1939) Reduction of CO2 with H2 in green plants. Nature 143: 204–205
4. Gaffron H1, Rubin J (1942) FERMENTATIVE AND PHOTOCHEMICAL PRODUCTION OF HYDROGEN IN ALGAE. See comment in PubMed Commons below J Gen Physiol 26: 219-240.
5. Jackson DD, Ellms JW (1896) On odors and tastes of surface waters with special reference to Anabaena, a microscopic organism found in certain water supplies of Massachusetts, Report Massachusetts State Board Health, 20: 410-420.
6. Schulz R (1996) Hydrogenases and hydrogen production in eukaryotic organisms and cyanobacteria. J Mar Biotechnol 4:16–22
7. Greenbaum E (1982) Photosynthetic hydrogen and oxygen production: kinetic studies. See comment in PubMed Commons below Science 215: 291-293.
8. Roessler PG1, Lien S (1984) Activation and de novo synthesis of hydrogenase in chlamydomonas. See comment in PubMed Commons below Plant Physiol 76: 1086-1089.
9. Happe T1, Naber JD (1993) Isolation, characterization and N-terminal amino acid sequence of hydrogenase from the green alga Chlamydomonas reinhardtii. See comment in PubMed Commons below Eur J Biochem 214: 475-481.
10. Skjånes K1, Lindblad P, Muller J (2007) BioCO2 - a multidisciplinary, biological approach using solar energy to capture CO2 while producing H2 and high value products. See comment in PubMed Commons below Biomol Eng 24: 405-413.
11. Nguyen AV1, Toepel J, Burgess S, Uhmeyer A, Blifernez O, et al. (2011) Time-course global expression profiles of Chlamydomonas reinhardtii during photo-biological Hâ,, production. See comment in PubMed Commons below PLoS One 6: e29364.
12. Geier SC, Huyer S, Praebst K, Husmann M, Walter C, Buchholz R (2012) Outdoor cultivation of Chlamydomonas reinhardtii for photobiological hydrogen production. J Appl Phycol 24:319–327.
13. Melis A1 (2007) Photosynthetic H2 metabolism in Chlamydomonas reinhardtii (unicellular green algae). See comment in PubMed Commons below Planta 226: 1075-1086.
14. Wykoff DD1, Davies JP, Melis A, Grossman AR (1998) The regulation of photosynthetic electron transport during nutrient deprivation in Chlamydomonas reinhardtii. See comment in PubMed Commons below Plant Physiol 117: 129-139.
15. Melis A1, Zhang L, Forestier M, Ghirardi ML, Seibert M (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga Chlamydomonas reinhardtii. See comment in PubMed Commons below Plant Physiol 122: 127-136.
16. Hemschemeier A1, Fouchard S, Cournac L, Peltier G, Happe T (2008) Hydrogen production by Chlamydomonas reinhardtii: an elaborate interplay of electron sources and sinks. See comment in PubMed Commons below Planta 227: 397-407.
17. Melis A1, Happe T (2001) Hydrogen production. Green algae as a source of energy. See comment in PubMed Commons below Plant Physiol 127: 740-748.
18. Mortensen LM1, Gislerød HR1 (2014) The effect on growth of Chlamydomonas reinhardtii of flue gas from a power plant based on waste combustion. See comment in PubMed Commons below AMB Express 4: 49.
19. Mandelik B, Newsome D, Kellogg P (1985) Kirk-othmer concise encyclopedia of chemical technology. Edited, M. Grayson, John Wiley & Sons, New York. 621-623.
20. Voordouw G1, Strang JD, Wilson FR (1989) Organization of the genes encoding [Fe] hydrogenase in Desulfovibrio vulgaris subsp. oxamicus Monticello. See comment in PubMed Commons below J Bacteriol 171: 3881-3889.
21. Adams MW1 (1990) The structure and mechanism of iron-hydrogenases. See comment in PubMed Commons below Biochim Biophys Acta 1020: 115-145.
22. Meyer J1, Gagnon J (1991) Primary structure of hydrogenase I from Clostridium pasteurianum. See comment in PubMed Commons below Biochemistry 30: 9697-9704.
23. Happe T1, Mosler B, Naber JD (1994) Induction, localization and metal content of hydrogenase in the green alga Chlamydomonas reinhardtii. See comment in PubMed Commons below Eur J Biochem 222: 769-774.
24. Benemann JR1, Berenson JA, Kaplan NO, Kamen MD (1973) Hydrogen evolution by a chloroplast-ferredoxin-hydrogenase system. See comment in PubMed Commons below Proc Natl Acad Sci U S A 70: 2317-2320.
25. Spruit CJP (1958) Simultaneous photoproduction of hydrogen and oxygen by Chlorella. Meded Landbouwhogesch Wageningen 58:1–17.

26. Winkler M, Hemschemeier A, Gotor C, Melis A, Happe T, et al.(2002) [Fe]-hydrogenase in green algae: photo-fermentation and hydrogen evolution under sulphur deprivation International Journal of Hydrogen Energy, 27, 1431-1439.

27. Nandi R1, Sengupta S (1998) Microbial production of hydrogen: an overview. See comment in PubMed Commons below Crit Rev Microbiol 24: 61-84.

28. Pinto FAL, Troshina O, Lindblad P (2002) A brief look at three decades of research on cyanobacterial hydrogen evolution. International Journal of Hydrogen Energy, 27: 1209-1215.

29. Greenbaum E (1980) Simultaneous photoproduction of hydrogen and oxygen by photosynthesis, Biotechnology and Bioengineering Symposium, 10, 1-13.

30. Greenbaum E (1988) Energetic efficiency of hydrogen photoevolution by algal water splitting. See comment in PubMed Commons below Biophys J 54: 365-368.

31. Benemann JR, Berenson JA, Kaplan NO, Kamen MD (1973) Hydrogen evolution by a chloroplast-ferredoxin-hydrogenase system. See comment in PubMed Commons below Proc Natl Acad Sci U S A 70: 2317-2320.

32. Rosenkrans AM, Krasna AI (1984) Stimulation of hydrogen photoproduction in algae by removal of oxygen by reagents that combine reversibly with oxygen. See comment in PubMed Commons below Biotechnol Bioeng 26: 1334-1342.

33. Pow T, Krasna AI (1979) Photoproduction of hydrogen from water in hydrogenase-containing algae. See comment in PubMed Commons below Arch Biochem Biophys 194: 413-421.

34. Mahro B, Grimme LH (1982) H2 photoproduction by green algae: the significance of anaerobic pre-incubation periods and of high light intensities for H2 photo-productivity of Chlorella fusca, Archives of Microbiology, 132, 82-86.

35. Mahro B, Grimme LH (1986) Improving the photosynthetic H2 productivity of the green alga Chlroella fusca by physiologically directed O2 avoidance and ammonium stimulation, Archives of Microbiology, 144, 25-28.

36. Randt , Senger H (1985) Participation of the two photosystems in light dependent hydrogen evolution in Scendesmus obliquus, Photochemistry and Photobiology, 42, 553-557.

37. Kosourov S, Tsygankov A, Seibert M, Ghirardi ML, et al. (2002) Sustained hydrogen photoproduction by Chlamydomonas reinhardtii: Effects of culture parameters. See comment in PubMed Commons below Biotechnol Bioeng 78: 731-740.

38. Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, et al. (2000) Microalgae: a green source of renewable H(2). See comment in PubMed Commons below Trends Biotechnol 18: 506-511.

39. Kosourov S, Makarova V, Fedorov AS, Tsygankov A, Seibert M, et al. (2005) The effect of sulfur re-addition on H2 photo-production by sulfur-deprived green algae. Photosynthesis Research, 85, 295-305.

40. Antal TK, Krendeleva TE, Laurinavichene TV, Makarova VV, Ghirardi ML, et al. (2003) The dependence of algal H2 production on Photosystem II and O2 consumption activities in sulfur-deprived Chlamydomonas reinhardtii cells. See comment in PubMed Commons below Biochim Biophys Acta 1607: 153-160.

41. Yoon JH, Shin JH, Kim MS, Sim SJ, Park TH, et al. (2006) Evaluation of conversion efficiency of light to hydrogen energy by Anabaena variabilis International Journal of Hydrogen Energy 31:721–727.

42. Lindblad P, Chirstensson K, Lindberg P, Fedorov A, Pinto F, et al. (2002) International Journal of Hydrogen Energy, 27, 1271-1281.

43. Howarth DC, Codd GA, (1985) The Uptake and Production of Molecular Hydrogen by Unicellular Cyanobacteria. Journal of General Microbiology, 131, 1561-1569

44. Smith GD, Ewart GD, Tucker W, (1992) Hydrogen production by cyanobacteria. International Journal of Hydrogen Energy, 17, 695-698.

45. Weare NM, Benemann JR (1974) Nitrogenase activity and photosynthesis in Plectonema boryanum. See comment in PubMed Commons below J Bacteriol 119: 258-265.

46. Huesemann MH, Hausmann TS, Carter BM, Gerschler JJ, Benemann JR (2010) Hydrogen Generation Through Indirect Biophotolysis in Batch Cultures of the Non-heterocystous Nitrogen-Fixing Cyanobacterium Plectonema boryanum. Appl Biochem Biotechnol 162: 208–220.

47. Benemann JR (1998) Processes analysis and economics of biophotolysis of water. A preliminary assessment, Technical report, IEA Agreement on the production and utilization of hydrogen. Springfield (VA), NTIS.

48. Redwood MD, Beedle MP, Macaskie LE, (2009) Integrating dark and light bio-hydrogen production strategies: towards the hydrogen economy. Rev Environ Sci Biotechnol 8:149–185.

49. Guana Y, Denga M, Yu X, Zhan W (2004) Two-stage photo-biological production of hydrogen by marine green alga Platymonas subcordiformis. Biochemical Engineering Journal 19: 69–73.

50. Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M, et al. (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga Chlamydomonas reinhardtii, Plant Physiol. 122: 127–135.

51. Hoffert MI, Caldeira K, Benford G, Criswell DR, Green C, et al.,(2002) Advanced Technology Paths to Global Climate Stability: Energy for a Greenhouse Planet. Science 298: 981-987.

52. Kheshgi HS, Prince RC, Marland G (2000) The potential of biomass fuels in the context of global climate change: Focus on transportation fuels. Annu. Res. Energy. Environ. 25: 199-244.

53. Pulz O (2001) Photobioreactors: production systems for phototrophic microorganisms. See comment in PubMed Commons below Appl Microbiol Biotechnol 57: 287-293.

Life-Cycle Assessment of Biodiesel Production from Microalgae: Energy, Greenhouse and Nutrient Balance

Amélia Delgado* and João Biché Danune

Companhia dos Mestres LDA, Technical and Scientific Consulting,Rua Ferreira Chaves nº8 1070-127 Lisboa, Portugal

***Corresponding author:** Amélia Delgado, Companhia dos Mestres LDA, Technical and Scientific Consulting, Rua Ferreira Chaves nº8 1070-127 Lisboa, Portugal, Tel: +351-919876849; E-mail: amelia.delgado@companhiamestres.com

Abstract

This review targets scientists, innovators, potential investors and other stakeholders interested in renewable energies, particularly on biodiesel production. Global warming is a fact that urges to be controlled. Together with the progressive depletion of the prevalent fossil fuel market reserves, the next years are expected to be characterized by increasing levels of global energy demand and Greenhouse Gases (GHG) emissions, mainly from emerging economies. Viable economical alternatives to fossil fuels are imperative and the production of biodiesel from microalgae is a promising alternative. These unicellular plants are able to fixate CO_2 - with the corresponding carbon market benefits - and produce fats and carbohydrates, which can be converted to biofuels. Microalgae grow much faster than other crops and do not compete with agriculture for food or land. Besides the positive operating results already verified in several companies, research is still targeting improvements in lipid production and downstream processing, optimizing energy consumption and GHG emissions. Currently the cultivation of microalgae solely for biodiesel production may not be economically viable. Yet, the adoption of complementary technologies (such as other renewable energy sources for power supply) and usages (like wastewater treatment) can allow for reduced operating costs and valuable byproducts generation.

Keywords

Carbon Market, CO_2 Biomitigation, Ecology Of Technology, Microalgae Cultivation, Return Of Investment Over Fossil Energy ($EROI_{Fossil}$)

Broader Context

Global awareness on climate changes in addition to the fact that fossil fuels will soon enter a decline phase[1] present challenges on security of energy supply and competitiveness.

According to IEA (International Energy Agency) [1], taking into account the impact

[1] [1]According to the report produced by International Energy Agency in 2013 (www.iea.org), an average conventional oil field can expect to see annual declines in output of around 6% per year

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

of measures already announced by governments to improve energy efficiency, support renewables, reduce fossil-fuel subsidies and, in some cases, to put a price on carbon, energy-related CO_2 emissions still rise by 20% to 2035. This leaves the world on a trajectory consistent with a long-term average temperature increase of 3.6°C, far above the internationally agreed 2°C target.

The global climate challenge requires the stabilization of atmospheric CO_2 levels as a matter of urgency [2].

Research on biofuel closely followed the price of petroleum but given the present scenario stronger efforts have been made, particularly in EU and USA to develop and implement safe, sustainable and carbon-neutral energy sources. Such energy sources have to be compatible with the existing infrastructures because fossil fuels will continue to play a vital role during the next decade, at least (figure 1).

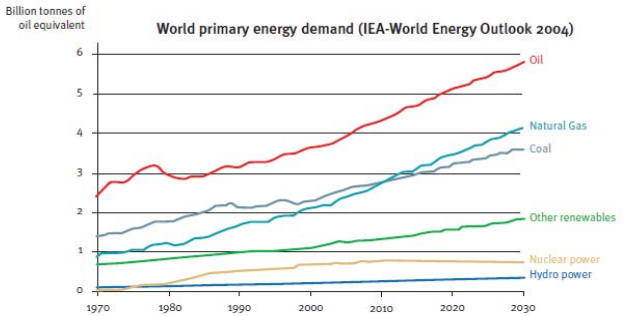

Figure 1: World primary energy demand.

(Reproduced from "the Zero Emission Fossil Fuel Power Plants Technology Platform (2006). A Vision for the Zero Emission Fossil Fuel Power Plants. Directorate-General for Research Sustainable Energy Systems. EU – Office for Official Publications of the European Communities. Luxembourg").

According to EU [2] over the next three decades fossil fuels will continue to dominate the power generation market to respond to the increased demand of emerging economies, particularly China, India and the Middle East, which drive global energy use one-third higher [3]. At the same time, the impacts of increased CO_2 emissions are becoming more evident .

The links between energy and development are illustrated clearly in Africa, where, despite a wealth of resources, energy use per capita is less than one-third of the global average in 2035 . Africa today is home to nearly half of the 1.3 billion people in the world without access to electricity and one-quarter of the 2.6 billion people relying on the traditional use of biomass for cooking [1]. Due to the semi-arid to tropical climates, vast areas of unused lands, poverty and food insecurity , Africa seems to have the necessary conditions to implement bioenergy systems. 9 countries (Mali, Niger, Senegal, Tanzania, Ethiopia, Angola, Mozambique, South Africa and Swaziland) have biofuel specific policies. Bioenergy options cross all sectors, end-uses, energy carriers, and implementation platforms. Consequently, policymakers need to consider the linkages across sectors and to set priorities [4].

The use of biomass-based energy resources includes:

First generation biomass sources, which are sugars or vegetable oils found in edible plants, as corn, soybean, canola or sugar cane. Biofuel precursors can be easily extracted using conventional technology and biofuels (mostly ethanol) are usually obtained through fermentation followed by chemical processes. Relevant limitations are the competition for the use of land and food supply as well as water footprint[2].

Second generation biomass sources are originated from non-edible sources and include lignocellulosic materials, as forestry or agricultural residues. Biofuel precursors are hard to extract because fermentable sugars are locked in by rigid polymers as lignin, hemicellulose and cellulose. Also included are sources as sewer, or used cooking oil that may require additional purification steps. Although more environmentally friendly, these processes are usually non-carbon-neutral and generally a net production of Greenhouse Gases (GHG) is commonly observed.

Third generation biofuels are biomass sources that correspond to processes which use CO_2 as a feedstock, most particularly the processes of producing biofuels from microalgae. These processes have been received increased attention from academy, industry and governmental agencies. In 2013, some systems are at demonstration stage or in transition to deployment stage, as are the cases of project "BIOFAT" in EU or project "OMEGA" in EUA[3].

It is noteworthy that algae farms can be installed on marginal lands or in off-shore waters, microalgae have much faster growth rates than terrestrial crops, they show highest productivity of biofuel's precursors, the requirements for fresh water are minimal and in some cases marine water can be used. And more important, algae, as any other plant, can use light to fixate atmospheric or captured CO_2 [2], reducing global emissions.

Feasibility of a given process depends on engineering, biology, economics, and environmental issues. For this reason, governmental agencies, mostly from EU and USA continue to invest in research in order to reduce capital and operating costs, making algae fuel production commercially attractive. The growth of the sector has been supported by Sectorial Associations, as Antenna (www.antenna.ch), Aprobio (http://www.aprobio.com.br) or NAA (http://www.nationalalgaeassociation.com/).

Holistic assessment of algal biofuel production systems can be of relevance to industrials, investors and decision-makers. It consists in observing resources, emissions and their impact on life cycle assessment, in order to know the sustainability of such systems.

How Do Algae Produce Biofuels' Precursors?

Microalgae are eukaryotic unicellular plants that naturally occur in fresh and marine waters. They fixate the greenhouse gas, carbon dioxide (CO_2) to grow photoautotrophically producing organic carbon compounds, as starch and lipids (triglycerides which alkane chains of 5 to 17 carbons).

Microalgae selected for biofuel production usually belong to the freshwater species *Spirulina* sp ., *Chlorella* sp., *Scenedesmus* sp., or to the marine species *Nannochloropsis* sp., *Dunaliella* sp. Photosynthesis is a process that converts light energy (usually from the sun) into chemical energy, stored in the chemical bounds of carbohydrates and fats. The overall equation of photosynthesis is

$CO_2 + H_2O + \text{photons} \rightarrow (CH_2O)n + O_2$

(carbon dioxide + water + light energy → carbohydrate + oxygen)

Photosynthesis occurs in two stages:

In the first stage, light-dependent reactions capture the energy of light primarily using the pigment chlorophyll. This and other related pigments are part of light-harvesting protein complexes located at the thylakoid membranes of the chloroplast. Each molecule of chlorophyll absorbs one photon and loses one electron . This electron goes through a transport chain that leads to the ultimate reduction of NADP to NADPH. In addition a proton gradient is formed across the thylakoid membrane, allowing for the concomitant synthesis of ATP, by a mechanism similar to respiration. The special pair chlorophyll molecule regains the lost

[2] Carbon dioxide Capture and Storage (CCS) is a technology with the potential to reduce the greenhouse effect and facilitate the use of fossil fuels simultaneously with biofuel production. An effort is being made to introduce CCS on a commercially viable basis (see ref. 2)

electron from water molecule (acting as electron donor) releasing oxygen (O_2). The equation that summarizes this step is:

$$2\ H_2O + 2\ NADP^+ + 3\ ADP + 3\ Pi + light \rightarrow 2\ NADPH + 2\ H^+ + 3\ ATP + O_2$$

The first stage of photosynthesis yields reducing intermediates (NADPH) , energy (ATP) and oxygen (O_2).

In the second stage, of light-independent reactions (or dark step) an enzyme named RuBisCO captures CO_2 from the atmosphere or (in the case of algae) from the liquid medium and in a process that requires NADPH, ATP, and CO_2 combines with a five-carbon sugar, ribulose 1,5-bisphosphate (RuBP), to yield two molecules of a three-carbon compound, Glycerate 3-Phosphate (GP), later converted to Glyceraldehyde-3-phosphate, a central metabolite, from which C-skeleton many other compounds can be synthesized.

$$3\ CO_2 + 9\ ATP + 6\ NADPH + 6\ H^+ \rightarrow C_3H_6O_3\text{-phosphate} + 9\ ADP + 8\ Pi + 6\ NADP^+ + 3\ H_2O$$

During the second stage of photosynthesis, CO_2 is reduced to carbohydrates, which are the precursors of every cell-forming or energy storage compound. As autotrophic organisms they are able to incorporate other elements as Nitrogen (N) and Phosphorus (P), as inorganic compounds (aqueous forms of mineral salts).

Photosynthesis and respiration can be seen as inverse overall reactions although sharing similar biochemical pathways and both occur in algae[3].

Photosynthetic efficiency varies with the frequency of the light being converted, light intensity, temperature and concentration of CO_2, and can vary from 0.1% to 8%. As unicellular algae have a much higher growth rate, during the time span of a regular terrestrial crop they produce a much higher quantity of biomass and storage materials .

Required Nutrients and Process Regulation

Typically, algae are grown as a monoculture in a liquid media consisting of an aqueous solution of mineral salts. As for most plants, main nutrients are inorganic nitrogen (N), phosphorus (P) and potassium (K) compounds. Trace minerals may be obtained from fresh water supply or added if necessary.

An example of such a culture medium is presented in the Table I, for the cultivation of *Spirulina* sp . (reference quantities for the preparation of 1L of standard fertilizer solution). Slightly salted and slightly hard waters can be used. Is advisable to determine hardness and pH of the water source in order to make corrections or find alternatives (in the absence of a lab, home aquarium kits can be used instead).

Ingredient	Chemical formula	g/L final volume	Remarks
Sodium bicarbonate	$NaHCO_3$	16	ex. baking soda
Potassium nitrate	KNO_3	2	or $NaNO_3$ instead (but in this case 0.5g K_2SO_4 should be added)
Salt	NaCl	1	Preferably coarse sea salt; or use brackish water when available
Ammonium or potassium phosphate	$(NH_4)_3PO_4$; $NH_4H_2PO_4$; $(NH_4)_2HPO_4$; KH_2PO_4	0.1	
Strong green tea	Complex solution	1 ml	
Iron sulphate	$FeSO_4.4H_2O$; $FeSO_4.7H_2O$	10-20mg	White or blue-green, respectively
Magnesium sulphate	$MgSO_4.\ 7H_2O$	0.1	

Table 1: General composition of a culture medium for freshwater microalgae.

[3] Further reading on photosynthesis and respiration:

- Blankenship RE (2008). Molecular Mechanisms of Photosynthesis (2nd ed.). John Wiley & Sons Inc.
- Biochemistry, Lubert Stryer [available online at: http://www.whfreeman.com/catalog/static/whf/stryer4/]

Adapted from Antenna Technologies - a teaching module for the production of Spirulina [available online: www.antenna.ch]

Besides nutrients, other process variables are temperature, pH, light and dark cycles, % inoculum, regularity of feeding and cell harvesting [5]. pH is an indirect measure of the condition of the culture, and it tends to increase proportionally to the biomass production. pH is mainly affected by the level of dissolved CO_2[6] and by the rates of the species of the nitrogen cycle: $NH_3/NO_2^-/NO_3^-$. Optimum and pH tolerance range of the cultivated species may slightly vary and should be kept within preset limits. Photosynthetic productivity may be enhanced by increasing the concentration of CO_2 [6], to which microalgae are much more tolerant when compared to terrestrial crops [7]. The CO_2 tolerance levels depends on the species (and most probably also on the strain) and may reach 100% [8].

According to Anjos *et al.* (2013) [9], CO_2 fixation rate is a function of CO_2 concentration and aeration rate. Bicarbonate ion (HCO_3^-) is the more common form in solution and suitable to cell uptake. These authors registered optimum values for CO_2 fixation (2.22 $gL^{-1}.d^{-1}$) by *C. vulgaris* P12, when using 6.5% CO_2 and 0.5 vvm aeration rate[6] after 7 days of cultivation at 30ºC.

The injection of captured CO_2 from combustion for algae growth has been the object of recent researches [8,10] and patents such as WO2013/022349 on Combining Algae Cultivation and CO_2 capture [11] or WO2012/131414 A1 (zero carbon dioxide and heat emission integrated system of power generation from natural renewable energy sources, organic waste reclamation and commodities production and method of conduction) [12].

Sewage, or wastewaters from aquaculture or animal breeding may be used with many advantages in supplying nutrients to algae, including C, N and P.

It has also been referred that microalgae may also dramatically increase the stocks of energy reserve compounds, particularly increasing the formation of lipid bodies, under cycles of N starvation [13, 14, 15, 16] as summarized in figure 2.

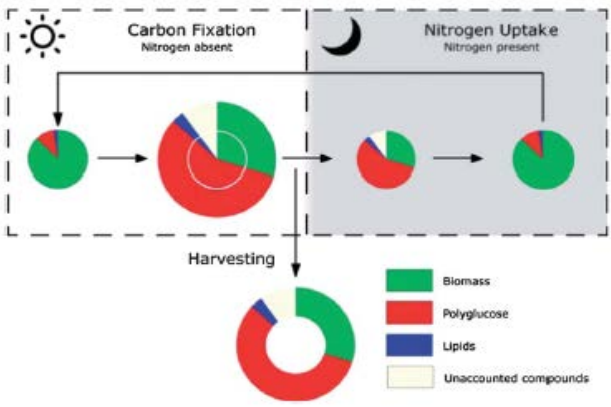

Figure 2: Fraction of biomass (green), lipids (blue), polyglucose (red), and unaccounted compounds (white) during light–dark cycles in the steady state after six weeks of cultivation. Pie-area is linearly related to total amounts, which are an average of three cycles.

(Reproduced from Mooij et. al. (2013) [see ref. 15] with the permission of Royal Society of Chemistry).

(Reproduced from Mooij et. al. (2013) [see ref. 15] with the permission of Royal Society of Chemistry).

It was recently suggested that the exploitation of these features - supplying large concentrations of CO_2 during the light phase, and restricting the nitrogen availability in

the dark phase of photosynthesis - are expected to improve productivities of biodiesel precursors [15]. This is in accordance with the implementation of a selective environment in favor of the most desirable strains, making process control easier and minimizing the impact of contaminant organisms in the stability of dominant population. As referred above, besides oils, algae also accumulate carbohydrates as energy reserves.

How Are Biofuels Produced From Cell Reserves?

Lipids or sugars can be used as precursors in biofuel production. After extracted and concentrated, glucose polymers can be fermented (by yeast) to ethanol. In the case of biodiesel production, the first step consists on drying the biomass to decrease their water content, followed by a solvent extraction step of triglycerides. n-Hexane or cyclo-hexane, methanol/chloroform or ethanol/n-hexane are the most common options. The obtained oil fraction undergo a transesterification reaction, consisting in breaking the bonds linking fatty acids to glycerol and replacing them with methanol [17]. In order to favor product formation, excess ethanol or methanol should be used because of the reversibility of the reaction. NaOH, KOH or $NaCH_3O$[4] are common catalysts. Glycerin is obtained as a valuable byproduct. The origins and destinations of solvents, lipid depleted biomass and CO_2 can be critical for the energy balance and the assessment of environmental impacts [18].

It is noteworthy that methanol can be produced from carbon monoxide and hydrogen directly, under the action of a reducing catalyst, generally sodium hydroxide (NaOH).

Biodiesel shows some different properties from fossil diesel fuel, particularly a higher viscosity. However it can replace it totally or partially in diesel-cycle engines [18]. In Brazil (where it is already a practice) the mixture of both fuel types is designated by B followed by the percentage of biodiesel in the mixture (for example B5 corresponds to a content of 5% biodiesel and 95% fossil-based diesel) [19].

On The Cultivation Of Microalgae:

The cultivation is initiated by seeding a flask or container (of suitable size) with the desired strain and allowing it to grow. Culture concentration can be generally inferred by the visual observation or by measuring the intensity of the color[5] . A pre-culture is thus obtained. The cultivation will proceed with culture dilutions, in the tanks every 2-4 days, generally doubling the volume.

Temperature should be above 20ºC, ideally near 35ºC, and the culture is agitated usually trough the bubbling of injected CO_2. This helps to spread the cells in the medium, maximizing the absorption of nutrients and the exposure to the sun. According to Antenna Technologies (www.antenna.ch) [5] minimum requirement correspond to manual agitation at least 4 times a day, although an electric pump would be desirable. The agitation should not be violent to avoid disruption of the cells .

The final yield on biodiesel depends on the amount of C incorporated into cell reserve materials. In its turn, CO_2 mass transfer efficiency directly depends on the type of injection device, on the gas flow rate and on the photosynthetic efficiency of the algae strain(s). Dissolved CO_2 can be assessed by pH measurements, as pH drops when the level of HCO_3^- increases[5].

As referred above N is a key variable in lipid formation. As a consequence, controlling total N levels (and ensuring that NO3- is the dominant species) may strongly impact process yield (figure. 2). When using wastewater to supply nutrients, the control of N feeding may present some challenges.

[4] Sodium methoxide
[5] As by recording the optical density at 750 nm, along growth.

In fact, according to Mooij *et al.,* (2013) [15], the production of algal biofuel should be regarded from an environmental biotechnology perspective. Same kind of "natural" approach applies to sewage treatment processes as applies to food fermentations (as cheese ripening), where mixed populations do exist. Dominant species, and consequently product yield, depends on process design and regulation. Desired species can be favored by manipulating process variables in accordance to their optimum growth requirements.

Another innovative approach has been developed by NASA [20]. The OMEGA's project brochure [21] states that "In OMEGA, oil-producing freshwater algae are grown in flexible, clear plastic photobioreactors (PBRs) attached to a floating infrastructure anchored offshore in a protected bay. Wastewater and CO_2 from coastal facilities provide water and nutrients. The surrounding seawater controls the temperature inside the PBRs and kills algae that escape from the system. The salt gradient between seawater and wastewater drives forward osmosis, to concentrate nutrients and facilitate algae harvesting. The OMEGA infrastructure also supports aquaculture below the surface and provides surfaces for solar panels and access to offshore wave generators and wind turbines. Integrating algae cultivation with wastewater treatment, CO_2 sequestration, aquaculture, and other forms of alternative energy creates ecology of technologies in which the wastes from one part of the system are resources for another" (Figure. 3).

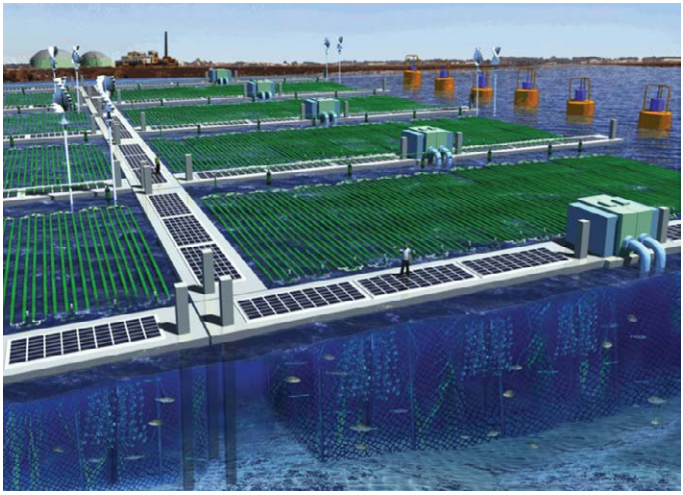

Figure 3: Artist's conception of the integrated OMEGA system, which may include algae cultivation for CO2 biomitigation and biofuel production, as well as electricity generation by other renewable sources (solar, wind, wave power systems), and a compatible aquaculture activity creating an "ecosystem of technologies"(reproduced from www.nasa.gov/ames/research).

Microalgae can be cultivated in a large range of scales: from home-made processes to algae farms of several hectares/acres. The process control can also range from visual observation and rough monitoring of main variables, to complex electronic automated systems. Thus, the more basic measurements for process control are:

a) optical density – to assess culture growth (it can be assessed by simple visual methods or accurately measured by a spectrometer)

b) temperature – should be periodically monitored through the use of simple thermometers or thermocouple probes. It should be kept within optimum culture values for optimum production

c) pH – it can be monitored trough installed probes or by using a portable pH meter or pH strips. pH is an indirect measure of several variables, mainly of dissolved CO_2 (pH lowers upon CO_2 injection).

d) $NO_3^-/NH_3/NO_2^-$ - The level of nitrogen can be measured accurately with complex instruments or roughly by using simple kits. The knowledge of the levels of total N, along dark and light cycles, helps in deciding about making corrections, for a better process control. As referred before NO_3^- is the preferred source of nitrogen and its supply affects cell growth, as well as the formation of lipid bodies.

e) Agitation – can be automated or manual. Agitation causes turbulence that avoids cell sedimentation and improves nutrients and gas exchanges.

For a more precise process control, additional variables should be monitored and automation is advisable.

Two types of vessels can be used:

Closed bioreactors*:* Tubular or flat panel photobioreactors enclosing the medium with suspended cells; reactors can be installed at open air or in green-houses, horizontally or vertically-oriented. This option may involve higher investment costs, allows automated process control, minimizes contaminations and the use of land, and results in higher productivity. An example of a process flow diagram of this type is presented in Figure 4**.** Some of these systems may not be adequate to underdeveloped regions, were any technical issue can become an obstacle.

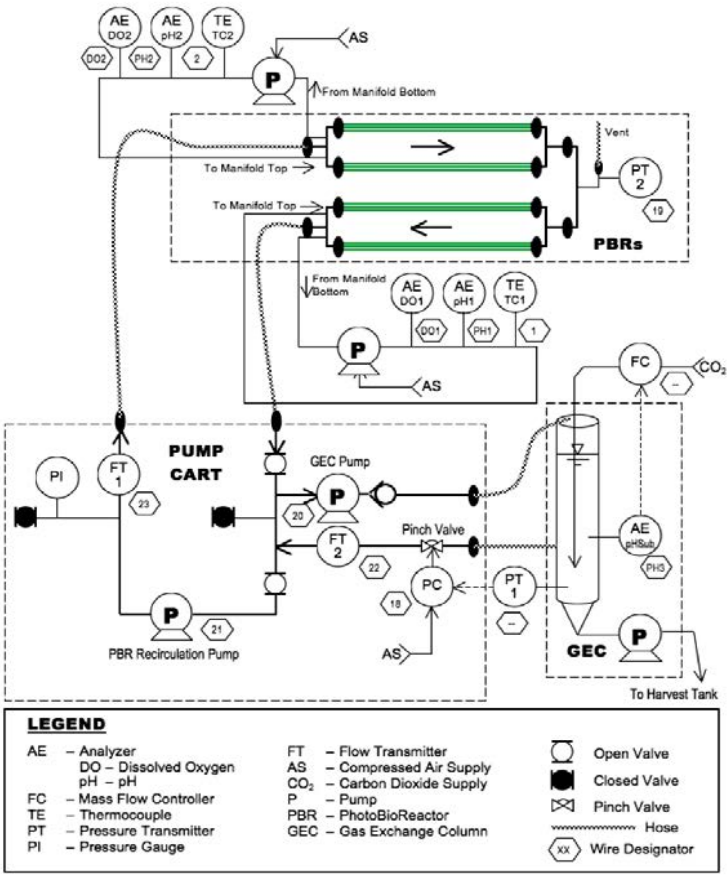

Figure 4: Example of a process flow diagram of a closed photobioreactor cultivation system. This particular example refers the to the OMEGA project, developed by NASA-AMES Research Center (reproduced from: http://www.nasa.gov/centers/ames/research/OMEGA/index.html).

Open bioreactors*:* pounds or tanks, usually installed at open air. This option involves much lower investment costs, may allow automated process control but it also works well just based on visual quality control and simple measurements with robust apparatus (as measuring cylinders, pH strips and kits). Contaminations can be a hurdle, if the process relies on a strain not adapted to the environment (the assumption that the desired strain will adapt and become dominant can fail). This extensive cultivation process requires larger areas but marginal lands can be used, since a fresh or slightly saline water source is available. If wastewater is used the water and nutrient demands are much lower. In any case, productivity is generally lower than with closed bioreactors. On the other hand, these systems can easily be installed in underdeveloped regions, due to their robustness (figure. 5).

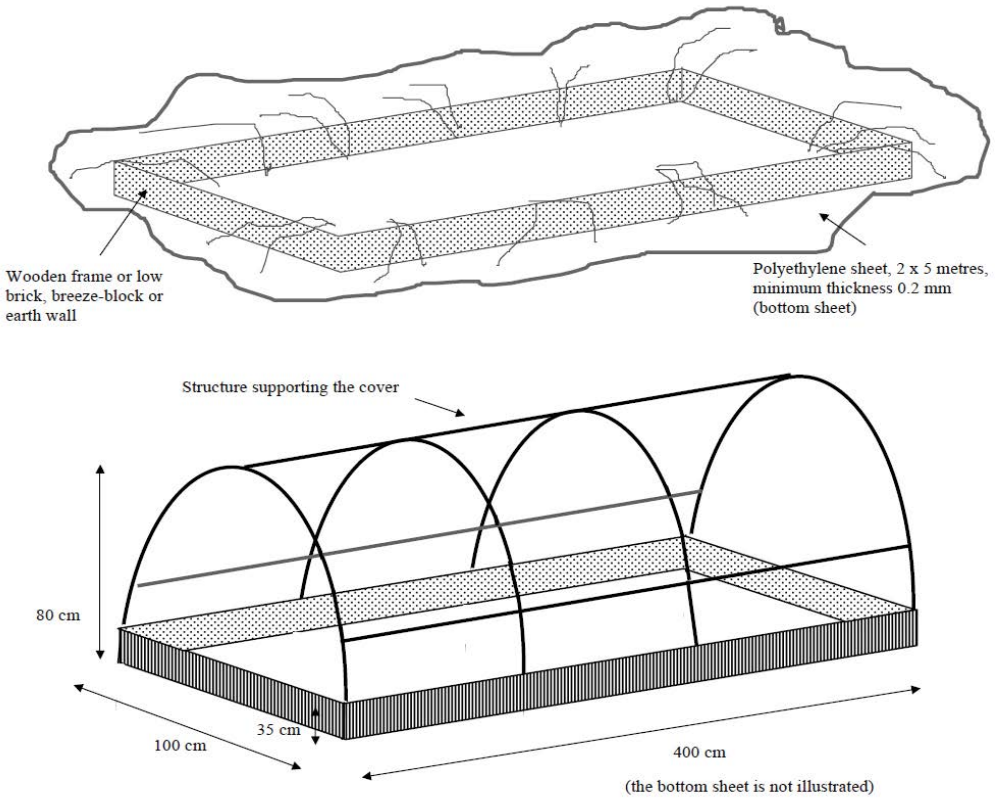

Figure 5: Construction of a simple covered tank, which can be covered when necessary to keep temperature and/ or protect from mosquitos and other pests (reproduced from Antenna Technologies – A teaching module for the production of spirulina, in: www.antenna.ch).

What's The Impact of the above Processes on Green-House Gases (GHG) Balance?

Kyoto protocol was signed in 1997 by 39 nations. It is an international agreement linked to the United Nations Framework Convention on Climate Change, which commits its Parties by setting internationally binding emission reduction targets. Its first commitment period started in 2008 and ended in 2012 targeting a 5% decrease of greenhouse gas emissions since 1990 [22]. Different objectives were set for each country, and that milestone was not reached, despite some positive effects of the treaty. Climate change is a fact now assumed

by governments. Even if the 5% reduction in emissions is reached during the next years, climate models foresee a slight impact (about 0.1 °C) on global warming [23].

Under the Kyoto's Protocol, countries must meet their targets primarily through national measures. However, the Protocol also offers them an additional means to meet their targets by way of three market-based mechanisms: International emissions trading, Clean development mechanism and join implementation. The allowed emissions are divided into "assigned amount units" (AAUs) and traded between countries, allowing for increasing emissions in change of purchasing credits on international market [22, 23]. Since carbon dioxide is the primary greenhouse gas (GHG), Carbon is now tracked and traded like any other commodity. This is known as the "carbon market" [22]. Other greenhouse gases that can be converted in carbon units are methane (CH_4), nitrous oxide (N_2O), CFC's and sulfur hexafluoride (SF_6). The equivalence has been based on the comparison of Global Warming Potential (GWP) between each gas and CO_2. It is assumed that GWP of CO_2 equals one unit, for a time spam of 100 years, and is taken into account the half-life period and the radiative forcing in atmosphere for each GHG. Some reference values are presented in Table 2.

Greenhouse gas	GWP relative to CO_2 (in ton of CO_2 equivalent)
Carbon dioxide	1
Methane	23
Nitrous oxide	298
perfluorocarbons	6500 - 8700
hydrofluorocarbons	140-11700
Sulphur hexachloride	23900

Table 2: Global Warming Potential (GWP) of main greenhouse gases (GHG), in a 100 years horizon *(Adapted from:* http://Scienceblogs.com.br/rastrodecarbono/2007/08/o-que-e-carbono-equivalente) A carbon equivalent has been fixed as an indirect tax, setting a price for carbon dioxide emissions. By definition 1Kg of CO_2 equals 0.2727 carbon units, accounting for the proportion of elemental carbon in the molecule. The carbon equivalent units of main GHG are presented in Table **3.**

Greenhouse gas	Carbon equivalent units
Carbon dioxide	0.273
Methane	6.27
Nitrous oxide	81.27
perfluorocarbons	1772 - 2372
Hydrofluorocarbons	38.2-3790
Sulphur hexachloride	6518

Table 3: Carbon units of main greenhouse gases (GHG) obtained from multiplying global warming potential (GWP) by 0.2727 *Adapted from:* http://Scienceblogs.com.br/rastrodecarbono/2007/08/o-que-e-carbono-equivalente.

The cultivation of bioalgae can mitigate CO_2 by fixating atmospheric or captured carbon, as explained above. CCS (carbon capture and storage) technologies have been improving trough the research efforts of some entities, particularly in EU and EUA. The storage and transportation of sequestered carbon still pose some economical and technical challenges. However it is feasible to use sequestered carbon in algae cultivation if the algae farm is installed nearby the CO_2 producing source and a connection between both compounds exists. As mentioned above, the overall process of producing biodiesel includes algae cultivation, oil extraction from it and subsequent biodiesel production. During algae cultivation, energy is spent in culture agitation and pumping but mostly particularly on biomass dewatering and further downstream processes. Global GWP of the process depends on whether renewable or fossil energy sources are used in these steps and also on the type of solvents and their destination, in the last process phases. The ways of assessing and improving the overall energy balance are discussed below.

Life Cycle Assessment (LCA)

The use of LCA models supported by sustainability metrics is herein discussed for the assessment of energy, Water Demand (WD) and GHG emissions. In this context, WD is

defined as the difference between the volume of water needed for algae cultivation per square meter during one year, and the annual precipitation in the region. GHG emissions can be calculated as the difference between the carbon content of harvested biomass (fixated carbon) and the GHG emissions of collateral and downstream processes.

Sensitivity analysis[9] of biodiesel production from algae have pointed bottlenecks in lipid productivity, type of cultivation process and downstream steps [24].

Currently the main motivation to implement this technology is to compete with fossil fuels. Thus, the index, Return Of Investment over fossil energy (EROI fossil) is defined as:

$EROI_{fossil}$ = Bioenergy output / non-renewable energy input [25]

In this context, for a project to be sustainable the above index should be higher than one, which means the process will generate C credits that can be traded in the carbon market.

Results of Zaimes and Khanna [24] indicate that the $EROI_{fossil}$ for microalgae biomass vary from 0.38 to 1.08, while Pardo-Cardenas *et al.* ,[18] registered larger variations in $EROI_{fossil}$, from slightly low values to significant reductions in GHG emissions (by EU sustainability criteria) being the more relevant reduction near 156% over the fossil reference. Despite the application of this methodology, Trent [20] was not able to calculate accurate values, for the OMEGA project. Although the methodology for the calculation of economic metrics is quite consensual, the associated degree of uncertainty, the residual number of large operating units among other factors, impair accurate assessments.

Results of these authors [18, 20, 25] suggest that improvements in algae-to-energy production are likely to occur via greater control over algal compositional inputs (favouring the accumulation of lipid bodies) and on the optimization of downstream processing.

Assuming the production of biodiesel in open pounds, using a sewage source of water and nutrients, the general flows, related to water demand are summarized in Figure 6. The chemical process of photosynthesis consumes water as a reactant, which means water must be periodically replaced. During the harvesting stage, process water from both flocculation and dewatering stages may be partially recycled back into the pounds (Figure 6). In short, the processes of microalgae cultivation have moderate water demands, when compared with some intensive agricultural crops. In addition wastewater can be used and partially purified in the process. In this case, the process delivers an environmental remediation service, which should be accounted for.

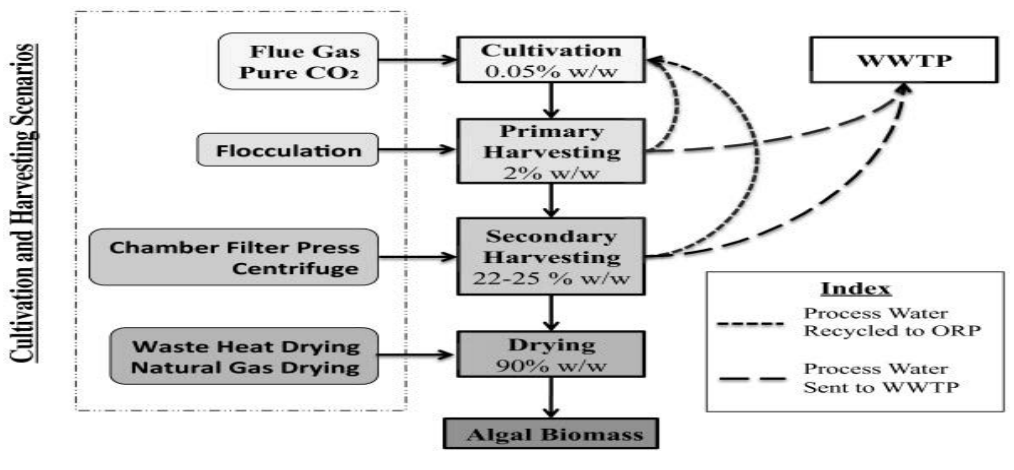

Figure 6: Microalgae biomass production chain and examined production pathways; (WWTP - wastewater treatment plant; ORP - oxidation reduction potential) (Reproduced from Zaimes and Khanna, 2013 [25] with the permission of Biomed Central).

Regarding water footprint, WD is negligible or very low when biodiesel production is coupled with wastewater treatment. On the other hand, the necessary pre-treatments of the wastewater, increase the fraction corresponding to overall energy demand as these costs are imputed to the system. Algae's direct water demands were found to be highly sensitive to geographic location [25]. Quantifying the direct component of the WD can help determine the impacts of biofuel production on regional water resources, and therefore it is an important criterion for the selection of optimal locations for algal cultivation.

Another common index is the Revenue Required (RR), which can be defined as the total revenue required to break-even, that is, the revenue required in addition to the revenue generated [20]:

$$RR = \frac{TLCC}{QWW}$$

Where

TLCC = annual total life cycle costs (including capital and operating costs – including costs of primary and secondary wastewater treatments)

QWW = annual output of the system (liters of wastewater processed per year)

The total revenue can be generated from the production of biodiesel, glycerol, naphtha as well as other added-value products.

When comparing biodiesel production by microalgae to traditional crops it is shown that oil content and annual productivity are much higher, while land use and water footprint are lower. However, in the case of biodiesel production by microalgae, production costs still need to be reduced and biodiesel yield need to be improved [18,20]. As referred above, the major energy consumption comes from dewatering and oil extraction stages. According to Pardo-Cárdenas *et al.,* [18] these values are strongly dependent on the solvent used for extraction. Other relevant environmental impacts (measured in EROI fossil) correspond to further downstream processing particularly the solvent recovery and the final destination of oil depleted biomass.

Despite some steps of biodiesel production are energy intensive, generally the processes are net GHG negative [18,25] and the next milestone is to improve the efficiency of carbon biomitigation procedures.

Moreover, a feasible way to decrease the $EROI_{fossil}$ is to combine distinct renewable energy sources to provide most of power to operate equipment, while continuing research efforts on technological improvements and on the search for innovative engineering solutions.

Concluding Remarks

Due to the inevitability of global warming and fossil fuel depletion, it is urgent to develop viable alternatives to fossil fuel and to reduce or to mitigate global GHG emissions. Microalgae may have a key role in solving both problems. Although some properties of biodiesel are different from diesel of fossil origin, the commercialization of mixtures has been successful in Brazil.

Microalgae cultivation aiming at producing biodiesel can generate extra incomes from extracting pharmaceuticals and welfare products. In addition exhausted biomass can be used as feed or fertilizer. The system may also provide valuable services as wastewater treatment, CO_2 biomitigation or environmental remediation.

When compared to other crops, microalgae present many advantages because of their faster growth rates, lower land and water requirements, and the possibility of using marginal lands or off-shore coastal areas. Downstream processes are still a bottleneck, being less efficient and requiring more energy to produce biodiesel than with other crops. Thus, the process of producing biodiesel from microalgae still poses some challenges of economic and technological nature.

When combined with other technologies, such as the use of other renewable energy sources, the overall economic and environmental indexes are more encouraging. General requirements are the vicinity of a source of CO_2 (as a power plant), the availability of wastewater and marginal lands or a protected coastal area. Commercial exploitation of these technologies is currently a reality. Nevertheless, research efforts are still ongoing to improve some steps and reduce costs, making these industries more and more competitive.

Microalgae cultivation is easier and cheaper in mild or tropical climates, because growth rates are highly dependent on temperature and light. Thus, Mediterranean areas, Latin America and certain zones of EUA, Australia and Africa are most suitable to install these facilities.

Although the production of GHG in Africa is negligible, the needs for energy are growing. In many areas the populations do not have access to electrical power and experience food security problems. Locally produced biodiesel can be a useful resource to fuel electric power generators.

In our opinion, it is possible to grow microalgae in small rural communities by implementing non-sophisticated systems. Open pound algae farms, combined with solar power generation systems, may provide these communities with energy and a cleaner environment (by providing sewage treatment), without competing with food production.

Africa has an ideal climate and offers large areas of marginal lands and ideal coastal sites. Some emerging economies are located in Africa and it may be feasible to implement more complex systems at certain sites, such as the "technological ecosystem" described in project OMEGA documentation [20, 21], alternating with more robust solutions in other locations.

In short, current studies show that cultivation of microalgae is still too expensive for biodiesel production alone, although biodiesel production costs exhibit economies of scale. However, there are economic and environmental advantages in coupling processes to obtain an overall negative GWP and thus potentiating incomes from trades in the carbon market. Despite some well established companies are commercially exploiting these technologies, the economic metrics are still not clear.

Acknowledgments

The authors wish to acknowledge the following organizations for granted permission to access and dissemination of information from their publications: EU Office for Official Publications of the European Communities; WIPO, OECD/IEA; FAO/UNEP, Royal Society of Chemistry, Antenna Technologies, NASA Ames Research Center. Authors wish to thank Dr. Jonathan Trent for his encouragement, particularly on the dissemination of OMEGA's project results and prospects.

References

1. The Zero Emission Fossil Fuel Power Plants Technology Platform (2006) A Vision for the Zero Emission Fossil Fuel Power Plants. Directorate-General for Research.
2. Sustainable Energy Systems. EU – Office for Official Publications of the European Communities. Luxembourg.
3. OECD/IEA (2013), World energy outlook 2013. International Energy Agency. Paris.
4. Richardson JW, Outlaw JL, Allison M (2010) The Economics of Microalgae Oil. AgBioForum 13: 119-130.
5. Antenna Technologies. A teaching module for the production of spirulina. Available on-line [www.antenna.ch].
6. Ducat DC, Avelar-Rivas JA, Way, JC, Silver, PA (2012) Rerouting carbon flux to enhance photosynthetic productivity. Appl Environm Microbiol 78: 2660-2668.
7. Hanagata N, Takeuchi T, Fukuju Y, Barnes DJ, Karube I (1992) Tolerance of microalgae to high CO2 and high temperature. Phytochemistry 31: 3345-3348.

8. ClimaTechWiki, a clean Technology platform. available on-line [http://climatetechwiki.org/technology/co2-mitigation-micro-algae];

9. Anjos M, Fernandes BD, Vicente AA, Teixeira JA, Dragone G (2013) Optimization of CO2 bio-mitigation by Chlorella vulgaris. Bioresource Technol 139: 149-154.

10. Wang B, Li Y, Wu N, Lan CQ (2008) CO2 bio-mitigation using microalgae. Appl Microbiol Biotechnol 79: 707-718.

11. Goether ELV, Van den Broeke LJP, Jahn J, Van den Bos WAP, et al. (2013) Combining Algae Cultivation and CO2 Capture. WIPO patent nº WO2013/022349.

12. Krisada K, Holasut S (2012) Zero carbon dioxide and heat emission integrated system of power generation from natural renewable energy sources, organic waste reclamation and commodities production and method of conduction. WIPO patent nº WO2012/131414 A1.

13. Simionato D, Maryse A, Block, NR, Jouhet, J, Maréchal E, et al. (2013) The Response of Nannochloropsis gaditana to Nitrogen de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus. Eukaryotic Cell 2013, 12:665.

14. Yong EB, Beardall (2003) Photosynthetic function in Dunaliella tertiolecta (chlorophyta) during a nitrogen starvation and recovery cycle. J. Phycol. 39: 897–905.

15. Mooij PR, Stouten GR, Tamis J, van Loosdrecht CM, Kleerebezem R (2013) Survival of the fattest, Energy Environ Sci, 6: 3404-3406.

16. Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U, et al. (2009) Algal lipid bdies: stress induction, purification, and biochemical characterization in wild-type and starchless Chlamydomonas reinhardtii. Eucariotic Cell 8: 1856-1868.

17. Lee RA, Lavoie J-M (2013) From first to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity. Animal Frontiers 3: 6-11.

18. Pardo-Cardenas Y, Herrera-Orozco I, González-Delgado A-D, Kafarov V (2013) Environmental assessment of microalgae biodiesel production in Colombia: comparison of three oil extraction systems. CT&F - Ciencia, Tecnología y Futuro 5: 85–100.

19. Aprobio, Associação dos Produtores de Biodiesel do Brasil. Available on-line [http://www.aprobio.com.br/]

20. Trent, Jonathan. (NASA Ames Research Center). (2012) OMEGA (Offshore Membrane Enclosures for Growing Algae) - A Feasibility Study for Wastewater to Biofuels. California Energy Commission. Publication number: CEC-500-2013-143.

21. OMEGA's project brochure, available on-line [http://www.nasa.gov/centers/ames/pdf/637997main_omega_brochure.pdf]

22. United Nations, Framework Convention on Climate Change. Available on-line [http://unfccc.int/kyoto_protocol/items/2830.php]

23. Parry IWH (1995) Pollution Taxes and Revenue Recycling. J Environm Econ Manag 29: S64–S77.

24. Delrue F, Setier PA, Sahut C, Cournac L, Roubaud A, Peltier G, Froment AK (2012) An economic, sustainability, and energetic model of biodiesel production from microalgae. Bioresour Technol 111:191-200.

25. Zaimes GG, Khanna V (2013) Microalgal biomass production pathways: evaluation of life cycle environmental impacts. Biotechnology for Biofuels 6: 88. Available on-line [http://www.biotechnologyforbiofuels.com/content/6/1/88]

Industrial Production of Microalgal Cell-Mass and Bioactive Constituents from Green Microalga-Botryococcus braunii

Ranga Rao A[1*], Sarada R[2], Ravishankar GA[3] and Phang SM[1]

[1]Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur, Malaysia

[2]Plant Cell Biotechnology Department, Central Food Technological Research Institute, (Constituent Laboratory of Council of Scientific & Industrial Research) Mysore, Karnataka, India

[3]Dayananda Sagar Institutions, Dr. CD Sagar Center for Life Sciences, Shavige Malleshwara Hills, 5th Floor, Kumaraswamy Layout, Bangalore, Karnataka, India

***Corresponding author:** Ranga Rao A, Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur, Malaysia-50603, Tel: +603-79674610; Fax: +603-79676994; E-mail: arangarao@um.edu.my

Abstract

The world is facing lack of alternative fuels. The demand for alternate energy is increasing every day and concurrently the depletion of fossil fuels has been so rapid that it could lead to energy crisis in the future. The energy production from photosynthetic micro-organisms such as algae is the solution to this issue, which is providing an eco-friendly alternative to meet energy requirements. Microalgae are potential source of nutrients and health promoting substances, as well as high valuable metabolites that are unique and of high commercial use. In this context, we selected microalgae *Botryococcus braunii* for the biofuel production, which contain 70% hydrocarbons in the biomass on the dry weight basis, and also it accumulates other bioactive compounds such as ether lipids, fatty acids, exo-polysaccharides and carotenoids, which are having high industrial applications. Our recent published results and also current literature on the effect of various culture conditions on biomass, hydrocarbon, lipids and fatty acid production in *B. braunii*, cultivation of *B. braunii* in raceway ponds and photobioreactors, downstream processing of hydrocarbons, bioactive molecules and their use in various applications, biological activities of *B. braunii* extracts with special reference to carotenoids were added to this book chapter. This chapter covers up to date information on the culture conditions, cultivation methods, biomass production, hydrocarbons, chemicals, bioactive constitutes and their biological properties, downstream processing of hydrocarbons from *B. braunii.*

Keywords: Bioactive compounds; Biomass; *Botryococcus braunii*; Hydrocarbons; Lipids; Photobioreactors; Raceway ponds

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

Introduction

Botryococcus braunii is a unicellular photosynthetic microalgae, member of the chlorophyceae (chlorophyta). It is producing large amounts of biomass, lipids, hydrocarbons and other bioactive molecules which can be used in renewable fuel. *B. braunii* is widespread in freshwaters, brackish lakes, reservoirs and ponds [1-3]. It is identified in several countries such as USA, Portugal, France, India, Japan, Philippines, Malaysia, and Thailand etc based on their geographical regions. The r-RNA (16s RNA) sequence of *B. braunii* is compared with other algae species *Characium vacultaum* and *Dunaliella parva* and found to be very close [4]. *B. braunii* is known to accumulate more hydrocarbons under various culture conditions [5-8]. It is divided into three different races-A, B and L based on the characteristics of hydrocarbon they produce. The race-A produces n-alkadienes and trienes (C_{25}-C_{31}), race-B produces botryococcenes (C_{30}-C_{37}) and race L produces tetraterpene (C_{40}) [3,9,10]. *B. braunii* also synthesizes lipids-fatty acids, triacylglycerol, and sterols [11]. Apart from hydrocarbons, *B. braunii* produces other bioactive molecules such as exo-polysacchrides and carotenoids [12,13]. Biomass, lipid and hydrocarbon content varied based on the culture conditions [5-8,12]. Hydrocarbons produced distillate fractionates-gasoline (67%), aviation (15%), diesel fraction (15%) and remaining residual oil by hydrocracking [9]. These fuels are reported to be free from nitrogen and sulfur oxides after combustion. Owing to its lipid and hydrocarbon production, these microalgae recognized for the renewable fuel [14]. Hydrocarbons and lipids are improved by supplying carbon-dioxide [8]. Further, research studies will be required on to improve the optimal culture conditions for the hydrocarbon and lipid production in *B. braunii* spp. The present book chapter is covered on the biomass, hydrocarbon and lipid production from *B. braunii* under various culture conditions, cultivation of *B. braunii* in raceway and photobioreactors, hydrocarbon extraction, bioactive constituents and their use in industrial applications, and also special attention to be paid to biological properties of *B. braunii*.

Morphology and Taxonomy

B. braunii cells were observed a pyramid shaped colloidal cell, green color, and together by a lipid biofilm matrix under microscopic, whereas some of the strains have dark green color with irregular colonies consisting of hundreds of elliptical cells interconnected by strand of tough mucilage. Sometimes *B. braunii* cells are attached to each other by a refringent material that sometimes links two or more distinct clumps of cells, but Metzger and Largeau [3] reported that the morphology of the alga varied in relation to age and culture conditions. *B. braunii* species are very difficult to identify since they have heterogeneity morphology. Many species of *B. braunii* were identified by Inter Simple Sequence Repeats (ISSR) or using standard species or nature of the hydrocarbon synthesis. *B. braunii* genomes are compared by using ISSR fingerprinting tools. ISSR is a potent tool to compare genomes for their identity. ISSR markers were used in the identification of taxonomical studies. Phylogenetic analyses of several strains of *B. braunii* were studied by 18s rRNA sequence [15,16]. 'A' and 'B' race cell sizes are bigger than 'L' race. 'A' race algal cells turns to pale yellow from green stage whereas 'B' and 'L' race algae cells turns to red orange and red brown color. The accumulation of carotenoids in the stationary phase of algae culture was observed. The biopolymers aliphatic and tetra-terpenoid compounds are accumulated in the cell wall of races [17]. These biopolymers are linked with ether bridges and fatty acid esters. Alkenyl phenol is

detected in 'A' race. These compounds are increased the solubility of phenolic moiety, prevent biological and chemical degradation in lipid region. Hydrocarbons are stored in outer cell wall of *B. braunii*. Some of the physiological and morphological characteristics of the race 'A', 'B' and 'L' are presented in (Table 1).

Race	Accumulated hydrocarbon	Colony color In stationary	Cell size (length x width)	Biopolymers	References
A	C_{25}-C_{31}	Pale yellow	13 x 7-9 µm	Aliphatic compounds	[9]
A	--	Green	11.0 x 6.4 µm	--	[69]
A	C_{13}-C_{26}	Green to Yellowish green to orange	3-13 µm	--	[70]
B	C_{30}-C_{37}	Red orange	13 x 7-9 µm	Aliphatic compounds	[9]
L	C_{40} H_{78}	Red-brown	8-9 x 5 µm	Tetra-terpenoid	[9]

Table 1: Some of the physiological and morphological characteristics of the 'A', 'B' and 'L' races of *B. braunii.*

Biosynthetic Pathway of *Botryococcenes*

In 'B' race produces botryococccenes (C_{30}) which contain higher levels of triterpenes. Isopenteyl disphosphate and dimethylallyl diphosphate are the fundamental precursors in the isoprepnoid biosynthesis pathway (Figure 1). Isopenteyl disphosphate produces in living organisms by the condensation of acetyl CoA through the mevalonate pathway. Casadevall et al., [18] reported that the mevalonate levels (2-^{14}C) were low in botryococcenes while using labeled experiments. Another experiment proved that botryococcenes and methylated squalenes levels were established by the non-mevalonate pathway after feeding the algae with glucose [19]. In this pathway pyruvate and glyceraldehyde-3-phosphate resulting from glycoclysis are condensed into 1-deoxy-D-xylulose-5-phosphate, in turn transposed into 2-C-methyl-D-erythritol-4-phosphate, the intermediate in IPP biosynthesis via the non-mevalonate pathway [20]. Squalene and botryococcene are triterpenes resulting from farnesyl moieties was reported by Poulter [21]. Actually farnesyl is accumulated into squalene and botryococcenes during a feeding experiment and it could be phosphorylated to its mono and diphophate esters with a cell free extract of *B. braunii* race B [22,23]. The role of farnesyl diphosphate as precursor of botryococcenes did not confirm by incubation of farnesyl diphosphate with cell free extract of *B. braunii*. The radio labeled precursor was incorporated into squalene but not into botryococcenes. This suggested that another derivative of farnesol was intermediate in botryococcenes biosynthesis reported by Inoue et al., [24]. Okada et al., [25] reported that farnesyldiphosphate is a precursor of botryococcenes. *Botryococcenes* synthase was inhibited by triton X-100 while it stimulates the squalene synthase [22]. Presqualene diphosphate as a common precursor in both squalene and botryococcenes synthesis was proved [26,27]. Either re-arranged or direct cleavage of cylopropane leads to squalene and botryococcenes. Still it is unknown that the single or two enzymes are responsible for squalene and botyrococcene synthesis. Jarstfer et al. [28] reported that recombinant yeast synthesize hydroxyl botryococcenes from presqualene diphosphate and squalene derivatives. The chemical structure of botryococcenes varied in relation to the strain origin, the methylation occurs at various positions depends on genetic factors. Alkylation of squalene in 'B' race, C_{31}-C_{34} higher homologues synthesis by methyl groups of methionine. The cyclo-botryococcenes synthesis is unknown; it may be from methylated botryococcenes or by methylation [29,30].

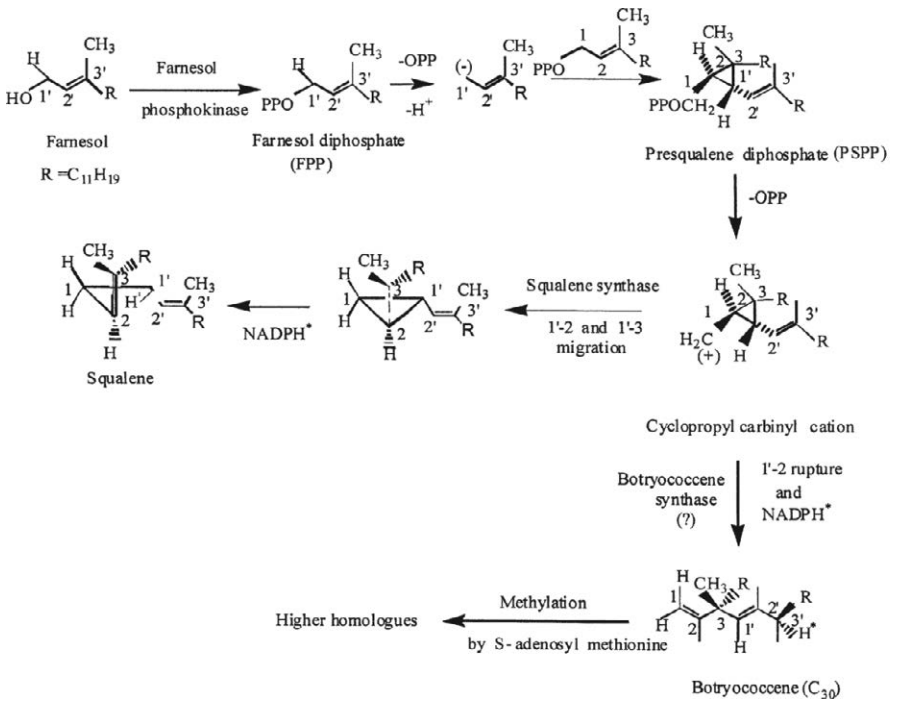

Figure 1: Biosynthetic pathway of *Botryococcenes* (C_{30}) (adapted from Banerjee et al. [9]).

Hydrocarbons in Races of *B. braunii*

B. braunii is divided into three different races-'A', 'B' and 'L' based on the characteristics of hydrocarbon they produces (Table 2 and Figure 2). The race-A produces n-alkadienes and trienes (C_{25}-C_{31}), race-B produces botryococcenes (C_{30}-C_{37}) and race L produces tetraterpene (C_{40}) [3,9,10] Race 'A' and 'B' were identified in continental, temperate and tropical lakes, whereas 'L' race was found in tropics. Hydrocarbon content varied strain to strain depending on the climatic zones. 'A' race exhibited around 20-60% of hydrocarbons on the dry weight basis which collected from the Bolivain strain from Lake Overjuyo and indigenous strains [8,11]. The hydrocarbon content in 'B' race was found to 40% on dry weight basis [31,32], whereas in race 'L' showed hydrocarbon content 8% in Thailand strains [33]. Relative percentage of hydrocarbon content and lipids in 'A', 'B' and 'L' races of *B. braunii* is presented in (Table 3).

Race A	Race B	Race L
$C_{25}H_{48}$	$C_{30}H_{50}$	$C_{40}H_{78}$
$C_{27}H_{48}$	$C_{31}H_{52}$	--
$C_{27}H_{51}$	$C_{32}H_{54}$	--
$C_{27}H_{52}$	$C_{33}H_{56}$	--
$C_{29}H_{54}$	$C_{34}H_{58}$	--
$C_{29}H_{56}$	$C_{35}H_{60}$	--
$C_{31}H_{58}$	$C_{36}H_{62}$	--
$C_{31}H_{60}$	$C_{37}H_{64}$	--

Table 2: Hydrocarbons produced by 'A', 'B' and 'L' races in *B. braunii*.

Strain name	Race	Hydrocarbon (%)	Lipid (%)	References
MCRC-Bb	A	20	26	[8]
N-836	A	22	28	[8]
CFTRI-Bb-1	A	34	25	[8]
SAG-30.81	A	46	--	[12]
LB-572	A	33	--	[12]
N-836	B	30	--	[13]
Yamanaka	A	16	--	[32]
Berkeley	B	37	--	[32]
Yayoi	B	33	--	[32]
Dawin	B	35	--	[32]
TRG	--	--	26	[32]
KMITL-2	--	--	55	[35]
Showa	B	39	--	[42]
765	--	24	13	[47]
IPPAS H-252	--	--	20	[58]
--	B	--	50	[90]
Kutz No LB 807/1Droop 1950 H-252	--	13	17	[152]
--	A	--	18	[153]
Jillamatong	A	20	--	[154]
Overjuyo-3	B	25	--	[154]
Paquemar	B	25	--	[154]
GUBITJTBB1	--	52	57	[155]
--	B	22	--	[156]
FACHB-357	B	19.4	42	[157]

Table 3: Hydrocarbon and lipid content in 'A', 'B' and 'L' races of *B. braunii* strains.

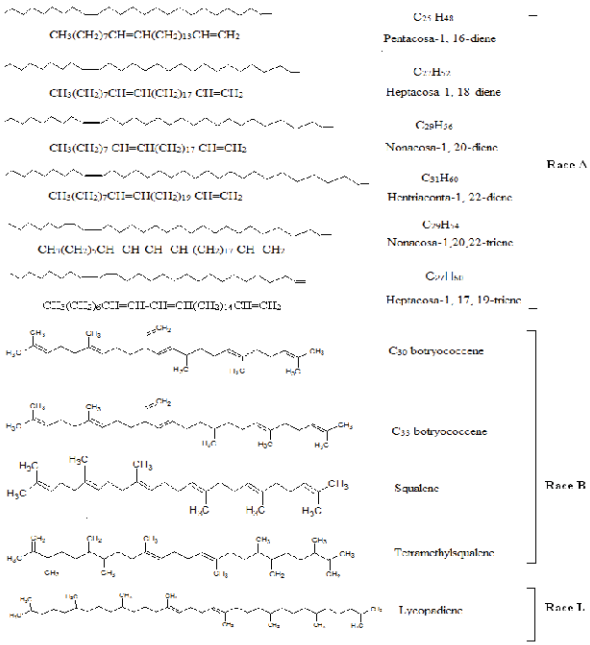

Figure 2: Types of hydrocarbons produced by 'A', 'B' and 'L' races of *B. braunii* (adapted from Metzger and Largeau, [3], Banerjee et al. [9].

Effect of Culture Conditions on *B. braunii*

The culture conditions such as pH, temperature, irradiance, nitrogen, phosphorus, carbon dioxide and salinity effects on the growth, biomass, hydrocarbon and lipid production of *B. braunii* were reported by various researchers [6-8,12,13,34-37].

Effect of pH, temperature, irradiance

The pH was important parameter which had great influence on the growth, biochemical composition and also the form of enzymes [38]. Culture pH is adjusted to 7.4 before inoculation, while increased in pH was detected during the growth of algae [7,8,14]. Usually, increase in pH was observed in the cultivation due to the consumption of dissolved carbon-dioxide for photosynthesis [7,8,14]. A change in pH was observed in carbon-dioxide and salinity cultures during the growth of algae [7, 8]. Effect of various pH (6.0-8.5) on the growth and hydrocarbon production in *B. brauni* strains (SAG-30.81 and LB 572) were reported by Dayananda et al. [34]. The results showed that *B. braunii* strains can be able to grow in the all tested pH, increased in biomass yield was observed at pH-6.0. The culture pH was increased; might be due to the utilization of biocarbonate by algae [7]. However culture pH did not effect on biomass and hydrocarbon production. Recently, Ren et al. [39] reported that the algal growth and lipid production were affected by the culture pH (6.0-11.0) in algae.

Some of the algae species can grew under higher temperature and irradiance [40-42]. Similarly, *B. braunii* grew the temperature at 30 ± 2°C under 1.2 ± 0.2 klux irradiance and 16:8 h light dark cycles [7,8] and it varies depends on the algal species. Temperature may effect on the fatty acid composition in the algae. Irradiance plays a major role in photosynthesis i.e. they convert CO_2 into organic compounds such as sugars, using the energy from light. *B. braunii* can be able to grow under high and low light intensities. High irradiance alters fatty acid synthesis to produce more saturated and mono-unsaturated fatty acids that mainly make up neutral lipids. Fatty acid composition changes especially in saturated one while increasing temperature in the culture [43]. Recently, Yoshimura et al. [42] reported that effect of various combinations of temperature (5-45°C) and irradiance (0-2000 µmol m^{-2} s^{-1}) on growth and hydrocarbon production in *B. braunii* strain (Showa), the maximum specific growth of 0.50/day was observed at 30°C with irradiance 850 µmol/m^2/s and hydrocarbon content was increased with specific growth rate.

Irradiance is the major factor which influence on growth and other high value products of algae. Low irradiance causes a reduction in dry weight while high irradiance causes biochemical damage to the photosynthetic machinery. Effect of light intensity on biomass and lipid content in *Botryococcus braunii* (KMITL-2) was investigated by Ruangsomboon [35]. The effect of light and dark cycles on growth and lipid contents in *Botryococcus braunii* (KMITL-2) were studied by establishing light and dark cycles (L:D, h:h) of 12:12, 14:10, 16:8, 24:0 under illumination of 87.5, 200, and 538 µE/m^2/s, at 25°C with constant bubbling of air. Results showed that the biomass yield was observed same at 24:0, 16:8 and 14:1 light and dark cycles, whereas the highest lipid content was obtained under 16:8 light and dark conditions. The biomass yield was found to be higher in 87.5 µE/m^2/s compared to 200 and 538µE/m^2/s. Major carotenoid lutein in *Botryococcus braunii* (LB-572) was enhanced at various light intensities reported by Rao et al. [44]. Similar results were observed when *Botryococcus braunii* exposed to various irradiance at light and dark cycles [36,45]. Effect of various conditions on the growth and hydrocarbon production of *B. braunii* strains are presented in (Table 4).

Strain name	Race	Temp(°C)	Irradiance (µmol/ m^2/s)	Light (h)	CO_2 (%)	Growth rate	Doubling time	Hydrocarbon (%)	References
LB-572	A	26	20	16	2	0.07	10.6	28	[7]
Gottingen (807/1)	A	25	131	14	1	0.3	2.3	44	[14]
Showa	B	25	240	12	2	--	--	38	[32]
KMITL-2	--	25	200	24	0	0.1	7	55	[35]
Showa	B	30	850	14	1	0.5	1.4	--	[42]
Showa	B	25,30	839	14	10	0.44	--	39	[42]
Showa	B	23-25	250	24	0	0.12	--	39	[46]
Showa	B	23-25	250	24	0.3	0.42	--	29	[46]
765	--	25	150	24	20	0.13	5.5	24	[47]
IPE-001	B	25	35	16	1	0.15	4.5	64	[80]
Yayoi	B	25	240	12	2	0.2	3.5	40	[122]
UC-58	--	25	250	24	1	0.42	1.7	--	[133]
CHN-357	--	25	303	12	0	0.2	3.5	10	[158]
NIES-836	--	25	303	12	0	0.09	7.7	35	[158]
UK-807-2	--	25	303	12	0	0.18	3.8	65	[158]

Table 4: Effect of culture conditions on the growth and hydrocarbon production of *B. braunii* strains (Adapted from Yoshimura et al. [42]).

Effect of Carbon dioxide (CO_2)

Carbon dioxide (CO_2) is necessary for microalgae to maintain the culture pH in medium. Recently, many studies were conducted on the carbon-dioxide effects on biomass, lipid, fatty acid composition and hydrocarbon production in *B. braunii* strains [8,42]. CO_2 favors the accumulation of lower chain botryococcenes (C_{30}-C_{32}) in *B. braunii* while sparged with ambient air contain higher botryococcenes (C_{33}-C_{34}) [46]. Methylation steps leading from C_{30} to C_{31} and C_{32} are faster in CO_2 enriched cultures than steps leading to C_{33}, C_{34} and higher homologues. In autotrophic media, growth and hydrocarbon content in *B. braunii* were improved by utilizing exogenous carbon source. We also reported that, the carbon dioxide effects on biomass, hydrocarbon and fatty acid profile in various indigenous species of *B. braunii* (LB-572, SAG 30.81, MCRC-Bb, N-836, CFTRI-Bb-1, and CFTRI-Bb-2) at 0.5, 1.0, and 2.0% (v/v) levels [8]. In the all tested levels, *B. braunii* was grown without any change in culture pH. Palmitic acid and oleic acid levels were increased in the strain *B. braunii* (LB-572) with CO_2 treatment at 1% and 2%. Hydrocarbon content was found to be 20% in the *B. braunii* LB-572, CFTRI-Bb-2, CFTRI-Bb-1, and N-836 strains, whereas it was less than 20% in the SAG 30.81. Another study evaluated the effect of carbon dioxide (2-20%) in *B. braunii*-765 [47]. The results showed that the strain can able to grow in all the CO_2 tested levels with aeration rate at 0.2 vvm, without any changes in culture pH. The maximum biomass yield was found to be 2.31 (g/L) at 20% CO_2 concentration on 25th day and also enhanced in hydrocarbon content and algal cell size with the increase of CO_2 levels.

Effect of Nitrogen

Nitrogen plays a major role in microalgae for the growth and lipid accumulation. It is supplied to *B. braunii* in the form of nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+), which regulates nitrogen metabolism in algae. Nitrogen deficiency lead to lipid accumulation in *B. braunii* was reported [48,49]. Zhila et al. [49] reported that *B. braunii* accumulates Triacylglycerols (TAGs) with higher amount of oleic acid under nitrogen limitation conditions, whereas changes in both fatty acid composition and polar lipids under nitrogen deficiency culture. Many algal species accumulates TAGs under nitrogen starvation, which contain more monounsaturated fatty acids [39]. Potassium nitrate, calcium nitrate, sodium nitrate and ammonium nitrate effects on hydrocarbon and fatty production in *B. braunii* (LB-572 and SAG 30.81) were evaluated by Dayananda et al. [34]. In the results, hydrocarbon

content was enhanced in both *B. braunii* (LB-572 and SAG-30.81) strains by potassium nitrate treated culture. Oleic acid is the major fatty acid was found in all the nitrate treated groups. Ammonia (NH_3) used as nitrogen source which causes the culture pH decline to 4, where the cells were damage due to nitrate reductase enzyme inactivation. Hydrocarbon content decreased when *B. braunii* exposed to NH_3, this indicates a diversion of acetyl Co-A from hydrocarbon synthesis pathway to amino acid synthesis pathway [50]. The effects of nitrogen (0-3.5 mg/L) on growth of *B. braunii* (764 and 765) at two different light intensities (60 and 110µmol/m^2) were evaluated by Sun et al. [51]. In the results observed that growth of *B. braunii*-764 with all nitrogen treatments were significantly higher at 110 µmol/m^2 compared to other treatments. Lack of nitrogen limits protein synthesis and thus increased lipid [52] or sometimes carbohydrates accumulation [53]. Biomass and lipid content in *Botryococcus* (KMITL-2) was enhanced by initial nitrogen concentration at 86 mg/L [35].

Effect of Phosphorus

Phosphorus is supplied to algae growth in the form of KH_2PO_4 and K_2HPO_4. Microalgae can absorb phosphate in the requirement of the cells. The phosphate can store in intracellular granules. The cells utilize phosphate when the extracellular phosphate supplies run out. Growth was enhanced in algae with the phosphate deficiency culture conditions [14]. Metabolites production in algae was increased by supplying phosphate. Phosphate released in the medium as the cell lyses in the declined stage of culture, while increased in the initial concentration of phosphate in the medium. Growth and hydrocarbon content in *B. braunii* was increased in the excess amount of phosphate, this may be changes in the nitrogen: phosphate (N: P) ratio in the culture medium, which influence on lipid accumulation in algae [14]. Effect of various phosphorus levels (0.15-0.77 mg/L) on growth of *B. braunii* (764 and 765) at 60 and 110 µmol/m^2 light intensities were studied by Sun et al. [51]. In the results, growth rate of *B. braunii* (764 and 765) at 60 µmol/m^2 showed no significant difference. The average growth rates of *B. braunii* (764) at 110 µmol/m^2 were significantly lower than other phosphorus treatments. Initial phosphorus concentration on growth and lipid content in *B. braunii* (KMITL-2) was observed, the results showed the maximum biomass was found to be 1.91 g/L at 444 mg/L phosphorous concentration, whereas the highest lipid content was found to be 54% at a phosphorus concentration of 222 mg/L [35].

Effect of Salinity

Microalgae are incredibly tolerant to changes in salinity. It is effected on microalgae in different ways osmosis stress, ion stress and changes of the cellular ion ratios due to the selective ion permeability of the membrane while changes in salinity. The better algae growing conditions for most species is at a salinity level that is slightly lower than that of their native habitat, which is obtained by diluting sea water with tap water [54]. Effect of various salinity concentrations on growth, biomass, hydrocarbons, lipids, fatty acids and secondary metabolites in race 'A' of *B. braunii* was reported by Ranga Rao et al. [7]. In the results, the biomass yield was enhanced and bioactive constituent changes were observed at all the tested levels of salinity. Fatty acids-palmitic acid (1.7 fold) and oleic acid (2 fold) were increased in both 34 mM and 85 mM salinity treated culture, whereas 2 fold increased in carotenoid content at 85mM salinity concentration. Maximum hydrocarbon content was observed in 51 mM and 68 mM of salinity. The lipid content of *B. braunii* in salt concentration was higher than control group (without salt) [55]. Similarly Vazquez-Duhalt and Arredondo-Vega [56] reported that the lipid content in cells of *B. brauni* (Austin & Gottingen) was enhanced in the presence and absence of NaCl. Earlier reported that, increase in salinity may results in a slightly increase in total lipid content of algae [47,53]. Secondary metabolites-carotenoids in *B. braunii* were enhanced by providing salt stress [6,7]. The effect of salinity on growth, biomass, lipid, fatty acid and hydrocarbon production in various *B. braunii* species were reported [36,57,58] Growth and lipid production of four *Botryococcus braunii* (TRG, KB, SK, and PSU) strains were tested, all the strains were survived higher salinity concentration, whereas growth of SK, TRG and KB strains was

decreased. The lipid content in SK, TRG and PSU strains were decreased when the salinity was increased [36]. Seawater containing medium was used for the hydrocarbon production in *B. braunii* (Showa) where the 90% hydrocarbon obtained without any pretreatment [57]. In another study conducted on the effect of 0.3 and 0.7 M NaCl on biomass yield, lipid content, and fatty acid profile of *Botryococcus braunii* (IPPAS H-252) in different phases of the culture, the culture growth was inhibited for first three days, due to considerable changes in the lipid and fatty acid profile. In the later phases of the culture, algae biomass increased, and the degree of unsaturation was increased, mainly due to rise in the content of polyenoic acid [58].

Cultivation of *B. braunii*

Raceway Ponds

Micro algal cultivation is promising method for the production of high value metabolites-biomass, lipids and hydrocarbons which are very used for the biofuel feed stock [59]. Algae are grown in raceway ponds and photo-bioreactors for the scale up studies (Figure 3). A raceway pond contains oval shape, closed loop channels, maintained depth in between 20 cm to 40 cm, and mixing is provided by paddle wheels. Raceway ponds are constructed by cement and plastics [60]. Continuous mixing gives equal light to the culture and also prevents sedimentation in pond. The raceway pond cultivation system is very cheap compared to closed system. This system needed investment in terms of light and other operations [61-63].

Contamination is the major problem in open and closed system due to the environmental fluctuations [64,65], and is required to monitor to keep culture in good condition. Contamination occurs in the ponds by other algae, bacteria or predator's-ciliates, rotifers, amoeba and cysts, and can be partially reduced or controlled by initial cell concentration, culture pH, chemical and physical treatments [65,66]. Agitation is essential as accumulation of biomass in one placed. Generally, cultures run in batch or continuous or semi-continuous. The batch cultivation was the best to maintain fresh algal inoculum as starting material for every batch. To obtain higher biomass yield and productivity depends the initial cell, biomass and nutrient concentrations, which plays a key role in maintaining healthy culture [67,68]. Our aim is to produce more biomass with minimum energy inputs, it is recommended to use marine forms to control or minimize contamination from other algal or zooplankton species.

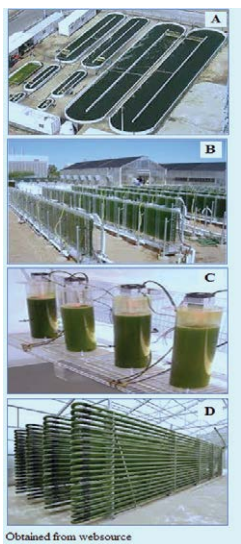

Figure 3: Common microalgae cultivation in raceway ponds (A), flat panel photobioreactors (B), bubble column photobioreactors (C) and tubular photobioreactors (D).

Recently a few research studies were conducted on the cultivation of *B. braunii* in raceway ponds [13,69,70]. *B. braunii* species-LB-572 and N-836 were grew in raceway and circular ponds reported by Ranga Rao et al., [13] in the results, the hydrocarbons of C_{20}–C_{30} carbon chain length were higher in raceway and circular ponds on 18th day cultivation was observed, whereas the major fatty acids were found to be palmitic and oleic acids in the both raceway and circular ponds. Ashok kumar and Regasamy [69] reported that *B. braunii* strains-Kutz, AP-103, AP-104 and AP-105 were isolated from the Indian freshwater bodies and cultivated them in raceway ponds for the biomass and lipid production, in the results observed that the maximum biomass, lipid, hydrocarbon content was found to be 1.8 g/L, 19% and 11% in AP-103 strain. In addition 33% carbohydrate and 18% protein contents were observed under raceway ponds cultivation. The major fatty acids- heptadecane, hexadecane, oleic, linolenic and palmitic acids were identified. Similarly, the maximum biomass yield in *B. braunii* (Mahabali) was found to be 2 g/L (w/w) on 14th day cultivation in raceway ponds reported by Dayananda et al. [70].

Photobioreactors

Photobioreactors (PBRs) are designed to grow microorganisms in closed system [71,72]. Recently they have received a great use in microalgae cultivation for the biomass production. PBRs can be optimized according to the biological and physiological characteristics of the algal species being cultivated. A uni-algal culture can be maintained in photobioreactors whereas in raceway ponds it is not possible. Environment sensitive algal species can easily grow in photobioreactors which gives more productivity. Based on their design, PBRs are considered to have several advantages compared to raceway ponds. In PBRs can be control various parameters such as pH, temperature, mixing, light, CO_2, O_2, prevent evaporation, reduce CO_2 losses, allow to higher cell densities and volumetric productivities. PBRs offer safe, protect environment and prevent contamination by microorganisms. PBRs have several disadvantages such as bio-fouling, overheating, oxygen accumulation, difficult in large scale, to install high operating cost, cell damage by shear stress and degradation of material used for the PBRs [73]. Biomass and lipid productivity in PBRs was higher than in raceway ponds [74,75]. Generally three parameters such as volumetric, areal and illuminated surface productivity used to calculate the biomass productivity in algae production systems [76].

PBRs-flat panel, tubular, bubble column, annular (cylindrical), air lifting is used for mass culture of algae. Tubular PBRs are made with plastic or glass tubes with U bends to capture more sunlight. The tubes diameter are 5-10 cm. Algal culture are circulated through mechanical pumps [60]. These PBRs have high surface volume; hence light capture is higher and gives higher biomass yield. Microalgae are successfully cultivated in tubular PBRs [77-79]. Tubular PBRs require a large land area for a given volume of reactor and this is a significant disadvantage. Bubble columns and airlift reactors are more compact than tubular devices can offer more advantages for large scale culture [77]. Airlift systems have been used for *Botryococcus, Porphyridum cruentum, Haematococcus pluvialis, Phaeodactylum tricornutum* [80-82]. Annular (cylindrical) PBRs vertically designed with aeration provided from the bottom and illuminated through transparent walls. They offer efficient light, temperature and agitation to algal cells. Mixing is very important that increases the cell concentration when exposed to light and minimize the dark volume of the reactor. It also enhances mass transfer between nutrients, facilitates dissipation of heat and prevents oxygen [60]. Efficient illumination can be achieved by internal light which distributes light into cultures [83]. Light and dark cycles can influence on photosynthesis. Enhancing productivity in cultivation of algae by supplying flashing light causes additional cost [84]. Now a day's LED photodiodes are used for illumination instead of fluorescent lights. Continuous illumination can be achieved by solar radiation during the day time whereas night time can use solar power driven LED lights to enhance productivity [85]. Light transmission can be increased by increasing the reactor that is exposed to light. Improving light penetration in PBRs was evaluated by Carvalho et al. [86]. Plastic bags are used for

microalgae outdoor cultivation system. Flat panel consist of joined transparent plates to store the culture on which the culture is illuminated from one or both sides and stirred by aeration. Flat panel PBR offers biomass production of photoautotrophic microorganisms. Flat vessels are made for outdoor mass cultivation of algae under photoautotrophs [87,88] which is very easy to build desired light path. The factors to be considered in the design of flat panels are the collection of solar radiation, to remove oxygen, to avoid nutrient gradients and to control temperature. In order to get irradiance 100 $\mu E/m^2/s$ in panel reactors, to maintain cell density 1 g/L and flat panel depth should be below 0.07 m. The optimum light path was 0.1 m for *Nannochloropsis sp.* in flat panel was reported by Zou and Richmond [89] where the biomass productivity reached 0.5 g/l/d. The flat panel reactors can achieve high biomass yield due to their light illumination surface area to volume. Mixing is provided by supplying CO_2 enriched air which used as carbon sources to increases cell density and biomass productivity.

Today, raceway ponds and PBRs used for *Botryococcus* culture for the biomass production [13,47,69,70,79]. Batch and continuous mode was tested in open and closed systems. Continuous mode is feasible for higher biomass production. Continuous culture mode in air lift PBRs used for *B. braunii* at small scale was reported by various authors [14,72,77,78]. Maximum hydrocarbon content was observed in bubble column photobioreactor by supplying 1% CO_2-enriched air with 10 klux irradiance [77,78]. In bubble column photobioreactors, the biomass yield was reached 7 kg/m^3 and hydrocarbon content was found to 50% in *B. braunii* culture was reported by Kojima and Zhang [90]. Compared with closed aseptic culture vessels, open pond reactors can provide a moderate surface to volume ratio at a much lower cost per unit volume [91]. However the culture conditions in open systems are less controlled than in closed reactors, consequently the biomass productivity was low compared with closed PBRs. In open culture systems contain mixed populations and not desired algae. Open culture system is the most reliable for producing large amounts of biomass, for extraction of lipids and hydrocarbons. Recently reported that the biomass productivity of *B. braunii* strains in raceway ponds [13,47,69,70] was comparatively same with the photobioreactors [47], this might be due to the strain difference, culture conditions (CO_2 supply, initial cell concentration and salinity) and also specific design of raceway ponds and photobioreactors. Based on the current literature, biomass and areal biomass productivity of *B. braunii* species in the cultivation of raceway and photobioreactors comparison with other algal species are presented in (Table 5).

Microalgae	Mode of cultivation	Biomass productivity or areal biomass productivity (g/L/dorg/m^2/d)	References
B. braunii (LB-572)	Raceway	0.11	[13]
B. braunii (N-836)	Raceway	0.05	[13]
B. braunii (765)	Photobioreactor	0.09	[47]
B. bruanii (AP-103)	Raceway	0.11	[69]
B. braunii (Mahabal)	Raceway	0.10	[70]
Phaeodactylum	Tubular	1.19	[71]
Sprulina	tubular	0.62	[84]
B. braunii (FACHB-357)	Single layer photobioreactors	5.50	[157]
B. braunii (LB-572)	Panel reactors	0.02	[155]
B. braunii (LB-572)	Biofilm photobioreactor	0.71	[160]
Chaetoceros	Bubble column	3.31	[161]
Chaetoceros	Air lift reactor	4.09	[161]
Chlorella kessleri	Tubular	0.12	[162]
Scenedesmus obliquus	Tubular	0.20	[162]
Spirulina	Tubular	0.39	[162]

Dunaliella	Flat plate	1.50	[163]
Haematococcus	Air lift reactor	0.80	[164]
Monodus	Bubble column	0.03	[165]
Strain name			
Nannochloropsis	Flat panel	0.22	[166]
Nannochloropsis	Flat plate	0.22	[166]
Nannochloropsis	Raceway	11.0	[167]
Nannochloropsis	Flat panel	27.0	[167]
Nannochloropsis	Tubular	25.0	[167]
Nannochloropsis salina	Raceway	0.20	[168]
Phaedactylum	Tubular	1.38	[169]
Phaeodactylum	Flat plate	1.38	[170]
Phaeodactylum	Tubular	1.19	[72]
Scenedesmus rubescens	Raceway	0.02	[171]
Scenedesmus sp.	Raceway	0.19	[172]
Spirulina platensis	Raceway	0.18	[173]
Synechocystis aquatilis	Flat panel	1.00	[174]

Table 5: Biomass and areal biomass productivity of *B. braunii* comparison with other algal species in raceway and photobioreactors cultivation.

Hydrocarbon Extraction

B. braunii produces variety of hydrocarbons which contain higher and lower carbon chain. Biomass can be harvested by filtration or centrifugation. Hydrocarbons are accumulated in outer cell wall. *B. braunii* cell wall is very thick and it is very difficult to extract or break [3]. Hydrocarbons can be recovered by solvent extraction process, high pressure, sonication or supercritical fluid extraction process. High pressure extraction for hydrocarbons used at bench scale. Hydrocarbons can easily extracted by non-toxic solvents which are inexpensive, easily available, and immiscible with water, low boiling point and reusable. For obtaining higher hydrocarbon recovery, dry algal cells can be used for the hydrocarbon extraction instead wet cells [3]. However, dewatering and drying of the biomass is not a cost-effective method for industrial applications. Wet biomass cells contain higher percentage of water; the algal cells reduce contact of the non-polar solvents with the outer cell wall [92]. Wet cells are aggregated and form clumps during the extraction process. Hydrocarbon recovery yield was influenced by physiological status of culture. Frenz et al. [93] reported that the hydrocarbon recovery was high in exponential growth phase of algae which was observed in photobioreactor cultivation. However, the strain, solvent selection, extraction time and temperature are the key parameters to get the efficient hydrocarbon recovery.

The biocompatible solvent selection is very critical because the presence of organic solvent can retard the cell growth. Based on the current literature, various solvents-hexane, n-octane, dodecane, acetone and ethyl acetate extraction methods were used for hydrocarbon recovery [93-97]. Terpenoids in *B. braunii* biomass was extracted by using solvent hexane [98]. Another solvent method, supercritical CO_2 extraction method used for hydrocarbons recovery, optimal time was found to be 30 min at pressure 30 MPa [99,100]. This extraction method was expensive, non-toxic, reusable, contains low viscosity and CO_2 allow quick extraction of solids. Algal hydrocarbons solubility in CO_2 extract was increased with increasing pressure [100]. Recently, Cho et al. [95] reported that n-octane was best for the hydrocarbon recovery when compared to dodecane, tetradecane and hexadecane solvents which gives 48% hydrocarbon recovery at 6h time. Three non-polar solvents-n-hexane, ethyl acetate and acetone at 50°C temperature with 10.3 MPa pressure used for hydrocarbon extraction [101], the results showed n-hexane was the efficient solvent extraction method for higher hydrocarbon recovery, further he confirmed accelerated solvent extraction (ASE)

was 6% higher than Sox-let method at 6 h and 60 ml solvent which saved time and solvent. Major hydrocarbons (C_{32}, C_{33}, C_{34}) recovered in dimethyl ether used as extraction solvent whereas in n-hexane extract was found to be C_{30} and C_{31} hydrocarbons reported by Kanda et al.[102] Dayananda et al. [12] reported that the hydrocarbon extraction was 33% in *B. braunii* (LB-572) and 46% in *B. braunii* (SAG-30.81) obtained using hexane extraction method. In our laboratory studies, hydrocarbon extracted for *B. braunii* strains using n-hexane showed 34%, 20% and 22% hydrocarbon recovered in CFTRI-Bb1, MCRC-Bb and N-836 strains of *B. braunii* reported by Rao et al. [7,8]. Based on these findings, n-hexane was the efficient solvent for the hydrocarbon extraction. Hydrocarbon recovery from various *B. braunii* species using solvent extraction methods is presented in (Table 6).

Strain name	Solvents/temp/pressure/extraction time	Hydrocarbon recovery (%)	Reference
CFTRI-Bb-1	n-hexane	34	[7]
MCRC-Bb	n-hexane	20	[7]
N-836	n-hexane	22	[7]
LB-572	n-hexane	33	[12]
SAG-30.81	n-hexane	46	[12]
Showa	n-hexane	39	[42]
Showa	n-hexane	90	[57]
UTEX-572	n-octane, 6h time	48	[94]
Showa	Hexane, at 50°C, 10.3 MPa pressure, 5 min	40	[101]
SAG 807-1	1,8-diazabicyclo-[5.4.0]-undec-7-ene/octanol (1:1)	16	[140]
SAG 807-1	1,8-diazabicyclo-[5.4.0]-undec-7-ene	15	[140]
SAG 807-1	1,8-diazabicyclo-[5.4.0]-undec-7-ene/ethanol (1:1)	12	[140]
Showa	n-hexane at 100°C	97	[175]

Table 6: Hydrocarbon recovery from biomass of *B. braunii* strains by using solvent extraction methods.

Bioactive Constitutes and their Use in Industrial Applications

B. braunii produces variety of bioactive molecules such as hydrocarbons, alkanes, ether lipids, fatty acids, polysaccharides and carotenoids which have high demand in industrial applications such as fuel, food and feed applications (Table 7). In addition, *B. braunii* is used in plant growth promoter studies in plant tissue culture systems. Carotenoids and exopolysaccharides from *B. braunii* showed various biological properties in *in vitro* and *in vivo* studies. These bioactive molecules are described briefly in the below.

Bioactive molecules	Industrial Application
Hydrocarbons	Fuels, electricity, natural gas, liquid fuel, petroleum gas,petrol, diesel, power plants, homes
Alkanes	Fuel oils, natural gas gasoline, polymers, paints, plastics, drugs, cosmetics, detergents,
Either lipids	Cytotoxic effects on tumor cells, chemical indicators in neoplasms, chemical mediators, Fat absorption, human food, animal feed
Fatty acids	Human nutrition, nutraceutical and pharmaceutical applications, Gene interactions, Acylation or proteins, Membrane fluidity, substrate specificity
Exopolysaccharides	Food, paint, laundry, textile, adhesive, binding agent, coating, emulsifying agent, stabilizer and thickening agent
Carotenoids	Antioxidant properties, anticancer activities , bioavailability , food, ingredients, feed, nutraceutical and pharmaceutical applications,

Table 7: Bioactive molecules from *B. braunii* and their industrial applications.

Source of Hydrocarbons

B. braunii contain three different races such as 'A', 'B' and 'L'. These are divided based on the characteristic of hydrocarbons they produce. Race 'A' produces odd numbered n-alkadienes and alkatrienes. Race A produces 60% of the olefins in the dry cell mass of the green state colonies [103]. Two unusual hydrocabrons-$C_{27}H_{51}$ and $C_{27}H_{48}$ tetrane reported in race A strain which was isolated from Lake Overjuyo, Bolivia. Chemical structures (30) and

hydrocarbons (50) were reported in 'A' race of *B. braunii* [11] and most of the compounds were identified monoenes, tetraenes and odd carbon numbered. The hydrocarbon distribution depends on the genetic factors which varied strain to strain during the cultivation under identical conditions [104]. Oleic acid is the main precursor of dienes and trienes reported by Templier et al. [105,106]. The 'B' race produces poly methylated unsaturated triterpenes, called botryococcenes and they can exist as isomers with the same carbons but different structures. Squalene and C_{31-} C_{34} methylated squalenes were synthesized by 'B' race in *B. braunii* [11,30]. These molecules were isolated from Bolivian strain of *B. braunii* [107]. The botyrococcene (C_{30}) was the precursor for higher homologous compounds reported by Metzger et al. [29]. *Botrycoccene* can accumulate around 20-80% in the dry cell biomass [108]. A 50 botryococcenes were purified in 'B' race reported by Metzger and Largeau, [11]. The 'L' race produces tetraterpene ($C_{40}H_{78}$), known as lycopadiene [109] and they can accumulates 2-8% of the dry cell biomass [33]. Lycopadiene was the sole hydrocarbon detected in *B. braunii* strains from Thailand and Ivory Coast [110].

Source of Alkanes

Alkanes are nothing but saturated hydrocarbons found in *B. braunii* [111]. Cell accumulates methylated aldehydes, is generated from fatty acids via methylation process by S-adenosyl methionine, and then converted them into aldehydes by fatty acyl reductase which requires ATP, CoA, and NADH for its activity. Fatty acyl reductase was solubilized in 0.1% octyl beta-glucoside, purified by blue A and pamitoyl agarose column chromatography and its molecular weight was found to be 35 kDa by SDS-PAGE [112,113]. Alkanes are obtained by the conversion of aldehydes which are generated from the fatty acids by methylation process; alkanes are losing one molecule of carbon in this process. Alkane's conversion was occurred via decarboxylation which requires decarbonylase enzyme activity in anoxic conditions. It is located in microsome, it may convert carbon monoxide in *B. braunii* into carbon dioxide which is necessary for organisms to accumulate higher amount of hydrocarbons and the enzyme can be inactivated by metal chelators. Saturated hydrocarbons such as docosane, hexacosane and heptacosane were reported in *B. braunii* (N-836) [114].

Source of Ether Lipids

'A', 'B' and 'L' races of *B. braunii* are producing variety of either lipids. Ether lipids made up with various triacylglycerols, glycerophosphatides and isopranoid dialkyldiglycerol tetraethers. 'A' race produces ether lipids like alkadienyl-O-alkatrienyl ether, resorcinolic either and alkenyl-O-botryalyl ether reported by various authors [115-117]. It is producing high amount of alkadienyl-O-alkatrienyl ether where the strain isolated from Lake Overjuyo, Bolivian and another from Lake Coat Herno from France [115]. These species contain 40% of ether lipids in the exponential phase of algae whereas in stationary phase their levels were low [118]. These ether lipids are close to hydrocarbons. However hydrocarbons levels were very low in these strains during the growth phase of algae. One more 'A' race strain was isolated from Lake Overjuyo, Bolivian which contains 35% of phenoxy ether lipids on the dry weight basis [117]. Another strain isolated from Maddingley brick Pits, UK which showed 5% of ether lipids on the dry biomass. Either lipids linked with phenoxy bonds or hydrocarbons which derived from alkadienes, thus give rise to high molecular weight lipid compounds. These lipids structurally derive from the coupling of alkadienes, alkenylresorcinols, alkenylhydroquinols or branched aldehydes by aldol condensation called botryals. Alkenyl-O-botryalyl ether is ether lipid which attached to hydroxyl group is esterified by oleic acid [117]. In 'B' race, ether lipids such diepoxy-tetramethylsqualene, botryolin A and botryoxanthin-A, B was reported [110,119-121). Minor components of botryolins were isolated from Ivory Coast. These triterpenoid triethers contain a tetramethylsqualene carbon skeleton. Braunixanthin-1,2 were isolated from the Japanese strain reported by Okada et al. [122]. They contain an alkylhdroquinol moiety mid chain bound by ether bridges

to enhinenone and a tetramethysqualene derivative. This contains a tetrahydrofuran ring, which derives from the cyclisation a diepoxy-tetramethylsqualene [110]. 'L' race produces various ether lipids, recently reported that diepoxy-lycopane, lycopanerol 'A', lycopanerol 'F' and lycopanerol 'H' isolated from *B. braunii* which linked with tetrahydrophyran ring containing lycopanes [110,119,123,124]. Lycopanerol 'F' found in the India and Ivory Coast strains which associated three tetrahydrofuran containing lycopanes with ether bridges. Lycopanerol 'H' found in the Ivory Coast strain which includes tetrahydrofuran and tetrahydropyran containing lycopene, alkylphenol, phenoxy bonds. Accumulation of lycopanerol in the 'L' race was found to be 10% on the dry weight basis. These compounds are formed as terpenoid, alkylphenol, resorcinol and non-terpenod with ether/phenoxy bonds. Each compound derived from the diepoxy-lycopane which containing one or three tetraterpenoid with tetrahydrofuran or tetrahydropyran [106].

Source of Fatty Acids

Microalgae species - *Botryococcus, Chlorella, Scenedesmus, Dunaliella, Nannochloropsis* and *Chlorococcum* etc accumulates high amount of lipids [61] Among the algal species, *Botryococcus braunii* is one considered to be a potential source for biofuel feedstock which produces high amount of fatty acids [6,7,13]. *Botryococcus* accumulates lipids in the range of 2-86% on the dry weight basis; this content was varied by culture conditions and specificity of alga [11]. Lipids contain various fatty acids such as palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic acid ($C_{18:3}$). Accumulation of fatty acids in the exponential phase was higher compared to stationary phase of algae. *B. braunii* contain methylated branched fatty acids and these are known to inhibit endothelial cell and leukocyte proliferation [113,125,126]. Fatty acids profile - $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{22:0}$ in *B. braunii* was reported in raceway and circular pond cultivation [13]. Enhanced oleic and palmitoleic acid content in *B. braunii* (LB-572) under salinity culture conditions [6].

Source of Exopolysaccharides

B. braunii produces Exopolysaccharides (EPS) but a few species are used in commercially importance. These are used in industrial applications such as food, paper, paint, laundry, textile and adhesive. Use of the polysaccharides depends on their rheological characteristics. Microbial polysaccharides have a great impact since their use in various applications. Polysaccharide production associated with pathogenicity of microorganisms. EPS produced by *Porphyridium curentum* used in commercial applications, whereas EPS produced by *Chlamydomonas Mexicana* considered to be soil conditioner [127,128]. Similarly, *B. braunii* is producing exoploysaccharides which accumulates in the ranges from 250 g/m^3 in 'A' and 'B' races and 1 kg/m^3 in 'L' race [14,129]. Cells in *B. braunii* are made up with mucilaginous polysaccharides; these are dissolved in culture medium, increases in viscosity of culture medium. Polymers-galactose, fucose, arabinose, rhamonse, uronic acids, and non-sugars are identified in *B. braunii*. Among the polymers, galactose is the major compound in the heterogeneouse polysaccharide. Some of the unusual sugars namely 3-O-methyl fucose, 3-O-methyl rhamnose and 6-O-methyl hexose were reported in *B. braunii*. EPS content 4-5.5 Kg/m^3 in *B. braunii* UC-58 was reported by Fernandes et al. [130]. The polysaccharide content was higher in decline phase of algae compared to exponential and stationary phase. Enhanced polysaccharide content in nitrogen deficiency conditions, where the degrading nitrogen contains macromolecules and accumulating polysaccharides and lipids. EPS content was declined, when urea and ammonia used as nitrogen source. The consumption of urea and ammonia causes decrease in culture pH, this gives low polysaccharide production. Optimum nitrate concentration was 8mM and temperature ranges from 25-30°C for polysaccharide production in *B. braunii*. EPS synthesis under continuous illumination was exhibit higher than cells grown under cyclic illumination [131]. The degree of biopolymer was decreased when the culture was exposed out of the optimal temperature. *Auriobacterium barkeri* associated with *B. braunii* culture, however they did

not effect on the exoploysaccharide synthesis [130]. Sulfated polysaccharides isolated from microalgae species, used in anti-adhesive property, inhibited HeLaS3 human cell line by algal polysaccharides [132]. EPS is a potential source of uronic and fucose, which can be used in reduce metal ions toxicity and substrate in chemical synthesis [133].

Source of Carotenoids

Microalgae are producing high value added products, in particularly carotenoids which have broad industrial applications still studies are required to improve their culture conditions or cultivation methods in order to be economically competitive in the market [134]. Some algal species accumulate high concentration of carotenoids under stress conditions [6,7,44]. Similarly, *B. braunii* is known to produce hydrocarbons, lipids, exopolysaccharides and carotenoids [135,136]. The accumulation of carotenoids in 'A', 'B' and 'L' race of *B. braunii* was reported by various authors [44,120-122,136-139]. *B. braunii* changes from green to brown, red orange and pale yellow due to the accumulation of secondary carotenoids. Carotenoids-β-carotene, echinenone, canthaxanthin, lutein, violaxanthin, loroxanthin, and neoxanthin are produced in 'B' and 'L' races in linear phase [138]. However, lutein is the major carotenoid (22-29%) reported in the linear phase of these races. Canthaxanthin and echinenone are the dominating carotenoids in the stationary phase [138]. Grung et al. [139] reported the presence of adonixanthin in 'L' race in stationary phase. Botryoxanthin-A [120], botryoxanthin-B, and α-botryoxanthin [121], braunixanthin 1 and 2 [122] are new carotenoids isolated from the 'B' race. Total carotenoid content in 'A' race of *B. braunii* was found to be 0.28% under sodium chloride stress conditions was reported by Ranga Rao et al. [6]. Carotenoids-violaxanthin, lutein, astaxanthin, zeaxanthin and β-carotene were characterized and quantified in 'A' race of *B. braunii*-LB-572 [44,140]. Biomarkers of 12 carotenoids in *B. braunii* were reported by Muntean et al. [136], however, the major carotenoid lutein was identified in 'A' race [140]. Further these carotenoids are characterized and identified by mass spectra [137]. Hydroxylation of hydrocarbon carotenoids is known to be responsible for the formation of 3-hydroxy cyclic carotenoids and epoxy carotenoids. The presence of traces of β-carotene in *B. braunii* may therefore be related to the conversion of these molecules to lutein. This may be a reason for the higher content of lutein in this alga. This alga represents a potential source of lutein, a commercially interesting carotenoid of application in aquaculture and poultry farming, as well as in the prevention of diseases related to age related macular degeneration.

Source of Elicitor

Enhancement of secondary metabolites by elicitation is one of the new strategies which are gaining commercial application. Elicitors are chemical compounds, either biotic or abiotic, which on contact with higher plants trigger the production of secondary metabolites [141]. Secondary metabolite production in plant tissue culture system was enhanced by various microalgal extracts such as *Spirulina, Haematococcus, Scenedesmus, Synechococcus, Nostoc* and *Botryococcus* [142-146]. Similarly, *B. braunii* extracts had some bioactive principles responsible for increasing secondary metabolite production in plant culture system [142,143]. Recently, the effect of *B. braunii* extracts on the growth and secondary metabolite production in *C. frutescens* callus culture was evaluated by Sharma et al. [142]. In the results, increase in seed germination, root, shoot and leaf length in both light and dark conditions in *C. frutescens* over the control group. The total chlorophyll and carotenoid content increased 2 fold after 15 days in *C. frutescens* culture at 8 mg/L *B. braunii* extract was when compared with control group. Major metabolites-vanillin and vanillylamine are intermediates in the capsaicin biosynthesis pathway, increased during the experimental period and this was reflected in the enhancement of capsaicin in the extract-treated callus cultures. Both capsaicin and vanillin content were increased in the *Capsicum* suspension cultures by using *B. braunii* extracts as elicitor. Vanillylamine in 1.5-fold, capsaicin in 2.5-fold and vanillin in 2-fold were higher than the control group after

incubation with *B. braunii*. Earlier *B. braunii* extracts showed growth-promoting effects on roots of *Tagetes erecta* at the 8ppm level reported by Murakami [143].

Biological Activity of *B. braunii*

Microalgae biomass is generally used in various applications such as food and feed applications. Since the biomass contains high value metabolites-carbohydrates, proteins, total carotenoids, phenolic, nucleic acids and fibre [147]. After extracting hydrocarbons and lipids from the biomass has high value co-products which have rich in nutritional benefits. It is used in various industrial applications such as aquaculture, poultry and nutraceutical in animal consumption. Microalgae products are available in the market with mixture of carotenoids; fatty acids which are utilized in the food industry. Algal biomass approved in commercial applications in several countries. Many research studies were conducted on use of algal biomass and carotenoids in food, feed and nutraceutical applications [134,148,149]. Microalgae *B. braunii* is known to produce high value compounds-carotenoids, fatty acids, lipids, polysaccharides and hydrocarbons from 'A', 'B' and 'L' races. Carotenoids-violaxanthin, astaxanthin, lutein, zeaxanthin, chlorophylls a and b, and α, β-carotene were identified in the *B. braunii*, among the carotenoids, lutein represents more than 75% of the total carotenoids. *B. braunii* extracts showed 70% antioxidant activity in DPPH and hydroxyl radical scavenging model systems and rat tissues at 10 ppm level of carotenoids [140]. Carotenoids in 'A' race of *B. braunii* showed biological activity in *in vitro* and *in vivo* models [137,140,150,151]. The antioxidant activity was enhanced in rats, due to the bioactive molecules in the biomass. Lutein in *B. braunii* biomass was enhanced bioavailability and antioxidant enzymes levels in rat tissues after feeding biomass as source of lutein [137,150]. Exoplolysaccharides (EPS) rich in *Botryococcus braunii* have various biological activities such as anti-adhesive against bacterial infections, cytotoxic effects and anti-tumor properties (Guzman Murillo and Ascencio, 2000). Antibacterial activity of *B. braunii* extracts were tested against important clinical bacterial isolates such as *Bacillus subtilus, Bacillus cereus, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus,Streptococcus fecalis* and *Yersinia enterocolitica* using agar-well diffusion assay method reported by Ranga Rao et al. [147]. Results proved that the ethyl acetate extract was showed highest inhibition against *E. aerogenes*. Based on the published data, *B. braunii* extracts can be used as bacteriostatic agents for suitable applications.

Conclusion

Based on the current studies, *B. braunii* produces higher amount of biomass, hydrocarbons, lipids and other bioactive constituents such as ether based lipids, fatty acids, polysaccharides and carotenoids which can be used for commercial applications. Various culture conditions were enhanced the growth, hydrocarbon content and other bioactive molecules in *B. braunii*. Raceway ponds and photobioreactors were developed for the scale-up studies. Contamination levels were minimized in culture by using photobioreactors. Hydrocarbon recovered by using various solvent extraction methods. Polysaccharides and carotenoids of *B. braunii* were showed biological properties in *in vitro* and *in vivo* models. *B. braunii* extracts used as elicitor in plant tissue culture system which enhanced secondary metabolite production in *capsicum frutescens*. Algal hydrocarbons have high octane rate to use as motor fuel. *B. braunii* can removed nitrates and phosphates from the wastewater. The current chapter could provide the production of hydrocarbons and other bioactive constitutes from *B. braunii* and their use in industrial applications.

Acknowledgments

The first author thanks the University of Malaya, Kuala Lumpur, Malaysia for providing financial support for the research grant.

References

1. Wake LV, Hillen LW (1981) Nature and hydrocarbon content of the blooms of the alga *Botryococcus braunii* occurring in Australian fresh water lakes. Aust J Mar Freshwater Res 32: 353-367.

2. Volova TG, Kalacheva GS, Zhila NO (2003) Specificity of Lipid Composition in Two *Botryococcus* Strains, the Producers of Liquid Hydrocarbons. Russian J Plant Physiol 50: 627-633.

3. Metzger P, Largeau C (2005) Botryococcus braunii: a rich source for hydrocarbons and related ether lipids. Appl Microbiol Biotechnol 66: 486-496.

4. Sawayama S, Inoue S, Yokoyama S (1995) Phylogenetic position of *Botryococcus braunii* based on small subunit of ribosomal RNA sequence data. J Phycol 31: 419-420.

5. Dayananda C, Sarada R, Bhattacharya S, Ravishankar GA (2005) Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii.* Process Biochem 40: 3125- 3131.

6. Sarada R, Ranga Rao A, Sandesh BK, Dayananda C, Anila N (2012) Influence of different culture conditions on yield of biomass and value added products in microalgae. Dyn Biochem Process Biotechnol Mol Biol 6: 77-85.

7. Rao AR, Dayananda C, Sarada R, Shamala TR, Ravishankar GA (2007) Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. Bioresour Technol 98: 560-564.

8. Ranga Rao A, Sarada R, Ravishankar GA (2007) Influence of CO_2 on growth and hydrocarbon production in *Botryococcus braunii.* J Microbiol Biotechnol 17: 414-419.

9. Banerjee A, Sharma R, Chisti Y, Banerjee UC (2002) *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit Rev Biotechnol 22: 245-279.

10. Barupal DK, Kind T, Kothari SL, Lee do Y, Fiehn O (2010) Hydrocarbon phenotyping of algal species using pyrolysis-gas chromatography mass spectrometry. BMC Biotechnol 10: 40.

11. Metzger P, Largeau C (1999) Chemicals of *Botryococcus braunii*. In: Cohen Z (Ed) Chemicals from microalgae. Taylor & Francis, London, pp: 205-260.

12. Dayananda C, Sarada R, Usha Rani M, Shamala TR, Ravishankar GA (2007) Autotrophic cultivation of *Botryococcus braunii* for the production of hydrocarbons and exopolysaccharides in various media. Biomass Bioener 31: 87-93.

13. Ranga Rao A, Ravishankar GA, Sarada R (2012) Cultivation of green alga *Botryococcus braunii* in raceway, circular ponds under outdoor conditions and its growth, hydrocarbon production. Bioresour Technol 123: 528-533.

14. Casadevall E, Dif D, Largeau C, Gudin C, Chaumont D, et al. (1985) Studies on batch and continuous cultures of *Botryococcus braunii:* hydrocarbon production in relation to physiological state, cell ultrastructure, and phosphate nutrition. Biotechnol Bioeng 27: 286-295.

15. Senousy HH, Beakes GW, Hack E (2004) Phylogenetic placement of *Botryococcus braunii* (Trebouxiophyceae) and *Botryococcus sudeticus* isolate UTEX 2629 (Chlorophyceae). J Phycol 40: 412-423.

16. Weiss TL, Johnston JS, Fujisawa K, Okada S, Devarenne TP (2011) Genome size and phylogenetic analysis of the A and L races of *Botryococcus braunii.* J Appl Phycol 23: 833-839.

17. Metzger P, Pouet Y (1995) *n*-Alkyl pyrogallol dimethyl ethers, aliphatic diol monoesters and some minor ether lipids from *Botryococcus braunii* a race. Phytochem 40: 543-554.

18. Casadevall E, Metzger P, Puech MP (1984) Biosynthesis of triterpenoid hydrocarbons in the alga Botryococcus braunii. Tetrahedron Lett 25: 4123–4126.

19. Sato Y, Ito Y, Okada S, Murakami M, Abe H (2003) Biosynthesis of the triterpenoids, botryococcenes and tetramethylsqualene in the B race of *Botryococcus braunii* via the non-mevalonate pathway. Tetrahedron Lett 44: 7035–7037.

20. Schwender J, Seemann M, Lichtenthaler HK, Rohmer M (1996) Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway in the green alga Scenedesmus obliquus. Biochem J 316: 73-80.

21. Poulter CD (1990) Biosynthesis of non-head-to-tail terpenes. Formation of 1'-1 and 1'-3 linkages. Acc. Chem. Res. 23: 70-77.

22. Inoue H, Korenaga T, Sagami H, Koyama T, Ogura K (1994) Phosphorylation of farnesol by a cell-free system from Botryococcus braunii. Biochem Biophys Res Commun 200: 1036-1041.

23. Inoue H, Sagami H, Koyama T, Ogura K (1995) Properties of farnesol phosphokinase of *Botryococcus braunii.* Phytochem 40: 377-381.

24. Inoue H, Korenaga T, Sagami H, Koyama T, Sugiyama H, et al. (1993) Formation of farnesal and 3-hydroxy-2,3-dihydrofarnesal from farnesol by protoplasts of *Botryococcus braunii.* Biochem Biophys Res Commun 196: 1401-1405.

25. Okada S, Devarenne TP, Murakami M, Abe H, Chappell J (2004) Characterization of botryococcene synthase enzyme activity, a squalene synthase-like activity from the green microalga *Botryococcus braunii*, Race B. Arch Biochem Biophys 422: 110-118.

26. White JD, Somers TC, Reddy GN (1986) The absolute configuration of (-)-botryococcene. J Am Chem Soc 108: 5352-5353.

27. White JD, Somers TC, Reddy GN (1992) Degradation and absolute configurationally assignment to C34 botryococcene. J Org Chem 57: 4991-4998.

28. Jarstfer MB, Zhang DL, Poulter CD (2002) Recombinant squalene synthase. Synthesis of non-head-to-tail isoprenoids in the absence of NADPH. J Am Chem Soc 124: 8834-8845.

29. Metzger P, Casadevall E, Pouet MJ, Pouet Y (1985) Structure of some botryococcenes: branched hydrocarbons from the B race of the green alga *Botryococcus braunii.* Phytochem 24: 2995-3002.

30. Huang Z, Poulter CD (1989) Tetramethylsqualene, a triterpene from *Botryococcus braunii* var. Showa. Phytochem 8: 1467-1470.

31. Metzger P, Berkaloff C, Casadevall E, Coute A (1985) Alkadiene and botryococcene producing races of wild strains of *Botryococcus braunii*. Phytochem 24: 2305-2312.

32. Okada S, Murakami M, Yamaguchi K (1995) Hydrocarbon composition of newly isolated strains of the green microalga *Botryococcus braunii.* J Appl Phycol 7: 555-559.

33. Metzger P, Casadevall E (1987) Lycopadiene, a tetraterpenoid hydrocarbon from new strains of the green alga *Botryococcus braunii.* Tetrahedron Lett 28: 3931-3934.

34. Dayananda C, Sarada R, Shamala TR, Ravishankar GA (2006) Influence of nitrogen sources on growth, hydrocarbon and fatty acid production by *Botryococcus braunii*. Asian J Plant Sci 5: 799-804.

35. Ruangsomboon S (2012) Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2. Bioresour Technol 109: 261-265.

36. Yeesang C, Cheirsilp B (2011) Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresour Technol 102: 3034-3040.

37. Zhu X, Ye L, Xu J, Huang H, Lin E (2013) Effects of Different Light Quality on Growth Rate and Accumulation of Organics in *Botryococcus braunii.* Bioprocess 3: 17-22.

38. Wong MT, Zhang D, Li J, Hui RK, Tun HM, et al. (2013) Towards a metagenomic understanding on enhanced biomethane production from waste activated sludge after pH 10 pretreatment. Biotechnol Biofuels 6: 38.

39. Ren HY, Liu BF, Ma C, Zhao L, Ren NQ (2013) A new lipid-rich microalga Scenedesmus sp. strain R-16 isolated using Nile red staining: effects of carbon and nitrogen sources and initial pH on the biomass and lipid production. Biotechnol Biofuels 6: 143.

40. Demura M, Ioki M, kawachi M, Nakajima N, Watanabe M (2013) Desciccation tolerance of *Botryococcus braunii* (Trebouxiophyceae, Chlorophyta) and extreme termperature tolerance of dehydrated cells. J Appl Phycol.

41. Gustavs L, Eggert A, Michalik D, Karsten U (2010) Physiological and biochemical responses of green microalgae from different habitats to osmotic and matric stress. Protoplasma 243: 3-14.

42. Yoshimura T, Okada S, Honda M (2013) Culture of the hydrocarbon producing microalga *Botryococcus braunii* strain Showa: optimal CO2, salinity, temperature, and irradiance conditions. Bioresour Technol 133: 232-239.

43. Renaud SM, Thinh LV, Lambrinidis G, Parry DL (2002) Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture, 211: 195-214.

44. Ranga Rao A, Sarada R, Ravishankar GA (2010) Enhancement of carotenoids in green alga-*Botryococcus braunii* in various autotrophic media under stress conditions. International J of Biomed Pharma Sciences 5: 87-92.

45. Solovchenko A, Khozin-Goldberg I, Didi-Cohen S, Cohen Z, Merzlyak M (2008) Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga *Parietochloris incisa*. J Appl Phycol 20: 245-251.

46. Wolf FR, Nonomura AM, Bassham JA (1985) Growth and branched hydrocarbon production in a strain of *Botryococcus braunii* (Chlorophyta). J Phycol 21: 388-396.

47. Ge Y, Liu J, Tian G (2011) Growth characteristics of *Botryococcus braunii* 765 under high CO2 concentration in photobioreactor. Bioresour Technol 102: 130-134.

48. Choi GG, Kim BH, Ahn CY, Oh HM (2011) Effect of nitrogen limitation on oleic acid biosynthesis in *Botryococcus braunii*. J Appl Phycol 23: 1031-1037.

49. Zhila NO, Kalacheva GS, Volova TG (2005) Effect of nitrogen limitation on the growth and lipid composition of the green alga *Botryococcus braunii* Kutz IPPAS H-252. Russ J Plant Physiol 52: 311-319.

50. Ohmori M, Wolf FR, Bassham JA (1984) *Botryococcus braunii* carbon/nitrogen metabolism as affected by ammonia addition. Arch Microbiol 140: 101-106.

51. Sun KF, Hu ZX, Duan SS (2010) Effects of nitrogen and phosphorus stress on the growth of *Botryococcus braunii.* Acta Hydrobil Sin 3: 517-524.

52. Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process 48: 1146-1151.

53. Hu Q (2004) Environmental effects on cell composition. In: Richmond A (ed.), Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Science, Victoria, 83-94.

54. Chen CY, Chang JS, Chang HY, Chen TY, Wu JH (2013) Enhancing microalgal oil/lipid production from *Chlorella sorokiniana* CY1 using deep-sea water supplemented cultivation medium. Biochem Eng J 77: 74-81.

55. Ben-Amotz A, Tornabene TG, Thomas WH (1985) Chemical profile of selected species of microalgae with emphasis on lipids. J Phycol 1: 72-181.

56. Vazquez Duhalt R, Arredondo-Vega BO (1991) Haloadaptation of the green alga *Botryococcus braunii* (race a). Phytochem 30: 2919-2925.

57. Furuhashi K, Saga K, Okada S, Imou K (2013) Seawater-cultured *Botryococcus braunii* for efficient hydrocarbon extraction. PLoS One 8: 66483.

58. Zhila N, Kalacheva G, Volova T (2011) Effect of salinity on the biochemical composition of the alga *Botryococcus braunii* Kütz IPPAS H-252. J Appl Phycol 23: 47-52.

59. Demirbas A, Fatih Demirbas M (2011) Importance of algae oil as a source of biodiesel. Energy Convers Manage 52: 163-170.

60. Brennan L, Owende P (2010) Biofuels from micro-algae- a review of technologies for production, processing, and extractions of biofuels and coproducts. Renew Sust Energy Revew 14: 557-577.

61. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25: 294-306.

62. Borowitzka M (2013) Techno-economic modelling for biofuels from microalgae, In: Algae for Biofuels and Energy. Springer Dordrecht 255-264.

63. Mutanda T, Ramesh D, Karthikeyan S, Kumari S, Anandraj A, et al. (2011) Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. Bioresour Technol 102: 57-70.

64. Wang H, Zhang W, Chen L, Wang J, Liu T (2013) The contamination and control of biological pollutants in mass cultivation of microalgae. Bioresour Technol 128: 745-750.

65. Mendes LBB, Vermelho AB (2013) Allelopathy as a potential strategy to improve microalgae cultivation. Biotechnology for Biofuels 6: 152.

66. Ranga Rao A, Yingchun G, Hu Z, Sommerfeld M, Hu, Q (2012) Comparative study of predators in mass cultures of *Chlorella zofingiensis*, *Scenedesmus dimorphus* and *Nannochloropsis oceanica* in ponds and photobioreactors. Algal Biomass Organization, Colorado, USA.

67. Wang J, Han D, Sommerfeld MR, Lu C, Hu Q (2012) Effect of initial biomass density on growth and astaxanthin production of *Haematococcus pluvialis* in an outdoor photobioreactor. J App Phycol 25: 253-260.

68. Becerra Dorame MJ, Lopez Elizas JA, Ocana FE, Aldaz NH, Voltolina D (2010) The effect of initial cell and nutrient concentrations on the growth and biomass production of outdoor cultures of *Dunaliella sp.* Ann Bot Fennici 47: 109-112.

69. Ashokkumar V, Rengasamy R (2012) Mass culture of *Botryococcus braunii* Kutz. under open raceway pond for biofuel production. Bioresour Technol 104: 394-399.

70. Dayananda, C, Kumudha A, Sarada R, Ravishankar GA (2010) Isolation, characterization and outdoor cultivation of green microalgae *Botryococcus sp.* Sci Res Essays 5: 2497-2505.

71. Acien Fernandez FG, Fernandez Sevilla JM, Molina Grima E (2013) Photobioreactors for the production of microalgae. Rev Environ Sci Biotehnol 12: 131-151.

72. Acien Fernandez FG, Fernández Sevilla JM, Sánchez Pérez JA, Molina Grima, E, Chisti Y (2001) Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance. Chem Eng Sci 56: 2721-2732.

73. Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: A review. Renew Sustain Ener Rev 14: 217-232.

74. Grobbelaar JU (2009) Factors governing algal growth in photobioreactors: the 'open' versus 'closed' debate. J Appl Phycol 21: 489-492.

75. Grobbelaar JU (2010) Microalgal biomass production: challenges and realities. Photosynth Res 106: 135-144.

76. Richmond (2004) Principles for attaining maximal microalgal productivity in photobioreactors: an overview. Hydrobiologia 512: 33-37.

77. Miron AS, Camacho FG, Gómez AC, Grima EM, Chisti Y (2000) Bubble column and airlift photobioreactors for algal culture. AIChE J 46: 1872-1887.

78. Miron AS, Gómez AC, Camacho FG, Grima EM, Chisti Y (1999) Comparative evaluation compact photobioreactors for large-scale monoculture of microalgae. J Biotechnol 70: 249-270.

79. Molina E, Fernández FGA, Camacho FG, Chisti Y (1999) Photobioreactors: light regime, mass transfer, and scale up. J Biotechnol 70: 231-247.

80. Xu L, Liu R, Wang F, Liu CZ (2012) Development of a draft-tube airlift bioreactor for *Botryococcus braunii* with an optimized inner structure using computational fluid dynamics. Bioresour Technol 119: 300-305.

81. Garcia-Malea Lopez MC, Del Rio Sanchez E, Casas Lopez JL, Acien Fernandez FG, Fernandez Sevilla JM, et al. (2006) Comparative analysis of the outdoor culture of *Haematococcus pluvialis* in tubular and bubble column photobioreactors. J Biotechnol 123: 329-342.

82. Converti A, Lodi A, Del Borghi A, Solisio C (2006) Cultivation of *Spirulina platensis* in a combined airlift-tubular system. Biochem Eng J 32: 13-18.

83. Kunjapur AM, Eldridge RB (2010) Photobioreactor design for commercial biofuel production. Ind Eng Chem Res 49: 3516-3526.

84. Posten C (2009) Design principles of photo-bioreactors for cultivation of micro-algae. Eng Life Sci 9: 165-177.

85. Briassoulis D, Panagakis P, Chionidis M, Tzenos D, Lalos A, et al. (2010) An experimental helical-tubular photobioreactor for continuous production of Nannochloropsis sp. Bioresour Technol 101: 6768-6777.

86. Carvalho AP, Silva SO, Baptista JM, Malcata FX (2011) Light requirements in microalgal photobioreactors: an overview of biophotonic aspects. Appl Microbiol Biotechnol 89: 1275-1288.

87. Hu Q, Guterman H, Richmond A (1996) A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs. Biotechnol Bioeng 51: 51-60.

88. Qiang H, Richmond A (1996) Productivity and photosynthetic efficiency of Spirulina plntensis as affected by light intensity, algal density and rate of mixing in a flat plate photobioreactor. J Appl Phycol 8: 139-145.

89. Zou N, Richmond A (1999) Effect of light path length in oudoor flat plate reactors on output rate of cell mass and of EPA in Nannochlorophsis sp. J Biotechnol 70: 351-356.

90. Kojima E, Zhang K (1999) Growth and hydrocarbon production of microalga *Botryococcus braunii* in bubble column photobioreactors. J Biosci Bioeng 87: 811-815.

91. Weissman JC, Goebel RP, Benemann JR (1988) Photobioreactor design: Mixing, carbon utilization, and oxygen accumulation. Biotechnol Bioeng 31: 336-344.

92. Magota A, Saga K, Okada S, Atobe S, Imou K (2012) Effect of thermal pretreatments on hydrocarbon recovery from *Botryococcus braunii*. Bioresour Technol 123: 195-198.

93. Frenz J, Largeau C, Casadevall E, Kollerup F, Daugulis AJ (1989) Hydrocarbon recovery and biocompatibility of solvents for extraction from cultures of *Botryococcus braunii.* Biotechnol Bioeng 34: 755-762.

94. Frenz J, Largeau C, Casadevall E (1989) Hydrocarbon recovery by extraction with a biocompatible solvent from free and immobilized cultures of *Botryococcus braunii.* Enzym Microb Technol 11: 717-724.

95. Choi SP, Bahn SH, Sim SJ (2013) Improvement of hydrocarbon recovery by spouting solvent into culture of *Botryococcus braunii*. Bioprocess Biosyst Eng 36: 1977-1985.

96. Sim SJ, An JY, Kim BW (2001) Two-phase extraction culture of *Botryococcus braunii* producing long-chain unsaturated hydrocarbons. Biotechnol Lett 23: 201-205.

97. An JY, Sim SJ, Kim BW, Lee JS (2004) Improvement of hydrocarbon recovery by two-stage cell-recycle extraction in the cultivation of *Botryococcus braunii*. J Microbiol Biotechnol 14: 932-937.

98. Eroglu E, Melis A (2010) Extracellular terpenoid hydrocarbon extraction and quantitation from the green microalgae *Botryococcus braunii* var. Showa. Bioresour Technol 101: 2359-2366.

99. Mendes RL, Nobre BP, Cardoso MT, Pereira AP, Palavra AF (2003). Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. Inorg Chim Acta, 356: 328-334.

100. Mendes RL, Coelho JP, Fernandes HL, Marrucho IJ, Cabral JMS (1995) Applications of supercritical CO_2 extraction to microalgae and plants. J Chem Technol Biotechnol 62: 53-59.

101. Deng L, Senseman SA, Gentry TJ, Zuberer D, Weiss TL (2012) Effect of selected herbicides on growth and hydrocarbon content of *Botryococcus braunii* (Race B). Ind Crop Prod 39: 154-161.

102. Kanda H, Li P, Yoshimura T, Okada S (2013) Wet extraction of hydrocarbons from *Botryococcus braunii* by dimethyl ether as compared with dry extraction by hexane. Fuel 105: 535-539.

103. Gelpi E, Schneider H, Mann J, Oro J (1970) Hydrocarbons of geochemical significance in microscopic algae. Phytochem 9: 603-612.

104. Metzger P, Templier J, Largeau C, Casadevall E (1986) An nalkatriene and some n-alkadienes from the A race of the green alga *Botryococcus braunii*. Phytochem 25: 1869-1872.

105. Templier J, Largeau C, Casadevall E (1984) Mechanism of non-isoprenoid hydrocarbon biosynthesis in *Botryococcus braunii*. Phytochem 23: 1017-1028.

106. Templier J, Largeau C, Casadevall E (1987) Effect of various inhibitors on biosynthesis of non-isoprenoid hydrocarbon biosynthesis in *Botryococcus braunii*. Phytochem 26: 377-383.

107. Achitouv E, Metzger P, Rager MN, Largeau C (2004) C31-C34 methylated squalenes from a Bolivian strain of *Botryococcus braunii*. Phytochemistry 65: 3159-3165.

108. Brown AG, Knights BA (1969) Hydrocarbon content and its relationship to physiological state in the green alga *Botryococcus braunii*. Phytochem 8: 543-547.

109. Metzger P, Allard B, Casadevall E, Berkaloff C, Coute A (1990) Structure and chemistry of a new chemical race of *Botryococcus braunii(chlorophyceae)* that produces lycopadiene, a tetraterpenoid hydrocarbon. J Phycol 26: 258-266.

110. Metzger P (1999) Two terpenoid diepoxides from the green microalga *Botryococcus braunii*: their biomimetic conversion to tetrahydrofurans and tetrahydropyrans. Tetrahedron 55: 167-176.

111. Sarada R, Dayananda C, Ranga Rao A, Shamala TR, Srinivas P (2012) Saturated and unsaturated hydrocarbon production from *Botryococcus braunii* spp. from Indian freshwater bodies and culture collection centers. In: Sahoo DB, Kaushik BD (eds). Algal Biotechnology and Environment. (1st edn), I.K. International Publishing House, Vedams eBooks (P) Ltd, New Delhi, India.

112. Metzger P, Villarreal-Rosales E, Casadevall E (1991) Methyl branched fatty aldehydes and fatty acids in *Botryococcus braunii*. Phytochem 30: 185-191.

113. Wang X, Kolattukudy PE (1995) Solubilization and purification of aldehyde-generating fatty acyl-CoA reductase from green alga *Botryococcus braunii.* FEBS Lett 370: 15-18.

114. Dayananda C, Sarada R, Srinivas P, Shamala TR, Ravishankar GA (2006) Presence of methyl branched fatty acids and saturated hydrocarbons in botryococcene producing strain of *Botryococcus braunii*. Acta Physiol Plant 28: 251-256.

115. Metzger P, Casadevall E (1991) Botryococcoid ethers, ether lipids from *Botryococcus braunii*. Phytochem 30: 1439-1444.

116. Metzger P, Casadevall E (1992) Ether lipids from *Botryococcus braunii* and their biosynthesis. Phytochem 31: 2341-2349.

117. Metzger P, Largeau C (1994) A new type of ether lipid comprising phenolic moieties in *Botryococcus braunii*. Chemical structure and abundance, and geochemical implications. Org Geochem 22: 801-814.

118. Villarreal-Rosales E, Metzger P, Casadevall E (1992) Ether lipid production in relation to growth in *Botryococcus braunii*. Phytochem 31: 3021-3027.

119. Metzger P, Rager MN (2002) Lycopanerols H, two high molecular weight ether lipids from *Botryococcus braunii* comprising an a-tocopherol unit. Tetrahedron Lett 43: 2377-2380.

120. Okada S, Mastuda M, Murakami M, Yamaguchi K (1996) Botryoxanthin-A a new member of the new class of carotenoids from green microalga *Botryococcus braunii*, Berkeley. Tetrahedron Lett 37: 1065-1068.

121. Okada S, Tonegawa I, Mastuda M, Murakami M, Yamaguchi K (1998) Botryoxanthin-B and alpha botryoxanthin-A from green microalga *Botryococcus braunii*. Phytochem 47: 1111-1115.

122. Okada S, Tonegawa I, Mastuda M, Murakami M, Yamaguchi K (1997) Braunixanthins 1 and 2, new carotenoids from the green microalga *Botryococcus braunii*. Tetrahedron 53: 11307-11316.

123. Metzger P, Aumelas A (1997) Lycopanerol-A, di-tetraterpenoid tetraether derivatives from green microalga *Botryococcus braunii* L race. Tetrahedron Lett 38: 2977-2980.

124. Rager MN, Metzger P (2000) Six novel tetraterpenoid ethers, lycopanerols B-G, and some other constituents from the green microalga *Botryococcus braunii*. Phytochemistry 54: 427-437.

125. Krishnan R (2003) Method of inhibiting angiogenesis. Patent No. WO-03092671.

126. Krishnan R, Collin PD (2003) Method of inhibiting rejection of transplanted material. Patent No. WO-03092672.

127. Koren KM, Rayburn WR (1984) Influence of growth status and nutrients on exocellular polysaccharides synthesis by the soil alga *Chlamydomonas mexicana*. J Phycol 20: 253-257.

128. Anderson DB, Eakin DE (1985) A process for the production of polysaccharides from microalgae. Biotechnol Bioeng Symp 15: 533-547.

129. Allard B, Casadevall E (1990) Carbohydrate composition and characterisation of sugars from the green microalga *Botryococcus braunii*. Phytochem 29: 1875-1878.

130. Fernandes HL, Tomme MM, Lupi FM, Fialho AM, Sa Correia I (1989) Biosynthesis of high concentration of an exopolysaccharide during cultivation of the microalga *Botryococcus braunii.* Biotechnol Lett 11: 433-436.

131. Lupi FM, Fernandes HML, Tomme MM, Sa Correia I, Novais JM (1994) Influence of nitrogen source and photoperiod on exopolysaccharide synthesis by the microalga *Botryococcus braunii*. Enzyme Microb Technol 16: 546-550.

132. Guzman-Murillo MA, Ascencio F (2000) Anti-adhesive activity of sulphated exopolysaccharides of microalgae on attachment of red sore disease-associated bacteria and helicobacter pylori to tissue culture cells. Lett Appl Microbiol 30: 473-478.

133. Lupi FM, Fernandes HML, Sa Correia I, Novais JM (1991) Temperature profiles of cellular growth exopolysaccharide synthesis by *Botryococcus braunii* Kutz. J Appl Phycol 3: 35-42.

134. Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101: 87-96.

135. Samorì C, Torri C, Samorì G, Fabbri D, Galletti P, et al. (2010) Extraction of hydrocarbons from microalga *Botryococcus braunii* with switchable solvents. Bioresour Technol 101: 3274-3279.

136. Muntean E, Muntean N, Dragos N, Bercea V (2008) Carotenoids as biomarkers in *Botryococcus braunii* algae. Res J Agri Sci 40: 49-54.

137. Ranga Rao A, Raghunath Reddy RL, Baskaran V, Sarada R, Ravishankar GA (2010) Characterization of microalgal carotenoids by mass spectrometry and their bioavailability and antioxidant properties elucidated in rat model. J Agric Food Chem 58: 8553-8559.

138. Grung M, Metzger P, Liaaen Jensen S (1989) Primary and secondary carotenoids in two races of green *Botryococcus braunii*. Biochem Sys Ecol 17: 263-269.

139. Grung M, Metzger P, Liaaen Jensen S (1994) Algal carotenoids 53; secondary carotenoids of algae 4; secondary carotenoids in the green alga *Botryococcus braunii*, race L, new strain. Biochem Sys Ecol 22: 25-29.

140. Rao AR, Sarada R, Baskaran V, Ravishankar GA (2006) Antioxidant activity of *Botryococcus braunii* extract elucidated in vitro models. J Agric Food Chem 54: 4593-4599.

141. Savitha BC, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA (2006) Different biotic and abiotic elicitors influence betalain production in hairy root cultures of Beta vulgaris in shake-flask and bioreactor. Process Biochem 41: 50-60.

142. Sharma A, Ranga Rao A, Dayananda C, Sarada R, Ravishankar GA (2010) *Botryococcus braunii*, a new Elicitor for Secondary Metabolite Production in Capsicum frutescens. Func Plant Sci Biotechnol 5: 9-13.

143. Murakami N (2000) Plant growth regulator composition and their manufacture. Jpn Kokai Tokkyo Koho 264: 809-814.

144. Mercier L, Lafitte C, Borderies G, Briand X, Esquerre Tugaye MT (2001) The algal polysaccharide carrageenans can act as an elicitor of plant defence. New Phytologist 149: 43-51.

145. Hanagata N, Uehara H, Ito A, Tekeuchi T, Karube I (1994) Elicitor of red pigment formation in carthamus tinctorius cultured cells. J Biotechnol 34: 71-77.

146. Fingerhut U, Webb LE, Soeder CJ (1984) Increased yields of Rhizobium japonicum by an extract of the green alga, Scenedesmus obliquus (276-3a). Appl Microbiol Biotechnol 19: 358-360.

147. Becker EW (2007) Microalgae in human and animal nutrition. In: A Richmond (editor). Hand book of micro algal culture. Blackwell, Oxford, UK, 312-351.

148. Milledge JJ (2011) Commercial application of microalgae other than as biofuels: A brief review. Rev Environ Sci Biotechnol 10: 31-41.

149. Plaza M, Herrero M, Cifuentes A, I*báñez* E (2009) Innovative natural functional ingredients from microalgae. J Agric Food Chem 57: 7159-7170.

150. Ranga Rao A, Baskaran V, Sarada R, Ravishankar G A (2013) In vivo bioavailability and antioxidant activity of carotenoids from microalgal biomass-A repeated dose study. Food Res Int 54: 711-717.

151. Ranga Rao A, Harshavardhan Reddy A, Aradhya SM (2010) Antibacterial properties of *Spirulina platensis*, *Haematococcus pluvialis*, *Botryococcus braunii* micro algal extracts. Curr Trends Biotechnol Pharm, 4: 809-819.

152. Kalacheva GS, Zhila NO, Volova TG (2001) Lipids from the green algae *Botryococcus* during staged growth in batch mode. Mikrobiologiia 70: 305-312.

153. Orpez R, Martinez ME, Hodaifa G, ElYousfi F, Jbari N (2009) Growth of the microalga *Botryococcus braunii* in secondarily treated sewage. Desalination 246: 625-630.

154. Li Y, Moore RB, Qin JG, Scott A, Ball AS (2013) Extractable liquid, its energy and hydrocarbon content in the green alga *Botryococcus braunii*. Biomass Bioener 52: 103-112.

155. Talukdar J, Kalita MC, Goswami BC (2013) Characterization of the biofuel potential of a newly isolated strain of the microalga *Botryococcus braunii* Kützing from Assam, India. Bioresour Technol 149: 268-275.

156. Khatri W, Hendrix R, Niehaus T, Chappell J, Curtis WR (2014) Hydrocarbon production in high density *Botryococcus braunii* race B continuous culture. Biotechnol Bioeng 111: 493-503.

157. Cheng P, Ji B, Gao L, Zhang W, Wang J, et al. (2013) The growth, lipid and hydrocarbon production of Botryococcus braunii with attached cultivation. Bioresour Technol 138: 95-100.

158. Li Y, Qin, JG (2005) Comparison of growth and lipid content in three *Botryococcus braunii* strains. J Appl Phycol 17: 551-556.

159. Bazaes J, Sepulveda C, Acién FA, Morales J, Gonzales L (2012) Outdoor pilot-scale production of *Botryococcus braunii* in panel reactors. J Appl Phycol 24: 1353-1360.

160. Ozkan A, Kinney K, Katz L, Berberoglu H (2012) Reduction of water and energy requirement of algae cultivation using an algae biofilm photobioreactor. Bioresour Technol 114: 542-548.

161. Krichnavaruk S, Powtongsook S, Pavasant P (2007) Enhanced productivity of *Chaetoceros calcitrans* in airlift photobioreactors. Bioresour Technol 98: 2123-2130.

162. De Morais MG, Costa Jav (2007) Carbondioxide fixation by Chlorella kessleri, C. vulgaris, Scenedesmus obliquus and Spirulina sp. cultivated in flasks and vertical tubular photobioreactors. Biotechnol Lett 29: 1349-1352.

163. Barbosa MJ, Zijffers JW, Nisworo A, Vaes W, van Schoonhoven J, et al. (2005) Optimization of biomass, vitamins, and carotenoid yield on light energy in a flat-panel reactor using the A-stat technique. Biotechnol Bioeng 89: 233-242.

164. Kaewpintong K, Shotipruk A, Powtongsook S, Pavasant P (2007) Photoautotrophic high-density cultivation of vegetative cells of *Haematococcus pluvialis* in airlift bioreactor. Bioresour Technol 98: 288-295.

165. Bosma R, van Zessen E, Reith JH, Tramper J, Wijffels RH (2007) Prediction of volumetric productivity of an outdoor photobioreactor. Biotechnol Bioeng 97: 1108-1120.

166. Richmond A, Cheng-Wu Z (2001) Optimization of a flat plate glass reactor for mass production of Nannochloropsis sp. outdoors. J Biotechnol 85: 259-269.

167. Jorquera O, Kiperstok A, Sales EA, Embiruçu M, Ghirardi ML (2010) Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresour Technol 101: 1406-1413.

168. Boussiba S, Vonshak A, Cohen Z, Avissar Y, Richmond A (1987) Lipid and biomass production by the halotolerant microalga Nannochloropsis salina. Biotechnol Bioeng 97: 1108-1120.

169. Hall DO, Fernández FG, Guerrero EC, Rao KK, Grima EM (2003) Outdoor helical tubular photobioreactors for microalgal production: modeling of fluid-dynamics and mass transfer and assessment of biomass productivity. Biotechnol Bioeng 82: 62-73.

170. Meiser A, Schmid-Staiger U, Trosch W (2004) Optimization of eicosapentaenoic acid production by Phaeodactylum tricornutumin the flat panel airlift (FPA) reactor. J Appl Phycol 16: 215-225.

171. Lin Q, Lin J (2011) Effects of nitrogen source and concentration on biomass and oil production of a Scenedesmus rubescens like microalga. Bioresour Technol 102: 1615-1621.

172. Ketheesan B, Nirmalakhandan N (2012) Feasibility of microalgal cultivation in a pilot-scale airlift-driven raceway reactor. Bioresour Technol 108: 196-202.

173. Richmond A, Lichtenberg E, Stahl B, Vonshak A (1990) Quantitative assessment of the major limitations on productivity of Spirulina platensis in open raceways. J Appl Phycol 2: 195-206.

174. Zhang K, Miyachi S, Kurano N (2001) Photsynthetic performance of a cyanobacterium in a vertical flat-plate photobioreactor for outdoor microalgal production and fixation of CO_2. Biotechnol Lett 23: 21-26.

175. Kita K, Okada S, Sekino H, Imou K, Yokoyama S, et al. (2010) Thermal pretreatment of wet microalgae harvest for efficient hydrocarbon recovery. Appl Energy 87: 2420-2423.

Development and Prospect of Microalgal Biodiesel

Qiang Wang*

Professor, Key Laboratory of Algal Biology (the LAB) Institute of Hydrobiology

***Corresponding author:** Qiang Wang, Professor,Key Laboratory of Algal Biology (the LAB), Institute of Hydrobiology, Chinese Academy of Sciences,Tel: 027-68780790: Mobile: 18602749963; E-mail: wangqiang@ihb.ac.cn

Introduction

Growing energy demand for fossil fuels comes from worldwide economic growth and development resulting in the sharp accumulation in CO_2 and nitrogen oxides (NOx) emissions, responsible for the majority of Greenhouse Gas (GHG) emissions worldwide, which bring some irreversible changes to the climate system including global warming. Without decisive action, energy-related GHG emissions will be more than double by 2050 [1]. This issue has led to the expansion of research and development on alternative energy with renewability and sustainability.

In recent years, renewable energy sources (e.g., hydro, bioenergy, solar, and wind) have received more attention and have been used by different nations to reduce consumption of fossil fuels and GHG emissions [2]. Compared with other forms of renewable energy, biofuels allow energy to be chemically stored, and also be used in existing engines and transportation infrastructures after blending to various degrees with petroleum diesel [2]. Based on these evidences, biofuels especially biodiesel will play a more crucial role as an alternative renewable energy in the near future to further diversify the global energy sources and gradually replace the fossil fuels. The feedstock selection for biodiesel production has evolved from the edible vegetable oils to the non-edible oils, and now focuses on microalgae [3].

Microalgae are a class of prokaryotic or eukaryotic photosynthetic unicellular organisms, with a wide range of species, high photosynthetic efficiency, fast growth, high oil content, strong adaptability to various environmental conditions. It has been reported that using microalgae for biodiesel production has lots of advantages in comparison with other available feedstocks [4-7]. For example, (1) Microalgae have much higher growth rate than other biodiesel feedstock, as the exponential growth rates can double their biomass as short as 3.5 h [8]; (2) Under certain conditions, many microalgae cells can accumulate a large amounts of neutral lipids and the highest content can reach more than 50% dry weight of biomass [9]; (3) Microalgae can be cultivated in saline/brackish water/coastal seawater therefore alleviating the load on freshwater sources; (4) Nutrients for microalgae cultivation can be obtained from a variety of wastewater sources, thus, providing the additional benefit of wastewater biological cleaning; (5)They can remove CO_2 and NOx from industrial flue

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

gases by biofixation, thereby reducing emissions of the major greenhouse gas; (6) They can also produce value-added co-products or by-products such as proteins, polysaccharides, pigments and residual biomass after oil extraction which can be converted into animal feed or organic fertilizer [10]; (7) Biodiesel derived from microalgae is biodegradable, renewable, non-toxic and burning without releasing NOx and sulfur oxides (SOx) that has already accumulated as a consequence of burning of fossil fuels. Given these advantages, the oleaginous microalgae, which have greater advantages compared to edible vegetable oils or non-edible oils, will be a most potential alternative for producing biodiesel [11].

Microalgae are currently being pursued as the superior raw material for biodiesel production, and many researches have been reported for all stages of microalgae biodiesel process chain. However, biofuels cannot compete economically in market due to its higher expense of production in comparison with fossil fuels. One promising way to make algal biofuel production more cost-effective is to couple environmental pollution control, including biological fixation of GHG (CO_2 and NOx) and wastewater treatment [12]. The objective of this review is to give an overview of the current status of microalgae used for biodiesel production, covering upstream (strain selection, cultivation and biomass harvesting) to downstream processes (lipids extraction, biodiesel conversion techniques). Then, several environmental benefits (e.g., CO_2 sequestration, NOx remove, and wastewater treatment) from microalgae and their combination with biodiesel production are presented. It is expected to provide an useful reference for revealing the potential and prospect of microalgae biofuel industry, and propelling the cost-effective and sustainable microalgae biodiesel industrialization (Table 1).

Technique	Advantages	Disadvantages
Sedimentation	Low power consumption Low requirement for skilled operators Useful as a pre-concentration step	Algal species specific Slow sedimentation rates Low cell recovery
Chemical Flocculation	Wide range of flocculants available Ease of use	Chemical contamination Removal of flocculants Highly sensitive to pH level Flocculants may be algal species specific
Flotation	Prone to harvest in mass culture	Algal species specific
Centrifugation	High harvesting efficiency Suitable for most algae types Rapid separation process Easy to operate	High capital and operational costs Cell damage Difficult bulk harvest
Filtration	Water and nutrition reuse Wide variety of filter and membrane types available	Fouling Slow process Suitable for large algal cells

Table 1: Comparison of microalgal harvesting methods [12].

Microalgae Biodiesel Production Process

Algal biodiesel production chain is mainly comprised of five processes including algal species selection, cultivation, harvest, lipid production, and biodiesel processing (Figure 1).

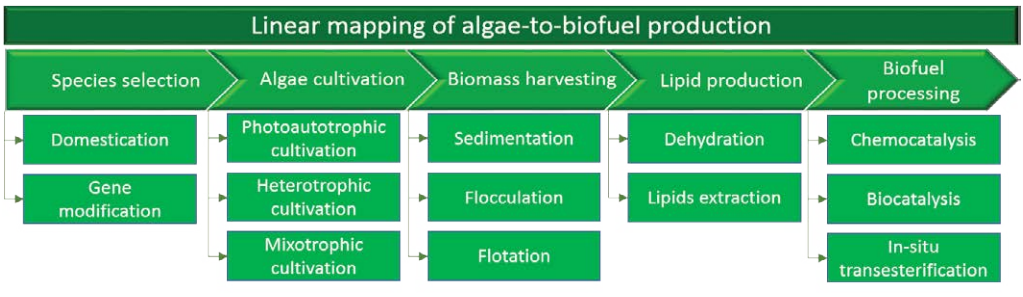

Figure 1: The overall microalgae biodiesel process.

Algal Species Selection

As the first and one of the most important steps of algae-based biofuel industry, microalgal strain selection affects the potential biofuel yield and determines which nutrients and cultivation units must be used for their growth [13]. . There are some key factors has to be considered in this step: (1) Fast growth rate increases yield per harvest volume in a certain period (productivity) and decreases production cost [14]; (2) High lipid content is one of the desirable features for using microalgae as biodiesel feedstock [15]; (3) Resistance to environmental conditions changes including light, temperature, competition from other microalgae species and/or bacterial [16]; (4) Feasibility for metabolic engineering strategies; (5) Ease of biomass harvest and further downstream processes (lipids extraction, biodiesel conversion techniques); (6) Possibility of obtaining other valuable products [16] .

It has been estimated that about 200,000–800,000 algal species existed, of which only about 35,000 species have been described [17]. Microalgae using as biodiesel feedstock should ideally show high biomass productivities and efficient biosynthesis of lipids, meaning not the single parameter (lipid content or growth rate) but the volumetric lipid productivity of the microalga should be the mainly selective criterion for biodiesel production. Researchers revealed a dominant correlation between biomass and volumetric lipid productivity (R^2 = 0.93), but no such relation was observed in regard to lipid content (R^2 = 0.01) [18]. Some potential microalgae reported for biodiesel production were *Chlorella*, *Scenedesmus* and *Botryococcus braunii*, which possessing oil levels between 20% and 50%, along with favourable productivities [19-21,12]; and *Chlorella* appears in particular to be a good option for biodiesel production [22]. Additionally, the marine microalgae (e.g. *Dunaliella* and *Nannochloropsis*) are more prone to mass production, as a high salinity prevents extensive contamination, while allowing sea water to be directly used instead of depleting fresh water resources [12]. Apart from screening microalgae for high biomass productivity and lipid content, the fatty acid profile of microalgae is also an important characteristic as it finally affects the quality of the biodiesel product. The total lipids composition of microalgal biomass is composed of a large variety of chemical fractions, ranging from triacylglycerols (TAGs) to sterols [23], among of which lipids containing TAGs and fatty acids composed by high proportion of palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0) and oleic acid (18:1) are preferred compounds for biodiesel production [19,20,24]. That is to say, microalgae containing high proportion of monounsaturated fatty acids (MUFA) and Saturated Fatty Acids (SFA) which have much more energy yield, superior oxidative stability, and higher cetane numbers are more suitable for biodiesel production [25].

The capability evaluation of microalgal species is crucial for algal species selection for biofuel production especially large-scale production. The general evaluation focuses on biomass production and biofuel generation potential of algal species in cell cultivation and biofuel processing. For example, six freshwater microalgal strains in the class of *Chlorophyceae,* including *Chlorococcum humicola*, *Didymocystis bicellularis*, *Monoraphidium contortum*, *Oocystis parva*, *Sphaerocystis* sp., and *Scenedesmus acutus* were compared for their biomass yield, lipid content, and lipid productivity, and the best strain was found to be *C. humicola* with the highest biomass yield of 0.113 g L^{-1} d^{-1}, the highest lipid content of 45.94%, and the highest lipid yield of 0.033 g L^{-1} d^{-1} [26].

In particular, the knowledge of the biochemical composition of microalgae is of most importance, which can provide information about the possibilities of their potential use as an alternative fuel source [27]. Molecular genetic technique is applied in the biochemical composition evaluation of microalgal species for biofuel production. The hydrocarbon compositions of 19 freshwater cyanobacterial species distributed among 13 genera were analyzed by [28] based on hydrocarbon profiles and phylogenetic analysis, and the results not only provide an evolutionary perspective with which to study the physiological function

of cellular hydrocarbons but also display the engineering capacity to molecularly design diversified hydrocarbon fuel products in cyanobacteria.

A good understanding of the decomposition of microalgae during thermochemical conversion, one of the most promising routes for biomass utilization, is important for developing efficient processing technology at an industrial scale, and thermogravimetric analysis coupled with mass spectrometry (TGA-MS) could be a useful technique to obtain information at real-time of mass loss and evolved gases for thermochemical processes [29]. For example, the thermal characteristics under oxidizing atmosphere of several species of microalgae: *Nannochloropsis gaditana*, *Scenedesmus almeriensis* and *Chlorella vulgaris* was investigated by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) coupled with Mass Spectrometry (MS), which concluded that these microalgae samples could be potential bio-energy feedstocks [29]. In a study by Rizzo et al., (2013)[30], *Chlorella* sp. and *Nannochloropsis* were analysed to settle a preliminary characterization of these microorganisms as a fuel and its thermogravimetric behavior, which can potentially be used in the design and modelling of thermochemical conversion processes of microalgae.

When the microalgae species obtained from the environment are not feasible for commercial production of biodiesel, the application of genetic methods in microalgae to develop organisms optimized for high productivity and energy value can be taken into account. It has been reported that overexpression of endogenous and exogenous genes encoding Polyunsaturated Fatty Acid (PUFA) desaturases of the blue-green alga *Synechocystis* sp. PCC 6803, can markedly increase accumulation of α-Linolenic Acid (ALA) and Stearidonic Acid (SDA) and decrease accumulation of linoleic acid and γ-Linolenic Acid [31]. However, several shortcomings have hindered the broader use of genetically engineered microalgae especially eukaryotic microalgae. For instance, few microalgal strains have been genetically modified due to a lack of specific molecular biology tools and the mechanisms underlying the regulation of both gene expression and the metabolic network are unclear. Furthermore, concerns of biological contamination have materialized in restrictive legislation [3].

Cultivation

Many reports described that both lipids content and fatty acids profile are affected by cultivation conditions [7,28,32]. There are four major types of cultivation conditions for microalgae: photoautotrophic [28,33], mixotrophic [34] and photoheterotrophic [35].

Main Cultivation Modes

It was reported that only photoautotrophic cultivation is technically and economically feasible to culture microalgae in commercial scale, typically at outdoor environment where sunlight is abundant and free [36]. Carbon constitutes half of the weight of the biomass, it is necessary to supply large amounts of carbon in the form of carbon dioxide due to its low price. Moreover, photoautotrophic microalgae are able to capture CO_2 and NOx from flue gases, an added advantage to the culture system. However, photoautotrophic method has its limitation especially in countries where sunlight intensity is not always suitable for photoautotrophic culture available throughout the year. The introduction of sufficient natural or artificial light to allow massive growth and dense populations is the main objective and a limiting factor of the cultivation [37].

Relatively high lipids yield and biomass productivity can be attained through heterotrophic cultivation mode, indicating that heterotrophic culture provides an absolutely good opportunity for large scale production by using conventional fermentation bioreactor. Higher cell density of 51.2 g L^{-1} and lipid concentration of 25.8 g L^{-1} were achieved by high-density fermentation of *Chlorella protothecoides* [38]. In another research, oil-rich biomass has been produced from heterotrophic fed-batch and semi-continuous cultures of *C. protothecoides* with pure glycerol as the main carbon source, and the results showed

that a maximum biomass concentration of 64 g L^{-1} was achieved in fed-batch mode and the highest value (around 50% of dry biomass) was obtained in semi-continuous mode [39]. In a study by Zheng et al., (2013) [40], high cell density culture of oleaginous microalga *Chlorella sorokiniana* with glucose as the carbon source resulted in algal biomass and lipid concentrations of 103.8 g L^{-1} and 40.2 g L^{-1} respectively, and the large amount of neutral lipids (92.9% of total lipids) and triacylglycerols (82.8% of neutral lipids) were ideal form of lipid for making biodiesel. However, the limited number of available heterotrophic algal species, potential contamination by bacteria, inhibition of growth by soluble organic substrates at low concentrations and the inability to produce some light-induced products (e.g. pigments) were the limiting factors of the heterotrophic cultivation mode for large scale production.

The limited light-induced products can be overcome through the following two ways, mixotrophic cultivation and two-step cultivation. For mixotrophic culture, microalgae are live either photoautotrophic or heterotrophic pathway depending on the concentration of organic compounds and light intensity available. Heterotrophic metabolism can obtain higher biomass but light induced products are very low, whereas adequate lighting is conducive to the synthesis of light induced product. Therefore, the artificial control of photoautotrophic and heterotrophic ratio [41], such as modulating light intensity, increasing CO_2 supply and reduce the addition rate of organic carbon to obtain higher biomass and light induced products. The two-step cultivation methods, namely the first heterotrophic or mixotrophic cultivation is to obtain higher biomass, and then transferred to autotrophic conditions where light induced to produce required metabolite products.

Large Scale Cultivation Systems

The two primary methods of microalgae cultivation in large scale cultivation are using open ponds and closed photobioreactors (PBRs). Currently, open ponds are cost efficient and over 90% of world microalgae biomass production was realized in large raceway ponds (Zhang et al., 2014) [42]. However, the very low culture density, low biomass productivity, contamination, susceptibility to weather events such as rainfall, and high evaporative losses are major drawbacks when commercial developments of microalgae biofuel are promoted [43-45]. To avoid the aforementioned drawbacks of open cultures, various PBR designs are proposed for biofuel production. PBRs appear in different common configuration: tubular reactors, flat plate reactors and column reactors (bubble or air-lift columns). Compared to open ponds, PBRs were closed to the atmosphere and protect the cultivated alga to be less prone, but not immune, to contamination; the growth parameters (e.g., temperature) could be better controlled; they allow to reach higher volumetric productivities and cell concentrations due to a higher surface-to-volume (S/V) ratio; closed systems eliminate or strongly reduce evaporation to save water; PBRs have not been engineered to the extent of other bioreactors in commercial use, existing sizable potential for further development and scale-up [46,47]. It is expected that algae productivities between 40 and 80 tons of dry matter per hectare per year can be realized in closed systems [48], which are promising for industrial scale production of microalgae. However, high capital and operating costs of PBRs is a major hurdle for their large-scale implementation. Although few of PBR designs have been explored at pilot level for biofuel production, they still await evaluation at industrial scale [49]. There is a dichotomy, need to be solved in future, between PBRs and open systems as the possible culture solution to be employed in large-scale microalgae biomass production.

Harvest

After cultivation, microalgae biomass has to be separated from its growth medium and recovered for downstream processing. Microalgae harvesting remain a major hurdle to industrial scale processing, and it was estimated to account for 20–30% of the total biomass

production cost [50]. An optimal harvesting technique should be independent of the cultured species, consume little energy and few chemicals and not damage the valuable products in the extraction process. When considering commercial-scale processes for dewatering and recovering algal biomass for further downstream processes, a traditional harvesting method may involve up to two steps, bulk harvesting (known as primary harvesting) to separate microalgae from suspension, such as sedimentation, flocculation and floatation, and thickening (known as secondary dewatering) to concentrate the microalgae slurry after bulk harvesting, such as centrifugation and filtration [50,36,51]. The choice of which harvesting technique to apply will depend on the species of microalgae and the final product desired.

Bulk harvesting

Sedimentation is a low cost harvesting method which is commonly applied for separating microalgae in water and waste-water treatment. On the one hand, due to its low power consumption and low requirement for skilled operators, sedimentation is useful as a pre-concentration step [52]. On the other hand, as sedimentation is low in rate and only suitable for harvesting of large size microalgae, flocculation is frequently used to increase the efficiency of gravity sedimentation [53].

Among the bulk harvesting methods, flocculation is frequently used to increase the efficiency of gravity sedimentation and commonly performed before secondary dewatering processes [53]. Addition of chemicals known as flocculants to counter the surface charge on the algae is widely applied, including inorganic coagulants (aluminum sulfate, ferric sulfate and ferric chloride) and organoclays [50,54]. However, the inorganic multivalent metal salts are toxic and expensive for commercial-scale culture of microalgae. The nontoxic organic polymer, such as polyacrylamide copolymers,chitosan and cationic starch [55,56] have been intensively investigated, but they are not economical for large-scale applications due to their higher price. Furthermore, the flocculant residuals in both algal biomass and harvested water are not only negative for later processing but also disadvantages for culture medium recycling. Thus, the flocculation technologies without addition of flocculants have been investigated. Induced by increasing medium pH, autoflocculation that flocculation of microalgae spontaneously can occur without the need for chemicals [57]. For example, harvesting efficiency higher than 90% of *Chaetoceros calcitrans* was achieved by adjusting the culture pH to 10.2 [58]. However, autoflocculation can only be applied for the limited strains and the high power that needs to be input in electrolytic method are not easy to scale-up and results in a temperature increase that may damage the system [59].

Flotation can capture particles with a diameter of less than 500 μm by collision between a bubble and a particle and the subsequent adhesion of the bubble and the particle [60]. Based on bubble sizes used in the flotation process, flotation is divided into Dissolved Air Flotation (DAF), dispersed flotation and electrolytic flotation. Microalgae strain *Tribonema minus* cultivated for 21 days in 40 L glass panel were harvested by DAF, and the recovery efficiency reached up to 95.57% [61]. The flotation process where microalgae float to the surface of medium is prone to harvest in microalgae mass culture, which has been used for specific strains. Although flotation has been mentioned as a potential harvesting method, there is very limited evidence of its technical or economic viability.

Thickening

The centrifugation process driving by a much greater force (gravity) to promote accelerated settling of microalgae cells can be used for almost all types of microalgae reliably and without difficulty [62]. Centrifugal recovery can be rapid and easy to operate, but it is only feasible if the targeted metabolite is a high-value product because the process is highly energy intensive [50]. In addition, using this technique at a large scale is problematic because of high power consumption, which increases production costs.

Filtration is a physical separation process by filter (membrane), which is characterized by their efficiency, reliability, and safety for the solid–liquid separation [63]. However, fouling limits the widespread use of membrane separation technology due to the reduction of permeation flux during the separation process.

Two types of thickening methods (without prior bulk harvesting), centrifugation and filtration, are too costly and energy intensive for large scale biofuels production. In this regard, the bulk harvesting technologies as pre-concentration step may play an important role in reducing the energy consumption during the thickening process of microalgae slurry [52].

Lipid Production

Dehydration

For lipid production, extensive drying of microalgae biomass is required as the presence of water interferes with the extraction and/or conversion of algal lipids to biodiesel, and the percentage of water contained in algal paste should not exceed 50% before oil extraction [64]. Spray drying, drum drying, freeze drying and solar drying are common methods for drying microalgae [65]. The former three drying methods are not economical and lead to a negative energy balance in large scale microalgae biodiesel production because of heavy dependency on fossil fuels. Solar drying is considered the economical method. However, it is not feasible in temperate countries where sunlight is limited at certain time of the year, and it requires large land areas for large-scale operations [51]. Thus, it is urgently needed to develop new technologies or approaches to ensure the sustainability of microalgae biofuel industry. In addition, as a large amount of thermal energy cost is required to dry microalgae, technology has been developed to recover the lipid from wet microalgae without drying, and direct liquefaction, supercritical fluid extraction, and dimethylether (DME) extraction have been reported as methods for recovering lipids from wet microalgae [66].

Lipids Extraction

Lipids and fatty acids are extracted from the microalgae biomass usually before biodiesel production. Lipids extraction efficiency is directly related to the overall process efficiency in biodiesel production, so develop efficient and cheaper extraction processes is necessary to reach industrial biodiesel production at appropriate costs. There are several oil extraction methods which have been used on microalgae [67]: methods assisted by mechanical disruption which uses cell homogenizers, ball mills, pressing systems, among others; enzyme-assisted extraction methods where microalgae cell wall is degraded by enzymes allowing lipids release; and other methods evaluated on microalgae including ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, autoclave and solvent based extractions.

Two general extraction processes, Hexane Soxhlet method and Bligh–Dyer method [68,69], are effective to extract microalgae lipids, and the hexane-based oil extraction is identified as the most adequate alternative of the routes assessed for scaling up from the energy point of view [67], but the extraction efficiency is highly dependent on microalgae strains. Liquefied DME is also promising as an extracting solvent. [70] proposed a simple method for the extraction of lipid from high-moisture microalgae by using DME without drying, cell disruption, and heating of the extractant, which was successfully achieved an extraction yield of 9.9–40.1% of the dry weight of the microalgae. Subcritical extraction greatly improved extraction efficiency. In addition, a water-miscible solvent of 1, 2-dimethoxyethane was also found for efficient extraction of algal oil from wet cells of *Botryococcus braunii* with a

high algal oil recovery above 96% [71]. Considering ethanol is relative cheap, easy recycling and safer than some other solvents, subcritical ethanol extraction was performed in total lipids extraction from microalgae paste. By using subcritical ethanol extraction, the drying process is unneeded, resulting in the less energy cost. For example, Chen et al., (2011) [34] applied subcritical ethanol extract lipids from wet microalgae paste of *Nannochloropsis* sp., which could reach 90.21% recovery rate of the total lipids. Moreover, the disadvantages of using chemical solvents, some of which are conventionally derived from non-renewable fossil fuels, are mostly related to their high toxicity towards human and surrounding environment. Several supercritical fluids mainly supercritical-CO_2 have received the most interest and applied to microalgae lipids extraction for biodiesel production recently [72-76], which is non-toxic and provide non-oxidizing environment to avoid degradation of extracts. Moreover, no separation step is needed since CO_2 is gaseous at ambient pressure [76]. However, high-cost of supercritical-CO_2 extraction prevent its development.

Biodiesel Processing

The main component of biodiesel is a mixture of fatty acid methyl ester, the length of carbon chain and degree of saturation determine the physical and chemical properties of fatty acid methyl esters, which finally determines the quality of biodiesel. The most accessible technology reported for biodiesel production currently is transesterification reaction (Figure 2), in which TAGs are reacted with short chain alcohol (e.g. methanol or ethanol) in the presence of catalyst and the final reaction products are biodiesel (fatty acid methyl esters, FAMES) and glycerol (by-product) (Lam et al., 2010) [77].This process has been widely used to reduce the high viscosity of triglycerides.

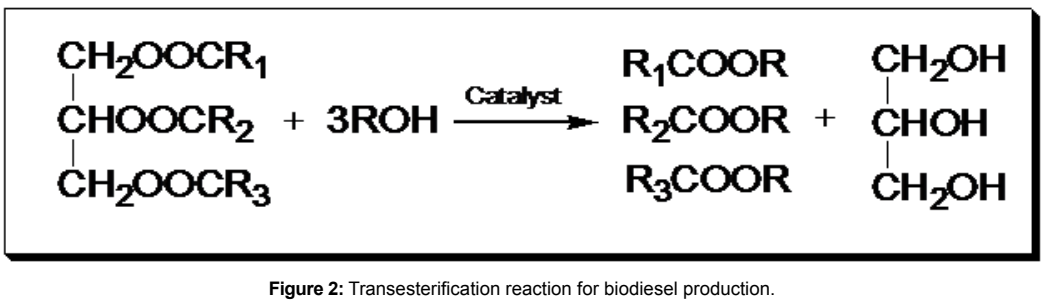

Figure 2: Transesterification reaction for biodiesel production.

Chemocatalysis

Alkaline catalysts have been the most commonly and economically used route to catalyze the transesterification reaction, in which the reaction can be catalyzed at low temperature and atmospheric pressure to achieve high conversion yield in short time (minutes) [78,4]. However, formation of soaps in the presence of the FFAs (> 1%) and water (> 0.06%) will lead to low biodiesel yield and increase the difficulty to separate biodiesel from co-product [79]. Acid catalysts have been proposed to overcome the limitations as the acid catalysts are not sensitive towards FFAs level in oil, and they can promote esterification (FFAs are converted to alkyl ester) and transesterification simultaneously [80]. Further, a two-step reaction has been proposed to produce biodiesel from lipids with a high FFAs content [81,82], in which microalgae lipids are initially subjected to acid pre-treatment so that FFAs level is reduced to less than 1% weight, followed by the second transesterification step performed by using alkaline catalysts. In a study, biodiesel production from *Tribonema minus* oil was followed by two-step catalytic conversion, and the conversion rate of TAGs reached 96.52% [83]. Nevertheless, high concentrations of the alkaline catalysts are necessary to neutralize

the acid catalysts from the first transesterification step, increasing the operational costs. Heterogeneous catalysis (e.g. CaO, MgO and mixed $CaO–Al_2O_3$, etc) has also been developed for biodiesel production as it is non-corrosive and can be recycled which minimize product contamination and number of water washing cycle. However, the use of heterogeneous catalysts can result in low biodiesel conversion yields in comparison with homogeneous catalysts [84,85].

Biocatalysis

Recently, enzymatic transesterification using lipases, another technique that catalytic effectiveness is not affected by high level FFAs and water in the reactors, has become more attractive for biodiesel production, as it allows the use of mild reaction conditions, avoids the generation of wastewater and eases the recovery of high purity glycerol [85]. However, the high cost of enzyme remains a barrier for its industrial applications. To decrease the cost, the enzyme could be immobilized in a suitable carrier, where lipases do not require excessive energy expenditures as immobilization facilitates its recovery and reuse [86]. For example, converting Scenedesmus incrassatulus lipids to FAMEs by Novozym 435 has obtained a higher FAMEs yield in a shorter reaction time (6 h) [86]. The immobilized Novozym 435 has been investigated widely and is reported to exhibit the best performance among different lipases sources [87,88].

In-Situ Transesterification

In contrast to traditional process of biodiesel production, in-situ transesterification (or direct transesterification), in which the lipids extraction and transesterification are carried out simultaneously, shows promise of both simplifying and reducing the cost of producing biodiesel from microalgae biomass [36]. However, the algal biomass needs to be dried prior to the in-situ transesterification procedure, because the presence of moisture at greater than 20% by mass in algal biomass significantly decreased biodiesel recovery when using in-situ transesterification [89,90] implied that algal biodiesel production from a dried feedstock might only prove sustainable if a low carbon solution such as solar drying was implemented to help reducing the water content of the feedstock. In recent years, improved in-situ transesterification process was explored to directly convert wet oil-bearing microalgae biomass into biodiesel. Dang-Thuan et al., (2013) [91] reported that C. *vulgaris* ESP-31 biomass (water content of 86–91%; oil content 14–63%) was pretreated by sonication to disrupt the cell walls and then directly mixed with methanol and solvent to carry out the enzymatic transesterification and achieve over 90% biodiesel conversion. In a study by Cerón-García et al., (2013)[39], biodiesel was obtained using a modified process based on transesterification of wet biomass paste, with a recovery of nearly 97%.

Economic Feasibility of Microalgal Biodiesel

The higher capital and operating costs of microalgae farming compared to conventional agriculture and the non-sufficiently positive energy balance (after accounting for energy requirements for water, CO_2 and nutrient supply, biomass harvesting and processing) result in the much higher price of algae-based biodiesel than normal diesel, which encumbers large-scale (commercial scale) manufacturing and applications of algae-based biodiesel [92-94,49]. There is no current industrial production of algae biodiesel in the world. The cost for microalgal biofuel production may be reduced by the following strategies (Figure 3): firstly, the alga are selected or reformed for increasing the lipid productivity; secondly, biofuel production from microalgae combined with processes such as wastewater and flue gas treatments, in which freshwater is replaced with wastewater and flue gases are used as a carbon and inorganic nutrient source for culturing microalgae; finally, the bio-refining

of microalgae for production of high value-added products will increase economic benefit for microalgal biodiesel production, in which cost of producing microalgal biofuel can be reduced substantially coupled with a biorefinery based production strategy.

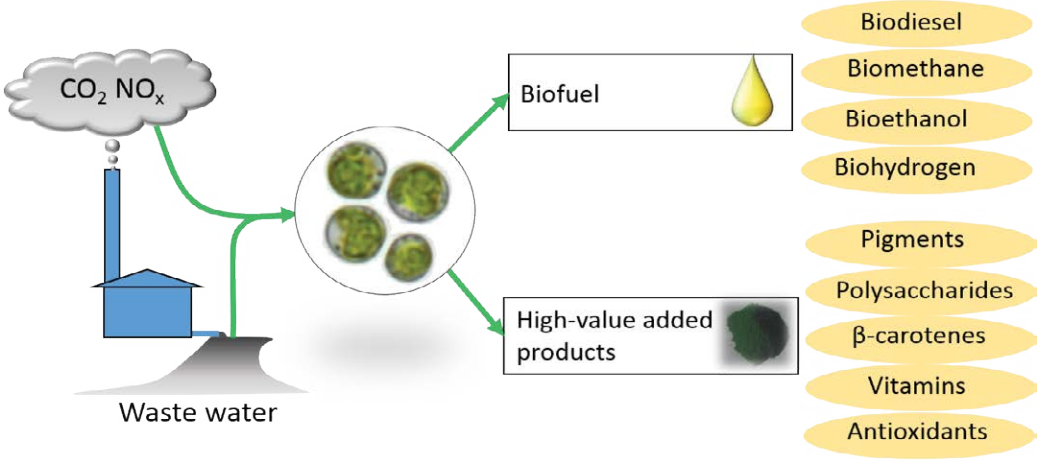

Figure 3: Biodiesel production integrated environmental pollution control and production of high value-added products.

Species

A major impediment to the utility of many algae species for producing biodiesel is the need for favourable conditions, for example, warm temperatures and high light intensities with long photoperiods [16]. Some of the main species of algae used in large scale commercial production, including *Chlorella*, *Haemattococcus*, *Dunaliella* and *Spirulina*, are generally restricted to geographic locations with warm climates and would be unable to grow at acceptable rates during the hot or cold seasons of the significantly hotter or cooler climates of the specific geographical area [95]. One solution to this problem is to identify indigenous algae that are adapted to local environment. For example, *Monoraphidium* sp. Dek19, collected from a local cold environment, was shown to be cold-tolerant and to grow to workable density at relatively low light intensities in sterilized treated effluent, indicating in general that searching for species of algae adapted to local environments is a good strategy for developing biodiesel feedstock [95]. In addition, native microalgae species may also perform better than most other species in commercial scale cultivation with wastewaters. In a study, selected five native strains (UM224, UM253, UM265, UM280, and UM284) with high growth rates and higher lipid productivities showed the ability to adapt to grow on concentrated municipal wastewater for biofuel production, which are considered highly promising compared with other strains reported in the literature [60].

If the alga can be selected, engineered or otherwise mutagenized into increasing the lipid productivity or other high value-added products, the production economics can be improved substantially. Among the wild species available in formal collections, high oil-producing microalgae species often couple with reduced growth rate. If the high growth rate and lipid content are combined, microalgae would become a promising feedstock for biofuel production, especially for biodiesel. When the wild species are not feasible for commercial production of biodiesel, the application of mutagenic or genetic method in microalgae to develop organisms optimized for high productivity and energy value can be taken into account. For example, an attempt to increase biomass production and lipid content with the use of chemically induced mutants was produced from wild type *Nannochloropsis* sp., and the mutants showed their comparative advantage over their wild type parent and their potential utilization by the algal biotechnology industry for the production of biofuels [96].

To enhance oil accumulation, Iwai et al.,(2014)[97] constructed a P deprivation-dependent overexpressor of a *Chlamydomonas* type-2 Diacylglycerol Acyl-Coa Acyltransferase (DGTT4), and the transformant strongly enhanced TAGs accumulation. However, several shortcomings at present, including very few strains of microalgae that underwent genetic modification, poor elucidation of the mechanisms underlying regulation of gene expression, lack of specific molecular biology tools, pending concerns of biological contamination–materialized in restrictive legislation, have hindered a broader utilization of genetically engineered microalgae [3]. Although progress in the mutagenic or genetic engineering of algae was too slow to develop an adequate number of optimized algal strains toward the synthesis of preferred products, a wide variety of new genetic tools, genome sequences, and high throughput analytical techniques will allow scientists to analyze metabolic pathways and conduct genetic modification with high precision.

Cultivation

One more significant option is through the cost reduction in process of microalgal cultivation. Microalgae grown at a large scale especially in open pond systems are susceptible to contamination by undesirable algae, bacteria, protozoa and zooplankton, which would costly to control at large scale. Bacterial contamination actively competes for nutrients and oxidise organic matter that could lead to growth inhibition even death of target microalgae [75]. Protozoa and zooplankton actively consume microalgae that result in collapse of algal concentration in relatively short periods of time [98]. Increase of pH beyond 8.3 can be done in order to prevent contamination by bacteria [99]. Moreover, a detergent and phenol procedure may be employed as a prevention method for bacterial contamination [100]. Contamination by protozoa, zooplankton and other algae may be reduced by utilisation of highly selective culture conditions. Algae that grow under extreme conditions, such as *Duniella salina* cultured well under high salinity [101] and *Spirulina* cultured well under high alkalinity [100], provide a competitive advantage thus limiting contamination by other microalgae, protozoa and zooplankton. In addition, closed systems are more likely to be run as continuous systems due to higher efficiency and significantly lower amounts of contamination of undesirable algae and other organisms [99]. Recently, the research work and commercial production mostly focus on the monoculture of a small number of species with high growth rate and lipid content. However, monocultures are susceptible to contamination, and strains with high lipid productivity are likely to be outperformed by other faster growing species [102,103]. Furthermore, when wastewater is used for monocultures, maintain a pure culture during the operation become more difficult because of constant airborne contamination in the open system and impacts from the wastewater [37]. Thus, mixed culture of algae can sustain themselves in wastewater treatment systems, and microalgae communities of higher diversity are considered to be more stable and lower invasion risk compared to monocultures [103].

The large consumption of water resources, inorganic nutrients (mainly N and phosphate) and CO_2 are costly for microalgae cultivation, most importantly, nutrient expenses make the major part of the cost of microalgae production and perplex the commercialization of algal biofuel [104,105]. Biofuel production from microalgae can be more environmentally sustainable, cost-effective and profitable if combined with processes such as flue gas and wastewater treatments [106,107].

Addressing global warming concern is one of the key global challenges and CO_2 acts as a principal effect to contribute the global warming. About 75% of the total anthropogenic CO_2 emissions were derived from fossil fuel burning [31]. The general technologies used to capture CO_2 are physical and chemical absorption, cryogenic distillation and membranes separation [108], which are energy consuming. CO_2 bio-fixation by microalgae has drawn much attention as an environmentally friendly CO_2 mitigation strategy. As the ability of CO_2 uptake by microalgae varies dramatically among algal species, it is necessary to select

suitable algal candidates to fix CO_2 from actual flue gases under a large collection of algal cultures although it is very time-consuming and technical challenging. Lam and Lee., (2012) [36] reported that *Scenedesmus obliquus* was able to tolerate high concentration of CO_2 up to 12% (v/v) with optimal removal efficiency of 67%. *Botryococcus braunii* was another appropriate for cultivation with CO_2 from flue gas and lipid productivity reached 20.65 mg L^{-1} d^{-1}. Liu et al., (2013) [36] described a high throughput screening method, which integrated a CO_2 mixer, GasPak bags and microplates charged with different CO_2 concentrations, to rapidly identify microalgae strains that can tolerate high CO_2 condition or flue gases from a large pool of culture collection species. Current methods for CO_2 feeding to algae cultures rely on the sparging of CO_2 directly from flue gases. However, due to low solubility of CO_2 in water, CO_2 feeding through sparging limits the amount of CO_2 absorbed in the liquid, leading to low carbon utilization efficiency [109]. Moreover, this system requires a considerable amount of energy for contacting of the CO_2 with the liquid phase effectively and achieving high enough CO_2 concentrations in the liquid, resulting in its limitation during scale-up [110]. Compared with open culture systems, closed photobioreactors could reduce CO_2 losses by prolonging CO_2 retention time and improving mass transfer efficiency, and structure designs of photobioreactors could further improve CO_2 dissolution efficiency and CO_2 utilization efficiency by microalgae [36]. In order to enhance CO_2 mass transfer rate as well as alleviate Dissolved Oxygen (DO) accumulation, a novel gas sparger of bubble tank was adopted in a PBR and the maximum CO_2 removal efficiency was 94% [111]. In contrast, absorption of CO_2 in alkaline solution can be used to stabilize the CO_2 as carbonate and then is fed to algae [112], which directly improves the efficiency of CO_2 uptake by the algae compared to direct injection of CO_2. Absorption liquids such as potassium carbonate are cheap and also reduce the energy requirement needed for the sparging of CO_2 into algae cultures [110]. However, it is necessary to select algae strains with high tolerance under alkaline conditions. For example,[113] reported an alga from alkaline hot spring could grow well over pH 11.5 and 50 °C.

NOx also compose a significant portion of flue gases, which restricted by legislation and needed to remove in an additional costly gas treatment step [114-115]. The conventional NOx treatments, physico-chemical DeNOx methods, are expensive and produce secondary wastes that often require further treatment [116]. Because N is one of the basic elements for algal production, notably, NOx can serve as an N source for microalgae and can be metabolized by microalgae [117]. Thus, a biological DeNOx (bio-DeNOx) method that uses microalgae may be noteworthy for flue gas treatment to reduce NOx emissions and merit further studies. NOx removal efficiencies varied among microalgae strains. Some microalgae species such as *Chlorella* sp. and *Scenedesmus* sp. [118,119,12] had shown the possible bio-DeNOx capacity. For NOx-removal by microalgae, NOx in flue gas are first dissolved in an aqueous phase, after which NOx are oxidized and assimilated by the algal cells. However, NO, which is the main component of NOx, is sparingly soluble in water, and the dissolution of NO into the microbial culture is the rate-limiting step for NO removal [119]. Some attempts have been reported that enhance the solubility of NO gas by using efficient complexing agent like metal-chelated EDTA [118]. However, the complexing agent used in cultivation is harmful for algae and expensive when commercial-scale culture of microalgae. In addition to enhancing the dissolution of NO in water, the initial fixation of massive NO or NOx into alkaline solution and then the cultivation of algal cells by using fixed nutrients is a possibly effective way of improving NO or NOx removal efficiency. In a study by Zhang et al., (2014) [12], the actual NOx fixed salts, which fixed by using alkaline solution from the caprolactam production plant of Sinopec's Shijiazhuang Refining & Chemical Company, were applied to *Chlorella* sp. C2 cultivation, and a 60% nitrite removal efficiency was obtained together with the production of 33% algae lipids.

Besides CO_2 and NOx, flue gases contain many other compounds such as H_2O, O_2, N_2, SOx, unburned carbohydrates (CxHy), CO, heavy metals, halogen acids and particulate matter (PM). Several chemical compounds (SOx, heavy metals, etc.) have shown to be toxic

to some microalgae[120]. To better engineer flue gas-fed microalgal cultures, the effects of all flue gas compounds on microalgae, tolerance of various microalgae to flue gas compounds and the interaction of flue gas compounds and microalgae need to be assessed [114,121].

In the pilot and large scale of microalgae culture, the use of huge amounts of fresh water is a serious problem, leading to the competition with land crops and human activities. As large amounts of nitrogen and phosphorus can be recovered from wastewater, microalgae production using wastewater as the nutrient source is a potential method to saves fresh water and offers added environmental advantages, which is effective in removing nitrogen, phosphorus and toxic metals from a wide variety of wastewaters, and producing cleaner effluents with high concentrations of dissolved oxygen [122]. Cabanelas et al., (2013) [123] used *Chlorella vulgaris* for nitrogen and phosphorus removal from municipal wastewater with the highest removal rates of 9.8 (N) and 3.0 (P) mg L^{-1} d^{-1}. Some microalgae widely used for nutrient removal from different wastewater streams are *Chlorella* sp. [124,20] and *Botryococcus braunii* [1]. Recently, some research results suggest that dual-use microalgae cultivation for wastewater treatment coupling with biofuel generation is an attractive option in terms of reducing the energy, nutrient and freshwater resource costs [83]. Zhou et al., (2012)[33] developed an effective hetero-autotrophic mode for improving wastewater nutrient removal, wastewater recycling and algal lipid accumulation with *Auxenochlorella protothecoides* UMN280, and the excellent nutrient removal efficiencies and lipids content were reached. Zhou et al.,(2013) [107] cultivated *Chlorella zofingiensis* in piggery wastewater, and achieved the maximum lipid productivity of 110.56 mg L^{-1} d^{-1}. Furthermore, effluents from anaerobic digestion, such as biogas fluid, can be utilized as a suitable nutrient source for microalgal culture, for example, *Chlorella* sp. is a microalga that could be utilized effectively in the simultaneous biogas upgrade and biogas fluid nutrient removal . Due to the complex characteristics of wastewater, the tests of growing algae in wastewater are mostly at laboratory scale, and require confirmation in long term and at relevant volume, as there is significant difference between laboratory-scale and large-scale production. Many issues including contamination, inconsistent wastewater components and unstable biomass production encumbers pilot and large scale algae cultivation [125]. Moreover, most previous studies have been carried out under suitable or optimal conditions. However, the cultivation performance of microalgae under outdoor and cold climate conditions was poor, showing the microalgae cultivation was region-dependent, which will hinder the scale-up and commercial application of algae cultivation for biodiesel production [126]. For example, total lipids ranged from 11% of dry weight in winter to 30% of dry weight with an increased amount of monounsaturated fatty acids in autumn when growing *Nannochloropsis oculata* outdoors in closed vertical flat photobioreactors [127]. Improve the stable performance of microalgal cultivation and nutrient removal under outdoor and cold climate conditions is an urgent issue needed to be solved to achieve sustainable development for the algal industry. In a study, mixotrophic culture with the addition of acetic acid (pH-regulation) was designed in an integrated approach combining freshwater microalgae *Chlorella zofingiensis* wintering cultivation in pilot-scale photobioreactors with artificial wastewater treatment, proved that pH adjustment using acetic acid was efficient in cultivating *C. zofingiensis* in wastewater in winter for biodiesel production and nutrient reduction [128].

Besides flue gas and wastewater, organic fertilizer derived from food waste, biomass or manure also contains high value of nutrients that can support microalgae growth. *Chlorella vulgaris* was cultivated using organic fertilizer as an alternative nutrient source for biodiesel production and the water was reutilized to re-cultivate *C. vulgaris*, it was found that *C. vulgaris* grown favourably under open environment and extracted lipids profile was proven to be suitable for making biodiesel [36]. Chen et al., (2014) [42] explored the feasibility of combining Swine Manure (SW) and mixed-culture algae (AW) for bio-crude oil production via hydrothermal liquefaction, and analysis of energy consumption ratios indicated that co-liquefaction of AW and SW was energetically feasible and could be an economically competitive system for bio-crude oil production.

Co-production

Besides microalgal biodiesel (the most studied microalgal biofuel type), microalgae can provide other several different types of renewable biofuels, including biomethane (also called as biogas) produced through anaerobic digestion of algal biomass [129], biohydrogen produced photobiologically [130] and bioethanol [131]. A study by Prajapati et al., (2014) [132] was focused on the comparative exploration of biogas production potential of three *Chlorella* sp. namely *Chlorella minutissima*, *Chlorella vulgaris* and *Chlorella pyrenoidosa*, among which *C. pyrenoidosa* was found to be the best in biogas generation and higher biogas yield of 0.464 ± 0.066 m^3 biogas kg^{-1} VS added with 57% (v/v) CH_4 content was obtained during 30 day digestion. A significant improvement in H_2 photoproduction was obtained in the marine green alga *Platymonas helgolandica* var. *tsingtaoensis* treated by carbonyl cyanide m-chlorophenylhydrazone (CCCP) and herbicide 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (Zhang et al., 2012)[130]. It is noteworthy that a study investigated the fermentation of *Chlorella vulgaris* for co-production of hydrogen and methane in a two-stage combined fermentation process (combination of dark fermentation for H_2 production from microalgae and CH_4 fermentation from residues following H_2 production process), which reported a seven-fold increase in H_2 production yields and the increase from 245 ± 2.46 to 414 ± 2.45 ml CH4 g-VS^{-1} in methane yields with enzymatic pre-treatment of the microalgae [133]. Martín and [131] optimized a process that integrated the use of glycerol to produce ethanol via fermentation within the simultaneous production of biodiesel and bioethanol from algae, increasing the production of bioethanol by at least 50%.

Except for lipids, various high-value chemical compounds such as pigments, antioxidants, β-carotenes, polysaccharides, vitamins and biomass can be extracted from microalgae, and they are largely used as bulk commodities in different industrial sectors (e.g. pharmaceuticals, cosmetics, nutraceuticals and functional foods) [16]. For example, β-carotene, a vitamin A precursor in health food, was the first high-value product commercially produced from *Dunaliella bardawil*, which was the predominant product in microalgal biotechnology and could be sold mostly in the human health food market [42] . Williams and Laurens (2010)[134] noted that 30% and 50% of the algal primary product mass was lost on producing cell proteins and lipids, respectively, and the increased lipid content reduces other valuable compounds in the biomass, and the "biofuel only" option is unlikely to be economically viable [134]. Realisation of algae-based biofuel plants is dependent on not only the scale-up economics of efficient algae growth, harvesting and oil extraction, but also available revenue streams from the oil and by-products of likely higher value than the fuel itself. Even the revenue from lower yield by-products, such as biofertilizers, may tip the balance between viable and non-viable commercialization [135]. For example, several species of algae, including the heterotrophic microalgae *Schizochytrium* sp. used to capture energy from glycerol byproducts from biofuel production, are high in total lipid and contain up to 52 % of the fatty acids as DHA [136].

Future Perspective

In the past few decades, extensive use of fossil fuels has led to global climate change, serious environmental pollution and health problems. Irreversible consumption of traditional fossil fuels has led to the global energy crisis. At the meantime, fossil fuel as the main energy source is not sustainable, and will further result in the accumulation of GHG in the atmosphere leading to global warming. With the urgent need to reduce carbon emissions and the lowered crude oil reserves, many countries are turning their attention to new, clean, sustainable energy development. The microalgae-based biofuels could be possible to completely replace fossil fuels. But before fully excavating the potential of microalgae for biodiesel production, there is still a long way to go. Constraints of using microalgae as a feedstock for biofuel production include the efficient biomass harvesting and oil extraction. Meanwhile, microalgae cultivation process requires strictly monitor light, nutrients, CO_2

and O_2 supply level control to ensure the best conditions for high algal biomass and oil production. There is no current industrial production of algae biodiesel in the world. The biggest challenge is that microalgae biodiesel does not have the economic competitiveness considering today's energy prices. To accelerate the commercialization and industrialization of microalgal biodiesel production, efforts can be made from three aspects to substantially reduce the cost: (1) Improving microalgal oil productivity by developing and applying new techniques to all links of the biodiesel production chain; (2) Producing microalgal biodiesel associated with environmental treatment processes such as wastewater and flue gas treatments; (3) Co-producing high value-added products.

Commercial-scale microalgae production still requires a lot of investment in research of all aspects including screening, domesticating or building rich energy-producing microalgae species; developing cost-effective algae production, harvesting and (wet) full utilization of microalgae biomass and efficient energy conversion technologies and equipment; construction of wastewater treatment based microalgal biofuels production system; establishing assessment mechanisms to evaluate the economic feasibility of microalgal biofuels. Despite the challenges, the microalgae are promising feedstock for biofuels production.

Acknowledgements

This work was supported jointly by the National Program on Key Basic Research Project (2012CB224803), the National Natural Science Foundation of China (31300030), Sinopec (S213049), the Natural Science Foundation of Hubei Province of China (2013CFA109) and the Knowledge Innovation Program of the Chinese Academy of Sciences (Y35E05).

References

1. Álvarez-Díaz PD, Ruiz J, Arbib Z, Barragán J, Garrido-Pérez C, et al. (2013) Factorial analysis of the biokinetic growth parameters and CO2fixation rate ofChlorella vulgarisandBotryococcus brauniiin wastewater and synthetic medium. Desalination and Water Treatment: 1-11.
2. Miao X1, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. See comment in PubMed Commons below Bioresour Technol 97: 841-846.
3. Amaro HM, Guedes A, Malcata FX (2011) Advances and perspectives in using microalgae to produce biodiesel. Applied Energy 88: 3402-3410.
4. Hossain AS, Salleh A, Boyce AN, Chowdhury P, Naqiuddin M, et al. (2008) Biodiesel fuel production from algae as renewable energy. American journal of biochemistry and biotechnology 4: 250-254.
5. Rosenberg JN1, Oyler GA, Wilkinson L, Betenbaugh MJ (2008) A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. See comment in PubMed Commons below Curr Opin Biotechnol 19: 430-436.
6. Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, et al. (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Research 1: 20-43.
7. Rodolfi L1, Chini Zittelli G, Bassi N, Padovani G, Biondi N, et al. (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. See comment in PubMed Commons below Biotechnol Bioeng 102: 100-112.
8. Chisti Y1 (2008) Biodiesel from microalgae beats bioethanol. See comment in PubMed Commons below Trends Biotechnol 26: 126-131.
9. Hu Q1, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, et al. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. See comment in PubMed Commons below Plant J 54: 621-639.
10. Chisti Y1 (2007) Biodiesel from microalgae. See comment in PubMed Commons below Biotechnol Adv 25: 294-306.
11. Hua L, WeiBo W, YongDing L, AiHua X, DunHai L, et al. (2011) The development of microalgae biodiesel and the utilization of oleaginous microalgae. Kezaisheng Nengyuan/Renewable Energy Resources 29: 84-89.
12. Zhang X1, Chen H, Chen W, Qiao Y, He C, et al. (2014) Evaluation of an oil-producing green alga Chlorella sp. C2 for biological DeNOx of industrial flue gases. See comment in PubMed Commons below Environ Sci Technol 48: 10497-10504.
13. Scott SA1, Davey MP, Dennis JS, Horst I, Howe CJ, et al. (2010) Biodiesel from algae: challenges and prospects. See comment in PubMed Commons below Curr Opin Biotechnol 21: 277-286.

14. El-Sheekh M1, Abomohra Ael-F, Hanelt D (2013) Optimization of biomass and fatty acid productivity of Scenedesmus obliquus as a promising microalga for biodiesel production. See comment in PubMed Commons below World J Microbiol Biotechnol 29: 915-922.

15. Xu J1, Hu H (2013) Screening high oleaginous Chlorella strains from different climate zones. See comment in PubMed Commons below Bioresour Technol 144: 637-643.

16. Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: A review. Renewable & Sustainable Energy Reviews 14: 217-232.

17. Ebenezer V1, Medlin LK, Ki JS (2012) Molecular detection, quantification, and diversity evaluation of microalgae. See comment in PubMed Commons below Mar Biotechnol (NY) 14: 129-142.

18. Talebi AF, Mohtashami SK, Tabatabaei M, Tohidfar M, Bagheri A, et al. (2013) Fatty acids profiling: A selective criterion for screening microalgae strains for biodiesel production. Algal Research 2: 258-267.

19. Bogen C1, Klassen V, Wichmann J, La Russa M, Doebbe A, et al. (2013) Identification of Monoraphidium contortum as a promising species for liquid biofuel production. See comment in PubMed Commons below Bioresour Technol 133: 622-626.

20. Cabanelas ITD, Ruiz J, Arbib Z, Chinalia FA, Garrido-Perez C, et al. (2013) Comparing the use of different domestic wastewaters for coupling microalgal production and nutrient removal. Bioresource technology 131: 429-436.

21. Chen Y, Wang JF, Zhang W, Chen L, Gao LL, et al. (2013) Forced light/dark circulation operation of open pond for microalgae cultivation. Biomass & Bioenergy 56: 464-470.

22. Phukan MM, Chutia RS, Konwar BK, Kataki R (2011) Microalgae Chlorella as a potential bio-energy feedstock. Applied Energy 88: 3307-3312.

23. Pruvost J1, Van Vooren G, Cogne G, Legrand J (2009) Investigation of biomass and lipids production with Neochloris oleoabundans in photobioreactor. See comment in PubMed Commons below Bioresour Technol 100: 5988-5995.

24. Wang H1, Gao L, Chen L, Guo F, Liu T (2013) Integration process of biodiesel production from filamentous oleaginous microalgae Tribonema minus. See comment in PubMed Commons below Bioresour Technol 142: 39-44.

25. Doan TTY, Sivaloganathan B, Obbard JP (2011) Screening of marine microalgae for biodiesel feedstock. Biomass and Bioenergy 35: 2534-2544.

26. Chaichalerm S, Pokethitiyook P, Yuan W, Meetam M, Sritong K, et al. (2012) Culture of microalgal strains isolated from natural habitats in Thailand in various enriched media. Applied Energy 89: 296-302.

27. Valdés F, Catalá L, Hernández MR, García-Quesada JC, Marcilla A, et al. (2013) Thermogravimetry and Py-GC/MS techniques as fast qualitative methods for comparing the biochemical composition of Nannochloropsis oculata samples obtained under different culture conditions. See comment in PubMed Commons below Bioresour Technol 131: 86-93.

28. Cheng HX, Tian GM, Liu JZ (2013) Enhancement of biomass productivity and nutrients removal from pretreated piggery wastewater by mixotrophic cultivation of Desmodesmus sp CHX1. Desalination and Water Treatment 51: 7004-7011.

29. López-González D, Fernandez-Lopez M, Valverde JL, Sanchez-Silva L (2014) Kinetic analysis and thermal characterization of the microalgae combustion process by thermal analysis coupled to mass spectrometry. Applied Energy 114: 227-237.

30. Rizzo AM, Prussi M, Bettucci L, Libelli IM, Chiaramonti D (2013) Characterization of microalga Chlorella as a fuel and its thermogravimetric behavior. Applied Energy 102: 24-31.

31. Nakanishi A, Aikawa S, Ho SH, Chen CY, Chang JS, et al. (2014) Development of lipid productivities under different CO2 conditions of marine microalgae Chlamydomonas sp. JSC4. See comment in PubMed Commons below Bioresour Technol 152: 247-252.

32. Zhang YM, Chen H, He CL, Wang Q (2013) Nitrogen starvation induced oxidative stress in an oil-producing green alga Chlorella sorokiniana C3. See comment in PubMed Commons below PLoS One 8: e69225.

33. Zhou W, Min M, Li Y, Hu B, Ma X, et al. (2012) A hetero-photoautotrophic two-stage cultivation process to improve wastewater nutrient removal and enhance algal lipid accumulation. See comment in PubMed Commons below Bioresour Technol 110: 448-455.

34. Chen H, Lao YM, Jiang JG (2011) Effects of salinities on the gene expression of a (NAD+)-dependent glycerol-3-phosphate dehydrogenase in Dunaliella salina. See comment in PubMed Commons below Sci Total Environ 409: 1291-1297.

35. Bhatnagar A, Bhatnagar M, Chinnasamy S, Das KC (2010) Chlorella minutissima--a promising fuel alga for cultivation in municipal wastewaters. See comment in PubMed Commons below Appl Biochem Biotechnol 161: 523-536.

36. Lam MK, Lee KT (2012) Microalgae biofuels: A critical review of issues, problems and the way forward. See comment in PubMed Commons below Biotechnol Adv 30: 673-690.

37. Perez-Garcia O, Escalante FM, de-Bashan LE, Bashan Y (2011) Heterotrophic cultures of microalgae: metabolism and potential products. See comment in PubMed Commons below Water Res 45: 11-36.

38. Xiong W, Li X, Xiang J, Wu Q (2008) High-density fermentation of microalga Chlorella protothecoides in bioreactor for microbio-diesel production. See comment in PubMed Commons below Appl Microbiol Biotechnol 78: 29-36.

39. Cerón-García MC, Macías-Sánchez MD, Sánchez-Mirón A, García-Camacho F, Molina-Grima E, et al. (2013) A process for biodiesel production involving the heterotrophic fermentation of Chlorella protothecoides with glycerol as the carbon source. Applied Energy 103: 341-34.

40. Liu C-Z, Zheng S, Xu L, Wang F, Guo C (2013) Algal oil extraction from wet biomass of Botryococcus braunii by 1,2-dimethoxyethane. Applied Energy 102: 971-974.

41. Ogbonna JC, Ichige E, Tanaka H (2002) Regulating the ratio of photoautotrophic to heterotrophic metabolic activities in photoheterotrophic culture of Euglena gracilis and its application to a-tocopherol production. Biotechnology Letters 24: 953-958.

42. Chen G, Qu S, Wang Q, Bian F, Peng Z, et al. (2014) Transgenic expression of delta-6 and delta-15 fatty acid desaturases enhances omega-3 polyunsaturated fatty acid accumulation in Synechocystis sp. PCC6803. See comment in PubMed Commons below Biotechnol Biofuels 7: 32.

43. Davis R, Aden A, Pienkos PT (2011) Techno-economic analysis of autotrophic microalgae for fuel production. Applied Energy 88: 3524-3531.

44. Richardson JW, Johnson MD, Outlaw JL (2012) Economic comparison of open pond raceways to photo bio-reactors for profitable production of algae for transportation fuels in the Southwest. Algal Research-Biomass Biofuels and Bioproducts 1: 93-100.

45. Zheng Y, Li T, Yu X, Bates PD, Dong T, et al. (2013) High-density fed-batch culture of a thermotolerant microalga Chlorella sorokiniana for biofuel production. Applied Energy 108: 281-287.

46. Moazami N, Ashori A, Ranjbar R, Tangestani M, Eghtesadi R, et al. (2012) Large-scale biodiesel production using microalgae biomass of Nannochloropsis. Biomass & Bioenergy 39: 449-453.

47. Arbib Z, Ruiz J, Alvarez-Diaz P, Garrido-Perez C, Barragan J, Perales JA, et al. (2013) Long term outdoor operation of a tubular airlift pilot photobioreactor and a high rate algal pond as tertiary treatment of urban wastewater. Ecological Engineering 52: 143-153.

48. Wijffels RH, Barbosa MJ, Eppink MHM (2010) Microalgae for the production of bulk chemicals and biofuels. Biofuels Bioproducts & Biorefining-Biofpr 4: 287-295.

49. Zittelli G, Rodolfi L, Bassi N, Biondi N, Tredici M, et al. (2013) Photobioreactors for Microalgal Biofuel Production. In MA Borowitzka, NR Moheimani, eds, Algae for Biofuels and Energy, Vol 5. Springer Netherlands, pp 115-131.

50. Molina Grima E, Belarbi EH, Acién Fernández FG, Robles Medina A, Chisti Y, et al. (2003) Recovery of microalgal biomass and metabolites: process options and economics. See comment in PubMed Commons below Biotechnol Adv 20: 491-515.

51. Sharma KK, Garg S, Li Y, Malekizadeh A, Schenk PM (2013) Critical analysis of current microalgae dewatering techniques. Biofuels 4: 397-407.

52. Uduman N, Qi Y, Danquah MK, Forde GM, Hoadley A, et al. (2010) Dewatering of microalgal cultures: a major bottleneck to algae-based fuels. Journal of renewable and sustainable energy 2: 012701.

53. Brennan L, Owende P (2010) Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable & Sustainable Energy Reviews 14: 557-577 .

54. Lee YC, Kim B, Farooq W, Chung J, Han JI, et al. (2013) Harvesting of oleaginous Chlorella sp. by organoclays. See comment in PubMed Commons below Bioresour Technol 132: 440-445.

55. Oh HM, Lee SJ, Park MH, Kim HS, Kim HC, et al. (2001) Harvesting of Chlorella vulgaris using a bioflocculant from Paenibacillus sp AM49. Biotechnology Letters 23: 1229-1234.

56. Vandamme D, Foubert I, Meesschaert B, Muylaert K (2010) Flocculation of microalgae using cationic starch. Journal of Applied Phycology 22: 525-530.

57. Beuckels A, Depraetere O, Vandamme D, Foubert I, Smolders E, et al. (2013) Influence of organic matter on flocculation of Chlorella vulgaris by calcium phosphate precipitation. Biomass & Bioenergy 54: 107-114.

58. Harith ZT, Yusoff FM, Mohamed MS, Din MSM, Ariff AB, et al. (2009) Effect of different flocculants on the flocculation performance of microalgae, Chaetoceros calcitrans, cells. African Journal of Biotechnology 8: 5971-5978.

59. Kim J, Ryu BG, Kim K, Kim BK, Han JI, et al. (2012) Continuous microalgae recovery using electrolysis: effect of different electrode pairs and timing of polarity exchange. See comment in PubMed Commons below Bioresour Technol 123: 164-170.

60. Zhou WG, Li YC, Min M, Hu B, Chen P, et al. (2011) Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresource Technology 102: 6909-6919.

61. Zhu L, Wang Z, Shu Q, Takala J, Hiltunen E, et al. (2013) Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment. See comment in PubMed Commons below Water Res 47: 4294-4302.

62. Pires JCM, Alvim-Ferraz MCM, Martins FG, Simoes M (2012) Carbon dioxide capture from flue gases using microalgae: Engineering aspects and biorefinery concept. Renewable & Sustainable Energy Reviews 16: 3043-3053.

63. Yan C, Zheng Z (2014) Performance of mixed LED light wavelengths on biogas upgrade and biogas fluid removal by microalga Chlorella sp. Applied Energy 113: 1008-1014 .

64. Kumar A, Ergas S, Yuan X, Sahu A, Zhang Q, et al. (2010) Enhanced CO(2) fixation and biofuel production via microalgae: recent developments and future directions. See comment in PubMed Commons below Trends Biotechnol 28: 371-380.

65. Richmond A (2008) Handbook of microalgal culture: biotechnology and applied phycology. In. John Wiley & Sons.

66. Saga K, Hasegawa F, Miyagi S, Atobe S, Okada S, et al. (2015) Comparative evaluation of wet and dry processes for recovering hydrocarbon from Botryococcus Braunii. Applied Energy 141: 90-95.

67. Peralta-Ruiz Y, González-Delgado AD, Kafarov V (2013) Evaluation of alternatives for microalgae oil extraction based on exergy analysis. Applied Energy 101: 226-236.

68. Demirbas A (2008) Production of biodiesel from algae oils. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects 31: 163-168.

69. Kanda H, Li P, Yoshimura T, Okada S (2013) Wet extraction of hydrocarbons from Botryococcus braunii by dimethyl ether as compared with dry extraction by hexane. Fuel 105: 535-539.

70. Kanda H, Li P, Ikehara T, Yasumoto-Hirose M (2012) Lipids extracted from several species of natural blue-green microalgae by dimethyl ether: Extraction yield and properties. Fuel 95: 88-92.

71. Liu A, Zhu T, Lu X, Song L (2013) Hydrocarbon profiles and phylogenetic analyses of diversified cyanobacterial species. Applied Energy 111: 383-393.

72. Mendes RL, Fernandes HL, Coelho JA, Cabral JM, Palavra AM, et al. (1994) Supercritical carbon dioxide extraction of hydrocarbons from the microalgaBotryococcus braunii. Journal of Applied Phycology 6: 289-293.

73. Andrich G, Zinnai A, Nesti U, Venturi F (2006) Supercritical fluid extraction of oil from microalga Spirulina (Arthrospira) platensis. Acta Alimentaria 35: 195-203.

74. Gouveia L, Nobre B, Marcelo F, Mrejen S, Cardoso M, et al. (2007) Functional food oil coloured by pigments extracted from microalgae with supercritical CO< sub> 2. Food Chemistry 101: 717-723.

75. Tang S, Qin C, Wang H, Li S, Tian S (2011) Study on supercritical extraction of lipids and enrichment of DHA from oil-rich microalgae. The Journal of Supercritical Fluids 57: 44-49.

76. Mouahid A, Crampon C, Toudji SAA, Badens E (2013) Supercritical CO2 extraction of neutral lipids from microalgae: Experiments and modelling. Journal of Supercritical Fluids 77: 7-16.

77. Lam MK, Lee KT, Mohamed AR (2010) Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: a review. See comment in PubMed Commons below Biotechnol Adv 28: 500-518.

78. Meher L, Vidya Sagar D, Naik S (2006) Technical aspects of biodiesel production by transesterification-a review. Renewable and Sustainable Energy Reviews 10: 248-268.

79. Hidalgo P, Toro C, Ciudad G, Navia R (2013) Advances in direct transesterification of microalgal biomass for biodiesel production. Reviews in Environmental Science and Bio-Technology 12: 179-199.

80. Velasquez-Orta SB, Lee JGM, Harvey AP (2013) Evaluation of FAME production from wet marine and freshwater microalgae by in situ transesterification. Biochemical Engineering Journal 76: 83-89.

81. Canakci M1 (2007) The potential of restaurant waste lipids as biodiesel feedstocks. See comment in PubMed Commons below Bioresour Technol 98: 183-190.

82. Francisco EC, Neves DB, Jacob-Lopes E, Franco TT (2010) Microalgae as feedstock for biodiesel production: carbon dioxide sequestration, lipid production and biofuel quality. Journal of Chemical Technology and Biotechnology 85: 395-403.

83. Sun X, Wang C, Li Z, Wang W, Tong Y, et al. (2013) Microalgal cultivation in wastewater from the fermentation effluent in Riboflavin (B2) manufacturing for biodiesel production. See comment in PubMed Commons below Bioresour Technol 143: 499-504.

84. Liu X, He H, Wang Y, Zhu S (2007) Transesterification of soybean oil to biodiesel using SrO as a solid base catalyst. Catalysis Communications 8: 1107-1111.

85. Lam MK, Lee KT (2012) Potential of using organic fertilizer to cultivate Chlorella vulgaris for biodiesel production. Applied Energy 94: 303-308.

86. Arias-Peñaranda MT, Cristiani-Urbina E, Montes-Horcasitas C, Esparza-García F, Torzillo G, et al. (2013) Scenedesmus incrassatulus CLHE-Si01: a potential source of renewable lipid for high quality biodiesel production. See comment in PubMed Commons below Bioresour Technol 140: 158-164.

87. Hernández-Martín E, Otero C (2008) Different enzyme requirements for the synthesis of biodiesel: Novozym 435 and Lipozyme TL IM. See comment in PubMed Commons below Bioresour Technol 99: 277-286.

88. Talukder MMR, Wu JC, Van Nguyen TB, Fen NM, Melissa YLS, et al. (2009) Novozym 435 for production of biodiesel from unrefined palm oil: Comparison of methanolysis methods. Journal of Molecular Catalysis B-Enzymatic 60: 106-112.

89. Sathish A, Smith BR, Sims RC (2014) Effect of moisture on in situ transesterification of microalgae for biodiesel production. Journal of Chemical Technology and Biotechnology 89: 137-142.

90. Azadi P, Brownbridge G, Mosbach S, Smallbone A, Bhave A, et al. (2014) The carbon footprint and non-renewable energy demand of algae-derived biodiesel. Applied Energy 113: 1632-1644.

91. Dang-Thuan T, Chen CL, Chang JS (2013) Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst. Bioresource Technology 135: 213-221.

92. Carioca JO, Hiluy Filho JJ, Leal MR, Macambira FS (2009) The hard choice for alternative biofuels to diesel in Brazil. See comment in PubMed Commons below Biotechnol Adv 27: 1043-1050.

93. Ahmad AL, Yasin NHM, Derek CJC, Lim JK (2011) Microalgae as a sustainable energy source for biodiesel production: A review. Renewable & Sustainable Energy Reviews 15: 584-593.

94. Jonker JGG, Faaij APC (2013) Techno-economic assessment of micro-algae as feedstock for renewable bio-energy production. Applied Energy 102: 461-475.

95. Holbrook GP, Davidson Z, Tatara RA, Ziemer NL, Rosentrater KA, et al. (2014) Use of the microalga Monoraphidium sp. grown in wastewater as a feedstock for biodiesel: Cultivation and fuel characteristics. Applied Energy 131: 386-393.

96. Anandarajah K, Mahendraperumal G, Sommerfeld M, Hu Q (2012) Characterization of microalga Nannochloropsis sp. mutants for improved production of biofuels. Applied Energy 96: 371-377.

97. Iwai M, Ikeda K, Shimojima M, Ohta H (2014) Enhancement of extraplastidic oil synthesis in Chlamydomonas reinhardtii using a type-2 diacylglycerol acyltransferase with a phosphorus starvation-inducible promoter. See comment in PubMed Commons below Plant Biotechnol J 12: 808-819.

98. Park JB1, Craggs RJ, Shilton AN (2011) Wastewater treatment high rate algal ponds for biofuel production. See comment in PubMed Commons below Bioresour Technol 102: 35-42.

99. Rawat I, Ranjith Kumar R, Mutanda T, Bux F (2013) Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. Applied Energy 103: 444-467.

100. Ogbonda KH, Aminigo RE, Abu GO (2007) Influence of temperature and pH on biomass production and protein biosynthesis in a putative Spirulina sp. See comment in PubMed Commons below Bioresour Technol 98: 2207-2211.

101. Chen M, Liu T, Chen X, Chen L, Zhang W, et al. (2012) Subcritical co-solvents extraction of lipid from wet microalgae pastes of Nannochloropsis sp. See comment in PubMed Commons below Eur J Lipid Sci Technol 114: 205-212.

102. Vasudevan PT1, Briggs M (2008) Biodiesel production--current state of the art and challenges. See comment in PubMed Commons below J Ind Microbiol Biotechnol 35: 421-430.

103. Chen G, Zhao L, Qi Y (2015) Enhancing the productivity of microalgae cultivated in wastewater toward biofuel production: A critical review. Applied Energy 137: 282-291.

104. Ketheesan B, Nirmalakhandan N (2011) Development of a new airlift-driven raceway reactor for algal cultivation. Applied Energy 88: 3370-3376.

105. Markou G, Georgakakis D (2011) Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: A review. Applied Energy 88: 3389-3401.

106. Jiang LL, Luo SJ, Fan XL, Yang ZM, Guo RB, et al. (2011) Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO2. Applied Energy 88: 3336-3341.

107. Zhou Y, Schideman L, Yu G, Zhang Y (2013) A synergistic combination of algal wastewater treatment and hydrothermal biofuel production maximized by nutrient and carbon recycling. Energy & Environmental Science 6: 3765-3779.

108. Abu-Khader MM (2006) Recent progress in CO2 capture/sequestration: a review. Energy Sources, Part A 28: 1261-1279.

109. Soletto D, Binaghi L, Ferrari L, Lodi A, Carvalho J, et al. (2008) Effects of carbon dioxide feeding rate and light intensity on the fed-batch pulse-feeding cultivation of< i> Spirulina platensis in helical photobioreactor. Biochemical Engineering Journal 39: 369-375.

110. Schipper K, van der Gijp S, van der Stel R, Goetheer E (2013) New Methodologies for the Integration of Power Plants with Algae Ponds. Energy Procedia 37: 6687-669.

111. Chai X, Zhao X (2012) Enhanced removal of carbon dioxide and alleviation of dissolved oxygen accumulation in photobioreactor with bubble tank. See comment in PubMed Commons below Bioresour Technol 116: 360-365.

112. Iancu P, Plesu V, Velea S (2012) Flue Gas CO2 Capture by Microalgae in Photobioreactor: a Sustainable Technology. In PS Varbanov, HL Lam, JJ Klemes, S Pierucci, JJ Klemes, eds, Pres 2012: 15th International Conference on Process Integration, Modelling and Optimisation for Energy Saving and Pollution Reduction, Vol 29. Aidic Servizi Srl, Milano, pp 799-804.

113. Hsueh HT, Chu H, Yu ST (2007) A batch study on the bio-fixation of carbon dioxide in the absorbed solution from a chemical wet scrubber by hot spring and marine algae. See comment in PubMed Commons below Chemosphere 66: 878-886.

114. Van Den Hende S, Vervaeren H, Boon N (2012) Flue gas compounds and microalgae: (bio-)chemical interactions leading to biotechnological opportunities. See comment in PubMed Commons below Biotechnol Adv 30: 1405-1424.

115. Arata S, Strazza C, Lodi A, Del Borghi A (2013) Spirulina platensis Culture with Flue Gas Feeding as a Cyanobacteria-Based Carbon Sequestration Option. Chemical Engineering & Technology 36: 91-97.

116. Jin YM, Veiga MC, Kennes C (2005) Bioprocesses for the removal of nitrogen oxides from polluted air. Journal of Chemical Technology and Biotechnology 80: 483-494.

117. Nagase H, Yoshihara K, Eguchi K, Okamoto Y, Murasaki S (2001) Uptake pathway and continuous removal of nitric oxide from flue gas using microalgae. Biochemical Engineering Journal 7: 241-246.

118. Jin H-F, Santiago DEO, Park J, Lee K (2008) Enhancement of nitric oxide solubility using Fe(II)EDTA and its removal by green algae Scenedesmus sp. Biotechnology and Bioprocess Engineering 13: 48-52.

119. Santiago DE, Jin H-F, Lee K (2010) The influence of ferrous-complexed EDTA as a solubilization agent and its auto-regeneration on the removal of nitric oxide gas through the culture of green alga< i> Scenedesmus sp. Process Biochemistry 45: 1949-1953.

120. Lee JH, Lee JS, Shin CS, Park SC, Kim SW, et al. (2000) Effects of NO and SO2 on growth of highly-CO2,-tolerant microalgae. Journal of Microbiology and Biotechnology 10: 338-343.

121. de-Bashan LE, Bashan Y (2010) Immobilized microalgae for removing pollutants: review of practical aspects. See comment in PubMed Commons below Bioresour Technol 101: 1611-1627.

122. Gómez C1, Escudero R, Morales MM, Figueroa FL, Fernández-Sevilla JM, et al. (2013) Use of secondary-treated wastewater for the production of Muriellopsis sp. See comment in PubMed Commons below Appl Microbiol Biotechnol 97: 2239-2249.

123. Nascimento IA, Marques SSI, Cabanelas ITD, Pereira SA, Druzian JI, de Souza CO (2013) Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria. Bioenergy Research 6: 1-13.

124. Gonzales L, Canizares R, Baena S (1997) Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae Chlorealla vulgaris and Scenedesmus dimorphus. Bioresource technology 60: 259-262.

125. Cai T, Park SY, Li Y (2013) Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renewable and Sustainable Energy Reviews 19: 360-369.

126. Slegers PM, van Beveren PJM, Wijffels RH, van Straten G, van Boxtel AJB, et al. (2013) Scenario analysis of large scale algae production in tubular photobioreactors. Applied Energy 105: 395-406.

127. Olofsson M, Lamela T, Nilsson E, Berge JP, del Pino V, et al. (2012) Seasonal Variation of Lipids and Fatty Acids of the Microalgae Nannochloropsis oculata Grown in Outdoor Large-Scale Photobioreactors. Energies 5: 1577-1592.

128. Zhu L, Hiltunen E, Shu Q, Zhou W, Li Z, et al. (2014) Biodiesel production from algae cultivated in winter with artificial wastewater through pH regulation by acetic acid. Applied Energy 128: 103-110.

129. Frigon J-C, Matteau-Lebrun F, Hamani Abdou R, McGinn PJ, O'Leary SJB, Guiot SR, et al. (2013) Screening microalgae strains for their productivity in methane following anaerobic digestion. Applied Energy 108: 100-107.

130. Zhang Y, Fan X, Yang Z, Wang H, Yang D, et al. (2012) Characterization of H2 photoproduction by a new marine green alga, Platymonas helgolandica var. tsingtaoensis. Applied Energy 92: 38-43.

131. Martín M, Grossmann IE (2014) Design of an optimal process for enhanced production of bioethanol and biodiesel from algae oil via glycerol fermentation. Applied Energy 135: 108-114.

132. Prajapati SK, Malik A, Vijay VK (2014) Comparative evaluation of biomass production and bioenergy generation potential of Chlorella spp. through anaerobic digestion. Applied Energy 114: 790-797.

133. Wieczorek N, Kucuker MA, Kuchta K (2014) Fermentative hydrogen and methane production from microalgal biomass (Chlorella vulgaris) in a two-stage combined process. Applied Energy 132: 108-117.

134. Williams PJIB, Laurens LML (2010) Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. Energy & Environmental Science 3: 554-590.

135. McMillan JR, Watson IA, Ali M, Jaafar W (2013) Evaluation and comparison of algal cell disruption methods: Microwave, waterbath, blender, ultrasonic and laser treatment. Applied Energy 103: 128-134.

136. Hauvermale A, Kuner J, Rosenzweig B, Guerra D, Diltz S, et al. (2006) Fatty acid production in Schizochytrium sp.: Involvement of a polyunsaturated fatty acid synthase and a type I fatty acid synthase. See comment in PubMed Commons below Lipids 41: 739-747.

Mass Cultivation of Phototrophic Microalgae

Peter E Zemke*

Utah State University, 4130 Old Main Hill, Logan, UT 84322-4130, USA

***Corresponding author:** Peter E. Zemke, Utah State University, 4130 Old Main Hill, Logan, UT 84322-4130, USA, Tel: 4352327708; E-mail: pezemke@gmail.com

Abstract

Cultivation systems for outdoor phototrophic cultivation of microalgae vary significantly in design, but they all provide the same environmental parameters microalgae require for growth. This chapter discusses how current and developing cultivation system concepts manage these environmental parameters, the engineering concepts behind cultivation system technologies, the current challenges to mass cultivation, and future technological opportunities.

Introduction

Microalgae and cyanobacteria can both be cultivated phototrophically outdoors. For convenience, “algae” will be used to refer to both microalgae and cyanobacteria in this chapter. Mass cultivation of algae outdoors is a viable biotechnology for many products and services, including wastewater treatment, high-value products such as carotenoids and essential fatty acids, and possibly biologicals [1] and biofuel feedstocks.

The cultivation systems for producing algae outdoors are numerous. The complexity of cultivation systems range from shallow unmixed ponds to precisely controlled optically-lit photobioreactors. Yet the purpose of all cultivation system designs is to provide environmental conditions that are necessary for photosynthetic growth. Consequently many of the same engineering fundamentals apply to all cultivation methods.

For the successful cultivation of algae, five general environmental parameters must be provided: containment, light, mixing, temperature control, and gas exchange. This chapter will briefly describe the most common cultivation systems currently in use, followed by a discussion of the various methods that are currently used to manage environmental parameters, and the engineering fundamentals for designing, selecting, and evaluating the performance of, cultivation systems.

Common Types of Cultivation Systems

There is an enormous variety of cultivation system configurations, and none are considered superior to another in every respect. Cultivation systems for algae are relatively new and are expected to continue to evolve toward more efficient and economical technologies. However, the majority of current cultivation system technologies can be categorized into three general types: open raceway ponds, vertical aerated photobioreactors, and tubular photobioreactors.

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

The configurations discussed herein are only examples, and do not imply the best or only possible configurations.

Open Raceway Ponds

Open raceway ponds, or high-rate algae ponds, are the simplest and most common type of cultivation system. They consist of a raceway typically 15-30 cm deep in which the media is circulated at a rate of about 20-30 cm/s, most often with a paddlewheel, although other means such as Archimedes screw pumps and air-lift designs have been employed as well [2]. Raceway ponds are popular because they are scalable, but have characteristically low volumetric biomass concentrations, and are prone to culture instability, pathogens, and predators. Algal species cultivated in open raceway ponds are typically extremophiles that favor high pH, salinity, or nutrient content to discourage competing organisms.

Vertical Aerated Photobioreactors

Vertical aerated photobioreactors are characterized by vertical or near-vertical transparent containers in which the media is circulated by air sparging. Vertical plate photobioreactors and (annular) column photobioreactors are two examples of this type of cultivation system. The optical path length (ratio of volume to illuminated surface area, see Section 4.1.2) is typically 10 cm or less, resulting in higher volumetric cell concentrations than open raceway ponds. Aeration is usually provided solely from the bottom of the photobioreactor. Aeration rates are measured in flow rate of air per volume of media and are often in the range of 0.3 to 1 L/L-min. Higher productivities have been demonstrated for very small optical path lengths (~1 cm) and very high aeration rates (~5 L/L-min) [3,4], but the high energy and maintenance required at these aeration rates are not practical for outdoor production. Higher productivities than open raceway ponds are common in vertical aerated photobioreactors [5], and since the media is enclosed in a container, contamination and culture stability are easier to regulate. However, these cultivation systems are capital-, energy-, and maintenance-intensive, which limit the scalability of these technologies.

Tubular Photobioreactors

Tubular photobioreactors consist of long runs of transparent tubes through which the media is pumped. Tube diameters are typically 5 to 8 cm, and the fluid is pumped at a velocity of 50 cm/s to maintain turbulence in the tubes. The most common tubular photobioreactors consist of long runs of rigid tubes laid horizontally or stacked in vertical rows, through which media is pumped from and to a central area. Large-scale tubular photobioreactors are of this configuration and have been employed for commercial production of high-value products. Other types of tubular photobioreactors have been demonstrated that consist of flexible tubing wrapped around a large cylinder [6,7], or thin-film tubes that float on large bodies of water [8]. The advantages and disadvantages of tubular photobioreactors are similar to vertical aerated photobioreactors: stable, rapid productivity and reduced contamination, but at high capital, energy, and maintenance costs.

Environmental Parameters

The function of a cultivation system is to create an ideal environment for algae to grow. The elements of the algal environment can be grouped into five general categories: containment, light, mixing, gas exchange, and temperature. Each of these parameters is discussed below.

Containment

This parameter is the most obvious of the parameters required for algal cultivation, but the requirement is not trivial as it has large implications on the design of cultivation systems.

Mass cultivation of algae is completed in an aqueous environment through which algae receives all of their nutrients and sunlight. The use of an aqueous media requires that the cultivation system provide a means of containment of the media. Open raceway ponds commonly consist of an excavated raceway that has been lined with a polymer film, with more costly raceway ponds consisting of rigid-walls constructed of fiberglass, concrete, or plastic. Photobioreactors utilize a variety of clear materials, including soft and hard plastics, and glass.

The means of containing the media is usually the most costly component of a cultivation system. A thin-film liner for open raceway ponds is expected to cost approximately 5 USD/m^2, which even with a presently optimistic production rate of 25-g/m^2-d and 25% lipid content, is the single largest economic barrier to biofuels feedstock production from algae [9]. For this reason, impermeable earthen materials (i.e. clay) have been proposed to line ponds. However, the impact of earthen liners on productivity, their durability and longevity, and permeability, are unclear.

The materials used to construct photobioreactors are significantly more expensive than raceway pond materials for the same growth area. This is primarily due to a combination of the hydrostatic pressure that the materials must resist and their relatively weak tensile strengths. Transparent thin-film polymers are becoming a popular means of reducing cost, but steel or some other rigid material is required to maintain the shape of the photobioreactor [10].

Light

Light is the requirement that makes phototrophic production of algae unique from other micro-biotechnologies. In heterotrophic processes, the energy source is provided in the form of organic carbon substrates. The volumetric concentration of the substrate is a variable that can be controlled (in most cases) for optimum production and stored in the media until the organisms consume them. In outdoor phototrophic cultivation, the energy source is sunlight. As a result, the areal energy concentration (energy per illuminated surface area of the cultivation system) is determined by the local weather, time, and season, and the energy entering the algal culture must be converted to electro-chemical energy almost instantaneously. There are three major implications arising from this difference.

Biomass Production is Proportional to Surface Area rather Than Volume

In heterotrophic cultivation, bioreactors most commonly take the form of large tanks. Biomass production is increased by increasing the media volume, and/or increasing the concentration of the substrate, if feasible. Since outdoor algae cultivation is limited by the quantity of sunlight, biomass production can only be increased by increasing the surface area that intercepts the sunlight. If light is the limiting substrate, the volume of media per area is of little to no consequence to the biomass productivity [11]. For this reason, algae cultivation systems are characterized by having very large surface area to volume ratios, hence why most photobioreactors are constructed to form thin tanks or tubes, and open raceway pond depths are generally limited to 20 to 30 cm. This has major implications to the economy of scale of outdoor cultivation of algae. For heterotrophic production, the biomass production of a bioreactor can be doubled by doubling the volume of the bioreactor. Since the surface area (material) increases as square of the characteristic length while volume increases as a cube of the characteristic length (square-cube law), the capital cost of doubling production then is roughly $2^{2/3}$, or 59% more. However, to double the productivity of a phototrophic cultivation system, double the cultivation surface area (material) is required, resulting in nearly a 100% increase in capital costs. This is a major challenge to reducing the cost of algae production. While phototrophic cultivation may have the advantage over heterotrophic cultivation of using a “free” energy source, the capital investment required to capture this energy is substantial.

The Rate of Growth Is Linear

Because of the capital investment required to capture sunlight, high rates of utilization are necessary to maximize the return on investment. Phototrophic cultivation systems therefore usually contain a sufficient photosynthetic pigment concentration to absorb ~100% of the photons the cultivation system intercepts, and environmental conditions are created such that light is the limiting substrate. Given these conditions, growth is limited by the quantity of photons received, consequently the biomass concentration increases linearly with time. For a thorough discussion of the biokinetics of growth in phototrophic cultures, see Kim et al. [12]. This differs from heterotrophic production in which growth in batch processes is exponential.

The Energy Efficiency of Phototrophic Systems Is Limited

In heterotrophic fermenters, the substrate concentrations are generally optimized to maximize production. In phototrophic cultivation systems, the sunlight intensity is an uncontrolled parameter. The optimum light intensity for algae is 200 μmol photons m^{-2} or less, while sunlight at the Earth's surface can exceed 1800 μmol m^2-s [13]. Prolonged exposure to direct sunlight is destructive to the pigments and photosynthetic machinery, which results in retarded growth or loss of the culture altogether [14,15]. Maintaining high pigment concentrations in the culture prevents this since all but the top-most layer is protected from the most intense sunlight [15].

To improve photosynthetic efficiency, phototrophic cultivation systems also utilize some method of light dilution, either spatial or temporal. Spatial light dilution is achieved by diffusing the sunlight to a larger surface area through optics or convoluted surfaces. This approach has demonstrated substantial improvements in photosynthetic efficiency [6,16], but at a significant and uneconomical increase in capital cost. The reason for this has to do with a low return on capital investment, which can be demonstrated as follows. A conservative estimate of the fraction of photons that can be utilized by a light-limited culture can be calculated using the Bush Equation [17]:

$$u_p = \frac{I_s}{I_o}\left[\ln\left(\frac{I_o}{I_s}\right)+1\right] \quad (1)$$

Where I_s is the saturating light intensity of the algae, and I_o is the incident light intensity. Assuming I_o/I_s is 10, then the maximum light utilization fraction is u_p = 0.33. In order to obtain a light utilization of u_p = 1, the surface area must be increased by a factor of 10 to make $I_o \approx I_s$; however, the utilization (and corresponding increase in productivity) only increases by a factor of 3. Recalling that the primary capital expense of a cultivation system is found in its surface area, the economics of convoluting the surface area to improve light utilization must be taken into account when considering spatial dilution.

The alternative to spatial light dilution is temporal light dilution, in which each algal cell is exposed to intense light for a duration on the order of milliseconds, and then concealed from the light for a sufficient time (on the order of tens of milliseconds) to allow the slower "dark" photosynthetic reactions to be completed before the cell is exposed to light again. Practically, this is accomplished through mixing, which will be discussed in detail in the following section.

Mixing

Mixing serves numerous requirements in cultivation systems. Algal cells are heavier than water and without mixing will settle to the bottom, although there are a few exceptions such as *Dunaliella* sp [18]. Insufficient mixing levels or areas in cultivation systems with poor

mixing (dead zones) often result in the formation of algal biofilms. Biofilms are undesirable in suspended-cell cultivation systems because cells in the biofilm use light less efficiently than suspended algae and their formation on illuminated surfaces blocks light from the suspended algae.

The obvious exception to the mixing requirement is cultivation systems in which a biofilm is desirable. Since mixing reduces the boundary layer around the cells to enhance nutrient transfer, reducing the boundary layer through turbulence is still desirable in biofilm cultivation systems, but not to the point of shearing the biofilm from the substrate.

Mixing serves a critical role for increasing photosynthetic efficiency. The photosynthetic pigments of algae have very high absorptivities. In a cultivation system with a typical biomass concentration (50 g/m^2 of illuminated area), the entirety of direct sunlight is absorbed within the first 10% of the culture, resulting in 90% of the culture deprived of light while the other 10% receives too much (Figure 1). Mixing of the culture cycles algal cells from the dark region to the lit region of the culture (photic zone) and back. For an efficiently mixed cultivation system, this results in a time-averaged light intensity that can be utilized by the algae much more efficiently than constant exposure to direct sunlight.

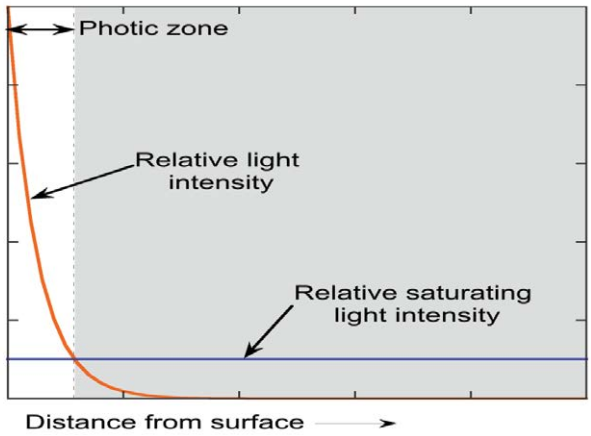

Figure 1: Typical light intensity profile in an algal culture.

Ideally, the ratio (φ) of light exposure duration (t_l) to the total light-dark cycle time (t_l + t_d) should be equal to the ratio of the algae's saturation light intensity to the incident light intensity:

$$\phi = t_l f = \frac{t_l}{t_l + t_d} \propto \frac{I_s}{I_o} \tag{2}$$

In addition, the frequency of the light-dark cycle, f, must be sufficiently high to prevent saturation of the photosynthetic reaction centers. This frequency is on the order of 10 Hz [19,20]. In randomly mixed cultivation systems φ and f vary significantly from cell to cell and with time. However, this variation is evidently not significant since the maximum photosynthetic efficiency can be demonstrated at light intensities up to 2000 µmol/m^2-s with optimized mixing [4]. Eq. 1 can be modified to account for mixed cultures as follows:

$$u_p = \frac{\gamma(\phi, f) I_s}{\phi I_o}\left[\ln\left(\frac{\phi I_o}{\gamma(\phi, f) I_s}\right) + 1\right] \tag{3}$$

Where γ is a function that quantifies the effectiveness of the mixing based on φ and f, similar to Terry's "integration function", Γ [20]. It is important to note that the value of γ is never more than 1, signifying that mixing does not increase the maximum rate of photosynthesis of the algae.

Temperature

Most algae of commercial interest are mesophilic, with survivable temperature ranges from freezing to 45°C and the optimal temperatures generally being in the range of 20 to 30°C. Outdoor cultivation systems are subject to extreme temperature and insolation variations, and must be capable of regulating the media temperature minimally within survivable temperature limits, and ideally within the optimal temperature range.

(Figure 2) demonstrates the flow of energy through a typical cultivation system. Insolation is the primary source of heating in cultivation systems. Algal cultures absorb practically 100% of the full spectrum of incident solar energy, but less than 5% (usually much less) of it is converted into biochemical energy [21]. The remaining energy becomes heat that must be expelled. Thermal convection alone to the outside air is insufficient, and photobioreactors can exceed 50°C on a sunny day even though the ambient air temperature never exceeds freezing. There are three primary ways that temperature is controlled in cultivation systems.

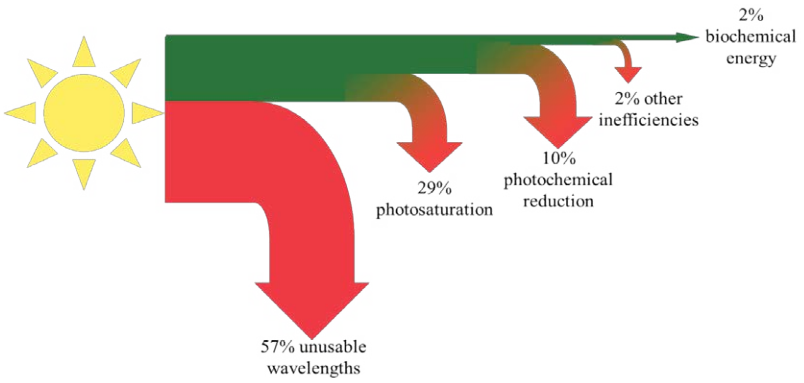

Figure 2: The fate of solar energy in an algal culture. Most of the energy contributes to the cultivation systems heat load while only a small fraction is converted to biochemical energy.

Thermal Mass

Thetemperature increase, ΔT, of a culture is inversely proportional to the mass of water in the culture:

$$\frac{dT}{d\tau} = \frac{\dot{E}_{net}}{\rho C_p d} \tag{4}$$

Where is the net areal thermal energy input (W/m^2) into the culture, ρ is the density of water, C_p is the specific heat of water (4.184 kJ/kg-K), and d is the optical path length of the cultivation system. If the optical path length is sufficient, the culture will not heat beyond the survivable temperature of the culture, and the stored heat can be dissipated by one of the other two temperature control methods discussed below. This strategy is common in nearly all open raceway ponds and many photobioreactor systems. While a higher volumetric cell density could be observed by maintaining an open raceway pond at a depth of 10 cm or less, typical depths are 15 to 20 cm, or more, to provide sufficient thermal mass to avoid overheating the culture.

Evaporative Cooling

While the thermal mass of an open raceway pond slows the rate of temperature increase of the culture, the heat must ultimately be rejected to the environment. For open raceway ponds, this occurs primarily through evaporative cooling. The large surface areas of open raceway ponds permit large quantities of water to evaporate into the adjacent air, taking thermal energy with it and reducing the temperature of the pond. The evaporation of 1 L of water takes with it 0.63 kWh of heat.

In photobioreactors, the air-water surface area is much smaller and therefore evaporative losses (and evaporative cooling) are much smaller. Evaporative cooling of photobioreactors can still be achieved by spraying water onto the surfaces of the photobioreactors. This is effective on thin-film or glass photobioreactors, but not generally sufficient for acrylic or polycarbonate photobioreactors because their thermal conductivities (20% that of glass) are too low to allow adequate heat transfer to the cooling water. Alternatively, the photobioreactors can be submerged in a pool that provides the thermal mass and evaporative cooling. This approach has been demonstrated by Solix Biosystem's cultivation system [22].

Internal cooling

Photobioreactors characteristically have very low volume to surface area ratios and therefore low thermal masses. Where a means of external heat transfer is not practical, heat is transferred from the media to a cooling fluid via a heat exchanger. The heat exchanger may be as simple as a corrosion-resistant tube submerged in the photobioreactor through which cooling fluid is pumped (Figure 3).

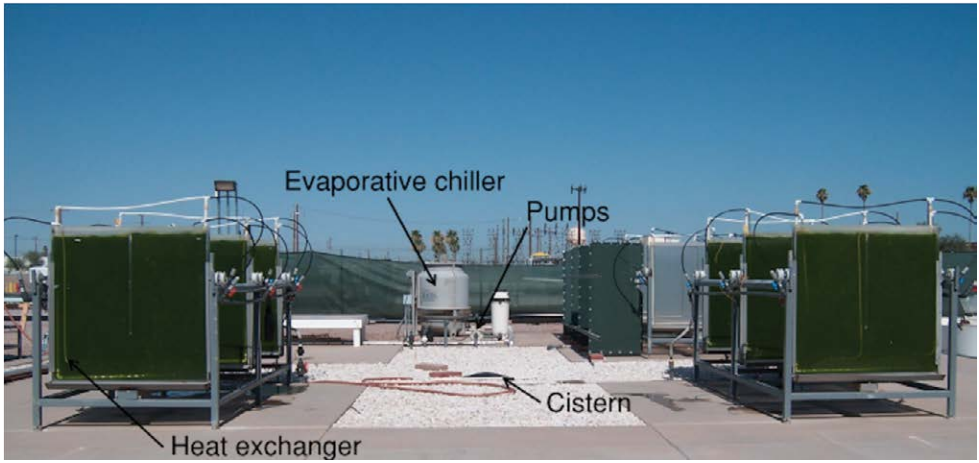

Figure 3: Temperature control system for vertical plate reactors. Cooling water is pumped through stainless steel tubes in the reactors from and to an underground cistern. An evaporative chiller expels heat from the cistern.

A large reservoir of cooling fluid provides the thermal mass that the photobioreactors lack. Depending on the location and season, this alone may be sufficient to regulate the temperature since the large surface areas of the photobioreactors can dissipate the heat to the surrounding air at night. However, an evaporative or vapor-compression chiller can be used to remove heat from the reservoir, if necessary.

In aerated photobioreactors, the heat transfer between the media and the air is negligible for mesophilic conditions. Specifically, injecting hot blower air or flue gas contributes little to the photobioreactor's thermal load.

The removal of heat from cultivation systems is a major challenge to outdoor

algae production. Because of the variations in thermal loads, it is difficult to maintain temperatures in their ideal range in open raceway ponds, resulting in poor productivity [23]. The evaporative water loss in open raceway ponds can be substantial [24], with as much as 25% of the water in a pond being lost to evaporation in arid regions, and 10% being typical. Assuming an evaporation rate of 10% per day and 25 g/m^2-d, the production of 1 kg of biomass requires 1000 L of makeup water. In photobioreactors, comparable quantities of water are required for evaporative chillers, or large amounts of energy for vapor-compression chillers. However, refrigeration chillers have the advantage of upgrading the heat to a higher temperature that may be more useful for process heat, although the economics of this have not been documented.

As a result, varieties of new approaches are being, or have been, developed to optimize temperatures and reduce cooling requirements. Pushparaj et al. [25] demonstrated that by circulating media between a photobioreactor and an open raceway pond, the temperatures of the two cultivation systems were more optimally maintained, resulting in better growth rates for *Spirulina sp.* Integrating the two cultivation systems reduces complexity and cost – i.e. the productivity of the open raceway pond is increased due to optimal temperatures, and the cost of the photobioreactor is reduced because the heat is sent to the open raceway pond rather than a reservoir and chiller.

Photosynthesis is only driven by photons within the wavelength range of 400-700 nm, which accounts of less than 50% of the sun's energy on the Earth's surface, but cultures absorb nearly 100% of the incident solar energy spectrum. The thermal load on the photobioreactors can therefore be reduced by as much as 50% by applying a spectrally selective material to the photobioreactor surfaces that reflects infrared radiation while transmitting visible radiation into the photobioreactor. Such materials include 3M's PR90EXT window film, as well as others. However, the economics of this approach have not yet been documented.

Finally, photobioreactors can be cooled by submersion in very large bodies of water such as the ocean where their presence does not result in a significant temperature increase of the body of water. NASA's OMEGA photobioreactor concept uses such an approach [8].

Gas Exchange

To produce 1 kg of biomass through photosynthesis, about 2 kg of CO_2 is required, and about 1.7 kg of O_2 is produced overall [26]. The transfer of CO_2 to and O_2 from, the medium is therefore a requirement of every cultivation system.

Even though the equilibrium O_2 concentration for water at standard temperature and pressure is only 8 mg/L, concentrations in excess of 100 mg/L can be observed in dense algal cultures as a result of photosynthesis [27]. Excessive oxygen concentrations are toxic to algae, and the rate of photoinhibition increases with increasing oxygen concentration [14].

CO_2 is necessary for photosynthesis, but also plays a significant role in the pH of the media. The pH of the medium increases as algae consume CO_2 for photosynthesis, and decreases as CO_2 is added. In order to maximize productivity, the CO_2 must be added at the appropriate rate to maintain the optimal pH [28].

The quantity of a gas that is transferred into or out of a liquid is proportional to the bubble surface area to volume ratio (a, cm^2/cm^3), the difference in concentration of the gas and liquid (C^*-C_L, mg/L), the CO_2 transfer coefficient (k_L, cm/s), and the time (Δt, s) the gas and liquid interact:

$$n = k_L a\left(C^* - C_L\right)\Delta t \quad (5)$$

Methods of gas transfer utilize one or more of these factors to facilitate a sufficient rate of CO_2 and O_2.

The large open surface area of open raceway ponds serves to provide a sufficient value of a for oxygen transfer out of the media. In vertically aerated photobioreactors, the surface area of the bubbles is sufficient to strip excess O_2 from the media. In tubular photobioreactors, an oxygen stripping tank/column removes O_2 by means of aeration or cascading the media through a flow of fresh air before the culture is recirculated through the tubes.

Since the concentration of CO_2 in air is less than 400 ppm, a more concentrated source is required for practical CO_2 transfer into the media, such as flue gas or commercially produced CO_2. The CO_2 is most often pressurized and sparged into the media by one of several means. The simplest means injects fine CO_2 bubbles (<2 mm) into the media with an air stone or similar fine-bubble diffuser. In vertically aerated photobioreactors, CO_2 is often mixed into the aeration air at concentrations of 1-2% v/v CO_2, thereby using the aeration distribution piping for CO_2 distribution as well. Aeration with straight flue gas has been demonstrated, but only certain species can tolerate the high CO_2 concentration [29]. In open raceway ponds, the shallow column of water does not permit sufficient time (Δt) for the CO_2 to be completely absorbed before it reaches the surface. To resolve this, some open raceway ponds are constructed with a CO_2 sump - a narrow deep trench used to add more media depth through which the CO_2 must travel. Alternatively, a floating hood may be located on the surface of the pond under which CO_2 is injected. The hood holds the concentrated CO_2 against the water surface to provide time for transfer into the media.

A more involved but efficient process for infusing CO_2 for all cultivation systems is the counter-current column - a vertical section of pipe in which media is pumped downward at a rate that nearly balances the CO_2 bubbles being injected in the bottom of the pipe [28].

Cultivation System Engineering

Design Considerations

The cell concentration, optical path length, and rate of mixing have been demonstrated to be critical, interdependent parameters in cultivation systems [3,11]. The considerations for selecting the values of these parameters will be discussed below.

Cell concentration

Determines the average light per photosynthetic unit. Too much light saturates the photosynthetic mechanisms, resulting in damage to the cells and/or poor photon utilization. However, increasing cell concentration results in a larger fraction of photosynthetic energy being used for cell maintenance. Therefore, there is an optimal cell concentration that balances light per cell with respiration for optimal productivity (Figure 4). The optimal cell concentration will depend on the strain and growth conditions and therefore must be determined experimentally.

Optical Path Length: is the depth through which light must penetrate the culture. It is usually reported as the ratio of the cultivation system volume to the surface area of one side of the cultivation system. For tubes the optical path length is the diameter of the tube, for panels it is the distance between the primary faces of the panels, and for open raceway ponds it is the depth of the pond. Volumetric cell or biomass density is ideally inversely proportional to optical path length - volumetric cell/biomass concentration generally increases with reducing the optical path length. In practice, the optical path length is also dictated by other considerations such as thermal mass, serviceability, manufacturability, pH stability, volumetric nutrient concentration, and mixing.

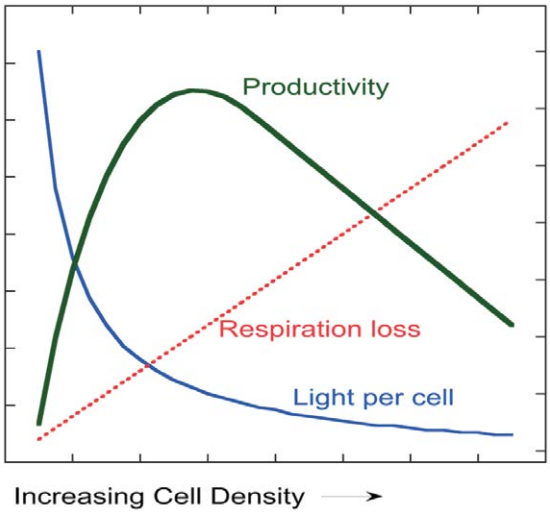

Figure 4: Light per cell, respiration loss, and productivity as a function of cell concentration.

The Rate of Mixing contributes to the frequency at which algal cells enter and exit the photic zone of the cultivation system. Ideally, the more rapid the rate of mixing, the more frequent the exposures, and the more efficient photon utilization becomes. But in practice, other considerations such as energy input, shear stress, and increased evaporative losses must be balanced as well.

Quantifying performance

In evaluating and comparing the performance of cultivation systems, metrics for quantifying growth are necessary. While the ultimate metric is economic (value of product versus cost to produce), productivity metrics are a valuable intermediate measurement.

Physically, growth is measured by quantifying either the product (biomass or some constituent of it), or the cell count, in a known volume of media, resulting in data in terms of mass of product per volume of media, or cell count per volume of media. From these data, numerous productivity metrics can be calculated in various ways.

The most obvious metric is volumetric productivity, which is the change in volumetric product or cell concentration over a period of time (g/L-d or cells/L-d). This metric works well for comparing cultivation systems that have identical optical path lengths, such as comparing the performance of different media or algae strains, but is misleading when the optical path lengths are different, such as between open raceway ponds and photobioreactors. For illustration, consider the comparison of an open raceway pond and a vertical plate reactor. The open raceway pond has a productivity of 0.1 g/L-d and a depth of 25 cm and the vertical plate reactor a volumetric productivity of 0.5 g/L-d and an optical path length of 5 cm. From these results the vertical plate reactor would appear to have productivity five times higher than the open raceway pond, but the open raceway pond contains five times the volume of media as the vertical plate reactor per illuminated area, and therefore the productivity per cultivation system surface area is identical. However, volumetric productivity is an important consideration when considering downstream processes as the relative amount of water that must be separated from the biomass determines the cost and method of the dewatering process [30].

The volumetric productivity and the optical path length of cultivation systems can be accounted by calculating the areal biomass productivity. This productivity is typically

reported in g/m^2-d or MT/ha-yr, similar to how agricultural productivities are reported. Areal productivity of the cultivation system can be calculated from the volumetric productivity by multiplying it by the volume to lit surface area ratio (L/m^2) of the cultivation system. In SI units, this ratio is equal to the optical path length of the cultivation system measured in mm (100 L/m^2 = 100 mm optical path length).

There are two types of areal productivities that can be reported: the areal productivity in terms of cultivation system surface area (aquatic productivity) or land area (terrestrial productivity). For horizontal cultivation systems such as open raceway ponds, the aquatic productivity only accounts for area covered by the media while the terrestrial productivity accounts for the surrounding service and utility access and pond median in addition to the media area. For open raceway ponds, the aquatic areal productivity is most frequently reported. For vertical photobioreactors, the aquatic productivity is calculated based on the photobioreactor surface area. The terrestrial productivity is calculated by multiplying the aquatic productivity by the ratio of ground area between the photobioreactors to their surface area [11] (Figure 5). While the terrestrial productivity is necessary for calculating the land necessary to produce a given quantity of product, or comparing the yield to agriculture, the aquatic productivity is economically more relevant since cultivation system surfaces generally cost two orders of magnitude more than the land on which they are placed.

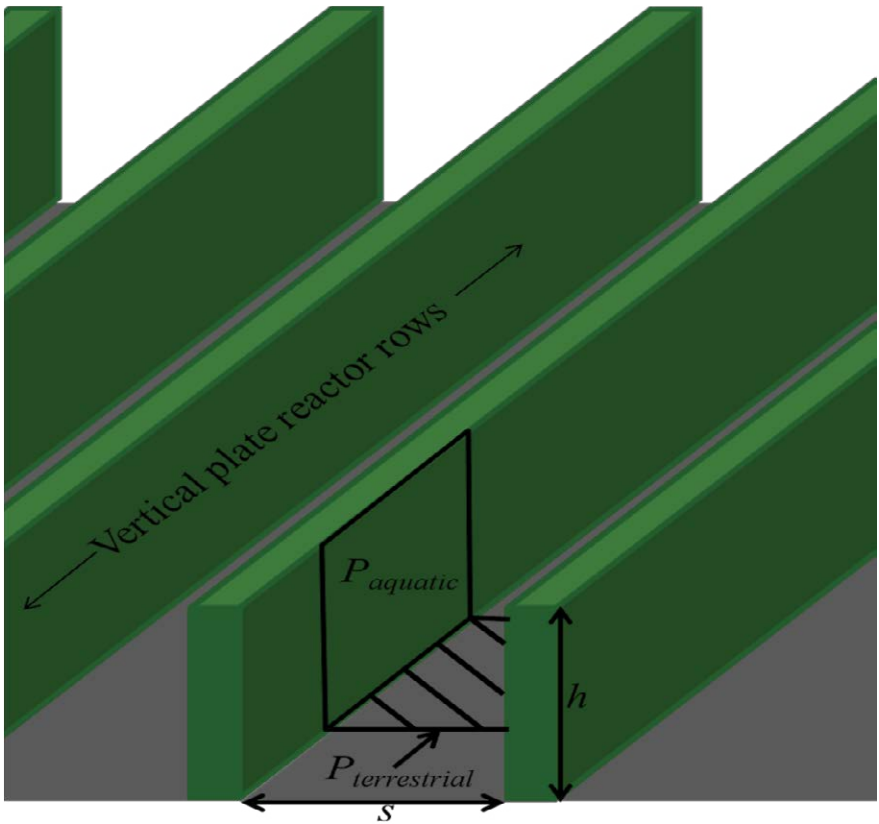

Figure 5: Illustration of aquatic and terrestrial productivity for a vertical plate reactor.

Comparing the areal productivity of cultivation systems can only be done if the cultivation systems are operated simultaneously at the same location since insolation varies widely by location, season, and sometimes from day to day. Therefore, a third useful metric is the photosynthetic yield. Photosynthetic yield takes into account the measurement of insolation

on the cultivation system surface in addition to volumetric density measurements. This metric permits the widest range of comparisons of growth conditions as photosynthetic yields from various locations, conditions, and seasons, can be fairly compared.

The result is the quantity of product normalized to the photons received (g/ mol photons) or energy received (g/kJ). If the energy content of the biomass is known, the net photosynthetic efficiency can be measured (% kJ biochemical/kJ solar).

Photosynthetic yield, and volumetric and areal productivities, can be calculated using a variety of time scales that will influence the result and the usefulness of the measurement. There are three methods of determining the productivity over a period of time: instantaneous, curve-fit, and time-average.

Instantaneous Productivity: It is calculated as the change in cell or biomass concentration (X) over a 24-hour period:

$$P_{inst} = \frac{X(t) - X(t - \Delta t)}{\Delta t} \tag{6}$$

While this is the most obvious method, differentiating the growth curve amplifies any "noise" or small measurement errors in the data, and will appear to give wildly unstable instantaneous productivities. For illustration, consider the cell concentrations of a typical batch growth of algae in a vertical plate reactor (Figure 6a). The cell densities increase in a linear fashion and the instantaneous productivities should be relatively constant. However, (Figure 6b) Line A shows the instantaneous productivities based on the data of (Figure 6a). Small deviations from linearity result in large deviations in productivity from day to day.

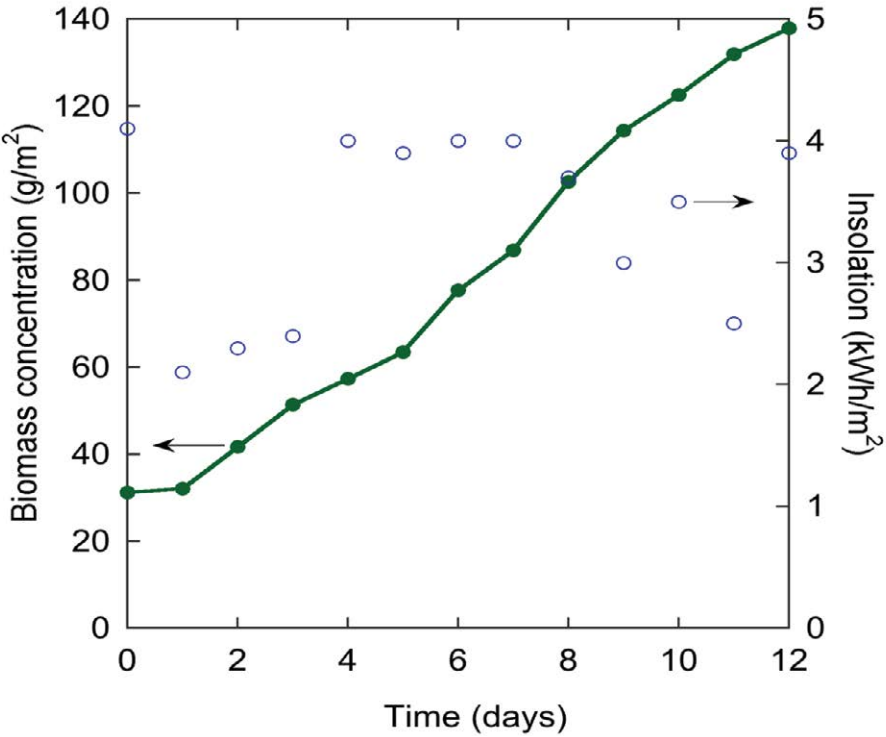

Figure 6a: Growth curve and daily insolation in a cultivation system.

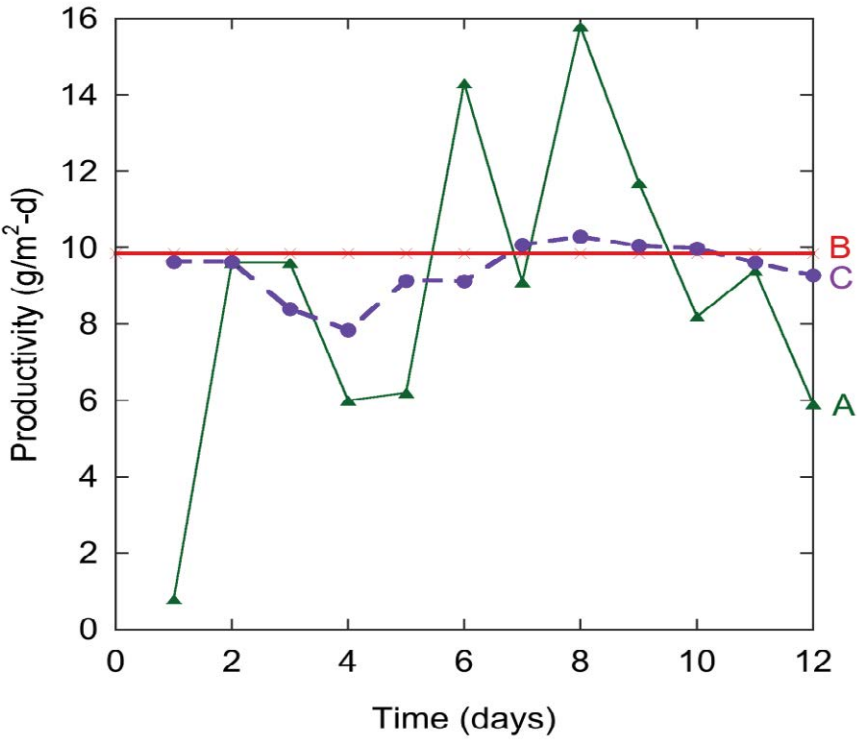

Figure 6b: Daily instantaneous productivity (Line A), (linear) curve-fit productivity (Line B), and time-averaged productivity (Line C).

Curve-Fit Productivity: Determines the productivity by fitting a curve to the data and taking the slope of the curve at the time of interest as the productivity at that time. This approach has the advantage that it eliminates data noise by fitting a smooth curve. However, the choice of curve can significantly influence the resulting productivities. If environmental conditions are constant throughout the experiment, a linear curve fit is the clear choice. However, changes in weather, nutrient concentration, and temperature are common throughout the course of a growth period, and a linear curve will not accurately represent deviations to productivity as a result of these changes (Figure 6b, Line B).

Time-Average Productivity: It is calculated similarly to instantaneous productivity, except that the change in product concentration is always calculated from the time the growth period started:

$$P_{t-ave} = \frac{X(t) - X(0)}{t} \tag{7}$$

This method is ideal for batch production since the day with length of time with the highest productivity indicates the optimal batch time, assuming sufficiently constant operating conditions. Since this method relies so heavily on the product concentration measurement at t = 0, this first measurement should be taken very diligently, and repeated several times to ensure an accurate initial concentration. It also has the disadvantage of instantaneous productivity for the first few days in that it is sensitive to noise, and the disadvantage of curve-fit productivity in that it does not fully account for changes to productivity that result from environmental changes.

Current Challenges and Future Opportunities

Outdoor cultivation of algae has many advantages that make it an attractive biotechnology for a variety of products and services. Algae can use land and CO_2 that would otherwise go unused, and algae can produce higher yields of several products than agriculture (e.g. carotenoids, polyunsaturated fatty acids). In certain locations, algae cultivation may have some value for wastewater treatment for removing nitrogen and phosphorous – if sufficient land and CO_2 is available - in place of processes that require large quantities of air and organic carbon.

However, there are many challenges associated with outdoor cultivation of algae that will require more technical advances in order for outdoor algae production to contribute a larger share of products, especially commodity products such as food, feed, and fuel.

The first major challenge relates to the poor economy of scale. Since little can be gained economically from scaling up, the price of materials used for cultivation systems must be minimized while productivity is maximized. Capital cost reduction will require advances in cultivation system design and materials to reduce the cost per cultivation surface area while still retaining the media and favoring healthy growth. While outdoor algal biomass production rates in excess of 50 g/m^2-d have been demonstrated, these rates are rarely sustained for more than a few days under ideal conditions [31]. Continued research into the growth dynamics of outdoor cultures, predator/pathogen mitigation, more efficient conversion of solar energy, and higher product contents will be crucial. A specific area of improvement is in mixing methods. Currently, temporal light dilution is accomplished through random mixing via a paddlewheel, turbulent flow, or aeration. The development of directed, rather than random, mixing to create light/dark cycles in cultivation systems will reduce energy consumption and increase photon utilization. This has been demonstrated successfully in open raceway ponds with hydrofoils [2], and in “weaved” tubular photobioreactors [32], but more practical designs are desirable.

Secondly, temperature control is a major practical limitation to algal cultivation. Large quantities of low-grade heat (30-40°C) must be expelled from cultivation systems. This requires either large quantities of water for evaporative cooling or electricity for refrigeration, thereby limiting the size and/or location of a cultivation facility to areas with cheap, abundant, and reliable water or electricity. Outdoor cultivation systems are subject to large fluctuations in thermal loads. In open raceway ponds and other cultivation systems with no or little active temperature control, productivity is hindered since temperature is not maintained within the optimal range. However, innovative cultivation systems, such as coupling photobioreactors with open raceway ponds, and sea-based systems, have shown that this obstacle can be overcome.

Finally, today’s algae species are still being “domesticated,” or genetically optimized for mass production. Much of the success of agriculture can be attributed to genetic breeding and selection to optimize crop yield and quality [33]. The discovery/development of strains that in man-made environments are more productive, more tolerant of environmental extremes (such as temperature), and resistant to pathogens and predators, will have a similar impact on the success of algal cultivation.

References

1. Tran M, Van C, Barrera DJ, Pettersson PL, Peinado CD, et al. (2013) Production of Unique Immunotoxin Cancer Therapeutics in Algal Chloroplasts, Proceedings of the National Academy of Sciences, 1101: 15-22.

2. Laws EA, Terry KL, Wickman J, Chalup MS (1983) A Simple Algal Production System Designed to Utilize the Flashing Light Effect, Biotechnology and Bioengineering 2510: 2319-2335.

3. Hu Q, Richmond A (1996) Productivity and Photosynthetic Efficiency of Spirulina platensis as Affected by Light Intensity, Algal Density, and Rate of Mixing in a flat plate photobioreactor, Journal of Applied Phycology 8: 139-145.

4. Hu Q, Zarmi Y, Ricmond A (1998) Combined effects of light intensity, light-path and culture density on output rate of *Spirulina platensis* (Cyanobacteria), European Journal of Phycology 33: 165-171.

5. Richardson JW, Johnson MD, Zhang X, Zemke P, Chen W, et al. (2013) A Financial Assessment of two alternative cultivation systems and their contributions to algae biofuels economic viability, Algal Research, in press.

6. Tredici MR, Zittelli GC (1998) Efficiency of sunlight utilization: tubular versus flat photobioreactors, Biotechnology and bioengineering 57: 187-197.

7. Fernandez FGA, Hall DO, Guerrero EC, Rao KK, Grima EM (2003) Outdoor production of *Phaeodactylum tricornutum* biomass in a helical reactor, Journal of Biotechnology 103: 137-152.

8. Wiley P, Harris L, Reinsch S, Tozzi S, Embay T, et al. (2013) Microalgae cultivation using Offshore Membrane Enclosures for Growing Algae (OMEGA), Journal of Sustainable Bioenergy Systems 3: 18-32.

9. Davis R, Fishman D, Frank ED, Wigmosta MS (2012) Renewable diesel from algal lipids: an integrated baseline for cost, emissions, and resource potential from a harmonized model, NREL/TP-5100-55431.

10. Rodolfi, L, Zittelli GC, Bassi N, Padovani G, Biondi N, et al. (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, Biotechnology and Bioengineering 102: 100-112.

11. P Zemke, M Sommerfeld, Q Hu (2013) Assessment of key biological and engineering design parameters for enhanced production of *Chlorella* sp. (Chlorophycease) in an outdoor photobioreactor, Applied Microbiology and Biotechnology 9712: 5645-5655.

12. Kim N-G, Suh S, Hur B-K, Lee C-G (2002) Simple monodimensional model for linear growth rate of photosynthetic microorganisms in flat-plate photobioreactors, Journal of Microbiology and Biotechnology 12: 962-971.

13. Goldman JC (1978) Outdoor algal mass cultures – II. Photosynthetic yield limitation. Water Res 13:119-136.

14. Baroli I, Gutman BL, Ledford HK, Shin JW, Chin BL et al. (2004) Photo-oxidative stress in a xanthophyll-deficient mutant of *Chlamydomonas*. Biol Chem 279: 6337-6344.

15. Wang J, Han D, Lu C, Sommerfeld M, Hu Q (2013) Effect of initial biomass density on growth and astaxanthin production of *Haematococcus pluvialis* in an outdoor photobioreactor. Journal of Applied Phycology 25: 253-260.

16. Dye DJ (2010) Spatial light dilution as a technique for conversion of solar energy to algal biomass, doctoral dissertation, Utah State University.

17. Burlew JS (1953) Current status of the large-scale culture of algae. In: Algal culture from laboratory to pilot plant. Washington DC: Carnegie Institute of Washington. 3-23.

18. Borowitzka LJ, Borowitzka MA (1990) Commercial production of B-carotene by *Dunaliella salina* in open ponds, Bulletin of Marine Science, 471: 244-252.

19. Rubio FC, Camacho FG, Sevilla JMF, Chisti Y, Grima EM, 2003. A mechanistic model of photosynthesis in microalgae, Biotechnology and bioengineering 81: 459-473.

20. Terry KL (1986) Photosynthesis in modulated light: quantitative dependence of photosynthetic enhancement on flashing rate, Biotechnology and Bioengineering 28: 988-995.

21. Blankenship RE, Tiede DM, Barber J, Brudvig GW, Fleming G, et al. (2011) Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement, Science 332: 805-809.

22. JC Quinn, T Yates, N Douglas, K Weyer, J Butler, et al. (2012) *Nannochlorposis* production metrics in a scalable outdoor photobioreactor for commercial applications, Bioresource Technology 117:164-171.

23. Sheehan J, Dunahay T, Benemann J, Roessler P (1998) A look back at the US Department of Energy's Aquatic Species Program – biodiesel from algae. Report no. NREL/TP-580-24190, National Renewable Energy Laboratory, Golden, CO.

24. Lundquist TJ, Woertz IC, Quinn NWT, Benemann JR (2010) A realistic technology and engineering assessment of algae biofuel production.

25. Pushparaj B, Pelosi E, Tredici MR, Pinzani E, Materassi R (1997) An integrated culture system for outdoor production of microalgae and cyanobacteria, Journal of Applied Phycology 9: 113-119.

26. Williams PJ le B, Laurens LML (2010) Microalgae as biodiesel and biomass feedstocks: review & analysis of the biochemistry, energetics, and economics, Energy and Environmental Science 3: 554-590.

27. Marquez FJ, Sasaki K, Nishio N, Nagai S (1995) Inhibitory effect of oxygen accumulation on the growth of *Spirulina platensis*, Biotechnology Letters 17: 225-228.

28. Becker EW (1994) Microalgae: Biotechnology and Microbiology, Ch. 10: Large-scale cultivation, 63-171.

29. Kurano N, Ikemoto H, Miyashita H, Hasegawa T, Hata H, et al. (1995) Fixation and Utilization of Carbon Dioxide by Microalgal Photosynthesis, Energy Conversion Management 36: 689-692.

30. Shuler ML, Kargi F (2002) Bioprocess engineering, basic concepts: second edition, Prentice-Hall, Upper Saddle River, NJ.

31. Morais MG, Radmann EM, Andrade MR, Teixeira GG, Brusch LRF, et al. (2009) Pilot scale semicontinuous production of *Spirulina* biomass in southern Brazil, Aquaculture 294: 60-64.

32. Carlozzi P (2003) Dilution of solar radiation through “culture” lamination in photobioreactor rows facing south-north: a way to improve the efficiency of light utilization by cyanobacteria (*Arthrospira platensis*), Biotechnology and Bioengineering 81: 305-315.

33. Diamond J (1999) Guns, germs, and steel: the fates of human societies, WW Norton & Co, NY.

Heterotrophic Growth of Micro Algae

Mala Khan[1], Rana Karmakar[1], Banani Das[2], Farah Diba[3], M H Razu[1*]

[1] Designated Reference Institute for Chemical Measurements, BCSIR, Dhaka-1205, Bangladesh

[2] Department of Environment, Ministry of Environment and Forest, Bangladesh

[3] Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

***Corresponding author:** Mamudul Hasan Razu, Designated Reference Institute for Chemical Measurements (DRiCM), Bangladesh Council of Scientific and Industrial Research (BCSIR), Qudrat-i-Khuda Road, Dhaka-1205, Bangladesh; Tel: +88-0172-1218185; E-mail: razu_ss86@yahoo.com

Abstract

Today cultured microalgae is used as producer of food ingredients such as omega-3 fatty acids or natural food colorants and dyes, food, fertilizer, bioplastics, chemical feedstock (raw material), pharmaceuticals, and algal fuel, and can also be used as a means of pollution control. Microalgae can grow 20 or 30 times faster than traditional food crops, and has no need to compete for arable land. Since micro algal production is central to so many commercial applications, there is a need for production techniques which increase productivity and are economically profitable. Where possible, heterotrophic growth overcomes major limitations of producing useful products from microalgae: dependency on light which significantly complicates the process, increase costs, and reduced production of potentially useful products. As a general role, and in most cases, heterotrophic cultivation is far cheaper, simpler to construct facilities, and easier than autotrophic cultivation to maintain on a large scale. This capacity allows expansion of useful applications from diverse species that is now very limited as a result of elevated costs of autotrophy; consequently, exploitation of microalgae is restricted to small volume of high-value products. In this literature, we present a general perspective of the field, describing the specific cellular metabolisms involved and the best-known examples from the literature and analyze the prospect of potential products from heterotrophic cultures.

Introduction

Recently, several photosynthetic microalgae have been identified as efficient biological systems for producing a wide variety of high-value chemicals and pharmaceuticals, such as phycobiliproteins, astaxanthin and polyunsaturated fatty acids (PUFAs) Consequently, several processes have been developed to obtain some of these compounds on a commercial scale; most of these developments are based on phototrophic growth using CO_2 as a carbon source [1,3]. Although most microalgae grow photoautotrophically, some are able to grow heterotrophically using organic substrates as sole carbon and energy sources The heterotrophic growth of microalga depends on the strain and culture conditions, and the

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

consumption of the carbon source depends on the transport or diffusion of the carbon source across the membrane, and the enzymatic processes required for its incorporation into the central carbon metabolism. A heterotroph (heteros = "another", "different" and trophe = "nutrition") is an organism that cannot fix carbon and uses organic carbon for growth [4]. Heterotrophs can be further divided based on how they obtain energy; if the heterotroph uses light for energy, then it is considered a photoheterotroph, while if the heterotroph uses chemical energy, it is considered a chemoheterotroph. In other word an organism that cannot synthesize its own food and is dependent on complex organic substances for nutrition.

Heterotrophic algae are algae that take up organic molecules as a primary source of nutrition. Compared to photoautotrophic growth, heterotrophic cultivation of microalgae eliminate light requirements, can significantly increase growth rates and cell mass, protein and lipid productivities [5–7]; bioreactor operation and maintenance is relatively simple and can be performed under strict axenic conditions; also cell masses obtained under heterotrophic conditions are higher because the energy density of the carbon source is higher in comparison with carbon dioxide [7] and cell densities can be increased using some culture strategies like fed-batch cultures, leading to a decrease in the costs of biomass harvesting [8,9]. One of the most notable advantages of the phototrophic cultivation is that under such condition microalgae fixes carbon dioxide and produces oxygen, contributing to the reduction of carbon emissions to the atmosphere [10]; while heterotrophic cultures use an organic carbon source, consumes oxygen and generates some CO_2 during this kind of cultivations. Furthermore, phototrophic cultures permit the use of non-potable water and not arable land and do not displace food crops cultures [10]. A microalga suitable for heterotrophic culture should have the following physiological abilities: divide and metabolize without light, grow on easily sterilized culture media, adapt rapidly to environmental changes and withstand the hydrodynamic stresses generated in stirred tank bioreactors and peripheral equipment [11, 12, 13].

A number of microalgae are capable of growing heterotrophically on organic substrates anf thus do not depend on sunlight for energy. Carbon in some form is necessary to provide the energy and carbon skeletons for cell growth. Heterotrophic algae derive energy from organic substrates [14]. Other carbon sources include carbohydrates such as fructose, sucrose, lactose and starch. C/N ration is an influencing factor with affects cellular lipid content as it controls the switch between lipid and protein syntheses. Nitrogen deficit (high C/N ratio) in the culture media triggers lipid accumulation [15]. Heterotrophic cultivation of some algae could result in higher biomass production and high lipid accumulation in cells. In microalgal culture, heterotrophic growth can be a cost effective alternative to photoautotrophic growth. This mode of culture eliminates the requirement for light and hence, offers the possibility of greatly increasing cell density and productivity. The heterotrophic growth processes can occur in open raceway ponds or closed bioreactor systems [20]. Different production schemes involving combinations of different growth regimes in various reactor configurations have been proposed in an effort to maximize biomass productivity. Although open raceway ponds require low energy inputs and lower capital costs, several issues such as contamination (i.e., by unwanted algal species as well as viral, bacterial and fungal pathogens) and low final biomass concentrations (often less than 1.0 g/L) increase production costs to levels that are still economically unviable [21]. Closed photobioreactors (CPBR) in different configurations have been proposed to address the contamination issues associated with open raceway systems. CPBRs also permit more stringent control of growth conditions and harvesting [22]. However, CPBRs often require extensive upfront capital investments compared to open raceway ponds and therefore face similar commercialization challenges. But in case of valuable product production it reduces the ultimate cost. Many photosynthetic microalgae have been reported to grow well in aerobic heterotrophic conditions like: *Chlorella protothecoides*, *C. vulgaris*, *C. sorokiniana*, *C. regularis*, and *C. pyrenoidosa*, *Scenedesmus sp*, *Haematococcus sp*, *Spirulina sp*, *Nitzchia laevis*, *Chlamidomonas reinhardtii*, *Scenedesmus obliquus*, *Synechocystis*, *Plectonema*

boryanum and *Nostoc* etc. with the introduction of organic compounds like glucose, peptone and acetate [23]. However, the sugar based heterotrophic system frequently suffers from problems with contamination.

This article analyzes the processes and cases solely where heterotrophic cultivation of microalgae is possible to explore the potential and usefulness of this approach. It presents cases of autotrophic growth only for comparison or when similar mechanisms operate under autotrophic and heterotrophic conditions. It focuses on: (1) Basic metabolic processes of the microalgae; (2) Environmental parameters affecting growth and metabolism; (3) Kinetic parameters, such as specific growth rates and biomass production, and (4) Actual and potential end-products and byproducts that can be obtained from heterotrophic microalgal systems. Finally, we discuss some promising avenues of research.

Metabolisms of Heterotrophic Algae Culture

An interesting feature observed in heterotrophic cultures is that there is a decrease in the overall chlorophyll content of the algae in course of its growth. It has been reported that there is up to 94% chlorophyll loss under heterotrophic growth. This could be attributed to the fact that there is reduction in the chlorophyll synthesis as photosynthesis is inhibited and the carbon in its organic form is directly incorporated from the various sugars fed to the algae [24]. This causes the microalgal cells to adapt to this kind of carbon assimilation where the synthesis of the unutilized chlorophyll is down regulated to conserve energy. Hence the metabolic regulation starts the biodegradation of chlorophyll in these heterotrophic cultures [25,26]. Furthermore, as chlorophyll pose some interference during the trans-esterification process for biodiesel production, the reduction in chlorophyll content during heterotrophy only favors the biodiesel production from microalgal feedstock. Besides providing the advantage of eliminating the light requirements, heterotrophy also gives a cultivation process that is much easier to control. The addition of external organic carbon in required amounts also generates a CO_2 rich environment that promotes growth of algae [24]. Due to the high cell densities achieved at the end of the heterotrophic process, the biomass harvesting is also cost effective [27]. A close study needs to be done with heterotrophy to obtain an enhanced cultivation technique for microalgal growth.

Metabolism of Organic Carbon Source

Glucose

Glucose is the most commonly used carbon source for heterotrophic cultures of microalgae, as is the case for many other microbial species. Far higher rates of growth and respiration are obtained with glucose than with any other substrate, such as sugars, sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols [28]. This may happen because glucose possesses more energy content per mol compared with other substrates. For example, glucose produces w2.8 kJ/mol of energy compared to w0.8 kJ/ mol for acetate [29]. Glucose promoted physiological changes in Chlorella vulgaris, which strongly affects the metabolic pathways of carbon assimilation, size of the cells, volume densities of storage materials, such as starch and lipids grains [30] and protein, chlorophyll, RNA, and vitamin contents [31]. Oxidative assimilation of glucose begins with a phosphorylation of hexose, yielding glucose-6-phosphate, which is readily available for storage, cell synthesis, and respiration. An equivalent of a single phosphate bond is required per mole of glucose assimilated into glucose-6-phosphate. In that process, an additional 30 equivalents of phosphate bonds are generated by aerobic oxidation of a mole of glucose [32]. Of the several

pathways used by microorganisms for aerobic glycolysis (Break down of glucose), apparently only two: the Embdene Meyerh of Pathway (EMP) and the Pentose Phosphate Pathway (PP pathway) have been shown in algae [33]. Under complete darkness heterotrophic growth, glucose is mainly metabolized via PP pathway can be seen in Figure 1 [34].

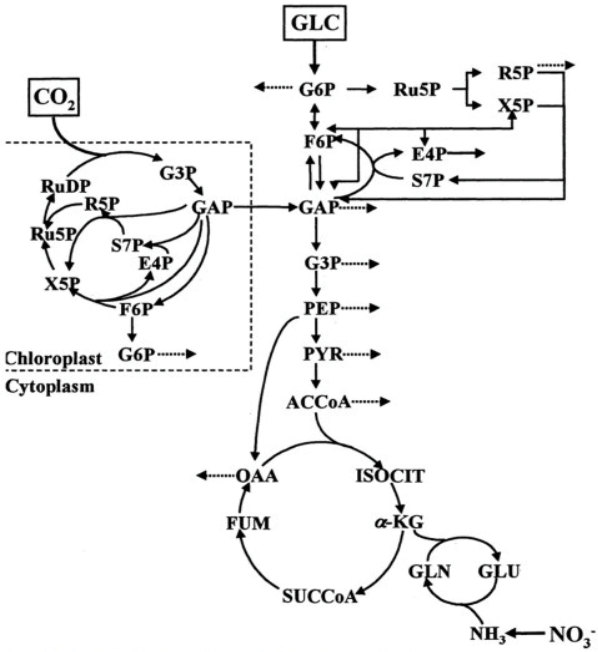

Figure 1: Glucose metabolism in heterotrophic microalgae. Compound abbreviations are following specified. GLC glucose, G6P glucose 6-phosphate, F6P fructose 6-phosphate, GAP 2 glyceraldehyde-3-phosphate, G3P glucose 3phosphate, PEP phosphoenolpyruvic acid, PYR pyruvate, ACCoA acetyl co-enzyme, ISOCIT isocitrate, α-KG alpha keto-glutamic acid, SUCCoA succinyl co-enzyme, FUM fumarate, OAA oxalo acetate, GLN glutamine, GLU glutamate, Ru5P ribose 5-phosphate, R5P ribulose 5-phosphate, X5P xylulose 5-phosphate, RuDP ribulose-1,5-diphosphate.

Carboxylic Acid (mainly acetate)

Uptake of dissolved carboxylic acids, such as acetic, citric, fumaric, glycolic, lactic, malic, pyruvic, and succinic under microalgal heterotrophic cultivation has been well known for decades [35]. Acetate (or acetic acid) is one of the most common carbon sources for many microbial species, including microalgae [32]. Once inside microalgal cells in the cytosol, the starting point for acetate assimilation is acetylation of coenzyme A by acetyl-CoA synthetase (EC 6.2.1.1) to form acetyl coenzyme A (acetyl-CoA) in a single-step catalyzed reaction using a single ATP molecule, as shown in Figure 1 [34]. Acetate (carried by coenzyme A) is generally oxidized metabolically through two pathways: (a) the glyoxylate cycle to form malate in glyoxysomes (specialized plastids in the glyoxylate cycle) and (b) through the Tricarboxylic Acid Cycle (TCA) to citrate in the mitochondria, which provides carbon skeletons, energy as ATP, and energy for reduction (NADH). However, acetate does not always promote growth. It could be toxic for many microorganisms at high concentrations, despite its common use for buffering high pH levels in bioreactors. Keeping the concentration of acetate at low levels is useful for the fed-batch configuration in cultures or pH-auxostat (pH is maintained as a constant).

Alcohols (mainly glycerol)

Heterotrophic growth using glycerol as a substrate has been demonstrated for several algae, despite the simplicity of glucose metabolism in microalgae. Most of these species

occur naturally in habitats of somewhat elevated osmolarity, such as seawater and saline pounds. Glycerol as an osmoticum (a substance that has the capacity of raising the osmotic strength of the solution and consequently keeps the osmotic equilibrium in cells) is an economical carbon source for an energy supply and carbon requirements and is a very compatible solute for enzymes and membranes, with almost no toxic effects even at high concentrations [36]. It is commonly used for long term preservation of microorganisms at low temperatures. Inside cells, glycerol is used as an osmo regulatory molecule. Glycerol is first phosphorylated using ATP and the glycerol phosphate is then oxidized to triose phosphate. Micro algae genomes contain genes encoding for glycerol kinase (EC 2.7.1.30), sn-glycerol-3-phosphate NAD oxidoreductase (EC 1.1.1.8) and and triose-phosphate (EC: 5.3.1.1) to convert glycerol into glyceraldehyde-3-phosphate and glycerate, which are intermediates in the EMP pathway of glycolysis to form pyruvate that enters the TCA cycle [33].

Metabolisms of Nitrogen Sources

After carbon, and apart from hydrogen and oxygen, nitrogen is quantitatively the most important element contributing to the dry matter of microalgal cells, accounting from 1 to 10% dry weight. Carbon and nitrogen metabolism are linked in microalgae because they share (a) carbon supplied directly from respiration of fixed CO2 (autotrophic growth) or assimilated organic carbon (heterotrophic growth) and (b) the energy generated in the TCA cycle and in the mitochondrial electron transport chain. The primary assimilation of inorganic nitrogen (ammonium) to form amino acids requires carbon skeletons in the form of keto-acids (2- oxaloglutarate and oxaloacetate) and energy in the form of ATP and NADPH to synthesize the amino acids glutamate, glutamine, and aspartate. In both autotrophic and heterotrophic growing cells, keto-acids, ATP, and NADPH are provided by the TCA cycle [37,38]. The metabolic pathways involved in nitrogen assimilation are depicted in Figure 2 [39].

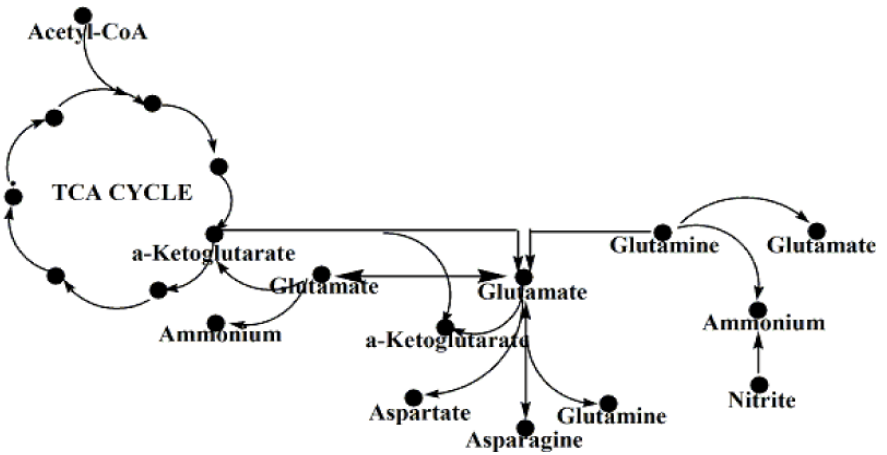

Figure 2: Typical nitrogen metabolic pathway in microalgae.

Metabolisms of Ammonium

Ammonium is the most preferred nitrogen source for algae. It is also the most energetically efficient source, since less energy is required for its uptake. Under autotrophic and heterotrophic conditions, ammonium is transported across the membranes by a group of proteins belonging to the Ammonium Transporter Family (AMT), a group of evolutionarily related proteins commonly found in bacteria, yeast, algae, and higher plants [40]. Several ammonium transporters, all belonging to the AMT family, have been identified in diatoms [41]. Ammonium is present in all compartments of the cell. Its concentration varies, depending on several factors including the concentration of ammonium in the neighboring compartment(s), the diferences in pH, and electrical potential between compartments. In

compartments where ammonium is not metabolized, such as the vacuole, the concentration of ammonium may approach its equilibrium value. In compartments in which ammonium is metabolized, such as the cytosol and plastids, the steady-state concentration of ammonium may be much lower than the predicted equilibrium [42].

Assimilation metabolism of ammonium under either authotrophic or heterotrophic conditions is catalyzed by glutamine synthetase (GS; EC 6.3.1.2), which produces glutamine, and glutamate synthase (GOGAT; EC 1.4.1.14), which produces two molecules of glutamate from glutamine plus one molecule of a-ketoglutarate [43-46]. Alternatively, ammonium is incorporated into glutamate by the reversible reductive amination of a-ketoglutarate, which is catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.2) [47]. Heterotrophic growth conditions do not affect uptake rates of ammonium and the expression of nitrogen assimilation enzymes but mixotrophic regimen does. For example, adding acetate to autotrophic *Scenedesmus obliquus* affects its rates of ammonium uptake. In autotrophy, uptake is 17.8 mmol cell^{-1} min^{-1} and is similar to that in heterotrophy (17.4 mmol cell^{-1} min^{-1}), but this is ~4 times lower than occurring under mixotrophy (65.9 mmol cell^{-1} min^{-1}) [48]. However, when the pH of the culture and other growth conditions are controlled, ammonium is a reliable nitrogen source [49]. For example, *P. tricornutum* grew well after adjusting the initial pH to 8 and a fed-batch configuration was used [50]. Another option to control pH and use ammonium as a nitrogen sources is to add a buffer.

Factor Related In Heterotrophic Growth of Microalgae

Heterotrophic cultivation is inappropriate for most microalgae and more species are obligate autotrophs than facultative heterotrophs [51,52]. Yet, some species are effectively grown in complete darkness and thus their cultures can be grown in conventional dark fermenters. Chen and Chen (2006) [53] listed the required initial characteristics that a microalgae species must have to be useful for heterotrophic cultivation: (a) Faculty of cell division and active metabolisms in absence of light. (b) Ability to grow in culture media with easy-to-sterile organic substrates where energy required for heterotrophic growth must be supplied by oxidation of part of the organic substrate [32]. (c) Ability to adapt to fast environmental changes, and (d) Capacity to resist hydro-mechanical stress inside the fermentors. However, it is impossible to precisely predict which specific substrates can be used or preferred by any given microalgae [33]. During respiration, oxygen is consumed and CO_2 produced. The respiration rate of any organic substrate is intimately geared to growth and cell division. Under optimal conditions, respiration rates are about 20%-30% of growth rates [54]. In microalgae, dark respiration of an organic substrate assimilated from the medium has rates varying from 0.01 to 0.6 d_1. This dark respiration plays two major roles in microalgae: (a) It serves as the exclusive source of energy for maintenance and biosynthesis under dark environment and (b) It provides essential carbon skeletons for biosynthesis under any growth condition. Under heterotrophic growth conditions, respiration rates equal or exceed the theoretical minimum cost of biomass synthesis. Values for CO_2 evolved per carbon (C) incorporated into new biomass (CO_2/C) equaled 0.4-1.4 for several *Chlorella* species and diatoms. This indicates that biomass synthesis during heterotrophic growth conditions can proceed at nearly the maximal theoretical efficiency.

Oxygen supply is a key factor in heterotrophic cultivation of microalgae. For example, the limitation of oxygen in a culture may reduce the specific growth rate of *Chlorella spp.* and thus lower the productivity of biomass when cell density is high [55]. Additionally, under cyclic cultures of autotrophic/ heterotrophic conditions, cell production of biomass of *Chlorella* is about 5.5 times higher than under autotrophic cycles alone, where cells were producing 16 times more ATP under heterotrophic culture [56]. In diatoms, heterotrophic growth is linked to their ability to maintain photosynthesis under dark environments using chloro-respiration to protect cells from photo damage after light returns; heterotrophic growth in this case is aided by high lipid accumulation, a product of reduced carbon in the absence of light [40].

Growth Characteristics of Heterotrophic Microalgae

The ability of a number of microalgal species to grow with organic carbon substrate has been demonstrated Previously [32,57]. However, the number of current commercially important microalgae that are capable of growth on organic carbon substrates in the dark, and where experience of fed batch cultivation has been gained, is very limited. Both the (growth) kinetic and stoichiometric characteristics of these microalgae, along with values for other microalgal species obtained from batch cultures, are summarized in Table 1 [58]. This table encompasses fast-growing species with a specific growth rate higher than 0.09 h−1 (e.g. *Chlorella*, *Crypthecodinium*, *Nitzia*, *Prototheca spp.*) and species that grow at about half the rate, but where a lot of cultivation experience is available (e.g. *Galdieria*, *Haematococcus*, *Nannochloropsis* or *Schizochytrium spp.*). These specific growth rates correspond to doubling times of between 7 and 15 h. Interestingly, the *Chlorella* genus exhibits a wide range of growth rates with glucose, which vary with species and growth conditions, such as temperature, pH or dissolved oxygen concentrations [59]. Furthermore, heterotrophic growth of *Dunaliella sp.* and *Nannochloropsis sp.* is possible but is not practicable due to its very slow growth [57].

Species		μ_{max} (h^{-1})	$Y_{x/s}$ ($gCDWg^{-1}$)	T (°C)	pH	Carbon sources	s_{inhib} ($g l^{-1}$)	Products
Chlorella vulgaris	P/H	0.180	0.55–0.69	36	6.0 – 7.5	Glucose (acetate, glutamate, lactate)	n.a.	Biomass
Crypthecodinium cohnii	H	0.089	0.56	25	7.2	Glucose (acetate)	>20	DHA
Dunaliella sp.	P/H	< 0.010	n.a.	26	7.5–8.3	Acetate, lactate, glucose, glutamate, glycerol	n.a.	Biomass beta-carotene
Euglena gracilis	P/H	0.045	0.43	25	2.8–3.5	Glucose, (acetate, alanine, aspartate, asparagine, ethanol, glutamate)	n.a.	Alpha-tocopherol
Galdieria sulphuraria	P/H	0.045–0.048	0.48–0.50	42	2	Glucose	>200	Phycocyanin
						Sugar beet molasses (fructose, sucrose)	>350	
Nannochloropsis oculata	P/H	< 0.007	n. a.	26	7.5–8.3	Glucose (ethanol)	n.a.	Biomass, EPA
Nitzschia alba	H	0.106	n. a.	30	n. a.	Lactate, succinate	n.a.	Biomass, EPA
						Glucose, glutamate		
Nitzschia laevis	H	0.017	0.44	20	8.2	Acetate, glucose	n.a.	EPA
Prototheca zopfii	H	0.330	0.81	21	7.2	Glucose (acetate)	n.a.	L-Ascorbic acid
Scenedesmus actus	P/H	0.040	n.a.	30	6	Glucose	>1	Biomass
Schizochytrium sp.	H	0.071	0.42	27	7	Glucose	>200	PUFA, DHA, GLA
Tetraselmis sueica	P/H	0.028	0.41	25	7.5	Acetate (glucose, glutamine, lactate)	n.a.	Lipids, PUFA n-3 HUFA

Table 1: Growth characteristics of microalgae in heterotrophic batch cultures.

μ_{max} maximum specific growth rate, $Y_{x/s}$ biomass yield determined in batch culture at given temperature (T), s_{inhib} substrate concentration resulting in a decrease of the specific growth rate and/or biomass yield with the particular substrate, *H* heterotroph, *P* (facultative) photoautotroph, *n.a.* not available, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *GLA* gamma-linolenic acid, *HUFA* highly unsaturated fatty acids, *PUFA* polyunsaturated fatty acids.

Cultivation of Microalgae in Stirred Bioreactors

The microalgal species which are currently attracting commercial interest grow under heterotrophic conditions and perform efficiently in conventional bioreactors in a similar

manner to bacteria or yeast [60]. Such sophisticated, safe and controllable bioreactor systems are used to produce novel high-value compounds with microalgae. In contrast, established microalgal products are mostly manufactured by traditional outdoor photoautotrophic technologies [61]. If a product is unique or is not obtainable in the desired quality or quantity by other means (such as extraction from animal or plant material or chemical synthesis), the superior heterotrophic growth characteristics become less critical. Performance losses may also be acceptable in cases where patent infringements need to be prevented. In order to be optimally suited for cultivation in stainless steel stirred bioreactors, a particular microalgal species should meet a number of desirable criteria showed in Table 2 [58]. The primary prerequisite is the ability to grow heterotrophically in an inexpensive, well-defined mineral medium with a high degree of resistance to mechanical and chemical stress.

	Prerequisites/benefits	Constraints
Bioreactor cultivation	Performance independent of climate Reduced downstream costs Enhanced productivity and/or titer Control of substrate concentrations Scalable process strategies Use of multi-purpose bioreactors Low land requirement Indoor and cGMP operation	High oxygen demand Sophisticated substrate feed control Rheological limitations (at high viscosity) Critical/toxic levels of metabolites High costs for (new) equipment
Culture media	Energy of light not required Defined (mineral) and inexpensive Easy to sterilize Non-corrosive (low salinity, acidity) Contamination protection (due to high salinity, extreme pH levels, high temperature > 40 °C)	Enhanced risk of contamination (organic carbon substrate, temperature, pH) Corrosion (high salinity, critical pH) Expensive ingredients (vitamins, amino acids) Non-defined composition (e.g. yeast extract)
Species	Available as axenic culture Reasonable specific growth rate Mechanical resistance Temperature achievable with conventional cooling (25–40 °C) Robust and resistant (to long periods of refrigeration, freezing, repeated cultivation, sudden condition changes)	Surface adhesion Aggregate formation Secretion of viscous metabolites Osmotic stress (at substrate over-dosing) Intracellular product harvest (hampered by rigid cell walls)

Table 2: Benefits and constraints of heterotrophic cultivation in stirred bioreactors.

Axenic Culture

An additional crucial prerequisite is the requirement for a monoculture in a long-term bioreactor operation. To date, this is still hampered by the dearth of axenic (pure) cultures of species isolated from the environment. In heterotrophic cultures, the advantage of preventing the growth of contaminants through selective photoautotrophic conditions is not a possibility. Thus, any (minor) contamination introduced with the inoculum could easily outgrow the desired microalgal species.

Composition of Culture Media and Micro algal Biomass

Culture Media

For natural phytoplankton (representing a heterogeneous consortium of microalgae), the proportions of the elements are typically derived from the 'Redfield ratio' dating back to 1934, suggesting a molar ratio of $C_{106}N_{16}P_1$ as described in Falkowski (2000) [62]. This has recently been further extended to include other important elements [63, 64]. For heterotrophic cultures of *Chlorella vulgaris*, a molar stoichiometry of $C_{3.96}H_{7.9}O_{1.875}N_{0.685}P_{0.0539}K_{0.036}Mg_{0.012}$ was determined [65], and this has been reflected in optimized media compositions for biomass production in high-cell-density fed batch processes. All of the major molecules in microalgae (i.e. proteins, carbohydrates, and lipids) contain carbon as the principal element, with oxygen, hydrogen and nitrogen at lower, or even zero,

concentrations. Typically, in media for heterotrophic cultures that support optimal growth, all of the constituents are supplied in stoichiometric excess to the organic carbon source. Applying stoichiometric principles to an established medium for photoautotrophic cultures of *Chlorella* spp. [66], the medium was shown to be deficient in iron, magnesium, sulphur and nitrogen. When optimised, a fivefold increase in biomass concentration was achieved [67]. For most of the microalgal species capable of heterotrophic growth, glucose or acetate is an adequate source of energy and carbon. In particular, glucose is used for the production of high-value compounds where the processes need to be reproducible for prospective regulatory approval for pharmaceutical manufacture. Nitrate, ammonia and/or urea are the preferred nitrogen sources at a bioreactor scale [68]. Tryptone, glycine and yeast extract have also been evaluated for their potential to enhance growth or product formation [69]. Moreover, growth data suggest that nitrogen source preference might vary between the species [69, 70]. Yeast extract, a complex component with a high carbon content, is not defined at the single-element level but is frequently used as a source of nitrogen, amino acids, vitamins and trace elements [71].

Biomass Composition

The growth regime together with the microalgae strain and supplied organic substrate greatly influences the molecular composition of microalgae biomass. The composition of biomass is usually reported as the percentage of biomass per dry weight of protein, lipids or carbohydrates or any other specific molecule. Table 3 [72] presents percentage rate of dry weight.

Compound (%)	Photo-autotrophic	Heterotrophic	Mixotrophic
Protein	29-60	10-40	32-40
Carbohydrates	10-20	15-45	10-30
Lipids (Total)	3-28	25-60	11-58
Ash	6.36±0.05	5.93±0.04	7.0±0.02
Other (nucleic acid and pigments)	10.42±0.65	11.20±0.61	9.14±0.78

Table 3: Biomass composition range obtained in photo-autotrophic, heterotrophic and mixotrophic cultivation of microalgae.

Advantage and Limitation of Heterotrophic Cultivation of Microalgae

Advantage

The advantages of heterotrophic cultivation of microalgae, in comparison with photo-autotrophic cultivation are the following:

(a). Higher growth rate and biomass density (also called biomass productivity)

(b). Higher lipid content per dry weight of cells (lipid productivity)

(c). Higher biomass productivity in per area of culture

(d). Cheaper and simple bioreactor design

(e). Easer scaling up process

(f). The possibility to manipulate biomass composition by changing the culture medium's organic substrate that stimulate specific metabolic and biosynthetic pathways and

(g). Potential to remove organic carbon and several types of nitrogen and phosphorus compounds from waste water

Limitation

Heterotrophic cultures have several major limitations:

(a) There is a limited number of microalgal species that can grow heterotrophically

(b) Increasing energy expenses and costs by adding an organic substrate

(c) Contamination and competition with other microorganism

(d) Inhibition of growth by excess organic substrate and

(5) Inability to produce light-induced metabolites.

Metabolic Products and Processes Using Heterotrophic Culture of Microalgae

The main driving force to grow microalgae commercially is harvesting metabolic products, feed for marine and terrestrial organisms, food supplements for humans, or to use the microalgae for environmental processes, such as wastewater treatment, fertilization of soils, biofuels, and phytoremediation of toxic wastes. The main attractiveness of heterotrophic cultivation is that it is potentially substantially cheaper.

Lipids

Several species of microalgae can be induced to overproduce specific fatty acids through relative simple manipulations of the physical and chemical properties of their culture medium. Accumulation of lipids in the microalgae cells, as well as for other oleaginous microorganisms (high oil producers), depends on diverse factors. These include growth temperature, pH, nutritional imbalances of carbon, nitrogen, phosphorous, and silicate, the growth regime (autotrophic, mixotrophic, or heterotrophic), the age of the culture,andthe specificmicroalgal strain [73, 16, 74]. For example, the lipid content in heterotrophically grown cells of *C. protothecoides* is as high as 55%, a quantity that is up to four times greater that autotrophically grown cultures under otherwise similar conditions [75]. In heterotrophic cultures, accumulation could be attributed to consumption of sugars at a rate higher than the rate of cell generation, which would promote conversion of excess sugar into lipids [76-78]. This process is often accomplished in two steps: exponential cell division leading to decreased growth from limits of nutrients, thereby leading to accumulation of lipids [79]. It might not be only related to higher lipid-synthesizing enzymes under nitrogen starvation, but to the cessation of other enzymes associated with cell growth and proliferation and operation of enzymes specifically related to accumulation of lipids [77, 80]. Another proposed mechanism for accumulating lipids under heterotrophic conditions used *E. gracilis* as a model. Under nitrogen starvation accumulation of lipids is attributed to mobilization of lipids from chloroplast membranes as chloroplastic nitrogen is relocated by 1, 5-biphosphate carboxylase/oxygensae (E.C. 4.1.1.39, Rubisco) [81]. After nitrogen starvation, microalgae, such as *C. pyrenoidosa*, *C. sorokiniana*, *Nitzschia alba*, *Skeletonema costatum*, *C. conhii*, accumulate large amounts of lipids, and diatoms respond to depleted silicates by accumulating lipids [40].

Polyunsaturated Fatty Acids

Long-chain polyunsaturated fatty acids (eicosapentaenoic acid, EPA, u-3, C20:5 and docosahexaenoic acid, DHA, u-3, C22:6) are two important fatty acids in early and old age metabolism in humans. They have been used in prevention and treatment of human diseases such as heart and inflammatory diseases and as nutritional supplements in humans and marine organisms in aquaculture. The nitrogen source affects production of EPA by the diatom *N. laevis* in heterotrophic cultures where nitrate and urea are preferred N sources for cell growth and EPA content. Tryptone and yeast extract were found to enhance EPA production [83]. Temperature also influences the fatty acids profile. When temperature is below the optimal growth temperature for the microalgae, more unsaturated fatty acids are metabolized, and the reverse effect occurs at higher temperatures. Reducing temperature by 10-15°C leads to a decrease in membrane fluidness. To compensate for decreasing fluidness, over-expression of the genes for desaturases (acyl-CoA desaturases, acyl-ACP desaturases, and acyl-lipid desaturases) promote desaturation of the membrane lipids. Use of acetic acid as a carbon

source for heterotrophic production of DHA in batch-fed cultures of high cell density of *C. cohnii* resulted in much higher lipid and DHA contents than in cultivation with glucose [83, 78].

Biodiesel

Biofuels from microalgae is an attractive option for microalgae biotechnology. Compared to all other applications, it is one of the most attractive, given the high prices of crude oil. Biodiesel is a suitable substitute for petroleum-based diesel fuel because of its multiple advantages for machines and the environment. heterotrophic microalgae cultivation represents a good source of LCFA [84]; so far, it is a less popular avenue for biodiesel production from microalgae. *C. protothecoides* is a suitable microalga for biodiesel production, heterotrophically using organic carbon sources. This species was able to produce quantities of lipids reaching ~50% of its dry weight. Enzymatic transesterification (converting lipids to biodiesel) was catalyzed by lipase, and the conversion rate reached close to 100% in several trials. One of the potential carbon sources for producing biodiesel heterotrophically is glycerol. Currently, glycerol is an inexpensive and abundant carbon generated as a by-product of biodiesel fuel production. Development of processes to convert this crude glycerol into higher-value products is needed. Given the highly reduced nature of carbon atoms in glycerol, fuel and reduced chemicals can be generated at higher yields than those obtained from common sugars, such as glucose [85, 86]. For example: *Schizochytrium limacinum* produced palmitic acid (16:0) as w45e60% of their dry weight when supplied with glucose, fructose, or glycerol [87, 88], which could potentially be used for biodiesel production.

Pigments

Naturally, all pigments are produced under autotrophic growth conditions, but surprisingly some are produced, and in large quantities, under heterotrophic dark conditions.

Carotenoids

Carotenoids from microalgae have been used for commercial purposes. Carotenoids are lipid-soluble pigments composed of isoprene units that are widely distributed in various classes of microalgae. Carotenoids are divided into two groups: those containing only hydrocarbons (not oxygenated) and xanthophylls that contain oxygen molecules [89]. Among the xanthophylls (zeaxanthin, canthaxantin, and lutein), lutein is considered the principal useful pigment of the group. It has high nutritional value and low toxicity and is used as a pigment for animal tissues (chicken skin and egg yolks coloring), food, cosmetics, and pharmaceutical products, such as an effective agent for prevention and treatment of a variety of degenerative diseases [90, 91]. Lutein is an intracellular product of *Chlorella*. This genus is used for production of lutein. Photoautotrophic systems produce low biomass; hence, heterotrophic cultivation represents an alternative. Increasing glucose concentration increases lutein production, but urea is currently the optimal source of nitrogen [90, 92-94]. Astaxanthin production by microalgae increases under stress conditions and is present in the esterified form and stored in lipid bodies outside the chloroplast, which enables green algae to accumulate a considerable amount [95]. *Haematococcus pluvialis* is the main producer of astaxanthin under autotrophic conditions but *C. zofingiensis* is superior in yield when heterotrophically cultivated with glucose [96]. The red microalgae *G. sulphuraria* can produce phycocyanin in carbon-limited but nitrogen-sufficient heterotrophic cultures; the content increases in the stationary phase. Although production of phycocyanin in this microalga is lower than in *S. platensis*, its ability to grow heterotrophically makes it a potential supplier of this pigment [97]. Another study found that this microalga produced more phycocyanin in heterotrophic batch-fed cultures of *G. sulphuraria* than is commonly attained in outdoor, sunlight-dependent cultures of *S. platensis* [98]

Waste Water Treatment

As mentioned earlier, tertiary wastewater treatment by microalgae is an old idea that so far has very limited application. This is directly related to the costs involved in treating very large volumes of wastewater in a timely manner under autotrophic conditions [99]. Heterotrophic wastewater treatment is a novel idea that so far has been studied at the laboratory scale. The most efficient carbon source for using *C. vulgaris* to treat wastewater heterotrophically was calcium acetate (Perez-Garcia et al., in press). Subjecting the autotrophic, immobilized microalgae-bacteria system for wastewater treatment [100-102] to heterotrophic conditions revealed even higher potential of the system to eliminate nutrients [103]. The new data cannot provide a solid prediction about the practical potential of this approach.

Genetic and Metabolic Engineering for Improving Existing Heterotrophic Microalgae

Genetic and metabolic engineering can be used to expand heterotrophic capabilities of strains to growth on a specific organic substrate or developing a metabolic pathway for a novel product synthesis by inserting the genes for these pathways. There is an increasing interest in use transgenic microalgae as green cell factories capable of producing biofuels and valuable proteins and carbohydrates [72]. Now-a-days few microalgae, including the green algae *Chlamydomonas reinhardii*, *Nannochloropsis gaditana*, and *Osterococcus tauri* or the diatoms *Phaeodactylum triconutum* are currently established as platforms for genetic and metabolic engineering [72]. Successful metabolic engineering of strains involves the sequencing of a wild type strain, a systematic description of the organism genotype and phenotype using "omics" techniqes, then the application of molecular and genetic techniques for metabolic engineering. Metabolic engineering of a specific strain has one of the following goals: a. expand metabolic capabilities by adding new genes to an organisms, b. add genes encoding for transporters, c. increase formation of a pre arabinose, citrate, fructose, malate, lactic acid, lactose, peptone, urea, fulvic acids, ethanol, methanol, and sucrose have been tested for heterotrophic cultivation of microalgae. Chlorella vulgaris culture on the above mentioned carbon sources reached significantly lower biomass concentration in comparison to culture on acetate or glucose [72].

Concluding and Future Prospects

Cultivation of microalgae that are primarily photosynthetic under heterotrophic dark conditions for production of economically useful metabolites or technological processes is a tempting option, given significant reductions in complexity of cultivation and costs. Because heterotrophic growth consumes simple, cheap, and available carbon sources (glucose, acetate, glycerol) that are commonly used by fermentation industries for other aims, it is predicted that adoption of this approach is an easy, uncomplicated task. Fortunately, some of the most common and best-studied microalgae, such as Chlorella, are also heterotrophs. This information can jump start research in heterotrophy, which is probably quite prevalent among microalgae [104,105]. Furthermore, with current developments in genomics, bioinformatics analyses, and genetic and metabolic engineering, new approaches in microalgae biotechnology, including heterotrophy, have emerged [106,107]. As a result of genetic engineering, some obligate photoautotrophs were transformed to heterotrophy through the introduction of sugar transporters. *Volvox carteri* was one of the first green algae to be transformed with the hexose/Hþ symporter gene derived from *Chlorella sp.* [108]. These examples of a simple genetic transformation of single gene of a sugar transporter in the outer membrane of microalgae show the

feasibility to convert microalgae from a photoautotrophic into a heterotrophic organism when sugar is present in the absence of light. Such genetic engineering is probably acceptable by society because microalgae cultivation can be independently managed without risk of environmental contamination.

Heterotrophic cultivation of microalgae is a niche of microalgae research field. Yet, the potential of expansion because of the advantages it offers are limitless. Only time will prove if this strategic approach will catch up with the industry.

Acknowledgments

The authors are thankful to the authorities of Designated Reference Institute for Chemical Measurements (DRiCM), Bangladesh Council of Scientific and Industrial Research (BCSIR) and Algasol Bangladesh Limited for providing required information and valuable suggestion.

References

1. Chen GQ, Chen F (2006) Growing phototrophic cells without light. See comment in PubMed Commons below Biotechnol Lett 28: 607-616.
2. Pereira H, Barreira L, Mozes A, Florindo C, Polo C, et al. (2011) Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae. See comment in PubMed Commons below Biotechnol Biofuels 4: 61.
3. Olaizola M (2003) Commercial development of microalgal biotechnology: from the test tube to the marketplace. See comment in PubMed Commons below Biomol Eng 20: 459-466.
4. Hogg, Stuart (2001) Essential Microbiology (2nd ed.) Wiley-Black well. p. 86. ISBN 978-1-119-97890-9.
5. Cheng Y, Zhou W, Gao C, Lan K, Gao Y, et al. (2009) Biodiesel production from Jerusalem artichoke (Helianthus tuberosus L.) tuber by heterotrophic microalgae Chlorella protothecoides. Soc Chem Ind, 84: 777–781.
6. O'Grady J, Morgan JA (2011) Heterotrophic growth and lipid production of Chlorella protothecoides on glycerol. See comment in PubMed Commons below Bioprocess Biosyst Eng 34: 121-125.
7. Perez-Garcia O, Escalante FME, de-Bashan LE, Bashan Y. (2011) Heterotrophic cultures of microalgae: metabolism and potential products. Water Res, 45(1): 11–36.
8. Chen F (1996) High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol, 14: 412–426.
9. Eriksen NT (2008) The technology of microalgal culturing. See comment in PubMed Commons below Biotechnol Lett 30: 1525-1536.
10. Gouveia L, Oliveira AC (2009) Microalgae as a raw material for biofuels production. See comment in PubMed Commons below J Ind Microbiol Biotechnol 36: 269-274.
11. Chen GQ, Chen F (2006) Growing phototrophic cells without light. See comment in PubMed Commons below Biotechnol Lett 28: 607-616.
12. Mojtaba A, Mohd S, Rosfarizan M, Raha A, Arbakariya B, et al. (2011) Improvement of medium composition for heterotrophic cultivation of green microalgae, Tetraselmis suecica, using response surface methodology. Biochem Eng J, 53: 187–195.
13. Chen F (1996) High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol, 14: 412–426.
14. Vazhappilly R, Chen F (1998) Eicosapentaenoic acid and docosahexanoic acid production potential of microalgae and their heterotrophic growth. J Am OIL Chem Soc, 75 (3): 393-397.
15. Pal D, Khozin-Goldberg I, Cohen Z, Boussiba S (2011) The effect of light, salinity, and nitrogen availability on lipid production by Nannochloropsis sp. See comment in PubMed Commons below Appl Microbiol Biotechnol 90: 1429-1441.
16. Wen ZY, Chen F (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. See comment in PubMed Commons below Biotechnol Adv 21: 273-294.

17. Engel N, Jenny TA, Mooser V, Gossauer A (1991) Chlorophyll catabolism in Chlorella protothecoides. Isolation and structure elucidation of a red bilin derivative. See comment in PubMed Commons below FEBS Lett 293: 131-133.

18. Engel N, Jenny TA, Mooser V, Gossauer A (1991) Chlorophyll catabolism in Chlorella protothecoides. Isolation and structure elucidation of a red bilin derivative. See comment in PubMed Commons below FEBS Lett 293: 131-133.

19. Hortensteiner S., Chinner, J., Matile, P., Thomas, H., Donnison, I.S., et al. (2000) Chlorophyll breakdown in Chlorella protothecoides: characterization of de-greening and cloning of de-greening-related genes. Plant Mol Biol, 42: 439–450.

20. Chen F, Johns MR (1991) Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic Chlorella sorokiniana. Appl Phycol, 3: 203–209.

21. Chen CY1, Yeh KL, Aisyah R, Lee DJ, Chang JS (2011) Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. See comment in PubMed Commons below Bioresour Technol 102: 71-81.

22. Brennan L; Owende P (2010) Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. 14: 557–577.

23. Nakas JP1, Schaedle M, Parkinson CM, Coonley CE, Tanenbaum SW (1983) System development for linked-fermentation production of solvents from algal biomass. See comment in PubMed Commons below Appl Environ Microbiol 46: 1017-1023.

24. Liam Brennan, Philip Owende (2009) Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sustain Energy Rev, 805: 1-21.

25. Xiong W1, Li X, Xiang J, Wu Q (2008) High-density fermentation of microalga Chlorella protothecoides in bioreactor for microbio-diesel production. See comment in PubMed Commons below Appl Microbiol Biotechnol 78: 29-36.

26. Howitt SM1, Udvardi MK (2000) Structure, function and regulation of ammonium transporters in plants. See comment in PubMed Commons below Biochim Biophys Acta 1465: 152-170.

27. Endo H, Nakajima K, Chino R, Shirota M (1974) Growth characteristics and cellular components of Chlorella regularis, heterotrophic fast growing strain. Agric. Biol. Chem. 38: 9-18.

28. Chen F, Johns MR (1991) Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic Chlorella sorokiniana. J. Appl. Phycol. 3: 203-209.

29. Griffiths DJ, Thresher CL, Street HE (1960) The heterotrophic nutrition of Chlorella vulgaris. Ann. Bot. 24: 1-11.

30. Boyle NR, Morgan JA (2009) Flux balance analysis of primary metabolism in Chlamydomonas reinhardtii. See comment in PubMed Commons below BMC Syst Biol 3: 4.

31. Martínez F, Orús MI (1991) Interactions between Glucose and Inorganic Carbon Metabolism in Chlorella vulgaris Strain UAM 101. See comment in PubMed Commons below Plant Physiol 95: 1150-1155.

32. Endo H, Nakajima K, Chino R, Shirota M (1974) Growth characteristics and cellular components of Chlorella regularis, heterotrophic fast growing strain. Agric. Biol. Chem. 38: 9-18.

33. Droop MR (1974) Heterotrophy of carbon. In: Stewart, W.D.P. (Ed.), Algal Physiology and Biochemistry. Blackwell Scientific, Oxford, UK, pp. 530-559.

34. Neilson AH, Lewin RA (1974) The uptake and utilization of organic carbon by algae: an essay in comparative biochemistry. Phycologia, 13: 227-264.

35. Dayanidhi Sarkar, Kazuyuki Shimizu (2015) An overview on biofuel and biochemical production by photosynthetic microorganisms with understanding of the metabolism and by metabolic engineering together with efficient cultivation and downstream processing, Bioresources and Bioprocessing, 2 (17): 1-19.

36. Bollman RC, Robinson GGC (1977) The kinetics of organic acid uptake by three Chlorophyta in axenic culture. J. Phycol 13: 1-5.

37. Richmond A (1986) Cell response to environmental factors. In: Richmond, A. (Ed.), Handbook for Microalgal Mass Culture. CRC Press, Boca Raton, FL., USA, pp. 69-99.

38. Lea PJ, Miflin BJ (2003) Glutamate synthase and the synthesis of glutamate in plants. Plant Physiol. Biochem. 41: 555-564.

39. Fernandez E, Galvan A (2007) Inorganic nitrogen assimilation in Chlamydomonas. See comment in PubMed Commons below J Exp Bot 58: 2279-2287.

40. Wenguang Zhou (2014) Potential Applications of Microalgae in Wastewater Treatments, Recent Advances in Microalgal Biotechnology, OMICS Group eBooks pp: 1-10.

41. Wilhelm C, Büchel C, Fisahn J, Goss R, Jakob T, et al. (2006) The regulation of carbon and nutrient assimilation in diatoms is significantly different from green algae. See comment in PubMed Commons below Protist 157: 91-124.

42. Allen AE, Ward BB, Song B (2005) Characterization of diatom (Bacillariophyceae) nitrate reductase genes and their detection in marine phytoplankton communities. J. Phycol. 41: 95-104.

43. Howitt SM, Udvardi MK (2000) Structure, function and regulation of ammonium transporters in plants. See comment in PubMed Commons below Biochim Biophys Acta 1465: 152-170.

44. Tischner R (1984) Evidence for the participation of NADP glutamate dehydrogenase in the ammonium assimilation of Chlorella sorokiniana. Plant Sci. Lett. 34: 73-80.

45. Kaplan D, Richmond AE, Dubinsky Z, Aaronson S (1986) Algal nutrition. In: Richmond, A. (Ed.), Handbook for Microalgal Mass Culture. CRC Press, Boca Raton, FL., USA, pp. 147-198.

46. Lu B, Yuan Y, Zhang C, Ou J, Zhou W, Lin Q (2005) Modulation of key enzymes involved in ammonium assimilation and carbon metabolism by low temperature in rice (Oryza sativa L.) roots. Plant Sci. 169: 295-302.

47. Vanoni MA, Curti B (2005) Structure--function studies on the iron-sulfur flavoenzyme glutamate synthase: an unexpectedly complex self-regulated enzyme. See comment in PubMed Commons below Arch Biochem Biophys 433: 193-211.

48. Inokuchi R, Kuma KI, Miyata T, Okada M (2002) Nitrogen-assimilating enzymes in land plants and algae: phylogenic and physiological perspectives. See comment in PubMed Commons below Physiol Plant 116: 1-11.

49. Combres C, Laliberte´ G, Sevrin Reyssac J, de la Nou¨ eJ (1994) Effect of acetate on growth and ammonium uptake in the microalga Scenedesmus obliquus. Physiol. Plantarum 91: 729-734.

50. de-Bashan LE, Antoun H, Bashan Y (2005) Cultivation factors and population size control uptake of nitrogen by the microalgae Chlorella vulgaris when interacting with the microalgae growth-promoting bacterium Azospirillum brasilense. FEMS Microbiol. Ecol. 54: 197-203.

51. Cero´n Garci´a MC, Ferna´ndez Sevilla JM, Acie´n Ferna´ndez FG, Molina Grima E, et al. (2000) Mixotrophic growth of Phaeodactylum tricornutum on glycerol: growth rate and fatty acid profile. J. Appl. Phycol., 12: 239-248.

52. Lee Y-K (2001) Microalgal mass culture systems and methods: their limitation and potential. J. Appl. Phycol. 13: 307-315.

53. Behrens PW (2005) Photobioreactor and fermentors: the light and the dark sides of the growing algae. In: Andersen, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, New York, USA, pp. 189-204.

54. Chen GQ, Chen F (2006) Growing phototrophic cells without light. See comment in PubMed Commons below Biotechnol Lett 28: 607-616.

55. Geider RJ, Osborne BA (1989) Respiration and microalgal growth: a review of the quantitative relationship between dark respiration and growth. New Phytol. 112: 327-341.

56. Wu Z, Shi X (2007) Optimization for high-density cultivation of heterotrophic Chlorella based on a hybrid neural network model. See comment in PubMed Commons below Lett Appl Microbiol 44: 13-18.

57. Yang C, Hua Q, Shimizu K (2000) Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/ dark-heterotrophic conditions. Biochem. Eng. J. 6: 87-102.

58. Gladue RM, Maxey JE (1994) Microalgal feeds for aquaculture. J. Appl. Phycol. 6: 131-141.

59. Fabian Bumbak, Stella Cook, Vilém Zachleder, Silas Hauser, and Karin Kova, et al. (2011) mBest practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations, Appl Microbiol Biotechnol. 91(1): 31–46.

60. Shi X, Zhang X, Chen F (2000) Heterotrophic production of biomass and lutein by Chlorella protothecoides on various nitrogen sources. See comment in PubMed Commons below Enzyme Microb Technol 27: 312-318.

61. Riesenberg D1, Guthke R (1999) High-cell-density cultivation of microorganisms. See comment in PubMed Commons below Appl Microbiol Biotechnol 51: 422-430.

62. Lee YK (1997) Commercial production of microalgae in the Asiae Pacific rim. J. Appl. Phycol. 9: 403-411.

63. Falkowski PG (2000) Rationalizing elemental ratios in unicellular algae. J Phycol 36(1):3–6

64. Ho TY, Quigg A, Finkel ZV, Milligan AJ, Wyman K, et al. (2003) The elemental composition of some marine phytoplankton. J Phycol., 39(6): 1145–1159.

65. Quigg A, Finkel ZV, Irwin AJ, Rosenthal Y, Ho TY, et al. (2003) The evolutionary inheritance of elemental stoichiometry in marine phytoplankton. See comment in PubMed Commons below Nature 425: 291-294.

66. Sansawa H, Endo H (2004) Production of intracellular phytochemicals in Chlorella under heterotrophic conditions. See comment in PubMed Commons below J Biosci Bioeng 98: 437-444.

67. Vonshak A (1986) Laboratory techniques for the cultivation of microalgae. In: Richmond A (ed) Handbook of microalgal mass culture. CRC, Boca Raton, FL, pp 117–145.

68. Mandalam RK, Palsson BO (1998) Elemental balancing of biomass and medium composition enhances growth capacity in high-density Chlorella vulgaris cultures. See comment in PubMed Commons below Biotechnol Bioeng 59: 605-611.

69. Grobbelaar JU (2004) Algal nutrition: mineral nutrition. In: Richmond A (ed) Handbook of microalgal culture: biotechnology and applied phycology. Blackwell, Oxford, pp 97–115.

70. Shen Y, Yuan W, Pei Z, Mao E (2010) Heterotrophic culture of Chlorella protothecoides in various nitrogen sources for lipid production. See comment in PubMed Commons below Appl Biochem Biotechnol 160: 1674-1684.

71. Xiong W, Li XF, Xiang JY, Wu QY (2008) High-density fermentation of microalga Chlorella protothecoides in bioreactor for micro biodiesel production. Appl Microbiol Biotechnol 78(1):29–36. doi:10.1007/s00253-007-1285-1

72. GRANT CL, PRAMER D (1962) Minor element composition of yeast extract. See comment in PubMed Commons below J Bacteriol 84: 869-870.

73. Aleš Prokop, Rakesh K Bajpai, Mark E, Zappi (2015) Microlagal heterotrophic and mixotrophic culturing for bio-refining: from metabolic route to Techno economics. Growth of Algal Biorefineries: Volume 2: Products and Refinery Design, pp: 74-77.

74. 5Ratledge C1, Kanagachandran K, Anderson AJ, Grantham DJ, Stephenson JC (2001) Production of docosahexaenoic acid by Crypthecodinium cohnii grown in a pH-auxostat culture with acetic acid as principal carbon source. See comment in PubMed Commons below Lipids 36: 1241-1246.

75. Chisti Y1 (2007) Biodiesel from microalgae. See comment in PubMed Commons below Biotechnol Adv 25: 294-306.

76. Xu H1, Miao X, Wu Q (2006) High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters. See comment in PubMed Commons below J Biotechnol 126: 499-507.

77. Chen F, Johns MR (1991) Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic Chlorella sorokiniana. J. Appl. Phycol. 3: 203-209.

78. Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. See comment in PubMed Commons below Adv Appl Microbiol 51: 1-51.

79. de Swaaf, ME, Sijtsma L, Pronk JT (2003) High-cell-density fedbatch cultivation of the docosahexaenoic acid producing marine alga Crypthecodinium cohnii. Biotechnol. Bioeng. 81: 666-672.

80. Leman J (1997) Oleaginous microorganisms: an assessment of the potential. See comment in PubMed Commons below Adv Appl Microbiol 43: 195-243.

81. Ganuza E1, Anderson AJ, Ratledge C (2008) High-cell-density cultivation of Schizochytrium sp. in an ammonium/pH-auxostat fed-batch system. See comment in PubMed Commons below Biotechnol Lett 30: 1559-1564.

82. Garcia-Ferris C, de los Rios A, Ascaso C, Moreno J (1996) Correlated biochemical and ultra-structural changes in nitrogen-starved Euglena gracilis. J. Phycol., 32: 953-963.

83. Wen ZY, Chen F (2001a) Optimization of nitrogen sources for heterotrophic production of eicosapentaenoic acid by the diatom Nitzschia laevis. Enzyme Microb. Technol. 29: 341-347.

84. Jiang Y, Chen F (2000) Effects of temperature and temperature shift on docosahexaenoic acid production by the marine microalga Crypthecodinium cohnii. J. Am. Oil. Chem. Soc. 77: 613-617.

85. Wen ZY1, Chen F (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. See comment in PubMed Commons below Biotechnol Adv 21: 273-294.

86. Yazdani SS1, Gonzalez R (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. See comment in PubMed Commons below Curr Opin Biotechnol 18: 213-219.

87. Murarka A1, Dharmadi Y, Yazdani SS, Gonzalez R (2008) Fermentative utilization of glycerol by Escherichia coli and its implications for the production of fuels and chemicals. See comment in PubMed Commons below Appl Environ Microbiol 74: 1124-1135.

88. Yokochi T, Honda D, Higashihara T, Nakahara T (1998) Optimization of docosahexaenoic acid production by Schizochytrium limacinum SR21. Appl. Microbiol. Biotechnol. 49: 72-76.

89. Chi Z, Pyle D, Wen Z, Frear C, Chen S (2007) A laboratory study of producing docosahexaenoic acid from biodieselwaste glycerol by microalgal fermentation. Process Biochem. 42: 1537-1545.

90. Cohen Z (1986) Products from microalgae. In: Richmond, A. (Ed.), Handbook for Microalgal Mass Culture. CRC Press, Boca Raton, FL., USA, pp. 421-454.

91. Shi XM, Chen F, Yuan JP, Chen H (1997) Heterotrophic production of lutein by selected Chlorella strains. J. Appl. Phycol. 9: 445-450.

92. Pulz O1, Gross W (2004) Valuable products from biotechnology of microalgae. See comment in PubMed Commons below Appl Microbiol Biotechnol 65: 635-648.

93. THERIAULT RJ (1965) HETEROTROPHIC GROWTH AND PRODUCTION OF XANTHOPHYLLS BY CHLORELLA PYRENOIDOSA. See comment in PubMed Commons below Appl Microbiol 13: 402-416.

94. Shi X, Zhang X, Chen F (2000) Heterotrophic production of biomass and lutein by Chlorella protothecoides on various nitrogen sources. See comment in PubMed Commons below Enzyme Microb Technol 27: 312-318.

95. Wang Y, Peng, J (2008) Growth-associated biosynthesis of astaxanthin in heterotrophic Chlorella zofingiensis (Chlorophyta). World J. Microbiol. Biotechnol. 24: 1915-1922.

96. Ip PF, Chen F (2005a) Production of astaxanthin by the green microalga Chlorella zofingiensis in the dark. Process Biochem. 40: 733-738.

97. Sloth JK, Wiebe MG, Eriksen NT (2006) Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga Galdieria sulphuraria. Enzyme Microb. Technol. 38: 168-175.

98. Schmidt RA, Wiebe MG, Eriksen NT (2005) Heterotrophic high cell-density fed-batch cultures of the phycocyanin-producing red alga Galdieria sulphuraria. See comment in PubMed Commons below Biotechnol Bioeng 90: 77-84.

99. de-Bashan LE, Bashan Y (2010) Immobilized microalgae for removing pollutants: review of practical aspects. See comment in PubMed Commons below Bioresour Technol 101: 1611-1627.

100. de-Bashan LE, Bashan Y, Moreno M, Lebsky VK, Bustillos, JJ (2002) Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae Chlorella spp. When co-immobilized in alginate beads with the microalgae-growth promoting bacterium Azospirillum brasilense. Can. J. Microbiol. 48: 514-521.

101. de-Bashan LE, Hernandez JP, Morey T, Bashan Y (2004) Microalgae growth-promoting bacteria as “helpers” for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res. 38: 466-474.

102. Hernandez JP, de-Bashan, LE, Bashan Y (2006) Starvation enhances phosphorus removal from wastewater by the microalgae Chlorella spp. co-immobilized with Azospirillum brasilense. Enzyme Microb. Technol. 38, 190e198.

103. Perez-Garcia O, de-Bashan LE, Hernandez JP, Bashan Y (2010) Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, and mixotrophic cultivation of Chlorella vulgaris immobilized with Azospirillum brasilense. J. Phycol 46: 800-812.

104. Tuchman N (1996) The role of heterotrophy in algae. In: Stevenson, R.J., Bothwell, M., Lowe, R.L. (Eds.), Algal Ecology. Freshwater Benthic Ecosystems. Academic Press, San Diego, CA, USA, pp. 299-319.

105. Hellebust, JA, Lewin J (1977) Heterotrophic nutrition. In: Werner, D. (Ed.), The Biology of Diatoms. Bot. Monogr., vol. 13. University of California Press, Los Angeles, CA, USA, pp. 169-197.

106. Hong, SJ, Lee CG (2007) Evaluation of central metabolism based on a genomic database of Synechocystis PCC6803. Biotechnol. Bioprocess Eng. 12: 165-173.

107. Boyle NR, Morgan JA (2009) Flux balance analysis of primary metabolism in Chlamydomonas reinhardtii. See comment in PubMed Commons below BMC Syst Biol 3: 4.

108. Hallmann A, Sumper M (1996) The Chlorella hexose/H+ symporter is a useful selectable marker and biochemical reagent when expressed in Volvox. See comment in PubMed Commons below Proc Natl Acad Sci U S A 93: 669-673.

Genetic Engineering of Microalgae

Ying-Fang Niu, Tan Huang, Wei-Dong Yang, Jie-Sheng Liu and Hong-Ye Li*

Key Laboratory of Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, College of Life Science, Jinan University, Guangzhou-510632, Chinal

***Corresponding author:** Hong-Ye Li, Key Laboratory of Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, College of Life Science, Jinan University, Guangzhou-510632, China, Tel: +86 20 85228470; E-mail: thyli@jnu.edu.cn

Abstract

In recent years, the pollution caused by excessive utilization of fossil energy, and the decreasing storage of fossil energy, both have stimulated the reemergence of alternative fuels like microalglal biofuels. Microalgae have been proposed to be the important source for the production of bioactive compounds, especially for the production of lipid-based biodiesel. High-lipid content microalgae strains could be domesticated and further genetically improved in order to redirect metabolic fluxes towards metabolic pathways responsible for lipid production. This review summarizes the current knowledge about metabolic engineering of microalgae for increasing the cellular lipid content, with a special emphasis on triacylglycerols for the production of biofuels. In addition, this work outlines the contribution of systems biology and genome-scale metabolic pathway modeling to genetic manipulation of algae, and visualizes their potential impact in the future.

Abbreviations

CaMV - Cauliflower Mosaic Virus; TAG - Triacylglycerol; CAT - Chloramphenicol Acetyltransferase; Fcp Fucoxanthin Chlorophyll a/c Binding Protein; NCBI - National Center for Biotechnology Information.

Introduction

The renewable energy becomes a most imminent issue due to the environmental pollution caused by combustion of fossil fuels. Biofuels are the only recyclable source of liquid fuel compounds and they will be a crucial component of renewable energy. Large-scale biofuels production based on agricultural crops takes up food feedstocks and occupies arable land, while algal biofuels could be an ideal candidate without these drawbacks. As a biofuel producer, microalgae possess many favorable traits. First, it can accumulate considerable oil bodies that are considered as a potential renewable feedstock for biofuel production. Besides, microalgae have much higher biomass productivity, thus researchers may develop methods to increase the production of value-added products. Also, algae culturing facilities could be located in aquatic environments, eliminating the utilization of arable land. These together have prompted intense interest in promoting the ability of microalgae to produce lipids, which can be converted into biodiesel easily [1].

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

Although microalgae have reemerged as the most promising feedstocks for biofuels, large-scale production of biofuels is hampered by the availability of few algal strains that can be selectively optimized for both high biomass productivity and high Triacylglycerol (TAG) content [2]. *Dunaliella salina, Chlorella sp., Chlamydomonas reinhardtii, Muriellopsis sp., Haematococcus pluvialis*, *Phaeodactylum tricornutum, Nannochloropsis sp.* are the potential economical and model microalgae at present. One potential solution is to modify highly oil-yielding microalgal strains by genetic engineering, based on the crucial enzymes that can be targeted for Triacylglycerol (TAG) accumulation and molecular understanding of lipid metabolic pathway in microalgae [3,4].

kThe algal fatty acid biosynthetic pathway has been deduced in silico by homology with those of bacteria and plants. Fatty acid synthase enzymes have been studied and notated in many sequenced algae, like *Chlamydomonas reinhardtii* [5,6]. Chloroplasts are the major site where fatty acid is produced. And in the algal chloroplast, fatty acids are biosynthesized by a type II FAS, a group of dissociated enzymes that elongate a growing fatty acid by two carbon units in a stepwise and repetitive pathway [7]. This process begins as the Acetyl-CoA Carboxylase (ACCase) carries out the carboxylation of acetyl-CoA to form malonyl-CoA, which has been considered the rate-limiting step in fatty acid biosynthesis in plants [8,9]. Malonyl-CoA gets loaded onto the Acyl Carrier Protein (ACP) which acts as a metabolic scaffold during fatty acid biosynthesis and must first get modified by a Phosphopantetheinyl Transferase (PPTase), In the ensuing steps, a group of enzyme, including b-Ketoacyl-ACP-synthase III, Ketoreductase (KR), Dehydratase (DH), and Enoyl Reductase (ER) enzymes, work collectively to form the precursors for fatty acids elongation. Then KASI elongates C4-C16 and KASII forms C18 from C16, respectively. And finally the mature fatty acid is either retained in the plastid, where it is transferred to glycerol-3-phosphate by an Acyl Transferase (AT) as the element of membrane lipids; or transported to the cytosol, where it is hydrolyzed by a Thioesterase Domain (TE), releasing the fatty acid from the FAS for plastid export.

And the biosynthesis of TAGs is based on the de novo fatty acid synthesis, some newly synthesized fatty acids are bound to release from plastids, in the cytosol they are converted to acyl-CoA esters and thereafter transferred to the ER for the storage TAGs [10]. TAGs consist of three FA chains esterified via the hydroxyl groups of a glycerol backbone. The Kennedy pathway is well characterized and one of the most straightforward TAG biosynthesis pathways; it consists of iterative acylation, adding to each hydroxyl group of glycerol initiating with glycerol-3- phosphate [11].

With the advent of established methods for characterization and manipulation of algal genomes, breeding and genetic engineering of microalgae will be available to develop economically viable strains of algae. Below we will discuss the recent advances in microalgal genetic engineering, including genetic transformation, stable heterologous gene expression, as well as other potential methods to use molecular genetics to promote microalgal biofuel production.

Advances of Metabolic Engineering in Microalagae

Products such as proteins, lipids, starch and alkanes from microalgae are of great interest [12,13] because of a vast amount of useful chemicals and fuels that can be derived from them [14]. The availability of an increasing variety of unicellular eukaryotic microalgae for genetic manipulation makes them promising producers of bioactive compounds. The eukaryotic algae possess a group of advantages for applications in the field of molecular biotechnology, with many of them already described in established bio-economy driven approaches. Fatty acid and triacylglycerol metabolic network of photoautotrophic cells has been determined many years ago [15,16]. Various researches [3,6] have been released when the genome of microalgae have been sequenced. Mechanism of Carbon acquisition by diatoms has been clearly described [16]. Producing algal biofuels exploits the ability of algae

to produce oils using only sunlight, carbon dioxide and water. Microalgae accumulate oil as nonpolar storage lipids, such as TAGs [1]. The photosynthetic and cellular membranes of algae also contain polar lipids, such as glycolipids, phospholipids and sterols. Oils from algae can yield biodiesel through transesterification [14]. These endeavor and achievements forms the foundation of our understanding of molecular biology of algae and pave the avenue of genetically modifying microalgae for oil accumulating.

For example, a strategy of blocking starch biosynthesis to direct carbon precursors into lipid biosynthesis has been carried out [17,18], and Trentacoste et al., report that the targeted knockdown of a multifunctional lipase/phospholipase/acyltransferase increased lipid yields without affecting growth in the diatom *Thalassiosira pseudonana* [19]. And in the case of the diatom *Phaeodactylum tricornutum*, Niu established stable nuclear transformation system, and increased the neutral lipid accumulation significantly in the *P. tricornutum* [13].

Algal biofuel producers have a higher productivity per ground area than agricultural crops do. While efficient production of non-volatile components in eukaryotic microalgae also requires fast secretion mechanisms. The separation of the product from the catalytic reaction site reduces the risk of protein degradation by cytosolic proteases, avoids product feedback inhibition and even offers the opportunity to produce and accumulate components that have the potential negative interference with the cell metabolism. Efficient product secretion is therefore also a crucial pre-condition for continuous cultivation strategies. The process of product secretion is well understood for proteins in eukaryotic algae. Protein secretion signals in *C. reinhardtii* are therefore already introduced in the most updated molecular engineering strategies. As a proof of concept regarding with the combination of synthetic biology, plate level protein abundance assay and recombinant protein quantification was recently used to demonstrate efficient nuclear gene expression combined with extracellular localization in two independent approaches [20,21]. In contrast, strategies for engineering efficient hydrocarbon secretion mechanisms into suitable eukaryotic hosts have not been sufficiently described and are therefore one of the most challenging tasks of algal biotechnology research in the near future.

In summary, the ability of eukaryotic microalgae to accumulate and store secondary metabolites in cellular compartments and to efficiently produce and secrete functionally active protein products make these organisms ideal hosts and powerful alternatives of cyanobacteria in industrial biotechnology. Promising strategies for the construction of more efficient algal strains in the future will be based on the availability of advanced synthetic biology tools for metabolic engineering via transformation of heterologous (prokaryotic and eukaryotic) genes in established eukaryotic hosts such as *C. reinhardtii*.

Methods for Transformation of Microalgae

Advances of Transformation of Microalgae

To date, microalgae have been transformed successfully through various transformation methods. The popular transformation methods are biolistic transformation and electroporation. In some cases, transformation resulted in the successful and stable expression of transgenes from either the nucleus or the plastid, but in most cases, only transient expression was observed. Until now, some efforts to increase the lipid yield of microalgae have been concentrated mainly on the optimization of growth and induction conditions, such as salinity, temperature, light, and nutrient depletion [22-24], while genetic modifications of microalgae to alter either lipid quantity or quality (i.e., composition) are rarely reported. The main reason is probably the lack of a generally applicable transformation protocol for microalgae. Since microalgae are such a diverse group of organisms, it cannot be guaranteed that a method that works for one species could be applied to another one. For example, some species such as *D. salina*, do not possess a rigid cell wall, whereas diatoms often have a very rigid silicate shell which make diatoms recalcitrant to artificial transformation, such as *P. triconutum*.

Available selective marker is another problem. Although auxotrophy markers are available for some species such as *C. reinhardtii*, stable transformation of other species still has to rely on transformed genes conferring resistance to antibiotics. These antibiotics might include some substances routinely used in the transformation of plants, such as zeocin and kanamycin. Also, heterologous gene expression in microalgae calls for preferential codon usage and appropriate promoter sequences to modulate expression. Undoubtedly this will be improved by an increasing number of fully sequenced and annotated microalgal genomes.

Above all, any protocol for the genetic transformation of a new microalgal strain has to be carefully modified to meet its specific requirements and overcome corresponding limitations. Despite the obstacles described above, genetic modification is still one of the main tools to study metabolic pathways in microalgae, and strongly contribute to our understanding of their metabolic biology. Genetic engineering is expected to be one of the main tools that will lead to sustainable and economically viable biofuels from [25-27].

Electroporation

Gene transformation by electroporation has been widely used in various cells for more than 30 years because of the simplicity and high efficiency of the procedure with a small amount of DNA (Neumann et al., 1982, Zimmermann et al., 1975). Transfer of exogenous genes by electroporation theoretically applies to all kinds of cells regardless of the cell's competence. The first application in cyanobacterium was performed in unicellular *Synechococcus sp.* NKBG042902-YG 1116 [28]. The electric field strength for cyanobateria was lower than that for fresh water strains. And the decrease in transformation efficiency due to the lowered electric field strength can be compensated by the enhancement of $CaCl_2$ pretreatment of algae. Aptly changing membrane structure could also increase the transformation efficiency, for example, we cultivate the diatom with f/2-si medium and get less rigid algal cell walls [29]. Efficient electroporation-mediated transformation was gained in both wild-type and cell wall-deficient eukaryotic *C. reinhardtti* strains [30]. The efficiency of electroporation was two orders of magnitude higher than that obtained with the glass beads method (Shimogawara et al., 1998). Currently, the electroporation transformation was established in many marine genera ranging from prokaryotic cells to eukaryotic red algae, green algae and diatoms.

Recently, the most popular microalga for potential biofuels production is known as the *Nannochloropsis sp.* and *Chlorella vulgar,* were successfully genetically transformed by electroporation, and several genes involved in the nitrogen metabolism were modified by the homologous recombination method [31,32]. Genetically engineered *P. tricornutum* was also acquired by microprojectile bombardment [33], where a variety of selectable markers and reporter genes were tested. Previously most of diatom transformation relied on microprojectile bombardment. However, microprojectile bombardment is not routinely available, and its high cost also limits its application in many laboratories. In contrast, electroporation is commonly used without the need of special consumables. The application of the electroporation technology in brown algae is constrained by undeveloped protoplast preparation and regeneration technologies, nevertheless this technique will rapidly advance subsequent functional researches and the application in biotechnologies [13,29].

Biolistic Transformation

Gene introduced by the biolistic method (microprojectile bombardment) has been proven to be the most efficient method and is highly reproducible in introducing exogenous DNA into algal cells. This protocol has been successfully applied for the transformation of many kinds of microalgal nuclear and chloroplast expression systems, thus we are not surprised that biolistic transformation remains one of the most useful tools in transgenic filed of microalgae regardless of their rigid cell walls or life cycle.

The biolistic transformation method has several advantages. First of all, various vectors can be employed in biolistic transformation. Because of limited understanding of the genetic

background of microalgae, it is difficult to construct helpful and useful algal endogenous vectors. Sometimes the vectors from *E. Coli* were used in algal biolistic transformations. Moreover, exogenous DNA can be introduced into different tissues and cells, including microbes, plants, and animals. More importantly, it is the only effective method that can reproducibly transform mitochondria, chloroplasts, and nucleus. The biolistic transformation procedure is mature and controllable, albeit the need of special pretreatment and expensive equipment (gene gun).

Nearly all of the physical and chemical parameters (rupture pressure, DNA concentration, particle travel distances, and vacuum degree) in the gene gun can be adjusted to fit different algal materials and acceptors. Marine cyanobacterium *Synechococcus sp.* NKBG15041c was transformed successfully using particle bombardment with Bacterial Magnetic Particles (BMPs) purified from one magnetic bacterium known as Magnet *ospirillum sp.*AMB-1. The particle is covered by a thin phospholipid and it can bind larger quantities of DNA than gold or tungsten particles [28]. Additionally, a more efficient transformation was obtained with BMPs than with gold particles. This experiment demonstrates the advantage of BMPs as a DNA carrier during biolistic processes for marine cyanobacteria.

To date, the available protocols for genetically engineering marine diatoms are seldom reported. Biolistic transformation is the only efficient tool to genetically manipulate marine diatoms. A stable nuclear transformation system was established and developed for several diatoms using particle bombardment in the 1990s [34,35]. At present, the utilization of several selectable marker and reporter genes for use in Diatom *Phaeodactylum tricornutum* was examined [33,36]. Transformation with particle bombardment have been established for various species of diatoms, including the centric diatom *T. pseudonana* [37], *Thalassiosira weissflogii* [36], *Chaetoceros sp.* [38], *C. cryptica* [34], and the pinnate diatoms *Navicula saprophila* [34], *P. tricornutum* [33,35,36,39], *Cylindrotheca fusiformis* [40].

Vector Construction and Gene Selection Strategies

Vector element construction is one of the crucial parts in determining the stability and frequency of exogenous DNA expression in microalgal expression systems. With the rapid advances of genome sequencing technologies, gene sequences of various microalgae was published and large-scale approaches have been developed to convert this sequence information into functional information. Generally, gene function can be annotated using various approaches, such as ectopic expression, gene silencing, protein sub-cellular localization examination, gene expression pattern analysis by promoter activity assay, structure-function analysis, and *in vitro* or *in vivo* biochemical assays [41-43]. Nevertheless, the conventional method for engineering expression constructs based on the restriction enzyme/ligase cloning method is extremely laborious and time consuming and is often hampered by lack of appropriate restriction sites; And accordingly, the production of constructs is a significant technical barrier for large-scale functional gene analysis in plants, even for most plant genes in model species such as *C. reinhardtii* and *P. tricornutum*. Therefore, new and effective technologies for "decoding" the acquired gene sequences are urgently needed.

Recently, the Gateway cloning system has been developed to facilitate large-scale production of gene constructs, and it is able to achieve rapid cloning of one or more genes into multiple destination vectors using site-specific recombination. The recombinational cloning systems are based on a two-step process [44]. Firstly, the DNA fragment of interest is cloned into a general donor vector by the GATEWAY BP reaction. Then, the DNA fragment flanked by two site-specific recombination sites (attB1 and attB2) in the donor plasmid can be transferred precisely into a variety of expression vectors by site-specific recombination reactions. Once the DNA products has been targeted into a donor vector, the transfer of the DNA constructs into an expression destination vector is a simple reaction by the GATEWAY LR reaction that requires no traditional restriction enzyme/ligase cloning. The recombinational cloning systems, especially the Gateway technology, have been widely used in the research community, and many Gateway-compatible

open reading frame entry (donor) clone collections and expression plasmids have been created for functional genomics in many organisms [45]. However, the cost of enzymes used in this way is comparatively expensive and the transformation efficiency was not as high as reported, especially it is hardly reported in diatom *P. tricornutum*, so a zero-background TA cloning vector can be constructed for genetic transformation of microalgae by taking advantage of the negative selection gene marker after transformation. Additionally, the technology can be adapted in many ways for developing specialized expression vectors allowing single-step assembly of PCR-amplified genes or fragments [46]. The plasmid is digested by XcmI because of its higher digested efficiency [46] and it can form two 3' T-overhangs.

Vector Construction: Promoter Selection and Codon Usage

The critical step for genetic transformation is to generate a plasmid vector containing all the genetic elements essential for expression. As shown in (Figure 1), a typical vector construction involves the manipulation of selection marker (usually antibiotic resistance gene) expression cassettes for either selection of plasmid in the bacteria or selection of gene transfer in the host organism, and an additional targeted gene expression cassette for expression in the host organism. However, the production of selection marker protein is not wanted after obtaining transgenic organism, and constitutive production of heterologous proteins in transgenic cells is sometimes problematic, since some unwanted proteins might affect normal growth of transgenic cells. One of the feasible solutions is to employ an inducible promoter to confine the expression of the selection marker protein in transgenic organism. Notably, development of inducible promoters is also essential for the design of transformation vectors allowing expression of the selection marker as well as the gene of interest in the target organisms.

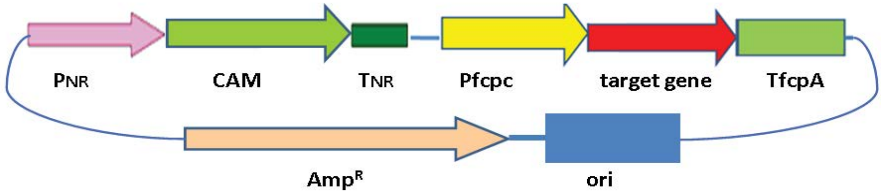

Figure 1: Schematic maps showing a typical microalgal transformation vector.

Annotations of The Plasmid Map Are Given Below: CAM: Reporter gene cassette encoding chloramphenicol acetyltransferase; PNR and TNR: Promoter and Terminator of the nitrate reductase gene from microalge; PfcpC: Promoter of microalgal fcpC gene; TfcpA: Terminator of microalgal fcpA gene; Amp^R: Ampicillin resistance gene cassette; ori: pBR322 ori for replication.

Some heterologous promoters widely used in plant transformation have been used in the genetic transformation of microalgae. For example, Cauliflower Mosaic Virus (CaMV) 35S promoter and p1'2' Agrobacterium promoter were tested to drive the expression of report gene GUS in dinoflagellates *Amphidinium* and *Symbiodinium* [47]. However, due to the unique nuclear characteristics of these microalgae, the expression of foreign genes under the control of heterologous promoters was affected, exhibiting relatively low expression levels and usually transient expression, not as expected as in higher plants, In contrast, RBCS2 promoter from unicellular green alga *Chlmydomonas reinhardtii* showed higher driving efficiency than 35S promoter in transgenic *Dunaliella salina* [48]. In the pennate diatom *Cylindrotheca fusiformis* and centric diatom *T. pseudonana*, relatively high transformation efficiencies were achieved using their own fucoxanthin chlorophyll a/c binding Protein (fcp) promoter or Nitrate Reductase (NR) promoter [37,40]. High efficiency transformation of the diatom *P. tricornutum* was achieved with a promoter from the diatom *Cylindrotheca*

fusiformis [39]. In the case of *Chlorella*, the development of genetic transformation has been still slow. Plant Ubiquitin gene promoter was used to drive the expression of a gene encoding the mature rabbit Neutrophil Peptide-1 (NP-1) in *Chlorella ellipsoidea* cells. In another study, *C. ellipsoidea* was transformed with a vector containing the flounder Growth Hormone gene (fGH) under the control of 35S promoter, and the phleomycin resistance Sh ble gene under the control of the *Chlamydomonas* RBCS2 gene promoter. Over 400 μg of fGH protein expression per liter culture containing 1 x 10^8cells/ml was estimated by ELISA [49]. However, the expression levels of these foreign genes in transformed *Chlorella* were still relatively low. Therefore, new and effective endogenous promoters are highly needed to be identified and isolated for efficient expression of foreign genes in *Chlorella*. The promoter of NR gene isolated and identified from the diatom *P. tricornutum* could drive the inducible expression of CAT report gene in transformed *C. vulgaris* in the presence of nitrate as the sole nitrogen source [32]. However, genetic transformation of diatom *P. tricornutum* is still encumbered by technical limitations of the present methods, the transformation efficiencies being lower compared with those of other pennate diatom, *C. fusiformis*, and centric diatom *T. pseudonana*. In these species, high transformation efficiencies have been achieved using their endogenous fucoxanthin chlorophyll a/c binding protein (fcp) promoter/terminator and Nitrate Reductase (NR) promoter/terminator that are derived from *C. fusiformis* and *T. pseudonana*, respectively [37,40]. High transformation efficiency of the diatom *P. tricornutum* was achieved with a promoter from the diatom *Cylindrotheca fusiformis* [39]. Therefore, new and effective endogenous promoters are urgently needed to be identified for efficient expression of foreign genes in *P. tricornutum*.

A plasmid DNA containing Green Fluorescent Protein (GFP) was transferred into a *Prochlorococcus* strain by interspecific conjugation with *E.coli*, and the expression of this protein was detected by Western blotting and cellular fluorescence [50]. Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells [51].In addition, a GFP reporter gene for *C. reinhardtii* chloroplast has been reported [52]. Besides, inducible EGFP expression under the control of the nitrate reductase gene promoter in transgenic *Dunaliella salina* [53] has been reported, and an inducible GFP expression system has also been established recently [29].

Genetic Engineering in Microalgae

Lipid metabolic network in microalgae is highly intricate, and fuller characterization of the metabolic pathways is needed before engineering microalgae for high-production of biodiesel. Genetic engineering plays a key role in transforming microbes into the desired cell factories.

These high productivity microalgal strains can be achieved by genetic engineering based on the crucial enzymes that can be targeted for fatty acid biosynthesis and molecular basis of lipid metabolic pathways [3,4]. Though the study on microalgae has reemerged because of the increasing interests in microalgal bioactive compounds and biofuel production, research into genetic manipulation and the function of key enzyme for microalgae has been rare due to some technical limitations, especially the lack of efficient genetic transformation tools.

Molecular characterization of several enzymes involved in lipid accumulation has been conducted in mammalian and plant [14,54-56]. However, such studies in microalgae are slow due to some technical limitations such as the lack of the transformation system. The exploit of microalgae has opened up new broad prospects for various kinds of bio-products. The primary requirement in microalgal bio-products industry is to have excellent microalgal strains. The ideal strain should have a combination of characteristics including fast growth, high productivity, strong resistance and suitable for large-scale cultivation. Genetic engineering is the effective approach to achieve such microalgal strains. The current studies present a trait-improved microalgal strain through this approach. Simkovsky et al., demonstrated that cyanobacteria without expression of O-antigen became resistant to

grazing by certain amoebae [57]. While some other researchers tried to boost photosynthesis efficiency through genetic engineering. For example, Justin et al., once introduced cyanobacterial carbon - concentration mechanism into a C3 crop species and enhanced the photosynthetic efficiency and yield of the crop species [58]. This could be a proof of concept for enhancement of lipid production by improving photosynthesis efficiency, apart from the strategy of redirecting the lipid biosynthesis based on a fully annotated pathway. In particular, a range of novel biological processes have been found in diatoms which were presumed to be acquired during evolution [59]. Thereby diatoms are also attractive for the discovery of novel metabolic pathways which are absent in other commonly studied model organisms [60].

Gene silencing can occur either through repression of transcription, termed post-transcriptional gene silencing, or through mRNA degradation [61,62]. Because of its high targeted specificity and efficiency, RNA interference has been proven to be a valuable tool for analyzing the biological function of the targeted gene and manipulating the metabolic process by sequence-specific knockdown [63]. A wide range of RNAi components, which promotes stable gene repression and the transient gene silencing, was identified in the algae, such as red alga *P. yezoensis* [64], diatom *P. Tricornutum* [65], *T. Pseudonana* [59], green alga *D. Salina* [66], and brown alga *Ectocarpus siliculosus* [67].

Perspective

Diminishing storage of fossil fuels and increasing concerns on their environmental pollution have stimulated the need for developing efficient and sustainable biofuels production strategies.

Large-scale biofuels production based on agricultural crops consumes food feedstocks and occupies limited arable land, while microalgal biofuels could alleviate these worries. Algae can be grown quickly on non-arable land to produce large quantities of lipids that can be converted to fuels easily. Though cost of biofuels production from algae remains high, it is a promising and sustainable biofuel source. With concerted effort, improvements can be made to obtain agriculturally and industrially available algae for biofuels production.

Industrial cultivation process produces a vast amount of algae per litre, but its cost remains high. Biofuels derived from agricultural cultivation could theoretically achieve cost efficiency that could compete with petroleum-based fuels, meanwhile bring about a reduced overall impact on environment. While its viability of commercial and industrial production at a sufficiently large scale is only recently subjected to test. Efforts engaged in process engineering and the use of transgenic methods aimed at developing domesticated algal crop strains, will benefit both strategies of increasing productivity and reducing input simultaneously. In the future, driven by the continuing rise of the prices of fossil fuels, corresponding technologies will be developed and favorable algal species (strains) will be acquired to allow algal biofuels to compete with other alternatives and traditional fuels.

Metabolic engineering of microalgae for increased lipid contents is still in its infancy. Although overexpression of single genes was utilized in other organisms to increase lipid yields, success in microalgae is rarely reported so far. Sequenced genomes, transformation techniques, promoters to drive gene overexpression, selection markers and many more are still under development, especially for non-model organisms. Additionally, it remains questionable if a single-gene overexpression/deletion will be sufficient enough to redirect the whole metabolic flux in cells, or whether a multigene and/or manipulation of a regulatory gene approach is more fruitful [12,26].

Genome-scale models incorporating a vast amount of transcriptomic, proteomic and metabolomic data will be used to identify bottlenecks that limit lipid biosynthesis. Basically bottlenecks could be an enzymatic reaction in the biosynthesis, metabolite supply, excessive

degradation or a combination of various factors. Then, any manipulation strategy could be tested *in silico* to verify its identity as a metabolic node and to identify secondary bottlenecks that need to be addressed. Finally, the organisms will be manipulated using genetic transformation, mutagenesis and breeding techniques, as has already been common for high-performance field crops. Apart from creating strains that can be used for the industrial production of biofuels, this approach will also greatly enhance our general biological understanding of microalgae. However, increasing the lipid content of microalgal cells is only one of the many steps on the road for ecologically sustainable and economically viable biofuels. The critical parameter is not lipid content per cell, but the overall lipid productivity [27,68]. Therefore, a favorable strain for biofuels production should also exhibit rapid and robust growth, at least under the specific cultivation conditions. Under these appropriate conditions, which may include, temperature, light regime, nutrient requirement, salinity and a sterile environment, its overall fitness should not be impaired too much by the mutation(s). On the other hand, a decreased fitness under wild conditions should be a desirable trait for the purpose of preventing the microalgal strain from spreading into natural ecosystems and causing environmental turbulence. This is especially true for genetically modified organisms, which face public opposition in many areas of the world.

Furthermore, genetic stability of the high-performance strain has to be guaranteed in order to obtain high lipid yields constantly. If cultures are subjected to continuous growth/harvesting cycles without re-inoculation from stock cultures, the general performance of the strain could be decreased because of genetic drift. The decrease could be quite pronounced if the introduced mutation slightly reduces the fitness of the strain; that is, there will be a selective pressure favoring any mutation counteracting the desired one. It is necessary to develop environmental friendly vectors, which includes searching for endogenous promoters, establishing a directional and stable foreign gene integration system based on algal homologous recombination, substituting antibiotic selection genes for an endogenous selection system of reverse mutation. In addition, it is critical to assess the risk that the transgenic algae might crossbreed with the domestic species/strains by a set of comprehensive tests in order to remove the potential threats to human health caused by the genetically transformed marine algae.

Microalgae exhibit enormous biodiversity and possess the potential for producing large quantities of biomass that contains high concentrations of lipids. Furthermore, harvesting microalgae and subsequent lipid extraction has to be efficient and simple. Here, high-lipid content microalgae that have been genetically engineered for the secretion of lipids could be a promising alternative that avoids sequential harvesting/extraction methods. Given the successes of lipid metabolic engineering in plant crops, not long before can we obtain rationally engineered microgalgae for biofuels production and value-added compounds, by incorporating increased knowledge about microalgae, refined metabolic network models and improved bioinformatic resources.

References

1. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25: 294-306.
2. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, et al. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54: 621-639.
3. Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010) Genetic engineering of algae for enhanced biofuel production. Eukaryot Cell 9: 486-501.
4. Williams N (2010) New biofuel questions. Curr Biol 20: 219-220.
5. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, et al. (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318: 245-250.
6. Khozin-Goldberg I, Cohen Z (2011) Unraveling algal lipid metabolism: Recent advances in gene identification. Biochimie 93: 91-100.
7. Ryall K, Harper JT, Keeling PJ (2003) Plastid-derived Type II fatty acid biosynthetic enzymes in chromists. Gene 313: 139-148.

8. Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1991) In vivo pools of free and acylated acyl carrier proteins in spinach. Evidence for sites of regulation of fatty acid biosynthesis. J Biol Chem 266: 1858-1865.

9. Post-Beittenmiller D, Roughan G, Ohlrogge JB (1992) Regulation of plant Fatty Acid biosynthesis : analysis of acyl-coenzyme a and acyl-acyl carrier protein substrate pools in spinach and pea chloroplasts. Plant Physiol 100: 923-930.

10. Yu WL, Ansari W, Schoepp NG, Hannon MJ, Mayfield SP, et al. (2011) Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae. Microb Cell Fact 10: 91.

11. Kennedy EP (1961) Biosynthesis of complex lipids. Fed Proc 20: 934-940.

12. Courchesne NM, Parisien A, Wang B, Lan CQ (2009) Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. J Biotechnol 141: 31-41.

13. Niu YF, Zhang MH, Li DW, Yang WD, Liu JS, et al. (2013) Improvement of Neutral Lipid and Polyunsaturated Fatty Acid Biosynthesis by Overexpressing a Type 2 Diacylglycerol Acyltransferase in Marine Diatom *Phaeodactylum tricornutum*. Mar Drugs 11: 4558-4569.

14. Radakovits R, Eduafo PM, Posewitz MC (2011) Genetic engineering of fatty acid chain length in Phaeodactylum tricornutum. Metab Eng 13: 89-95.

15. Campbell D, Hurry V, Clarke AK, Gustafsson P, Oquist G (1998) Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiol Mol Biol Rev 62: 667-683.

16. Roberts K, Granum E, Leegood RC, Raven JA (2007) Carbon acquisition by diatoms. Photosynth Res 93: 79-88.

17. Li Y, Han D, Hu G, Dauvillee D, Sommerfeld M, et al. (2010) *Chlamydomonas starchless* mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. Metab Eng 12: 387-391.

18. Li Y, Han D, Hu G, Sommerfeld M, Hu Q (2010) Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. Biotechnol Bioeng 107: 258-268.

19. Trentacoste EM, Shrestha RP, Smith SR, Glé C, Hartmann AC, et al. (2013) Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. Proc Natl Acad Sci USA 110: 19748-19753.

20. Lauersen KJ, Berger H, Mussgnug JH, Kruse O (2013) Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*. J Biotechnol 167: 101-110.

21. Rasala BA, Lee PA, Shen Z, Briggs SP, Mendez M, et al. (2012) Robust expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide. PLoS One 7: 43349.

22. Horvatić J, Persić V (2007) The effect of Ni2+,Co2+, Zn2+, Cd2+ and Hg2+ on the growth rate of marine diatom *Phaeodactylum tricornutum Bohlin*: microplate growth inhibition test. Bull Environ Contam Toxicol 79: 494-498.

23. Liu ZY, Wang GC, Zhou BC (2008) Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour Technol 99: 4717-4722.

24. Yang ZK, Niu YF, Ma YH, Xue J, Zhang MH, et al. (2013) Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation. Biotechnol Biofuels 6: 67.

25. Georgianna DR, Mayfield SP (2012) Exploiting diversity and synthetic biology for the production of algal biofuels. Nature 488: 329-335.

26. Schuhmann H, DK Lim, PM Schenk (2012) Perspectives on metabolic engineering for increased lipid contents in microalgae. Biofuels 3: 71-86.

27. Blatti JL, Michaud J, Burkart MD (2013) Engineering fatty acid biosynthesis in microalgae for sustainable biodiesel. Curr Opin Chem Biol 17: 496-505.

28. Matsunaga T, Takeyama H, Nakamura N (1990) Characterization of cryptic plasmids from marine cyanobacteria and construction of a hybrid plasmid potentially capable of transformation of marine cyanobacterium, *Synechococcus sp.*, and its transformation. Appl Biochem Biotechnol 24: 151-160.

29. Niu YF, Yang ZK, Zhang MH, Zhu CC, Yang WD, et al. (2012) Transformation of diatom *Phaeodactylum tricornutum* by electroporation and establishment of inducible selection marker. Biotechniques 0: 1-3.

30. Brown LE, Sprecher SL, Keller LR (1991) Introduction of exogenous DNA into *Chlamydomonas reinhardtii* by electroporation. Mol Cell Biol 11: 2328-2332.

31. Kilian O, Benemann CS, Niyogi KK, Vick B (2011) High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis sp.* Proc Natl Acad Sci USA 108: 21265-21269.

32. Niu YF, Zhang MH, Xie WH, Li JN, Gao YF, et al. (2011) A new inducible expression system in a transformed green alga, *Chlorella vulgaris*. Genet Mol Res 10: 3427-3434.

33. Zaslavskaia LA, JC Lippmeier, PG Kroth, AR Grossman, KE Apt (2000) Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. Journal of Phycology 36: 379-386.

34. Dunahay T, E Jarvis, PG Roessler (1995) Genetic transformation of the diatoms *cyclotella cryptica* and *navicula saprophila*. Journal of Phycology 31: 1004-1012.

35. Apt KE, Kroth-Pancic PG, Grossman AR (1996) Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. Mol Gen Genet 252: 572-579.

36. Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C (1999) Transformation of Nonselectable Reporter Genes in Marine Diatoms. Mar Biotechnol (NY) 1: 239-251.

37. Poulsen N, PM Chesley, N Kröger (2006) Molecular genetic manipulation of the diatom *Thalassiosira pseudonana* (Bacillariophyceae). Journal of Phycology 42: 1059-1065.

38. Miyagawa-Yamaguchi A, T Okami, N Kira, H Yamaguchi, K ohnishi, et al. (2011) Stable nuclear transformation of the diatom *Chaetoceros sp.* Phycological research 59: 113-119.

39. Miyagawa A, T. Okami, N Kira, H Yamaguchi, K Ohnishi, et al. (2009) High efficiency transformation of the diatom *Phaeodactylum tricornutum* with a promoter from the diatom *Cylindrotheca fusiformis*. Phycological Research 57: 142-146.

40. Poulsen N, Kröger N (2005) A new molecular tool for transgenic diatoms: control of mRNA and protein biosynthesis by an inducible promoter-terminator cassette. FEBS J 272: 3413-3423.

41. Kumar A, Hirochika H (2001) Applications of retrotransposons as genetic tools in plant biology. Trends Plant Sci 6: 127-134.

42. Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol 133: 462-469.

43. Earley KW, Haag JR, Pontes O, Opper K, Juehne T, et al. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J 45: 616-629.

44. Marsischky G, LaBaer J (2004) Many paths to many clones: a comparative look at high-throughput cloning methods. Genome Res 14: 2020-2028.

45. Aliverti A, Pandini V, Pennati A, de Rosa M, Zanetti G (2008) Structural and functional diversity of ferredoxin-NADP(+) reductases. Arch Biochem Biophys 474: 283-291.

46. Chen S, Songkumarn P, Liu J, Wang GL (2009) A versatile zero background T-vector system for gene cloning and functional genomics. Plant Physiol 150: 1111-1121.

47. Leon R, E Fernandez (2007) Nuclear Transformation of Eukaryotic Microalgae: Historical Overview, Achievements and Problems. Adv Exp Med Biol 616: 1-11.

48. Sun Y, Yang Z, Gao X, Li Q, Zhang Q, et al. (2005) Expression of foreign genes in Dunaliella by electroporation. Mol Biotechnol 30: 185-192.

49. Kim DH, Kim YT, Cho JJ, Bae JH, Hur SB, et al. (2002) Stable integration and functional expression of flounder growth hormone gene in transformed microalga, *Chlorella ellipsoidea*. Mar Biotechnol (NY) 4: 63-73.

50. Tolonen AC, Aach J, Lindell D, Johnson ZI, Rector T, et al. (2006) Global gene expression of *Prochlorococcus ecotypes* in response to changes in nitrogen availability. Mol Syst Biol 2: 53.

51. Soboleski MR, Oaks J, Halford WP (2005) Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. FASEB J 19: 440-442.

52. Franklin S, Ngo B, Efuet E, Mayfield SP (2002) Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. Plant J 30: 733-744.

53. Li J, L Xue, H Yan, H Liu, J Liang (2008) Inducible EGFP expression under the control of the nitrate reductase gene promoter in transgenic *Dunaliella salina*. Journal of Applied Phycology 20: 137-145.

54. Jako C, Kumar A, Wei Y, Zou J, Barton DL, et al. (2001) Seed-specific over-expression of an Arabidopsis cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. Plant Physiol 126: 861-874.

55. Park J, Rho HK, Kim KH, Choe SS, Lee YS, et al. (2005) Overexpression of glucose-6-phosphate dehydrogenase is associated with lipid dysregulation and insulin resistance in obesity. Mol Cell Biol 25: 5146-5157.

56. Moellering ER, Benning C (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryot Cell 9: 97-106.

57. Simkovsky R, Daniels EF, Tang K, Huynh SC, Golden SS, et al. (2012) Impairment of O-antigen production confers resistance to grazing in a model amoeba-cyanobacterium predator-prey system. Proc Natl Acad Sci USA 109: 16678-16683.

58. McGrath JM, Long SP (2014) Can the cyanobacterial carbon-concentrating mechanism increase photosynthesis in crop species? A theoretical analysis. Plant Physiol 164: 2247-2261.

59. Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, et al. (2004) The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science 306: 79-86.

60. Siaut M, Heijde M, Mangogna M, Montsant A, Coesel S, et al. (2007) Molecular toolbox for studying diatom biology in *Phaeodactylum tricornutum*. Gene 406: 23-35.

61. Angaji SA, Hedayati SS, Poor RH, Madani S, Poor SS, et al. (2010) Application of RNA interference in treating human diseases. J Genet 89: 527-537.

62. Xu G, Sui N, Tang Y, Xie K, Lai Y, et al. (2010) One-step, zero-background ligation-independent cloning intron-containing hairpin RNA constructs for RNAi in plants. New Phytol 187: 240-250.

63. Dafny-Yelin M, Chung SM, Frankman EL, Tzfira T (2007) pSAT RNA interference vectors: a modular series for multiple gene down-regulation in plants. Plant Physiol 145: 1272-1281.

64. Liang C, Zhang X, Zou J, Xu D, Su F, et al. (2010) Identification of miRNA from *Porphyra yezoensis* by high-throughput sequencing and bioinformatics analysis. PLoS One 5: 10698.

65. De Riso V, Raniello R, Maumus F, Rogato A, Bowler C, et al. (2009) Gene silencing in the marine diatom *Phaeodactylum tricornutum*. Nucleic Acids Res 37: 96.

66. Jia Y, Xue L, Liu H, Li J (2009) Characterization of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from the halotolerant alga *Dunaliella salina* and inhibition of its expression by RNAi. Curr Microbiol 58: 426-431.

67. Cock JM, Sterck L, Rouzé P, Scornet D, Allen AE, et al. (2010) The Ectocarpus genome and the independent evolution of multicellularity in brown algae. Nature 465: 617-621.

68. Larkum AW, IL Ross, Kruse O, Hankamer B (2012) Selection, breeding and engineering of microalgae for bioenergy and biofuel production. Trends Biotechnol 30: 198-205.

Algae Harvesting: Challenges, Progress and Perspectives

Xuezhi Zhang*

[a]Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China 430072

***Corresponding author:** Xuezhi Zhang, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China 430072, Tel.: +86 27 6878 0929; fax: +86 27 6878 0917; E-mail: zhangxuezhi@ihb.ac.cn

Abstract

Algae harvesting remains one of the bottlenecks preventing large scale algal cultivation for the production of biomass, biofuel, and bioproducts. In this chapter, the recent advances of algae harvesting using sedimentation, flotation, filtration, centrifugation, ultrasonic harvesting, and magnetic harvesting were reviewed. The modeling of algae harvesting processes, including membrane harvesting, sedimentation harvesting and dissolved air flotation harvesting are introduced, and these models contributed the knowledge towards the understanding of the harvesting processes, to the identification of key parameters determining the harvesting efficiency. Factors affecting algae harvesting are summarized, including algal cell properties and culture media characteristics. Future perspectives on algae harvesting are discussed in terms of water and nutrient recycling, evaluating and improving the quality of the harvested biomass, as well as techno-economic analyzing of the harvesting processes.

Challenges in Algae Harvesting

Different studies showed that harvesting costs contribute more than 30% of the total cost of algal production in open ponds [1,2]. The main reason is the large amount of water processed during the harvesting operation. To produce one gallon of biodiesel using the open race way pond, 23,000 liters of water needs to be processed, assuming a final dry weight of 1 g L^{-1}, with the lipid content at 30%. Pumping this large amount of water through the harvesting equipment consumes huge energy.

Algae water separation is also a challenge because the algae have small sizes (2-50 μm diameters), similar densities to water [3] and negative charge. Some species are sensitive to shear forces, and in some applications the use of chemicals is limited to prevent biomass contamination. Moreover, the characteristics of algal cells and culture media vary with algal species and cultivation process [3,4], indicating there is no universal harvesting methods suitable for all applications. Algae harvesting processes not only affect the extraction and bioprocessing, but influence the cultivation process as a result of recycling separated water and nutrients. While taking all the above factors into considerations, high quality biomass needs to be generated in a short time, with affordable cost for commercialization.

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

Factors Affect Algae Harvesting

The operational parameters of each algae harvesting method affect algae water separation, and need to be optimized according to algal properties and culture media characteristics. Understanding these factors would help process optimization. In this section several key physical and biochemical properties of algae are summarized.

Cell Size and Density

Microalgae have a typical size ranging from 2 to 50 μm. The harvesting of small size algae species, such as *Chlorella* and *Nannochloropsis* (2-3 μm) usually has more challenges. Higher centrifuge force, more coagulant, or small sized filters are required for the harvesting of small sized algae species. Algal species with a larger size, such as *Spirulina or Haematococcus* can be easily harvested using a screen, or by sedimentation. Various methods of coagulation were used to aggregate microalgae cells to increase the effective "particle" size and hence ease sedimentation, centrifugal recovery, and filtration [5]. Changes in cell size during the growth cycle were also observed under stressed conditions, such as predation stress, high light intensity, or growth inhibit.

The algae particles have a density very close to water, usually between 1010-1030 kg m^3, depending on the species, as well as their growth conditions. The density of cell has much effect on the sedimentation, flotation, and centrifugation harvesting, while has little effect on the filtration harvesting. Sea water usually has a density around 1020 kg m^3 (at the salinity of 3.5%) and the less density difference between marine algae species and the media make the centrifugation harvesting of marine species more difficult. Unlike the cell/flocs size, changes in the density of algae or culture usually cannot be easily achieved, and this parameter has rarely been manipulated for algae harvesting.

Cell surface properties

Cell surface charge is one of the main factors for the stability of microalgae in the culture. At neutral pH, microalgae cells have a slightly negative charge because of the presence of proton-active carboxylic, phosphoric, hydroxyl, and amine functional groups [6,7]. This negative charge separates the cells and prevents their aggregation. Zhang et al. studied the changes in the surface properties of *Chlorella zofingiensis* at different growth phases [8]. They found that the concentrations of a surface functional group decreased as culture aged (Figure 1A), resulting in the reduced zeta potential and cell aggregations (Figure 1B). When harvested using dissolved air flotation, to achieve the 90% harvesting efficiency, the highest Al^{3+} dosage was required for the culture in the exponential growth phase, while the lesser Al^{3+} dosage was required for the stationary phase culture, and the least amount of Al^{3+} was required for the declining phase culture. Exploring algal cell surface properties and understanding how to manipulate these properties to favor algae harvesting will have significant impact on the algae harvesting, and needs to be addressed in future research.

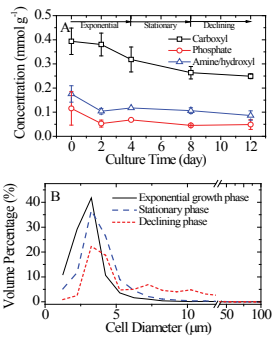

Figure 1: Change in algal surface functional groups concentrations (A) and algal cell size distributions (B) in different growth phases.

Culture Media Characteristics

Algal culture media generally compose an appropriate source of nutrients, pH buffer substance, metal chelates such as Na_2EDTA. Culture media characteristics affect algae harvesting in different ways. Marine culture has a high concentration of ions and its density is higher than freshwater. The high ionic strength affects the coagulant dosages. Ferric coagulants are preferred, as aluminum coagulants are too soluble in seawater [9]. The presence of high concentrations of Mg^{2+} in marine culture (~1330 mg L^{-1} at 3.5% salinity) makes marine algae easy to be coagulated with pH increase [10].

Presence of organic matters affects the coagulation based harvesting, and membrane harvesting. During the growth cycle, concentrations of organic matters increase as a result of cell metabolism and cell lysis. Zhang et al. reported that the organic matters are the major membrane foulants than algal cells or bacteria when harvesting *Chlorella zofingiensis* using 50 KDa ultrafiltration membrane [11]. Particularly, the small sized organic matters (7-11 KDa), and/or the hydrophilic non-acid fractions were the major membrane foulants. The organic matters compete with algal cells for coagulant, and more Al^{3+} is required in the presence of organic matters comparing with the Al^{3+} dosage for the culture in the absence of organic matters [8].

Recent Advances in Algae Harvesting

Microalgae can be harvested by sedimentation, flotation, centrifugation, filtration or some novel methods such as electro-coagulation, ultrasonic harvesting or magnetic separation [6,12,13]. Unfortunately, none of the common approaches and processes have proven to be economically suitable for all the large-scale microalgae harvesting [14] because the harvesting was affected by a variety of factors, such as the algal strain and their growth phases, characteristic of culture media. The selection of harvesting techniques should be based on the understanding of algae properties, the media characteristics, the application of the harvested biomass, and the unit operations individual harvesting technique. A brief description of the current available algae harvesting methods and technologies is provided below.

Sedimentation

Sedimentation is a commonly used and inexpensive process for algae harvesting [15]. Coagulation is usually used to increase their size and make them easily settled to the bottom of a water column. The mechanism of harvesting includes charge neutralization, floc formation, and floc settlement. Batch sedimentation can be conducted in the process tanks, or reactors, and the process can be operated continuously using relatively simple settling tanks. Figure 2 shows the continuous sedimentation unit at AzCATI, which has a 3000 L settling volume with laminar settling grids installed. The sedimentation unit can be operated in the flow rate of 1000~4500 L h^{-1}. After sedimentation, there were still algae in the sedimentation effluent, and more algae was found with higher process flow rate. Typically, the concentrated algae slurry has a solid content between 2%-5%, and further dewatering using centrifuge or filter press is needed.

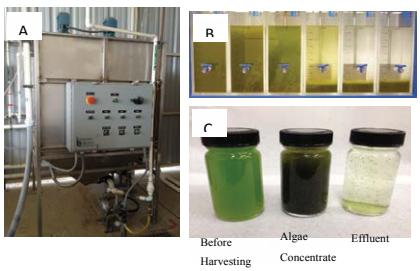

Figure 2: Algae harvesting using sedimentation: continuous sedimentation unit (Integrated Engineers Inc.) at AzCATI (A), sedimentation jar tester (B), and products before and after sedimentation harvesting (C).

Algal cells settled slowly naturally due to the small sizes and densities close to the culture media. Algae cells have negative charges, which prevents the cell aggregation. The surface charge can be reduced or neutralized by adding coagulants such as multivalent cations or cationic polymers to the culture. Comparing with multivalent cations (Al^{3+} or Fe^{3+}), chitosan, an edible nontoxic natural biopolymer can proved effective coagulation with various microalgae [16, 17]. However, the high price prevents its application in large scale harvesting. Microalgae can be self-flocculated (autoflocculation) with a pH increase [10, 18,19]. As the pH increases, formation of magnesium hydroxide, calcium phosphates and substituted forms precipitates the algal cells by charge neutralization and/or sweep floc [20]. It has been reported that Mg^{2+} in fresh culture media may not be sufficient and that additional Mg^{2+} was required for the autoflocculation [10]. Algae harvesting with Mg^{2+} did not introduce extrinsic inorganic ions, which avoids the contamination of biomass or separated media.

Laboratory scale electro-coagulation/flocculation was reported as a novel harvesting technique. Non-sacrificial anodes were first used for electro-coagulation/flocculation, in which the negatively charged algae cells move towards the anode and flocs were formed as a result of lost negative charges. As a result 95 % of the original micro-algae in suspension was separated [21]. Since the coagulants are not required, the using of non-sacrificial electrode has some advantage avoiding the biomass impurity. However, the non-sacrificial electrodes are prone to fouling [12]. Sacrificial Al or Fe anodes have been tested for electro-coagulation/ flocculation harvesting of *Chlorella* and *Phaedactylum*. Aluminium anodes were proved to be better than Fe anodes, and Al concentration in the micro-algal biomass and growth media for recycling was lower than with the use of alum [22]. Although electro-coagulation- flocculation may be a potential technology, there are concerns about increased power consumption in scale-up as the distance between electrodes greatly influences power consumption. Methods to reduce energy and electrode costs are still necessary for practical application [23].

Flotation

Flotation process has been extensively applied in algae separation, which has been proved more efficient than sedimentation for algal-water separation [24]. The hydraulic loading rate of flotation systems is higher than that of a sedimentation tank with a tube settler [25]. Because of these advantages, flotation has increasingly been used for algae harvesting [26-28]. In flotation-harvesting, air bubbles are introduced into a flotation tank or basin where they adhere to algal flocs generated by coagulation/flocculation. The algal flocs attached with air bubbles have a lower density than water and float to the surface where they can be collected by a skimming device. With respect to the different methods of producing the air bubbles, the flotation process can be classified into three types: dispersed air flotation, also known as forth flotation, dissolved air flotation, and electroflotation [24, 29,30].

Dissolved air flotation (DAF) uses small air bubbles (40 to 80 microns) generated by releasing pressurized air-saturated water into the flotation tank/basin to increase efficiency [24]. In a study using alum and PAC as coagulants, over 80% of 18 algal species was removed from water by dissolved air flotation [13]. Cationic surfactant molecules have been used to increase the hydrophobicity of algae surfaces by means of a charge neutralization process. At a concentration of 90%, removal was achieved using a CTAB (cetyl trimethylammonium bromide) concentration of 40 mg L^{-1} [31]. Zhang et al. critically evaluated DAF harvesting of *Chlorella zofingiensis* with regard to algae concentration, culture conditions, type and dosage of coagulants, and recycle ratio. Harvesting efficiency increased with coagulant dosage and leveled off at 81%, 86%, 91% and 87% when chitosan, Al^{3+}, Fe^{3+}, and CTAB were used at dosages of 70, 180, 250, and 500 mg g^{-1}, respectively. At high biomass concentrations, the available bubble number becomes a limiting factor, and more bubbles (or higher recycle ratio than 20%) are required [3].

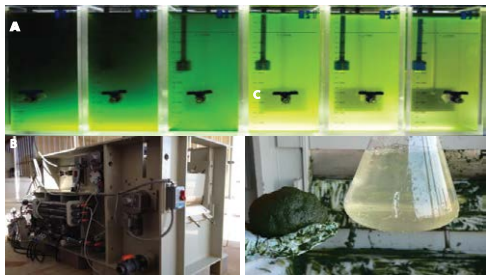

Figure 3: Algae harvesting using DAF: DAF jar tester (A), continuous DAF unit (World Water Works Inc.) at AzCATI (B), and harvested biomass and separated media(C).

Figure 3 shows the DAF jar testers being used for the optimizing of coagulant type and dosages, and the continuous DAF harvesting unit at AzCATI. The DAF unit was equipped with a Nikuni pump, instead of an air saturator for the generation of air bubbles, and the unit is capable of harvesting algae at 450-1100 L h^{-1}. After collecting the floated biomass using a rake, the rotary screen further removes water from the biomass, resulting in a solid content higher than 8%, which might eliminate the centrifugation dewatering process

Filtration

Screens are probably the most common filters and in most cases the least expensive. Microstrainers, vibrating screen filters, and filter presses are the main types of screens used for microalgae harvesting. The principle of screen harvesting is to introduce algae biomass onto a screen of a given pore size. The efficiency of the screening operation depends on the spacing between screen openings and algal size. Larger microalgal species require larger openings, resulting in faster flow rates and a lower cost. The California Department of Water Resources reported that up to 30 percent of the algal removal occurred using screens of pore sizes 25 and 35 microns [32]. Extensive off-lake screens (nylon mesh with a dimension of 20 m^2) or on-lake barges has been successfully used to harvest *Aphanizomenon flos-aquae* from Upper Klamath Lake, the largest freshwater lake system in Oregon [33].

Although the screen is an inexpensive way for algae harvesting, its productivity is low due to gravity's effect on water penetration. At higher microalgal concentrations, the screen is blocked easily. Hu et al. has invented a novel backwashable algae harvesting screen (Figure 4), in which the air was used periodically to backwash and scour the screen surface [34].

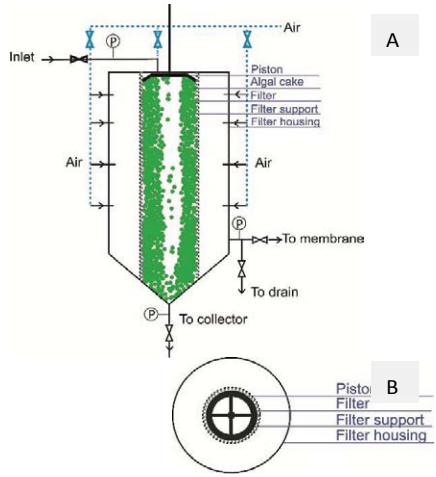

Figure 4: Algae harvesting using automatic screen filter, side view (A), and top view (B

During the filtration process, algal suspension flows through the screen and algae biomass cakes form onto the filter surface. The permeate flow drops gradually as the process continues, and the pressure before and after the screen (or filter) is monitored. When the pressure difference is too high, the feed flow is stopped and the biomass collection and screen cleaning program starts. The procedure includes: 1) drain remaining water inside the filter housing back to the feed tank; 2) use scraping device to remove the algae cake layer to an algae container; 3) periodically backwash the screen using water and/or pressurized air from the permeate side. With this invention, the screen harvesting can be operated automatically and more efficiently.

Membrane algae harvesting has been expanded in an incredible pace recently. According to their pore size, membranes can be divided to microfiltration, ultrafiltration, and nanofiltration. Rossignol et al. investigated the use of polymer membranes for continuous recovery of two marine microalgae, *Haslea ostrearia* and *Skeletonema costatum*. A polyacrylonitrile ultrafiltration membrane of 40 kDa molecular weight cutoff (MWCO) was found satisfactory for recovering the algal cells [35]. Petrusevski et al. examined the application of the tangential flow filtration system for the concentration of living freshwater phytoplankton. Samples were concentrated 5 to 40 times using a 0.45 pm pore-size membrane to concentrate algae from large volumes of reservoir water characterized by low algal biomass [32]. Using a PVC ultrafiltration membrane, Zhang et al. introduced the air assisting backwash to alleviate membrane fouling, and concentrated *Scenedesmus quadricauda* 150 times, reaching a final dry weight of 155 g L^{-1} [36].

Figure 5 shows the PVC membrane unit at AzCATI, algae before harvesting (1.1 g L^{-1}), algal concentrate (60.5 g L^{-1}), and membrane permeate. The membrane has 50 KDa MWCO, and the filtration area is 30 m^2. The average permeate flow is 500 L m^{-2} h^{-1}.

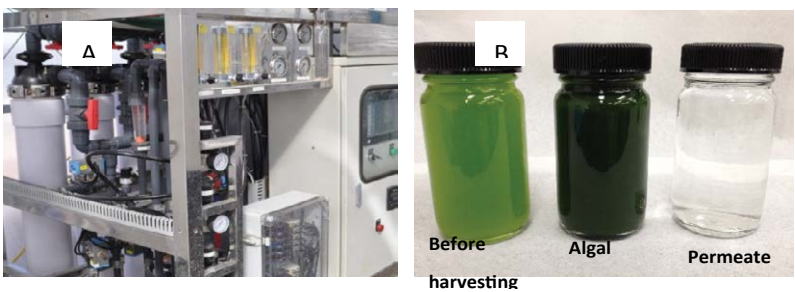

Figure 5: Membrane algae harvesting: membrane algae harvesting unit (Litree Purifying Technology Co., Ltd) at AzCATI (A), and the products before and after membrane harvesting (B).

Membrane algae harvesting is a physical separation process, and thus the contamination of the biomass by chemicals is avoided. Most importantly, membrane filtration is capable of removing protozoans, bacteria and viruses from algal culture media, while retaining residual nutrients, which enables the recycling of algal culture media. As membrane manufacturing techniques improve and the range of applications expand, the cost of membrane filters has steadily decreased, which makes the membrane algae harvesting more affordable.

Ultrasonic Harvesting

Ultrasound has been used to separate algae, which incorporates high frequency (on the order of MHz) and low amplitude ultrasound to aggregate algae cells [37]. In an acoustic field, the algal cells are moved and aggregated into the area in which the cell experiences no shear stress. An acoustic wave with a resonance frequency of 2.1 MHz was applied

to the continuous separation of the freshwater microalgae *Monodus subterraneus UTEX* 151 [37]. Zhang et al., applied the ultrasound to the freshwater microalgae *Microcystis aeruginosa* coagulation using Polyaluminum Chloride (PAC). The settling rate increased when ultrasound was used, due to the destruction of the gas vacuoles inside cells. 1-5 s sonication was sufficient to improve the flocculation performance of algal cells [38].

A pilot-scale ultrasonic algae harvester was tested using *N. oculata.* by National Alliance for Advanced Biofuels and Bioproducts (NAABB) scientists (Figure 6). The unit operated at 45-225 L h^{-1} achieved a typical concentration factor of 6, and a peak concentration factor of 18.

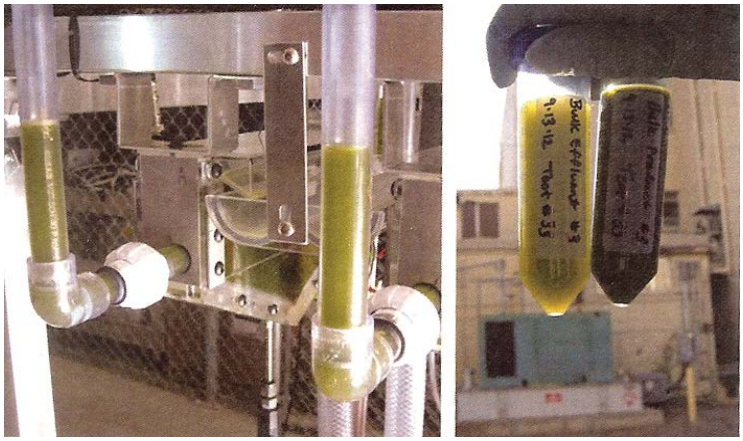

Figure 6: Ultrasonic algae harvesting. Photo courtesy of National Alliance for Advanced Biofuels and Bioproducts (NAABB).

Magnetic Harvesting

Magnetic separation has been demonstrated as a powerful tool to capture algae by adsorption to submicron-sized magnetic particles. Cerff et al. studied the harvesting of fresh water algae *Chlamydomonas reinhardtii and Chlorella vulgaris* as well as marine algae *Phaeodactylum tricornutum and Nannochloropsis salina* using silica-coated magnetic particles. Separation efficiencies higher than 95% were obtained for all algae while maximum particle loads of 30 and 77 gram magnetic particles per gram algae before separation were measured for *C. reinhardtii* and *P. tricornutum* at pH 8 and 12, respectively. Separation efficiencies depended on particle concentration, pH and media composition [39].

Hu et al., reported the efficient microalgae harvesting using polyethylenimine (PEI) coated Fe_3O_4 nanoparticles. The functional magnetic nanocomposites were 12 nm in diameter and 69.77 emu g^{-1} of saturation magnetization. A harvesting efficiency of 97% of *Chlorella ellipsoidea* cells was achieved within 2 min with a nanocomposite dosage of 20 mg L^{-1} [40]. Harvesting efficiency was found to increase with temperature. The adsorption capacity of the Fe_3O_4-PEI nanocomposites for the microalgal cells reached up to 93.46 g dry weight g^{-1} of nanocomposites through electrostatic attraction and nanoscale interactions between the nanocomposites and the microalgal cells [40]. Hu et al. also invented a magnetic separator for algae harvesting which can be operated in both batch and continuous models [41]. The magnetic separator consists of a permanent magnet drum, separation chamber and scraper blade (Figure 7). In the batch operation, more than 95% of the *Chlorella ellipsoidea* cells are harvested within 40 seconds. In the continuous operation mode, the harvesting efficiencies reached more than 95% when the flow rates through the separation chamber were less than 100 mL min^{-1}. Further increasing flow rates reduced the harvesting efficiency [41].

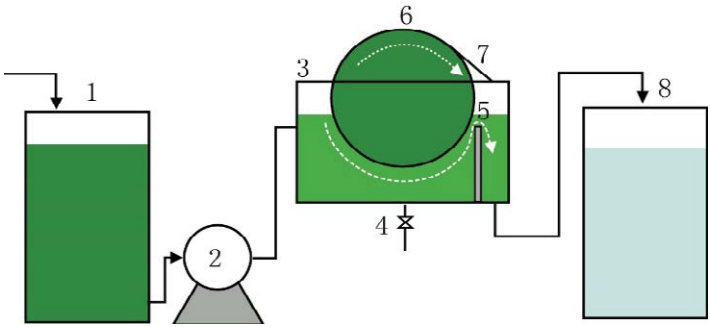

Figure 7: Schematic illustration of magnetic separation system: magnetic particles mixed in algae suspension (1), peristaltic pump (2), harvesting tank (3), batch process valve (4), baffle (5), magnetic separator (6), scraper blade (7), and separated media (8). Drawing modified from [41].

Centrifugation

Using a centrifuge increases the separation force on algal cells and thus extends efficiency for algae harvesting and dewatering. Large volume and continuous flow centrifuges are well commercialized, including basket centrifuge, disc bowl centrifuge and decanter centrifuges. Centrifugation is a preferred method of harvesting algal cells for producing high value bioproducts. Centrifugation would not be a practical approach for algae-for-fuel application without pre-concentration because of its high capital cost and time- and energy-consumption. Besides, the centrifugal process exposes cells to high gravitational and shear forces, which may damage the cell structure.

Recently Evodos has invented a novel centrifuge also known as dynamic settler, using spiral plate technology (SPT). The SPT technology is based on thin layer dewatering combined with a large settling area under centrifugal force. The maximum length of the trajectory to be travelled by the algae cells in the direction of the area under centrifugal force is ~ 3-6 mm and a settling area has been increased to about 65 m^2 (Type 250), which dropped the energy consumption significantly. Before each biomass discharge, the residual water will be pumped out of the centrifuge, and thus the ash free solid content in the discharged biomass is usually higher than 25%. Figure 8 shows the Evodos unit (Type 25), its SPT technology, and the harvested biomass.

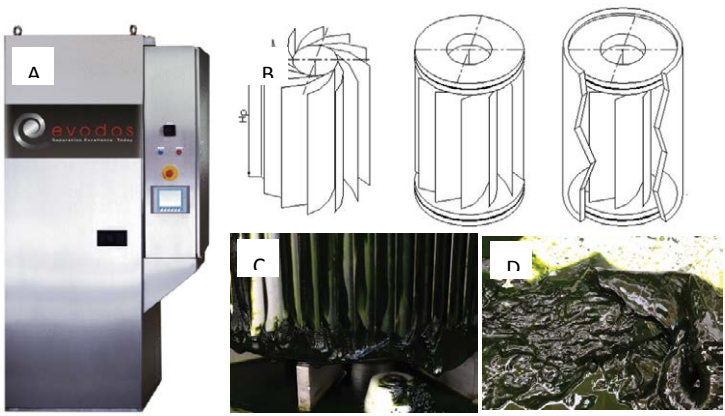

Figure 8: Algae harvesting using Evodos centrifuge: dynamic settler Type 25 (Evodos) at AzCATI (A), drawing of the Spiral Plates, courtesy of Evodos (B), algal biomass on collected the plates (C), and the biomass harvested (D).

Algae Harvesting Process Modeling

The algae harvesting process is usually developed on the basis of empirical considerations combined with on-site testing. Modeling of algae harvesting would help to identify key parameters involved in the harvesting process and be valuable in efforts to achieve high harvest efficiencies. The modeling of algae harvesting using membrane filtration, dissolved air flotation and sedimentation, respectively, is reviewed below:

Membrane Algae Harvesting Modeling

A membrane harvesting model was developed by Zhang et al. to predict the flux decline and final concentration based on a resistance-in-series analysis and a cake development [36]. During the membrane harvesting process, the algae cake layer is developed across the membrane surface, and thus increases the membrane resistance. This cake layer is periodically removed by backwashing to restore membrane flux. While the organic foulants attached on the membrane surface or inner membrane pores cannot be removed by this backwashing process, resulting in the gradual decrease of membrane flux. The permeate flow rate can be described by Darcy's law, taking all the resistances into consideration (Figure 9), as shown in the following equation:

$$Q = \frac{A\Delta P}{\mu(R_m + R_c + R_{ir})} \qquad \text{Eq. (1)}$$

where Q ($m^3 \cdot s^{-1}$) is the permeate flow rate, A is membrane filtration area (m^2) , ΔP is the TMP (transmembrane pressure, Pa), μ is the viscosity of permeate (Pa·s), R_m is the inherent membrane resistance (m^{-1}), R_c is the cake resistance (m^{-1}), and R_{ir} is the fouling resistance due to adsorption, or chemical bonding (m^{-1}).

The permeate flow rate through a membrane at any time can be calculated using the pre-determined inherent membrane resistance, R_m, using pure water. The cake resistance, R_c , can be calculated through thickness of specific resistance, and thickness of algae cake layer as calculated from the steady state flux, and backwashing irreversible, R_{ir}, can be calculated by predetermined backwash efficiencies $r = \frac{Q_n}{Q_{n-1}}$. The net force exerted on an algal particle, F, is the sum of negative direction forces such as permeation drag (F_d) move algae toward the membrane surface, and positive forces such as Brownian diffusion (F_B), shear-induced diffusion (F_s), and lateral inertial lift (F_l) shift algae away from the membrane surface. At a steady state, the flux (J_s) can be calculated as $J_s = v_B + v_s + v_l$ with v is the kinematic viscosity ($m^2 \cdot s^{-1}$). Flux decline due to fouling of membrane is illustrated in Figure 10.

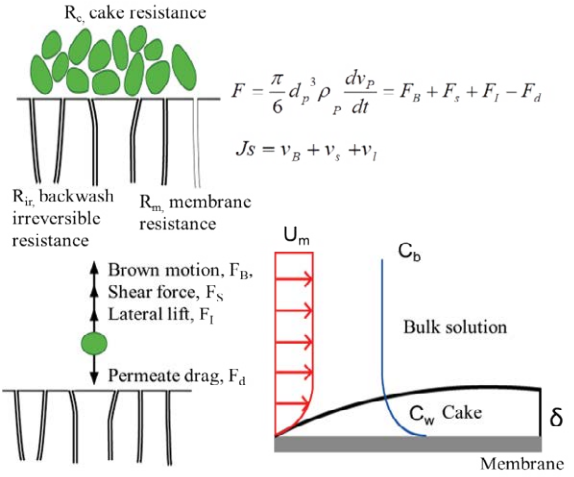

Figure 9: Illustration of the membrane algal harvesting modeling concepts.

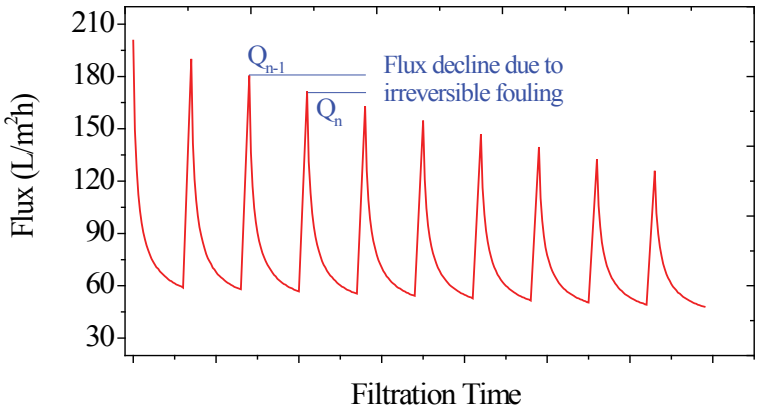

Figure 10: Calculation of backwashing efficiency from membrane flux decline curves.

This model was verified for the *Scenedesmus quadricauda* harvesting, in which algal suspension was concentrated 150 times, with the calculated harvesting rate and average permeate flux of 46 $g \cdot m^{-2} \cdot h^{-1}$ and 45.5 $L \cdot m^{-2} \cdot h^{-1}$, respectively. The changes in flux and volumetric concentration factor predicted by the model agreed well with the experimental data (R^2=0.993). The flux decline predicted by the model under different conditions was consistent with the experimental results. Given the several key parameters, such as algae cell size, concentration, transmembrane pressure (TMP), cross flow velocity, specific cake growth rate and resistance, the model is capable of predicting the volume reduction factor (VRF) and process time for any algae species, or any membranes, which would be very useful for the design and optimization of membrane algae harvesting process.

DAF Algae Harvesting Modeling

Zhang et al. reported a DAF algae harvesting model based on the algae flocs-bubble collision and attachment efficiencies, with the consideration of mass-based floc size distributions and a bubble limitation. The total mass based harvesting efficiency, η_{DAF}^{m}, can be calculated through the following equations:

$$\Delta(\delta F_n) = r_{fb}\left[1 - \exp(-\lambda t B_n)\right] \quad \text{Eq. (2)}$$

$$r_{fb} = \min\left[1, \max\left(0, \frac{v_{fb} t_r}{h}\right)\right] \quad \text{Eq. (3)}$$

$$\lambda = 0.25 \alpha \eta_T v_b \pi d_b^2 \quad \text{Eq. (4)}$$

$$\eta_{DAF}^{m} = \int_{d_p}^{\infty} \Delta(\delta F_n)\delta F_m \quad \text{Eq. (5)}$$

where δF_n is the number fraction of flocs that are actually harvested; t is the floc-bubble contact time (s), B_n is the bubbles available ($\#\cdot L^{-1}$) to interact with flocs of size d_f, t_r is the allowed rise time (s) and h is the water depth (m), λ is a rate expression in inverse concentration units ($m^3 \cdot s^{-1}$). a is the floc-bubble attachment efficiency, v_b is the bubble rise velocity ($m \cdot s^{-1}$) and d_b is the bubble diameter (m), and δF_m is mass-based size distribution.

In the rate expression, η_T is the floc-bubble collision frequency (η_T) used in computing the floc-bubble interaction frequencies, which is the sum of various bubble collisions due to Brownian diffusive motions (h_D), interception by bubbles as derived from particle

trajectory analysis (η_I), sweep by settling flocs (η_S), and an inertial motion contribution that is particularly associated with flocs traveling at greater velocity than the local fluid (large particles, high levels of turbulence) (η_{IN}).

During the model calculation, bubbles were allocated to the flocs from larger to smaller sizes, until all bubbles were assigned, as larger flocs had a better chance to collide with bubbles. The number and mass of flocs in a given size range collected by bubbles was then calculated according to floc-bubble collision efficiency and attachment efficiency, using the available bubble number at the given floc size range. Small flocs are collected at reduced (or zero) efficiency as bubbles are depleted. The final mass-based harvesting efficiency was then calculated with the integral of the mass of the flocs collected in all floc size ranges.

The model has been verified with the experimental data obtained in the DAF harvesting of *Chlorella zofingiensis* [3]. The harvesting efficiencies predicted by the model agreed reasonably with experimental data obtained at different Al^{3+} dosages, algal concentrations, and recycle ratios. The model revealed the importance of coagulation to increase floc-bubble collision and attachment, and the preferential interaction of bubbles with larger flocs, which limit the availability of bubbles to the smaller sized flocs. On the basis of the experimental data and model predictions, three critical conditions necessary to achieve a high DAF harvesting efficiency are identified: 1) flocs of similar or larger sizes than the DAF bubbles (55 μm) should be generated to improve bubble-floc collision efficiency; 2) the surface of algal flocs after coagulation should be favorable for bubble attachment; and 3) a sufficient number of bubbles is necessary to capture the flocs generated when harvesting algal biomass with a higher initial concentration, which can be achieved by increasing the recycle ratio or altering the floc size distribution. The influence of algae cell size, flocs size, initial concentration, floc fractal dimension, recycle ratio, and floc-bubble attachment efficiency can be also predicted by the DAF algae harvesting model.

Sedimentation Harvesting Modeling

A combined sedimentation model is developed by Salim et al., [42], based on the Stokes' law, Smoluchowski's flocculation model, and population balances. The settling tank was modelled as a cascade of ideal mixers (Figure 11) with $z = 1$ for the top mixer and $z = z_{max}$ for the bottom mixer. Multiple particle sizes are included in the model to simulate a polydisperse solution consisting of particles with equal density. Particles were divided into limited number (six) of size classes, $1 < i < i_{max}$ with size class 1 containing single cells of 1–3 μm and size class i_{max} containing flocs of 395–579 μm. The population balances were solved numerically using an Euler approximation for the time steps.

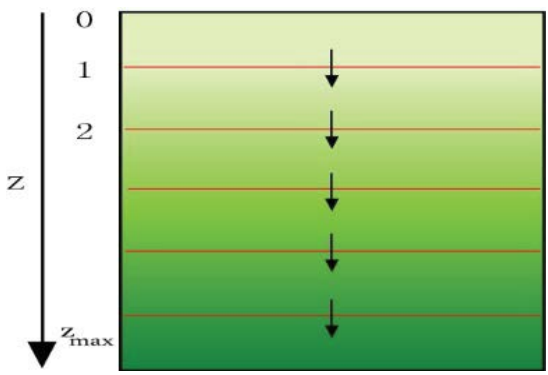

Figure 11: Schematic overview of a cascade of ideal mixtures in the sedimentation tank. Drawing modified from [42].

The velocity of an individual falling algae cell or floc in water can be predicted with Stokes' law:

$$v_{\infty,i} = \frac{1}{18} \cdot \frac{\Delta\rho}{\eta} \cdot g \cdot d_i^2 \qquad \text{Eq. (6)}$$

where d_i is the diameter of particle (m), $\Delta\rho$ is the density difference between the particle and the liquid (g L^{-1}), η is the viscosity of the liquid (Pa·s) and $v_{\infty,i}$ is the velocity of a spherical particle of class size i in a dilute solution (m s^{-1}).

The balance over mixer z for particles in size class i gives:

$$C_{i,z,t+1} = C_{i,z,t} + \left(\frac{v_{i,z-1,t} \bullet C_{i,z-1,t} - v_{i,z,t} \bullet C_{i,z,t}}{\Ä z} + r_{i,z,t}\right) \bullet \Ä t \qquad \text{Eq. (7)}$$

where $C_{i,z,t}$ and $v_{i,z,t}$ are the concentration and velocity of particle class i at position z at time t, respectively. The boundary conditions are $v_{i,0} = 0$ and $v_{i,z\max} = 0$.

The production rate of particles in size class i is calculated with Smoluchowski's model using Eq. (8):

$$r_{i,z,t} = \frac{1}{2}\alpha \bullet \sum_{f=1}^{i-1} \frac{G}{6} \bullet (d_f + d_j)^3 \bullet C_{f,z,t} \bullet C_{i-f,z,t} - \alpha \bullet \sum_{j=1}^{\infty} \frac{G}{6} \bullet (d_i + d_j)^3 \bullet C_{i,z,t} \bullet C_{j,z,t} \qquad \text{Eq. (8)}$$

where f, i and j are the numbers of cells in a floc, G is the shear rate (s^{-1}), C_f, C_i and C_j are the concentrations of flocs with f, i and j cells, respectively and d_f, d_i and d_j are the diameters of the colliding particles (m). The first term in this equation describes formation of particles in size class i due to collision of two smaller particles; the second term describes disappearance due to collision of particles in size class i with other particles. Particles in size class 1 cannot be formed and particles in size class i_{max} cannot disappear.

For any given algae species with known density, minimum external porosity, single cell size and initial number concentration, as well as the known process parameters, such as initial size distribution and shear rate, the model is able to predict the time needed to reach a desired concentration in a sedimentation tank, or to predict the final concentration after settling for a certain amount of time. The concentration of the algae cells as function of the time and the position in the sedimentation tank can be predicted. The model was validated with experimental data for *Ettlia texensis*. The concentration changes measured in time at different heights in the sedimentation vessel agreed well with model predictions. According to the model, harvesting algae with an initial concentration of 0.26 g dry weight L^{-1} and a tank height of 1 m, 25 hours of settling is required to reach a final concentration of 5.2 g dry weight L^{-1}. The model expended the knowledge on the understanding of the algae flocculation /sedimentation harvesting, and can be used for sedimentation process design with its capability of predicting the settling time and/or concentration after settling,

Algae Harvesting Perspectives

Reduce the Cost of Algae Harvesting

Harvesting costs contribute more than 30% of the total cost of algal production in open ponds [1, 2], and algae harvesting remains one of the major constrains limiting the large scale algae cultivation. Lots of efforts have been directed on the harvesting cost reduction. It is very practical to combine two or more of these methods to obtain a greater separation rate at lower costs. For example, the combination of flocculation–sedimentation with centrifugation can significantly reduce process costs [43]. The optimization of a pre-concentration step before the dewatering process is another promising approach towards lowering harvesting costs [44]. Other directions to reduce the harvesting cost include the selection of the best harvesting methods based on the characteristics of the target strain, and developing novel harvesting method.

Water and Nutrients Recycling

Huge amount of water needs to be processed in the production of algae biomass, biofuel, and bioproducts. For example, to generate one kg of algae biodiesel, 3726 kg water is required without recycling. Recycling harvest water reduces the water usage by 84% [45]. When recycling the separated water, unused nutrients can also be recycled, which will significantly save the resources and make the algae biomass production more sustainable. In practice, centrifuge separated media has been returned to the pond after partial harvesting. However, there are still algae, bacteria, and possible virus in the returned effluent which may cause growth inhabitation or culture contamination. Rodolfi et al., found that when grown in the centrifugation separated recycled media, the culture productivity of *Nannochloropsis* reduced significantly, as a result of cell aggregation [46]. Another research on *Chlorella zofingiensis* indicated that the dry biomass of algae growing in sedimentation separated cultural media for three times was about 80% less than that of algae growing in fresh media [47].

Comparing with centrifugation, sedimentation, or flotation, membrane harvesting results in the highest recycled water quality, which is free of algae, bacteria and virus if the membrane pore size is small enough. Although membrane filtration is very effective to remove particular substances, the presence of small sized organic matters may inhibit algae growth, and thus accumulation of bioproducts. Further treatment of the recycled media using activated carbon adsorption, or advanced oxidation, may be considered for growth inhibitor removal.

Improving the Quality of Harvested Biomass

During the harvesting process, the biochemical composition in the biomass may change, depending on the processing time and operational environment. The bacterial population may also increase during the harvesting process. Fast harvesting in a well-controlled environmental is preferred, especially for the production of high value bioproducts. Loss of intracellular components due to cell damage under high shear force caused by centrifugal pump or centrifuge has been reported [48]. The using of the inorganic coagulants brings the possible contamination of the harvested biomass or separated water, which burdens the biomass processing and treatment for water recycling. Zhang et al. compared the metal concentrations in the recycled media and harvesting biomass during, Al^{3+}, Fe^{3+}, Mg^{2+} coagulation-DAF harvesting of *Chlorella zofingiensis*. When Al^{3+} was used as coagulant, Al^{3+} residual was found in the DAF separated culture media with a concentration of 5.7 mg L^{-1}. When Fe^{3+} was used, 93.2 mg Fe^{3+} g^{-1} dry biomass was found after washing the harvested biomass with 0.1 M HCl three times, while Mg^{2+} and Al^{3+} in the harvested biomass can be easily removed using acid washing [49]. Currently, there are few reports on the quality of the harvested biomass, and more studies are needed to evaluate and improve the quality of biomass during the algae harvesting process.

Techno-Economic Modeling Of the Algae Harvesting

Techno-economic models are a very useful tool for us to gain greater understanding of informed decision making in the algae industry. Many techno-economic model of algal biofuels and bioproducts production have been developed, which guided the direction future technique development [45, 50, 51]. Algae cell properties and media characteristics vary with the types of production species used, and the cultivation strategies. The best suitable harvesting process needs be selected based on the usages of the bioproducts, the quality of the biomass, the processing time, and most importantly, the economics of harvesting. A detailed techno-economic model on algae harvesting is critically important to understand the harvesting process, and select the suitable harvesting technique.

Parameters such as algae properties, media characteristics, and operational conditions need to be considered into the techno-economic model. With the integration of the harvesting processing model, mass balance, and energy balance, the model should be capable to predict the harvesting time, energy consumption, and cost for any given harvesting scenario. Sensitivity analysis would help to identify the key parameters in the harvesting process, thus help the optimization of the harvesting process.

References

1. Zittelli GC, Rodolfi L, Biondi N, and Tredici MR (2006) Productivity and photosynthetic efficiency of outdoor cultures of Tetraselmis suecica in annular columns. Aquaculture, 261(3): 932-943.
2. Salim S, Bosma R, Vermuë MH, Wijffels RH (2011) Harvesting of microalgae by bio-flocculation. See comment in PubMed Commons below J Appl Phycol 23: 849-855.
3. Zhang X, Hewson JC, Amendola P, Reynoso M, Sommerfeld M, et al. (2014) Critical evaluation and modeling of algal harvesting using dissolved air flotation. See comment in PubMed Commons below Biotechnol Bioeng 111: 2477-2485.
4. Henderson R, Parsons SA, Jefferson B (2008) The impact of algal properties and pre-oxidation on solid-liquid separation of algae. See comment in PubMed Commons below Water Res 42: 1827-1845.
5. Elmaleh S, Coma J, Grasmick, A and Bourgade L, et al. (1991) Magnesium Induced Algal Flocculation in a Fluidized-Bed. Water Science and Technology, 23(7-9): 1695-1702.
6. Molina Grima E, Belarbi EH, Acién Fernández FG, Robles Medina A, Chisti Y, et al (2003) Recovery of microalgal biomass and metabolites: process options and economics. See comment in PubMed Commons below Biotechnol Adv 20: 491-515.
7. Hadjoudja S, Deluchat V, Baudu M (2010) Cell surface characterisation of Microcystis aeruginosa and Chlorella vulgaris. See comment in PubMed Commons below J Colloid Interface Sci 342: 293-299.
8. Zhang X, Amendola P, Hewson JC, Sommerfeld M, Hu Q, et al. (2012) Influence of growth phase on harvesting of Chlorella zofingiensis by dissolved air flotation. See comment in PubMed Commons below Bioresour Technol 116: 477-484.
9. Edzwald JK, Haarhoff J (2011) Seawater pretreatment for reverse osmosis: chemistry, contaminants, and coagulation. See comment in PubMed Commons below Water Res 45: 5428-5440.
10. Wu Z, Zhu Y, Huang W, Zhang C, Li T, et al. (2012) Evaluation of flocculation induced by pH increase for harvesting microalgae and reuse of flocculated medium. See comment in PubMed Commons below Bioresour Technol 110: 496-502.
11. Zhang W, Zhang W, Zhang X, Amendola P, Hu Q, et al. (2013) Characterization of dissolved organic matters responsible for ultrafiltration membrane fouling in algal harvesting. Algal Research-Biomass Biofuels and Bioproducts,. 2(3): 223-229.
12. Uduman N, Qi Y, Danquah MK., Forde GM and Hoadley A, et al.,(2010) Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable and Sustainable Energy, 2010. 2(1).
13. Milledge JJ and Heaven S (2013) A review of the harvesting of micro-algae for biofuel production. Reviews in Environmental Science and Bio-Technology 12(2): 165-178.
14. Hoffmann JP (1998) Wastewater treatment with suspended and nonsuspended algae. Journal of Phycology 34(5): 757-763.
15. John J, Milledge SH (2013) A review of the harvesting of micro-algae for biofuel production. Reviews in Environmental Science and Bio/Technology, 12(2): 165-178.
16. Sirin S, Trobajo R, Ibanez C and Salvado J (2012) Harvesting the microalgae Phaeodactylum tricornutum with polyaluminum chloride, aluminium sulphate, chitosan and alkalinity-induced flocculation. Journal of Applied Phycology, 24(5): 1067-1080.
17. Xu Y, Purton S, Baganz F (2013) Chitosan flocculation to aid the harvesting of the microalga Chlorella sorokiniana. See comment in PubMed Commons below Bioresour Technol 129: 296-301.
18. Spilling K., Seppala J and Tamminen T (2011) Inducing autoflocculation in the diatom Phaeodactylum tricornutum through CO2 regulation. Journal of Applied Phycology, 2011. 23(6): 959-966.
19. Vandamme D, Foubert I, Fraeye I, Meesschaert B, Muylaert K, et al. (2012) Flocculation of Chlorella vulgaris induced by high pH: role of magnesium and calcium and practical implications. See comment in PubMed Commons below Bioresour Technol 105: 114-119.
20. Baya DT, Effebi KR, Tangou TT, Keffala C, Vasel JL, et al. (2013) Effect of hydroxyapatite, octacalcium phosphate and calcium phosphate on the auto-flocculation of the microalgae in a high-rate algal pond. See comment in PubMed Commons below Environ Technol 34: 2407-2414.
21. Poelman E, DePauw N, and Jeurissen B (1997) Potential of electrolytic flocculation for recovery of micro-algae. Resources Conservation and Recycling, 19(1): 1-10.

22. Vandamme D, Pontes SC, Goiris K, Foubert I, Pinoy LJ, et al. (2011) Evaluation of electro-coagulation-flocculation for harvesting marine and freshwater microalgae. See comment in PubMed Commons below Biotechnol Bioeng 108: 2320-2329.

23. Lee AK., Lewis DM and Ashman PJ (2013) Harvesting of marine microalgae by electroflocculation: The energetics, plant design, and economics. Applied Energy 108: 45-53.

24. Edzwald JK (2010) Dissolved air flotation and me. See comment in PubMed Commons below Water Res 44: 2077-2106.

25. Crittenden JC, Trussell RR, Hand DW, Howe KJ and Tchobanoglous G (2005) Water treatment: principles and design, 2nd ed. Hoboken, NJ.: John Wiley & Sons, Inc.

26. Wiley PE, Brenneman KJ, Jacobson AE (2009) Improved algal harvesting using suspended air flotation. See comment in PubMed Commons below Water Environ Res 81: 702-708.

27. Cheng, WP, Chen WY and Yu RF (2010) PACl coagulation for the solid-liquid separation of highly concentrated algae suspensions. Desalination and Water Treatment 6(1-3): 290-297.

28. Xu L, Wang F, Li HZ, Hu ZM, Guo C , et al. (2010) Development of an efficient electroflocculation technology integrated with dispersed-air flotation for harvesting microalgae. Journal of Chemical Technology and Biotechnology, 2010. 85(11): 1504-1507.

29. Matis KA (1995) Flotation science and engineering New York: Marcel Dekker.

30. Hanotu J, Bandulasena HC, Zimmerman WB (2012) Microflotation performance for algal separation. See comment in PubMed Commons below Biotechnol Bioeng 109: 1663-1673.

31. Chen YM, Liu JC, and Ju YH (1998) Flotation removal of algae from water. Colloids and Surfaces B-Biointerfaces, 12(1): 49-55.

32. Petrusevski B, Bolier G, Vanbreemen AN and Alaerts GJ (1995) Tangential flow filtration - a method to concentrate fresh-water algae. Water Research, 29(5): 1419-1424.

33. Carmichael WW, Drapeau C and Anderson DM (2000) Harvesting of Aphanizomenon flos-aquae Ralfs ex Born. & Flah. var. flos-aquae (Cyanobacteria) from Klamath Lake for human dietary use. Journal of Applied Phycology,12 (6): 585-595.

34. Hu Q, Sommerfeld M, Zhang X and Kale A (1999) Algae filtration systems and methods. 2011: US.

35. Rossignol N, Vandanjon L, Jaouen P and Quemeneur F (1999) Membrane technology for the continuous separation microalgae/culture medium: compared performances of cross-flow microfiltration and ultra-filtration. Aquacultural Engineering, 20(3): 191-208.

36. Zhang X, Hu Q, Sommerfeld M, Puruhito E, Chen Y (2010) Harvesting algal biomass for biofuels using ultrafiltration membranes. See comment in PubMed Commons below Bioresour Technol 101: 5297-5304.

37. Bosma R, van Spronsen WA, Tramper J and Wijffels RH (2003) Ultrasound, a new separation technique to harvest microalgae. Journal of Applied Phycology 15(2-3): 143-153.

38. Zhang GM, Wang B, Zhang P Y, Wang L,Wang H, et al. (2006) Removal of algae by sonication-coagulation. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering, 41(7): 1379.

39. Cerff M, Morweiser M, Dillschneider R, Michel A, Menzel K, et al. (2012) Harvesting fresh water and marine algae by magnetic separation: Screening of separation parameters and high gradient magnetic filtration. Bioresource Technology, 118: 289-295.

40. Hu YR, Guo C, Wang F, Wang SK, Pan F, et al .(2014) Improvement of microalgae harvesting by magnetic nanocomposites coated with polyethylenimine. Chemical Engineering Journal, 242: 341-347.

41. Hu YR, Guo C, Xu L, Wang F, Wang SK, et al. (2014) A magnetic separator for efficient microalgae harvesting. See comment in PubMed Commons below Bioresour Technol 158: 388-391.

42. Salim S, Gilissen L, Rinzema A, Vermuë MH, Wijffels RH, et al. (2013) Modeling microalgal flocculation and sedimentation. See comment in PubMed Commons below Bioresour Technol 144: 602-607.

43. Schlesinger A, ED Bar-Gil A, Carmely H, Einbinder S, Gressel J, et al. (2012) Inexpensive non-toxic flocculation of microalgae contradicts theories; overcoming a major hurdle to bulk algal production. Biotechnology Advances, s30(5): 1023-1030.

44. Ana I Barros, ALG Manuel Simões, José CM Pires b (2015) Harvesting techniques applied to microalgae: A review. Renewable and Sustainable Energy Reviews, 41: 1489–1500.

45. Yang J, Xu M, Zhang X, Hu Q, Sommerfeld M, et al. (2011) Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. See comment in PubMed Commons below Bioresour Technol 102: 159-165.

46. Rodolfi L, Zittelli GC, Barsanti L, Rosati G, Tredici MR, et al. (2003) Growth medium recycling in Nannochloropsis sp. mass cultivation. See comment in PubMed Commons below Biomol Eng 20: 243-248.

47. Zhu LD, Takala J, Hiltunen E, Wang ZM (2013) Recycling harvest water to cultivate Chlorella zofingiensis under nutrient limitation for biodiesel production. Bioresource Technology, 144: 14-20.

48. Harun R, Singh M, Forde GM and Danquah MK. (2010) Bioprocess engineering of microalgae to produce a variety of consumer products. Renewable & Sustainable Energy Reviews, 14(3): 1037-1047.

49. Zhang XZ, Wang L, Hu Q Harvesting algal biomass using magnesium coagulation-dissolved air flotation. Bioresource Technology, Submitted.

50. DOE (2010) National algal biofuels technology roadmap.

51. Lardon L, Hélias A, Sialve B, Steyer JP, Bernard O, et al. (2009) Life-cycle assessment of biodiesel production from microalgae. See comment in PubMed Commons below Environ Sci Technol 43: 6475-6481.

Recent Advances in Microalgal Proteomics

Michael T Guarnieri*

National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO-80401, USA

***Corresponding author:** Michael T Guarnieri, National Renewable Energy Laboratory, 15013 Denver West Parkway, MS 3323 Golden, CO-80401, USA, Tel: 3033847921; E-mail: Michael.Guarnieri@nrel.gov

Abstract

Driven by the genomics revolution, functional, post-genomic analyses, such as transcriptomics, metabolomics, and proteomics, have become essential techniques for examination of mRNA, metabolite, and protein dynamics in algal systems. Such analyses are now providing insight into genomic architecture and metabolic capacity, and elucidating temporal regulatory mechanisms governing industrially relevant algal phenotypes and cellular responses to an array of environmental stimuli. Proteomic analyses, in particular, are playing a critical role in unraveling the prominent post-transcriptional regulatory mechanisms in algae. In turn, algal proteomics is laying the groundwork for implementation of algal strain-engineering strategies and helping bring to bear the full potential of microalgal biocatalysts. Herein, advances in microalgal proteomics are reviewed.

Introduction

As interest in alternatives to petroleum-derived fuels and chemicals has intensified, so too has interest in cyanobacteria and eukaryotic microalgae (henceforth collectively referred to as “algae”) as bio-production platforms. These organisms are emerging as “multi-use feedstocks” for an array of applications, ranging from biofuels and biomaterials to cosmetics and nutraceuticals. In turn, the utilization of algae as biocatalysts is seeing unprecedented growth. Omic analyses are driving this growth, playing an integral role in genome-wide modeling, elucidation of biosynthetic regulatory mechanisms, and hypothesis-driven strain engineering.

High-throughput sequencing technologies have played a major role in genomic and transcriptomic analyses [1], providing invaluable insight into genome architecture and gene expression, respectively, in an array of algae. A wealth of knowledge has been gained through the genomic sequencing and transcriptional profiling of the model eukaryotic and prokaryotic algae, *Chlamydomonas reinhardtii* [2] and *Synechocystis* PCC 6803 [3], respectively. However, given the prevalence of post-transcriptional regulation in algal systems, with translational regulation of chloroplast gene expression observed in a number of species [4], proteomic analyses are emerging as an essential tool for complete examination of these systems, and will likely continue to play a unique and prominent role in bringing to bear the full potential of microalgae as biocatalysts.

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

Just as high-throughput sequencing technologies are playing a major role in advancing genomic and transcriptomic analyses, so too are novel technologies driving the advancement of proteomic analyses. Improvements in labeling strategies and separation technologies, coupled with ever-improving mass spectral detection and bioinformatic data analysis suites, are opening the door for facile, quantitative, genome-wide proteomic analysis [5]. Among the recent advances are an array of enhanced modifications to conventional label-free proteomic strategies, such as Difference Gel Electrophoresis (DIGE) and Gel-based Liquid Chromatography/Mass Spectrometry (GeLC/MS) [6,7], as well as more contemporary label-based strategies, such as Reductive Isotope Di-Ethylation (RIDE) [8], Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) [9], and isobaric Tags for Relative and Absolute Quantitation (iTRAQ) [10]. This chapter focuses upon the application of these technologies to examine advances in algal proteomics, with a particular focus on biotechnological implications thereof.

Eukaryotic Algae Proteomics

Despite the relative wealth of genomic and transcriptomic data available across a diverse range of algal species, available proteomic data is comparatively sparse. In eukaryotic algae, proteomic analyses have primarily been applied to the model organism *Chlamydomonas reinhardtii,* which will serve as the primary focus of this section. These analyses have largely focused upon elucidation of subcellular compartment composition and sub-proteome mapping, or proteome-wide responses to environmental stress. More recently, proteomic analyses of non-model organisms, which often possess more desirable biocatalyst traits, have been undertaken (discussed further below).

Among the first proteomic analyses performed in algae was a comprehensive characterization of algal mitochondria [11]. *C. reinhardtii* mitochondria were isolated and analyzed via Blue Native-Polyacrylamide Gel Electrophoresis (BN-PAGE). Complexes involved in oxidative phosphorylation were resolved and the oligomeric state of these complexes was determined. Complexes were further resolved into their subunits by a 2D SDS-PAGE approach. Hippler, et al. similarly employed two-dimensional gel electrophoresis coupled to immuno-blotting (2D-IS) and mass spectrometry in order to characterize the thylakoid membranes isolated from wild-type and mutant strains of *C. reinhardtii* [12]. Their method facilitated the separation and characterization transmembrane-spanning light-harvesting complex proteins. Comparative analyses between wild-type and mutants deficient in photosystem I showed differential LHC abundance. The light harvesting complex was further characterized by Stauber, et al., wherein gene tagging revealed differentially processed N-termini in light harvesting proteins, as well as potential post-translation phospho-regulation, results which lend insight into the prominent differential processing and post-translational modifications observed in many algal systems [13].

Schmidt and colleagues examined the *C. reinhardtii* eyespot, the "visual system" governing phototaxis in flagellate green algae [14]. The group employed nano-liquid chromatography in combination with electrospray ionization-tandem mass spectrometry (nano-LC-ESI-MS/MS) in order to identify over 200 proteins in purified eyespots. The proteomic analysis was coupled with functional analysis, through silencing of a putative phototactic signaling kinase, Casein Kinase 1 (CK1), resulting in reduced levels of flagella and eyespot. In addition CK1 silencing disrupted circadian control of phototaxis, flagellar formation, and hatching.

Cytoskeletal organelles and components thereof have also been intensely examined in *C. reinhardtii*. Centriole component analysis was undertaken by Keller, et al., wherein "naked" centrioles were isolated and analyzed via multidimensional proteomics [15]. These cytoskeletal organelles are critical for proper centrosome formation, and are necessary for cilia and flagella development. Interestingly, the analysis revealed an array of human disease gene products encoding protein components of the centriole. Similarly, Pazour, et al. examined purified flagella from *C. reinhardtii*, employing a GeLC/MS/MS approach to

identify nearly 700 proteins, including nearly all known flagellar proteins, with functionality primarily associated with motor and signal transduction components [16]. As observed with Keller's work, numerous proteins with homologues associated with human disease were identified.

With the recent intensified interest in algal biofuels, a number of proteomic analyses have focused upon microalgal lipid accumulation. Moellering, et al. isolated lipid droplets from *C. reinhardtii* and employed a GeLC/MS/MS approach to examine droplet-associated proteins [17]. Their analysis identified over 250 proteins in a lipid droplet-enriched fraction, including a protein unique to green algae, referred to as Major Lipid Droplet Protein (MLDP). RNAi was employed to examine the functionality of this protein, demonstrating its role in lipid droplet size, and suggesting a potential role as a marker for lipid droplets and triacylglyceride accumulation. Interestingly, a number of proteins associated with lipid biosynthesis and protein trafficking were shown to be directly associated with lipid droplets, providing the first evidence that lipid droplet themselves may play an indirect role in carbon flux, and a direct role in lipogenesis. Related, the *C. reinhardtii* Carbon Concentrating Mechanism (CCM) was investigated by Baba and colleagues using isoelectic focusing coupled to 2D SDS-PAGE and fluorescent staining [18]. Under high-CO_2 condition (3% above ambient air levels), extracellular proteins increased with gametogenesis-related and hydroxyproline-rich glycoproteins displaying the largest increase in abundance. Additionally, flagellar components were induced under high-CO_2 conditions.

The model marine diatom, *Phaeodactylum tricornutum*, has also been proteomically-queried for lipid accumulation mechanisms. Label-free proteomics have been applied to *P. tricornutum* in low-and high-lipid states, revealing major changes in branched-chain amino acid metabolism, glycolysis, and lipid metabolism [19]. This work found Methylcronotyl-CoA Carboxylase (MCC2), to be the most differentially regulated gene upon transition to a lipid accumulation state. This analysis was functionally complemented, wherein MCC2 inhibition was knocked down by RNA interference. The resultant phenotype displayed altered flux to branched chain amino acids and incomplete nitrogen utilization, in turn leading to decreases in both biomass and TAG accumulation. *P. tricornutum* was further examined under nitrogen deprivation, revealing increased abundance in proteins regulating nitrogen assimilation and fatty acid biosynthesis [20]. Interestingly, these abundance increases corresponded with a decrease in photosynthesis and lipid catabolism enzymes. Such analyses provide critical insight linking molecular and physiological changes, and provide strain-engineering targets for enhanced TAG and biomass accumulation in diatoms.

Sub-proteomic analyses have also been widely employed in model diatoms. Frigeri and colleagues examined the isolated cell wall of *Thalassiosira pseudonana*, the first diatom with a sequenced genome, using a modified GeLC/MS/MS approach [21]. This study marked the first examination of a diatom proteome, identifying 31 cell wall proteins. mRNAs coding for these proteins were examined during synchronized cell cycle progression, with nearly 30% of the genes examined displayed similar expression patterns as known genes involved in silica polymerization (silaffins). Additionally, the identification of glutamate acetyltransferase, a critical component of the urea cycle and amine metabolism led the authors to analyze patterns of mRNA expression for genes involved in polyamine biosynthesis. Functional analyses confirmed the role of polyamines in silica structure.

Algal responses to environmental stress have been extensively examined via proteomic analyses. In order to monitor proteome dynamics in *C. reinhardtii* in response to heat shock, Muhlhaus and colleagues employed a novel approach involving full ^{15}N labeling and mass spectrometry-based shotgun proteomics [22]. Using this approach the group identified over 3,000 proteins. Over 240 proteins were shown to be differentially abundant under heat stress. Proteins increasing in abundance were primarily comprised of chaperones, as well as proteins involved in chromatin remodeling, signal transductions, photosynthesis, and apoptosis. Proteins decreasing in abundance were enriched in carbon flux into protein

biosynthesis, providing insight into algal stress acclimation as it relates to nitrogen utilization (discussed further below).

The iron-deprivation response was examined in *C.reinhardtii* through a combined comparative proteomics and spectroscopic approach [23]. The results revealed reduced photosynthetic electron transfer in iron-deficient cells, despite a significant increase in the antenna size of photosystem II. A number of stress-related chloroplast proteins were also induced under iron starvation. The authors concluded that iron deprivation "induces a transition from photoheterotrophic to primarily heterotrophic metabolism" lending insight into the regulatory hierarchy of organellar iron allocation.

Recently, integrated transcriptomic and quantitative proteomics were applied to *T. pseudonana* to examine the phosphorus stress response [24]. Over 100 proteins were differentially abundant under phosphorus deplete conditions. Coordinated changes in abundance of transcripts and proteins were observed for multiple biochemical pathways, notably including glycolysis and translation-related genes and proteins. The data reveal the importance of polyphosphate production on cellular phosphorous allocation and transport. Additionally, an increase of metalloenzymes and remodeling of sulfolipids lent further insight into the mechanistic switch to utilize dissolved organic phosphorous.

A critical process consideration for large-scale implementation of algal biocatalysis is the utilization of water, with great interest being directed towards the examination of algal species capable of saltwater cultivation. A series of analyses by Liska, Katz, and colleagues examined the hypersaline adaptability of the halotolerant green alga *Dunaliella sp.* [25,26]. Comparative proteomics examined high-salt-induced proteins using 2D-DIGE, identifying nearly 80 differentially abundant proteins. These proteins were involved in diverse functional ontology classifications, ranging from Calvin cycle and starch mobilization to protein biosynthesis. The results revealed an enhancement of photosynthetic CO_2 assimilation and carbon repartitioning towards glycerol synthesis, in order to provide an osmoprotectant, providing critical insight into algal halotolerance.

The work above primarily focuses upon model eukaryotic alga, namely, algae with well-characterized nuclear or organellar genomes. Comparatively, proteomic data for non-model organisms is minimal, though methods to circumvent the lack of genomic sequence data are emerging. Wang and colleagues performed one of the first large-scale proteomic analyses of an unsequenced organism, employing a "cross-species protein identification strategy" in order to examine the proteome of the *Haematococcus pluvialis* cell wall [27]. Conserved motifs and domains were examined to aid in peptide identification. However, it was noted that post-translational modifications and multiple amino acid substitution differences across species dramatically reduced the identification rates. Our lab has recently employed GeLC/MS/MS to explore the nitrogen deprivation-induced lipid accumulation response in the oleaginous microalga, *Chlorella vulgaris*, using a transcriptomic sequence data as a reference database [6]. Our results revealed a pronounced increase in both fatty acid and triacylglyceride biosynthetic proteins upon nitrogen deprivation, and demonstrate the utility of a working transcriptome in the absence of genomic sequence data. More recently, we have examined the time-course dynamics of the *C. vulgaris* soluble proteome, identifying an array of cell cycle and cell signaling proteins that may play a role in coordinating nitrogen sensing, cell cycle progression, and carbon partitioning [7]. Similar nitrogen deprivation proteomic analyses in *C. reinhardtii* observed alterations in general stress responses and carbon assimilation processes, also observing increases in fatty acid and TAG biosynthetic machinery under nitrogen limited conditions. Complementary metabolomic analysis also revealed altered nitrogen: carbon ratios in both amino acid metabolism and the TCA cycle [28]. Garnier and colleagues also observed alterations to the fatty acid biosynthetic machinery under nitrogen deprivation in the non-model marine alga, *Tisochrysis lutea* [29]. Utilizing 2-DE/MS, they compared wild-type and a lipid over-accumulating mutant of *T. lutea*, identifying 17 differentially expressed proteins attributed

to nitrogen depletion. These proteins spanned an array of metabolic pathways, including lipid and amino acid biosynthesis, further suggesting universal mechanisms of nitrogen deprivation-induced lipid accumulation.

Beyond lipid accumulation mechanisms, proteomic efforts are now underway to examine high- and low-light tolerance mechanisms in algae, critical for effective deployment in open pond systems in which light intensity will vary by both region and season. McKew, et al, examined the *E. huxleyi's* plasticity and adaptation to "suboptimal" and "supraoptimal" light intensity [30]. LC-MS/MS shotgun proteomics revealed differential abundance of proteins involved in photosynthesis, with nearly two-fold increases in PSI : PSII protein ratios, and dramatic (>5-fold) increases in light-harvesting proteins under low light intensity. Conversely, photoprotective proteins were nearly 3-fold higher under high light intensity.

Cyanobacterial Proteomics

Similar to proteomic analyses in eukaryotic algae, cyanobacterial proteomics have primarily been applied to the model organism *Synechocystis* PCC 6803 (PCC 6803), again heavily focused on organelle composition and stress responses. The outer membrane of PCC 6803 was isolated and characterized via 2D gel isoelectric focusing by Huang and colleagues [31]. Forty-nine proteins were identified corresponding to 29 different gene products. All of the identified proteins contained N-terminal signal peptides, though nearly 40% of the proteins were of unknown function. An array of porins and transporters were identified, as well as a number of outer membrane associated proteases. Similarly, the plasma membrane of PCC 6803 was examined by Pisareva, et al. 1D SDS/PAGE MALDI-TOF MS was utilized to identify 51 proteins from a purified plasma membrane fraction [32]. The majority of the proteins examined were newly identified, with representation from chemotaxis, metalloprotease, and secretion ontologies and a significant fraction predicted to be integral, transmembrane helix-containing proteins.

The composition and dynamics of membrane protein complexes were examined in PCC 6803 by 2D blue native/SDS PAGE MS. Approximately 20 distinct membrane protein complexes were resolved from photoautotrophically grown wild-type cells [33]. Four distinct complexes containing type I NAD(P)H dehydrogenase subunits were identified. An array of trophic growth modes was examined, including photoautotrophic, mixotrophic, and heterotrophic, in the presence of varied concentrations of CO_2, iron, and salt. Iron depletion was found to have the most profound effect on photosystem I. Conversely, the NDH complexes were most affected by changes in CO_2 and trophic mode.

Cyanobacterial halotolerance and high-salt stress responses have also been extensively examined via proteomic analyses. Fulda and colleagues employed ^{35}S-methionine labeling coupled to 2DE and PMF to examine differential abundance of protein induced by salt shock or accumulated after long-term salt acclimation [34]. Similar to the salt stress response observed in eukaryotic microalgae (discussed above), biosynthetic enzymes governing glucosylglycerol were upregulated under high-salt conditions. Additionally, an array of universal stress response proteins, such as heat-shock and anti-oxidative proteins, were upregulated. The resultant data were compared to transcriptomic analyses, revealing a high concordance between some transcript and protein levels. However, a large percentage (42%) of up-regulated proteins were not differentially abundant at the transcript level, further underscoring the significance of post-transcriptional regulation observed in both eukaryotic microalgae and cyanobacterial systems. Pandhal and colleagues further explored cyanobacterial salt tolerance by comparatively examining PCC 6803 and a closely related halotolerant *Euhalothece* sp. BAA001 [35]. A ^{15}N in vivo labeling approach was coupled to quantitative iTRAQ under increasing salt concentration. Antioxidative enzymes and putative transcriptional regulators involved in stress response were implicated as key players in halotolerance.

Quantitative 2D DIGE has also been employed to examine the heat stress response of PCC 6803. Suzuki, et al., subjected cells to high heat (44°C) and found high correlation at both the transcriptional and protein levels for chaperonins and proteases, such as members of the groEL and Hsp families [36]. However, similar to the salt-stress response, they observed that levels of some proteins, such as elongation factors, are primarily regulated post-transcriptionally, suggesting both transcriptional and translational regulation plays a prominent role in heat shock responses. Slabas and colleagues further examined the heat shock response of PCC6803 by comparatively examining wild type and Histidine Kinase 34 (Hik34) knockout mutants with enhanced thermal tolerance [37]. Quantitative 2D-DIGE/MS revealed increased abundance of heat shock proteins in wild types cells when subjected to heat stress. Interestingly, thermal tolerant mutants displayed higher abundance of heat shock proteins under both low- and high-heat conditions, suggesting Hik34 negatively regulates heat shock responsiveness in PCC 6803.

As in eukaryotic microalgae, biofuel applications are being explored in cyanobacteria, and proteomic examination of hydrocarbon production and tolerance in these systems is underway. In order to examine the low cellular tolerance of PCC 6803 to alkanes, Liu and colleagues employed iTRAQ-LC-MS/MS to examine the response to hexane [38]. Ubiquitous stress responses were induced by hexane addition, leading to the upregulation of a number of transporters, sulfur relay system-, and oxidative stress-related proteins. The results lay the foundation for metabolic engineering strategies to enhance alkane stress tolerance in *Synechocystis*.

Perhaps the most comprehensive proteomic analyses performed in PCC 6803 includes a recent time-course shotgun characterization of the proteome in response to alterations of 33 environmental conditions, including nitrogen, iron, sulfate, and phosphate deprivation, varied trophic growth modes, and cold and salt stress [39]. The resultant datasets spanned over 50% of the predicted genome. Notably, quantitative analyses revealed a universal stress response involving the activation of atypical carbon and nitrogen acquisition pathways.

Significant proteomic efforts have also been dedicated to the *Arthrospira* genus of cyanobacteria (commonly referred to as "*Spirulina*"), widely cultivated as a food source and dietary supplement. Indeed, proteomic analyses have had a large impact on strain engineering strategies targeting elucidation and enhancement of *Spirulina* composition for nutraceutical applications (for extensive review, please refer to [40]). Wang and colleagues employed 2DE and peptide mass fingerprinting to compare protein expression profiles under varied salt stress conditions. This analysis identified a number of proteins proposed to confer high salt tolerance to *Arthrospira sp.*, including ABC transporters and Signal Recognition Particles (SRPs) [41]. Hongsthong and colleagues conducted a series of proteomic analysis, employing 2D-DIGE/MS, to examine *Arthrospira* responsiveness to both cold- and heat-induced stress [42,43]. Interestingly, the data revealed a relationship between temperature and nitrogen assimilation. Wattiez's group recently employed label-free proteomics to examine the *Spirulina* nitrogen deprivation response, revealing novel insight into long-term survival mechanisms and alternative nitrogen utilization [44]. They further examined the cyanobacterial responsiveness to light/dark diurnal cycles using a novel 3D LC-MS/MS approach, wherein immobilized metal affinity chromatography was successfully employed to remove the highly-abundant allophycocyanin [45].

Beyond *Synechocystis sp.* and *Spirulina sp.*, significant proteomic efforts have also been dedicated to the cyanobacterial genus *Synechococcus*. Koksharova and colleagues employed MALDI-TOF MS to comparatively examine wild-type and cell division mutants of *Synechococcus sp.* [46]. Proteins related to myriad biological functions, including cell division, chromosome segregation, photosynthesis, and protein synthesis, were differentially regulated in cell division mutants, underscoring the pleiotropic response to cell cycle inhibition. Subproteomic analyses of *Synechococcus sp.* carboxysomes and metalloproteins [47,48], proteome responsiveness to nutrient deprivation [49] and temperature alteration

[50], and proteomic regulation of circadian rhythm [51] have further bolstered the proteomic knowledge base surrounding this high-potential cyanobacterial genus. Additional proteomic efforts have extended to other non-model cyanobacteria. For example, Perez-Gomez and colleagues recently examined phycobilisome composition in *Fremyella diplosiphon* and *Tolypothrix* PCC 7601 using integrated nano-LC/ESI/MS/MS [52], elucidating the localization of rod-linker proteins and oxidoreductases in phycocyanin and phycoerythrin. A number of studies have also examined differential toxicity in strains of *Microcystis aeruginosa* [53,54]. Surprisingly, a number of unrelated pathway components were implicated in the analysis, including glycolytic and Calvin cycle proteins, providing novel insight into microcystin (toxin) metabolism.

Discussion

Though a myriad of algal proteomic analyses have been discussed in this chapter, this is far from an exhaustive review of algal proteomics conducted to date. Rather, this chapter is intended to convey the impact proteomics has had on elucidating our fundamental understanding of algal biology, both basic and applied. In addition to delineating phylogenetic relationships and evolutionary linkages through characterization of organelle and subproteome complements, the genome-wide proteomic complement of an organism allows for a more complete definition of metabolic regulation, capacity, and ultimately, suitability as a biocatalyst. As algae continue to emerge as promising biocatalysts for an array of biochemical and bioproducts, proteomics will play an increasingly important role in strain exploration and optimization.

A number of the comparative proteomic experiments discussed above employ nutrient deprivation as a means to examine algal stress responses, and the link between these responses and phenotypic manifestation is just beginning to be defined. For example, in many cases, nutrient deprivation has been shown to lead to lipid accumulation in oleaginous algae, though knowledge of the interplay between nutrient sensing, carbon partitioning, and cell cycle regulation remains incomplete. By understanding the networks regulating these responses, hypothesis-driven strain engineering strategies can be developed, targeting stress phenotypes in the absence of environmental cues, in turn streamlining bioprocesses. Similarly, resistance to environmental stress, such as that observed in halotolerant algae, can lend insight into genetic engineering strategies targeting saltwater tolerance in a freshwater alga, again having massive bioprocess implications. Additionally, microalgae offer relatively unexplored platforms as model systems. Insights into omic architecture and stress response mechanisms can potentially be applied to higher plants, such as the development of nutrient and drought resistant terrestrial food crops. Lastly, beyond diatoms and chlorophytes, a wealth of proteomic data is now available for dinoflagellates, which comprise a large fraction of marine plankton [55-57]. Though biotechnological applications for dinoflagellates have yet to be fully examined, these data are nonetheless valuable resources for the examination of unicellular photosynthetic organisms.

Technological advances in peptide labeling, separations, and detection will continue to facilitate proteomic analyses with increasingly higher throughput and quantitative capability. Bioinformatic advances, which are beyond the scope of this chapter, will also play an integral role in advancing this field. Additionally, integration of proteomic data with genomic, transcriptomic and metabolomics data will be essential to fully define algal systems. However, the importance and power of algal proteomics is clear, and will continue to be an increasingly important component of algal systems biology.

References

1. Morozova O, Marra MA (2008) Applications of next-generation sequencing technologies in functional genomics. Genomics 92: 255-264.

2. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, et al. (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318: 245-250.

3. Kaneko T, Tabata S (1997) Complete genome structure of the unicellular *cyanobacterium Synechocystis sp.* PCC6803. Plant Cell Physiol 38: 1171-1176.

4. SP Mayfield, CB Yohn, A Cohen, A Danon (1995) Regulation of Chloroplast Gene-Expression. Annu Rev Plant Phys 46: 147-166.

5. Eisenberg D, Marcotte EM, Xenarios I, Yeates TO (2000) Protein function in the post-genomic era. Nature 405: 823-826.

6. Guarnieri MT, Nag A, Smolinski SL, Darzins A, Seibert M, et al. (2011) Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga. PLoS One 6: 25851.

7. Guarnieri MT, Nag A, Yang S, Pienkos PT (2013) Proteomic analysis of *Chlorella vulgaris*: potential targets for enhanced lipid accumulation. J Proteomics 93: 245-253.

8. Barrios-Llerena ME, Pritchard JC, Kerr LE, Le Bihan T (2011) The use of a novel quantitation strategy based on Reductive Isotopic Di-Ethylation (RIDE) to evaluate the effect of glufosinate on the unicellular algae *Ostreococcus tauri*. J Proteomics 74: 2798-2809.

9. Martin SF, Munagapati VS, Salvo-Chirnside E, Kerr LE, Le Bihan T (2012) Proteome turnover in the green alga *Ostreococcus tauri* by time course 15N metabolic labeling mass spectrometry. J Proteome Res 11: 476-486.

10. J Longworth, J Noirel, J Pandhal, PC Wright, S Vaidyanathan (2012) HILIC- and SCX-based quantitative proteomics of *Chlamydomonas reinhardtii* during nitrogen starvation induced lipid and carbohydrate accumulation. J Proteome Res 11: 5959-5971.

11. R van Lis, A Atteia, G Mendoza-Hernandez, D Gonzalez-Halphen (2003) Identification of novel mitochondrial protein components of *Chlamydomonas reinhardtii*. A proteomic approach. Plant Physiol 132: 318-330.

12. M Hippler, J Klein, A Fink, T Allinger, P Hoerth (2001) Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from *Chlamydomonas reinhardtii.* Plant J 28: 595-606.

13. Stauber EJ, Fink A, Markert C, Kruse O, Johanningmeier U, et al. (2003) Proteomics of *Chlamydomonas reinhardtii* light-harvesting proteins. Eukaryot Cell 2: 978-994.

14. Schmidt M, Gessner G, Luff M, Heiland I, Wagner V, et al. (2006) Proteomic analysis of the eyespot of *Chlamydomonas reinhardtii* provides novel insights into its components and tactic movements. Plant Cell 18: 1908-1930.

15. Keller LC, Romijn EP, Zamora I, Yates JR 3rd, Marshall WF (2005) Proteomic analysis of isolated *chlamydomonas centrioles* reveals orthologs of ciliary-disease genes. Curr Biol 15: 1090-1098.

16. Pazour GJ, Agrin N, Leszyk J, Witman GB (2005) Proteomic analysis of a eukaryotic cilium. J Cell Biol 170: 103-113.

17. Moellering ER, Benning C (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryot Cell 9: 97-106.

18. Baba M, Suzuki I, Shiraiwa Y (2011) Proteomic analysis of high-CO(2)-inducible extracellular proteins in the unicellular green alga, *Chlamydomonas reinhardtii*. Plant Cell Physiol 52: 1302-1314.

19. Ge F, Huang W, Chen Z, Zhang C, Xiong Q, et al. (2014) Methylcrotonyl-CoA Carboxylase Regulates Triacylglycerol Accumulation in the Model Diatom *Phaeodactylum tricornutum*. Plant Cell 26: 1681-1697.

20. Yang ZK, Ma YH, Zheng JW, Yang WD, Liu JS, et al. (2014) Proteomics to reveal metabolic network shifts towards lipid accumulation following nitrogen deprivation in the diatom *Phaeodactylum tricornutum*. J Appl Phycol 26: 73-82.

21. Frigeri LG, Radabaugh TR, Haynes PA, Hildebrand M (2006) Identification of proteins from a cell wall fraction of the diatom *Thalassiosira pseudonana*: insights into silica structure formation. Mol Cell Proteomics 5: 182-193.

22. T Muhlhaus, J Weiss, D Hemme, F Sommer, M Schroda (2011) Quantitative shotgun proteomics using a uniform ^{15}N-labeled standard to monitor proteome dynamics in time course experiments reveals new insights into the heat stress response of *Chlamydomonas reinhardtii*. Mol Cell Proteomics 10: M110.004739.

23. Naumann B, Busch A, Allmer J, Ostendorf E, Zeller M, et al. (2007) Comparative quantitative proteomics to investigate the remodeling of bioenergetic pathways under iron deficiency in *Chlamydomonas reinhardtii*. Proteomics 7: 3964-3979.

24. Dyhrman ST, Jenkins BD, Rynearson TA, Saito MA, Mercier ML, et al. (2012) The transcriptome and proteome of the diatom *Thalassiosira pseudonana* reveal a diverse phosphorus stress response. PLoS One 7: 33768.

25. Katz A, Waridel P, Shevchenko A, Pick U (2007) Salt-induced changes in the plasma membrane proteome of the halotolerant alga *Dunaliella salina* as revealed by blue native gel electrophoresis and nano-LC-MS/MS analysis. Mol Cell Proteomics 6: 1459-1472.

26. AJ Liska, A Shevchenko, U Pick, A Katz (2004) Enhanced photosynthesis and redox energy production contribute to salinity tolerance in *Dunaliella* as revealed by homology-based proteomics. Plant Physiol 136: 2806-2817.

27. Wang SB, Hu Q, Sommerfeld M, Chen F (2004) Cell wall proteomics of the green alga *Haematococcus pluvialis* (Chlorophyceae). Proteomics 4: 692-708.

28. Lee do Y, Park JJ, Barupal DK, Fiehn O (2012) System response of metabolic networks in *Chlamydomonas reinhardtii* to total available ammonium. Mol Cell Proteomics 11: 973-988.

29. Garnier M, Carrier G, Rogniaux H, Nicolau E, Bougaran G, et al. (2014) Comparative proteomics reveals proteins impacted by nitrogen deprivation in wild-type and high lipid-accumulating mutant strains of *Tisochrysis lutea*. J Proteomics 105: 107-120.

30. McKew BA, Lefebvre SC, Achterberg EP, Metodieva G, Raines CA, et al. (2013) Plasticity in the proteome of *Emiliania huxleyi* CCMP 1516 to extremes of light is highly targeted. New Phytol 200: 61-73.

31. Huang F, Parmryd I, Nilsson F, Persson AL, Pakrasi HB, et al. (2002) Proteomics of *Synechocystis sp.* strain PCC 6803: identification of plasma membrane proteins. Mol Cell Proteomics 1: 956-966.

32. Pisareva T, Shumskaya M, Maddalo G, Ilag L, Norling B (2007) Proteomics of *Synechocystis sp.* PCC 6803. Identification of novel integral plasma membrane proteins. FEBS J 274: 791-804.

33. Herranen M, Battchikova N, Zhang P, Graf A, Sirpiö S, et al. (2004) Towards functional proteomics of membrane protein complexes in *Synechocystis sp.* PCC 6803. Plant Physiol 134: 470-481.

34. Fulda S1, Huang F, Nilsson F, Hagemann M, Norling B (2000) Proteomics of *Synechocystis sp.* strain PCC 6803. Identification of periplasmic proteins in cells grown at low and high salt concentrations. Eur J Biochem 267: 5900-5907.

35. Pandhal J, Ow SY, Wright PC, Biggs CA (2009) Comparative proteomics study of salt tolerance between a nonsequenced extremely halotolerant *cyanobacterium* and its mildly halotolerant relative using in vivo metabolic labeling and in vitro isobaric labeling. J Proteome Res 8: 818-828.

36. Suzuki I, Simon WJ, Slabas AR (2006) The heat shock response of *Synechocystis sp.* PCC 6803 analysed by transcriptomics and proteomics. J Exp Bot 57: 1573-1578.

37. Slabas AR, Suzuki I, Murata N, Simon WJ, Hall JJ (2006) Proteomic analysis of the heat shock response in *Synechocystis* PCC6803 and a thermally tolerant knockout strain lacking the histidine kinase 34 gene. Proteomics 6: 845-864.

38. Liu J, Chen L, Wang J, Qiao J, Zhang W (2012) Proteomic analysis reveals resistance mechanism against biofuel hexane in *Synechocystis sp.* PCC 6803. Biotechnol Biofuels 5: 68.

39. Wegener KM, Singh AK, Jacobs JM, Elvitigala T, Welsh EA, et al. (2010) Global proteomics reveal an atypical strategy for carbon/nitrogen assimilation by a *cyanobacterium* under diverse environmental perturbations. Mol Cell Proteomics 9: 2678-2689.

40. Klanchui A, Vorapreeda T, Vongsangnak W, Khannapho C, Cheevadhanarak S, et al. (2012) Systems biology and metabolic engineering of Arthrospira cell factories. Comput Struct Biotechnol J 3: 201210015.

41. Wang H, Yang Y, Chen W, Ding L, Li P, et al. (2013) Identification of differentially expressed proteins of Arthrospira (Spirulina) plantensis-YZ under salt-stress conditions by proteomics and qRT-PCR analysis. Proteome Sci 11: 6.

42. Hongsthong A, Sirijuntarut M, Prommeenate P, Lertladaluck K, Porkaew K, et al. (2008) Proteome analysis at the subcellular level of the *cyanobacterium* Spirulina platensis in response to low-temperature stress conditions. FEMS Microbiol Lett 288: 92-101.

43. Kurdrid P, Senachak J, Sirijuntarut M, Yutthanasirikul R, Phuengcharoen P, et al., (2011) Comparative analysis of the Spirulina platensis subcellular proteome in response to low- and high-temperature stresses: uncovering cross-talk of signaling components. Proteome Sci 9: 39.

44. Deschoenmaeker F, Facchini R, Leroy B, Badri H, Zhang CC, et al. (2014) Proteomic and cellular views of *Arthrospira sp.* PCC 8005 adaptation to nitrogen depletion. Microbiology 160: 1224-1236.

45. Matallana-Surget S, Derock J, Leroy B, Badri H, Deschoenmaeker F, et al. (2014) Proteome-Wide Analysis and Diel Proteomic Profiling of the *Cyanobacterium Arthrospira* platensis PCC 8005. PLoS One 9: 99076.

46. Koksharova OA, Klint J, Rasmussen U (2007) Comparative proteomics of cell division mutants and wild-type of *Synechococcus sp.* strain PCC 7942. Microbiology 153: 2505-2517.

47. Long BM, Badger MR, Whitney SM, Price GD (2007) Analysis of carboxysomes from *Synechococcus* PCC7942 reveals multiple Rubisco complexes with carboxysomal proteins CcmM and CcaA. J Biol Chem 282: 29323-29335.

48. Barnett JP, Scanlan DJ, Blindauer CA (2012) Protein fractionation and detection for metalloproteomics: challenges and approaches. Anal Bioanal Chem 402: 3311-3322.

49. Cox AD, Saito MA (2013) Proteomic responses of oceanic *Synechococcus* WH8102 to phosphate and zinc scarcity and cadmium additions. Front Microbiol 4: 387.

50. Mackey KR, Paytan A, Caldeira K, Grossman AR, Moran D, et al. (2013) Effect of temperature on photosynthesis and growth in marine *Synechococcus spp.* Plant Physiol 163: 815-829.

51. Guerreiro AC, Benevento M, Lehmann R, van Breukelen B, Post H, et al. (2014) Daily rhythms in the cyanobacterium Synechococcus elongatus probed by high-resolution mass spectrometry based proteomics reveals a small-defined set of cyclic proteins. Mol Cell Proteomics .

52. Pérez-Gómez B, Mendoza-Hernández G, Cabellos-Avelar T, Leyva-Castillo LE, Gutiérrez-Cirlos EB, et al. (2012) A proteomic approach to the analysis of the components of the phycobilisomes from two cyanobacteria with complementary chromatic adaptation: Fremyella diplosiphon UTEX B590 and Tolypothrix PCC 7601. Photosynth Res 114: 43-58.

53. R Alexova, PA Haynes, BC Ferrari, BA Neilan (2011) Comparative protein expression in different strains of the bloom-forming *cyanobacterium Microcystis aeruginosa*. Mol Cell Proteomics 10: M110003749.

54. Tonietto A, Petriz BA, Araújo WC, Mehta A, Magalhães BS, et al. (2012) Comparative proteomics between natural Microcystis isolates with a focus on microcystin synthesis. Proteome Sci 10: 38.

55. Wang DZ, Zhang YJ, Zhang SF, Lin L, Hong HS (2013) Quantitative proteomic analysis of cell cycle of the dinoflagellate *Prorocentrum donghaiense* (Dinophyceae). PLoS One 8: 63659.

56. Li C, Zhang Y, Xie ZX, He ZP, Lin L, et al. (2013) Quantitative proteomic analysis reveals evolutionary divergence and species-specific peptides in the *Alexandrium tamarense* complex (Dinophyceae). J Proteomics 86: 85-96.

57. Wang DZ, Li C, Zhang Y, Wang YY, He ZP, et al. (2012) Quantitative proteomic analysis of differentially expressed proteins in the toxicity-lost mutant of *Alexandrium catenella* (Dinophyceae) in the exponential phase. J Proteomics 75: 5564-5577.

Transformation of Chloroplasts of *Chlamydomonas* for the Production Of Therapeutic Proteins

Pérez España Victor Hugo[1], López Pérez Pablo Antonio[1], Romero Cortes Teresa[1], Peralta Gil Martín[1], Duran Figueroa Noé [2], Badillo-Corona Agustín[2]

[1]Escuela Superior de Apan, Universidad Autónoma del Estado de Hidalgo, Carretera Apan-Calpulalpan Km 8, Chimalpa, Apan, Hidalgo, México

[2]Unidad Profesional Interdisciplinaria de Biotecnología, Instituto Politécnico Nacional, Av. Acueducto SN Col. Barrio la Laguna Ticomán, 07340 México City, México

***Corresponding author:** Pérez España Victor Hugo1, Escuela Superior de Apan, Universidad Autónoma del Estado de Hidalgo, Carretera Apan-Calpulalpan Km 8, Chimalpa, Apan, Hidalgo, México

Abstract

Chlamydomonas reinhardtii can be considered as a viable alternative for the production of various products of industrial interest. Integration of transgenes into the chloroplast genome of this algae has important advantages over others expression systems. Combined with the efficacy, safety, eases to store the products obtained for a considerable time, make of these algae a practical and sustainable expression system of recombinant proteins. The biological behavior of protein obtained by the modification of the chloroplasts of *Chlamydomonas* exhibit the same behavior that natively produced. Therefore, the development of tools and transformation methods on this expression system, will contribute directly to diminish the cost of production of biopharmaceutical products for use in humans and animals. In this chapter, we review the most recent progress in the engineering of chloroplast of *C. reinhardtii* directed to produce therapeutic proteins.

Keywords: Algae*; Chlamydomonas*; Chloroplast Transformation; Therapeutic Proteins

Introduction

In the field of biopharmaceutical products, the new trends are directed to improve the benefits in terms of cost, safety and coverage [1]. It is particularly important in developing countries where the access to appropriate therapeutics is very limited. The term "biopharmaceutical" was coined specifically to describe products generated or produced by molecular biology methods [2], such products include antibodies, growth factors, antigens, blood-related proteins, hormones, etc.

Traditionally, the organisms mainly used for expression of recombinant proteins have been bacteria, yeasts and mammalian cells [2-4]. Molecular pharming is a system that combines

Recent Advances in Microalgal Biotechnology
Edited by: Dr. Jin Liu, Dr. Zheng Sun and Dr. Henri Gerken

traditional agriculture with molecular biotechnology. In terms of costs, the production of proteins in land plants has shown to be an attractive alternative [5,6]. In this platform, have been expressed a variety of therapeutic proteins and biological compounds, for example, hormones [7], vaccines [8] and antibodies [9]. However, nuclear expression in plants have three major drawbacks, 1) the time required for to have a stable transgenic line and for to produce usable quantities of the protein; 2) the potential contamination to others crops via pollen and 3) transgene silencing. Plastid genomes have been successfully engineered for avoiding some of these drawbacks [10, 11].

The eukaryotic microalga *Chlamydomonas reinhardtii*, represent an excellent alternative for recombinant protein production. This microalga combines the beneficial features of the plant system without the mentioned drawbacks and offers others desirables characteristics, like: faster growing, potential to scale up rapidly, can be cultivated in containment to reduce the risk of gene flow, don't require cultivable lands, grow photosynthetically using the light energy and CO_2 as carbon source, have sexual and asexual reproduction, etc. This microorganism has the machinery to fold and assemble proteins, has been classified as Generally Recognized as Safe (GRAS) organisms by the Food and Drug Administration (FDA). Actually, exist transformation protocol for the three genetic compartments (nucleus, mitochondria and chloroplast). The antigens produced by these algae can be stored for a long time (1.5 years at room temperature) in lyophilized cells and their rigid cell wall protect the antigens from rapid degradation in the stomach [12, 13].

The Figure 1, compare the cost of production of therapeutic antibodies in different expression platforms, it shows that the cost of production in algae is much cheaper that in CHO cells, transgenic chickens/eggs, transgenic goats/milk, and even lower than in microbes and plants [14- 17].

Nowadays, there are a number of papers that reports transgenic strains of *C. reinhardtii* that express therapeutic proteins in their chloroplasts (Table 1), those proteins show similar biological activity to the native protein, demonstrating the huge potential as a robust platform for the production of recombinant proteins. In this chapter we review the most representative cases of production of therapeutic proteins in *Chlamydomonas* chloroplasts, presenting a general protocol of transformation and discuss the potential of this technology in the future years.

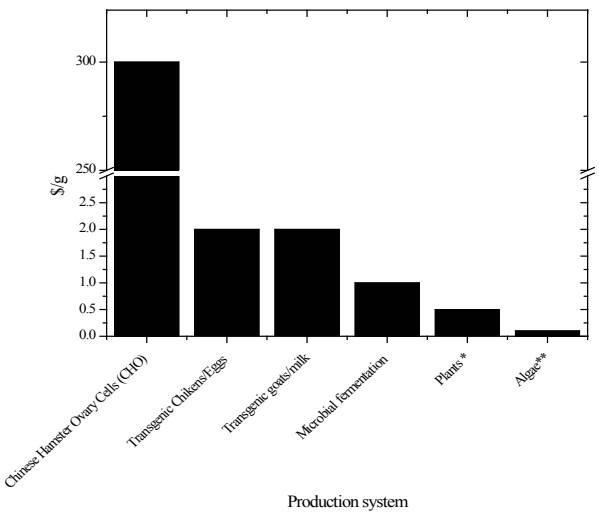

Figure 1: For the cost of production several assumptions were made: *The expression level is 1% of the dry weight; final yield is 18.75 kg purified protein per acre. ** Are not considered costs associated with energy, purification, bioreactor operation, waste treatment, equipment depreciation, and others [14-17]

Recombinant Therapeutic Protein	Origin organism	Yield (%)	Construct (cassette)	Assay Activity	Principal Remarks
Antibody HSV8-lsc, large simple chain antibody directed against glycoprotein D	Herpes Simplex Virus (HSV)	NR	*atp*A 5´ HSV8lsc *rbc*L 3´ *rbc*L 5´ HSV8lsc *rbc*L 3´	*in vitro*	Here, was shown, by first time the efficient expression of a unique large single-chain (lsc) antibody in chloroplasts of *C. reinhardtii*. Were achieved high levels of protein accumulation using either of two chloroplast promoters and 5' and 3' UTRs elements.
Protein VP1 fused to Cholera Toxin B (CTB)	Foot-and-mouth disease virus (FMDV)	3 – 4 TSP	*atp*A 5´ CTB-VP1 *rbc*L 3´	*in vitro*	This study demonstrated that CTB-VP1 fusion protein could be highly expressed in chloroplasts. Binding assays showed that the *C. reinhardtii* chloroplast-expressed CTBVP1 fusion protein could bind to the intestinal membrane GM1- ganglioside receptor, indicating the feasibility of using transgenic green alga as a mucosal vaccine source.
Tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL)	*Homo sapiens*	0.43 - 0.67 TSP	*atp*A 5´ TRAIL *rbc*L 3´	NR	The expression vector, p64TRAIL, containing the cDNA coding for the soluble TRAIL (sTRAIL), was constructed with *clp*P-*trn*L-*pet*B-*chl*L-*rpl*23-*rpl2* as *C. reinhardtii* plastid homologous recombinant fragments and spectinomycin-resistant *aadA* gene as a select marker.
Mammary-associated serum amyloid (M-SAA)	*Bos taurus*	0.25 TP 3 TSP 5 TSP	*psb*D 5´ M-SAA *psb*A 3´ *psb*A 5´ M-SAA *psb*A 3´ + *psb*D-*psb*A	*in vitro* HT29 cells	The mammalian coding region of bovine mammary-associated serum amyloid (M-SAA) achieve expression levels of recombinant protein above 5% of total protein
Structural protein E2	Classical Swine Fever Virus (CSFV)	1.5 - 2 TSP	*rbc*L 5´ E2 *atp*A 3´	*in vivo*, subcutaneously in mice	The results showed that C. *reinhardtii* chloroplast could be an effective system for giving rise to accumulation of the CSFV E2 glycoprotein. In order to identify the expressed protein immunogenicity, animal immune experiments by infection and gavage were conducted.

Glutamic Acid Decarboxilase 65 (GAD65)	*Homo sapiens*	0.25 - 0.3 TSP	*rbc*L 5´ hGAD65 *rbc*L 3´	*in vitro* serum and spleen cells of mice	In this study, was demonstrated that *C. reinhardtii* is a superior expression platform for the production of hGAD65. The ability to produce low cost hGAD65 in large quantities will facilitate the development of immunoassays useful for screening, monitoring and treatment large numbers of individuals with type 1 diabetes.
IBDV-VP2 **IHNV-G** **PNV-VP2** **VP2** **IPNV-VP2 SBC (LecA) p57** **PCV2** **VP-2C** **VP28**	NR	4-0.8 (TCP) < 0.5 TCP < 0.3 TCP 1-0.1 TCP 1-0.2 TCP < 0.5 TCP 0.9-0.2 TCP < 0.5 TCP 21-0.2 TCP	*psb*A 5´ transgene *psb*A *atp*A 5´ transgene *rbc*L	NR	Here, were identified four factors that limit recombinant protein yield in the chloroplast of *Chlamydomonas*: • First, the transgenic protein should be codon optimized with codons that are used by highly expressed proteins. • Second, it is necessary to control or limit the activity of proteases, particularly ATPdependent proteases. • Third, by applying the ‘‘transformosome’’ concept, unique genotypes can be selected that permit high expression of foreign proteins. • Fourth, the protein may be toxic to the cell.
Heavy chain human monoclonal antibody against anthrax protective antigen 83 (HC-83K7C) **Light chain human monoclonal antibody against anthrax PA83 (LC-83K7C)**	*Homo sapiens*	0.01 dwt	*psb*A 5´ HC *rbc*L 3´ *psb*A 5´ LC *psb*A 3´	*in vitro*	Here, was shown that the *C. reinhardtii* is capable of synthesizing and assembling a full-length IgG1 human monoclonal antibody (mAb) in transgenic chloroplasts.

D2 fibronectin-binding domain fused to the cholera toxin B (CTB-D2)	*Staphylococcus aureus*	0.7 TSP NR	*rbc*L 5´ CTB-D2 *rbc*L 3´ *atp*A 5´ CTB-D2 *rbc*L 3´	*in vivo* oral immunization of mice	In this study, was obtained the stable expression of the D2 fibronectin-binding domain of *Staphylococcus aureus* fused with the cholera toxin B subunit (CTB), under the control of *rbcL* promotor and UTRs regions. Importantly, the alga vaccine was stable for more than 1.5 years at room temperature.
Fibronectin domain 14 (14FN3) **Vascular Endothelial Growth Factor (VEGF)** **High Mobility Group protein B1 (HMGB1)**	*Homo sapiens*	3 TSP 2 TSP 2.5 TSP 0.15 TSP 0.1 TSP 1 TSP	*psb*A 5´ Transgene *psb*A *atp*A 5´ Transgene *rbc*L 3	*in vitro*	In this work, was tested whether the algal chloroplast could support the expression of a diverse set of current or potential human therapeutic proteins. All of the algal chloroplast expressed proteins are soluble and showed biological activity comparable to that of the same proteins expressed using traditional production platforms.
acrV **vapA**	*Aeromonas salmonicida*	0.8 TP 0.3 TP	*psa*A 5´ exon1 acrV/ vapA *rbc*L 3´	NR	In this work, were obtain acrV and vapA proteins, which encode antigens from the fish pathogen *Aeromonas salmonicida*.
Phytase (appA)	*Escherichia coli*	43% and 41%	*atpA* 5′ appA *rbc*L 3′	NR	In the present study, was successfully created transgenic microalgae to express functional *E. coli* AppA phytase. Further optimization of the protein yield in the transgenic Chlasate and the formulation in the diet are necessary to lower the production cost.
Pfs25 **Pfs28**	*Plasmodium falciparum*	0.5 TSP 0.2 TSP	*psbA* 5′ Pfs25/Pfs28 *psbA*	NR	In this work, was tested whether algal chloroplasts can produce malaria transmission blocking vaccine candidates, *Plasmodium falciparum* surface protein 25 (Pfs25) and 28 (Pfs28). Here was demonstrate that algae are the first recombinant system to successfully produce an unmodified and aglycosylated version of Pfs25 or Pfs28.

Single chain antibody that recognizes the CD22 surface protein from B-cells fused to Domains II and III of exotoxin A (PE40) (αCD22PE40) Dimeric version of αCD22PE40	*Pseudomonas aeruginosa*	0.3 -0.4 TSP	*psbA* 5´ αCD22-PE40 *psbA* 3´	*in vitro* Cell cultures	In this work, was generated a series of chimeric proteins containing a single chain antibody (scFv) targeting the B-cell surface antigen CD22, genetically fused to the eukaryotic ribosome inactivating protein, gelonin, from *Gelonium multiflorm*. Authors show that the addition of an Fc domain of a human IgG1 to these fusion proteins results in the production of assembled dimeric immunotoxins, containing two cell binding scFvs and two gelonin molecules. Additionally, they demonstrate that these algal expressed proteins are capable of binding and reducing the viability of B-cell lymphomas, while treatment of T-cells.
Surface protein 25 fused to the β subunit of the cholera toxin (CtxB-Pfs25)	*Plasmodium falciparum*	0.09 TSP	psbA 5′ Pfs25 EGF 1-4 psbA 3´	*In vivo* oral vaccination	Eukaryotic green algae are devoid of endotoxins, human pathogens, or other known toxic compounds and provide a stable storage and delivery mechanism. Furthermore, lyophilized whole cells are a significant cost advantage over injectable vaccines and alternative oral vaccination strategies that use formulated nanoparticles. For example, algal biomass can be produced for about $3/kg, meaning that a therapeutic dose could cost just a few cents. A more thorough understanding of mucosal adjuvant proteins is paramount to developing oral vaccines moving forward. Ideally, antigens couldbe paired with mucosal adjuvants that elicit an appropriate immune response that confers long-lived protection from disease.

Single chain antibody targeting the CD22 receptor from B-cells fused to the gelonin (αCD22Gel)	*Gelonium multiflorm*	0.2 – 0.3 TSP	*psbA* 5′ *psbA* 3′	NR	They were developed transgenic strains of algae that produced either the monovalent or divalent gelonin based immunotoxins. Here in, it was shown that gelonin based immunotoxins are produced as soluble proteins by *C. reinhardtii* chloroplasts that are enzymatically active and capable of binding to target cancer B-cells and reducing their viability

Table 1: Overview of recombinant therapeutic proteins expressed in chloroplast of *Chlamydomonas* (modified from TSP= % Total Soluble Protein; TCP= % Total Cell Protein is equivalent to 2X TSP [36]; TP= % Total Protein; NR= Not Reported

Chloroplasts of *Chlamydomonas*

Chlamydomonas reinhardtii contains a single large chloroplast that occupies approximately 40-60% of the cell volume. The chloroplast genome (cpDNA o plastome) is a circular molecule of approximately 203 kpb divided by 21.2 kpb inverted repeats into two single-copy regions of 80 kpb containing 99 genes [18]. Each chloroplast contains approximately 80 copies of its genome [19]. Is the site of important biosynthetic pathways like the photosynthesis and it is able to accumulate significant quantities of soluble proteins [20].

Chloroplasts of *C. reinhardtii*, have proven to be efficient and easily transformed. Plastid transformation occurs via homologous recombination, unlike nuclear transformation where is difficult predict the insertion site in the genome. Boyton *et al.*,[21] reported the first event of transformation of the *Chlamydomonas* chloroplast almost three decades ago. In 1988, they used the method called "biolistic", which consist in to bombard microprojectiles coated with some cloned plasmid. Fortunately, the chloroplast genome has been sequenced, and databases are available for searching requirements. Now, can be identified UTR´s and promoter sequences [22], required for design transformation vectors and facilitate the translation of foreign proteins through the optimization of the use of codons [23- 25].

Integration of transgenes into the chloroplast genome of algae has important advantages, for example: a) it enables to control the site of insertion of exogenous DNA through homologous recombination machinery [26], b) multiple transgenes can be expressed as a single operon; c) the constructs are not silenced by the nuclear machinery of silencing and therefore d) keep high levels of expression. Another advantage is, in the case of antibodies, the absence of glycosylation in the chloroplasts may enhance its efficacy and safety. The aglycosylated antibodies lack the ability to fix complement or recruit killer cells. The glycosylation appears to have little impact on antibody assembly or antigen binding, plastids possess a distinct set of proteases compared with other cellular compartments [27], allowing accumulation of proteins that would be degraded in the cytoplasm. Therefore, the production of antibodies in chloroplast would be an ideal system for many situations [28, 29].

The potential of *Chlamydomonas* chloroplasts as an excellent expression platform has been shown in recent works where express some fully actives therapeutic proteins in this alga. For reach an efficient production of recombinants proteins in the chloroplasts, some aspects should be considered like the codon bias, promoters, 5´and 3´ untranslated regions, transgenes, strains, etc. Here we review some of them.

Codon Optimization

Due at the degeneracy of the genetic code, there are many alternatives to encode the same protein. Where each amino acid is coded by among one and six synonymous codons, this redundancy causes than certain codons are used more frequently than others. Often, the

DNA sequence used to encode a protein in one organism is quite different from the sequence that would be used to encode the same protein in another organism [30]. This unequal use of synonymous codons for coding amino acids between organisms, and even within genes of the same organism is called codon bias.

In some studies, was found that the organisms use most frequently the codons translated by most abundant tRNA and the genes with that codons are efficiently translated and have high expression levels, which correlates positively with protein production cost [31-33]. This suggested that the natural selection could act on codon bias on three ways: 1) increasing translation efficiency in highly expressed genes, 2) regulating the translation of the proteins that can be harmful for the cell at high levels and 3) reducing the misincorporation in the translation of proteins biosynthetically expensive [33].

C. reinhardtii chloroplast genome has an elevated AT content and noted codon bias, with A or U preferred at the third position [34-36]. Unlike higher plant chloroplasts, *Chlamydomonas* chloroplasts appear do not use TGA stop codon [37].

For determinate the codon usage affects in the level of expression, Franklin *et al.* compared the accumulation of the protein GFP in strains transformed with the codon-optimized GFP and strains transformed with non-optimized GFP. The results shown an increase 80-fold of the strains with codon-optimized protein GFP, indicating that codon bias has a profound effect on the expression of heterologous protein in the *Chlamydomonas* chloroplast [35].

Constructions Used In Transformation of Chloroplasts

Availability of marker genes, regulator regions (promoters, terminators, 5´ y 3´ UTRs), are of key importance for heterologous expression [22]. For *Chlamydomonas* there are available a variety of regulator regions that control the expression of transgenes in the chloroplasts, the promoters and UTRs most used are from genes *psbA*, *atpA*, *rbcL* and *psbD* (Table 1). According to reports, the best results of expression have been using the promoter *psbA* in the strain CC741 mt+ [38], reaching yields of until 21 % of Total Cell Protein (TCP).

Plastid expression vectors have a similar structure, consisting principally of: two flanking regions for homologous recombination, a cassette of expression with a promoter for the RNA transcription and 5´and 3´ Untranslated Regions (UTRs) for mRNA stability of the transgene. Additionally, are required selection markers against antibiotics, the most used are *aad*A and *aphA*-6 genes which confer resistance against spectinomycin and kanamycin, respectively. In the Figura 2C, it shows a schematic representation of a common cassette of expression with the selection gene in a replication plasmid.

Transformation Methodology

The first step for production of recombinants proteins in an organism host is, to introduce stably genetic material in any of the three compartments (nucleus, chloroplast and mitochondria). In *Chlamydomonas,* fortunately there are protocols for each one [21, 39, 40].

Boynton *et al.,* (1988) realized the first event of transformation in the chloroplast genome of *Chlamydomonas* using particle bombardment (biolistics). This method consists in introduce inert particles cover with DNA but preserving the cellular viability. Actually, this method is the most efficient and reliable for introduce DNA into the plasmid of *C. reinhardtii.* Exist others methods considerably cheaper and easier than microparticle bombardment [41,42]. A protocol of transformation by biolistic is described in [43] , some modifications can be made for particular requirements. In Figure 2, chloroplast transformation by biolistic is schematized.

Cell growth and media

C. reinhardtii strains and plasmids can be obtained from the *Chlamydomonas* Resource Center (http://chlamycollection.org/).

1. Inoculate the strain of *Chlamydomonas* into 50 ml starter culture liquid Tris-Acetate-phosphate (TAP) medium (see http://www.chlamy.org/media.html) and grow under low light to a density of 1.0 x 10^6 cells/ml.
2. Transfer an aliquot of starter culture into a 250 ml flask and grow in TAP to a concentration of 1.0 x 10^6 cells/ml. To achieve high transformation efficiency, never use cultures above 2.0 x 10^6 cells/ml.
3. Centrifuge the culture in 50 ml tubes at 3,000 rpm for 5 min at room temperature.
4. Decant supernatant and resuspend the pellet in 1 ml of fresh medium.
5. Plate 0.2 ml on selective agar medium in a laminar flow chamber. Allow excess liquid to absorb into the plate while perform DNA preparation.

DNA Preparation

6. Place ethanol sterilized macrocarrier membranes in the holders.
7. Mix 10 µg of transformation plasmid with 50 µl binding buffer into a sterile Eppendorf tube.
8. Add 60 µl (3 mg) Seashell S550d carrier gold to the mixture and incubate on ice for 1 min.
9. Add 100 µl precipitation buffer and incubate on ice for 5 min.
10. Mix briefly 10 s by vortex
11. Centrifuge for 15 s at 10,000 rpm to collect gold particles.
12. Remove the supernatant and wash with 500 µl cold ethanol.
13. Mix 10 s by vortex, remove supernatant, and add 50 µl ice-cold ethanol again.
14. Resuspend pellet with a 1–2 s burst from a sonicator probe tip at minimum amplitude.
15. Aliquot 10 µl portions of DNA/gold mixture onto individual macrocarrier membranes.

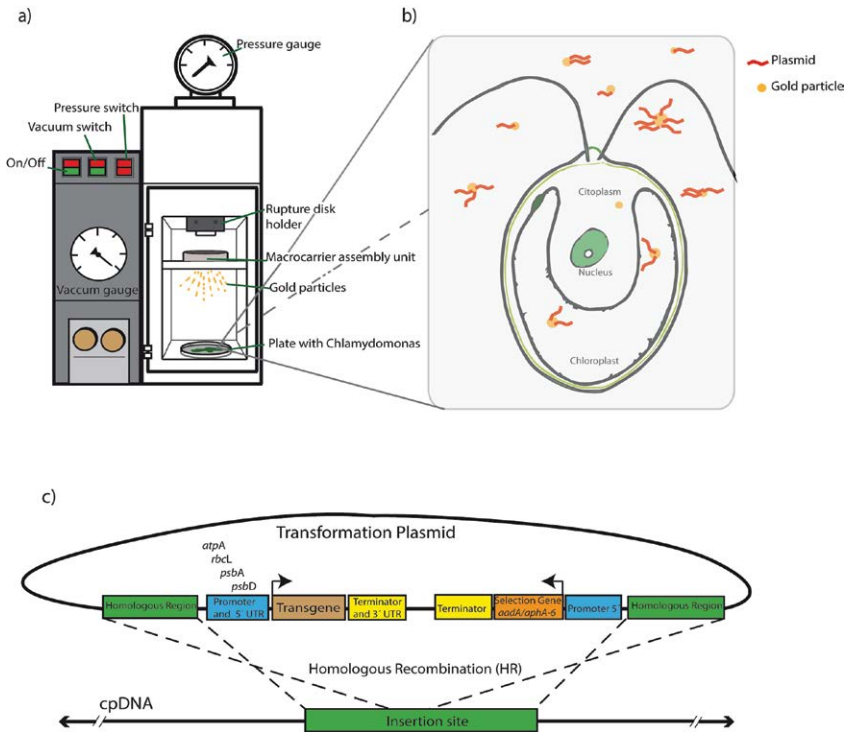

Figure 2: Chloroplasts transformation by biolistic and homologous recombination. a) Schematic representation of a particle bombardment device. The device uses helium gas to accelerate gold particles towards algae, b) Gold particles are coated with a plasmid carrying the genes of interest and when accelerated penetrate the cells. c) When the plasmids carrying the transgenes penetrate the cells and chloroplasts, integrate into the plastid genome by homologous recombination between regions present in the plasmid and in the chloroplast genome (CpDNA).

Bombardment

16. Turn on the particle delivery system. The helium pressure should be at least 1,750 psi.
17. Turn on vacuum pump connected to particle delivery system.
18. Clean all biolistic components and bombardment chamber with 75 % ethanol and allow to dry.
19. Place a 1,350 psi rupture disc into rupture disc holder and screw tightly into particle delivery system.
20. Place a stopping screen into the center of the macrocarrier assembly unit (see Figure 2a).
21. Follow with prepared gold-coated macrocarrier membrane and holder placed gold side down.
22. Place assembly unit in the highest chamber slot nearest to the rupture disc.
23. Place plate with cells in the second slot from the bottom. Uncover plate.
24. Close chamber door and turn on the vacuum switch until pressure reaches 28 in. Hg.
25. Press and hold pressure switch until rupture disc breaks.
26. Press vacuum switch to vent. When pressure reaches 0 in. Hg, open the door and immediately cover the plate.
27. Recover transformations in low light (<1,500 lux).

Difficulties for Stable Expression of Transgenes in Chloroplasts

C. reinhardtii has many desirable features with tremendous potential for convert in an excellent recombinant protein expression system. However, exist different factors that could to limit the expression yield and stability of heterologous proteins in the chloroplast. These difficulties for foreign gene expression in microalgae can be due to the inadequate choose of regulatory sequence, effects of the "transformosome" or particular state genomic of each transformant, biased codon usage, instability of the mRNA or protein.

Four possible factors that can affect the expression of transgenes in chloroplasts have been determinate by Surzycki *et al.*,[38]. The first one is the codon optimization, in chloroplasts, there are two principal classes of genes that are undergoing translational selection [44]. Some authors commit the error of optimize the codon bias using the average of the total of genes instead of select the most frequent codons for each gene class.

The second factor is the protein degradation. Surzycki *et al.* investigated the role of proteases on yield of recombinant proteins. They inhibited the protein synthesis in chloroplasts with chloramphenicol, with or without cyanide m-chlorophenyl-hydrazone (CCCP), which uncouples chloroplast and mitochondrial energy production. The results shown that the recombinant protein has a half-time of 16.9 h when the cells are treated with chloramphenicol, and accumulate with a half-time of 1.5 h without chloramphenicol, further protein degradation is about three times slower in the presence of the uncoupler. These results indicate that proteolytic degradation of recombinant proteins is ATP-dependent [38].

Transformation by homologous recombination with a single construct does not guarantee equal expression level of the independent transformants. The differences in expression levels of transgenic proteins in transformants obtained using the same vector, could be due to the unique characteristics or transformation-associated genotypic modifications, referred as "transformosome". In each event of insertion of a DNA fragment in the chloroplast genome, additional insertions could occur within the nuclear genome creating different transformosomes [38]. This would affect the recombinant protein accumulation in each of the transformants.

The fourth factor that limits the recovery of recombinant proteins for some genes, could be the protein toxicity. The constitutive high-level expression of transgenes can interfere with

the cell metabolism, inducing reduced growth and phenotypic alterations, with consequent reductions in biomass and protein production [45]. In these cases, the possibility of regulating transgene expression in transformed cells expressing recombinant proteins is highly desirable.

Expression of Therapeutic Proteins

Molecular pharming or biopharming, is the production of pharmaceutically important and commercially valuable proteins in plant cells [46,47-60]. Many investigators are migrating to produce recombinant proteins with pharmaceutical activity in *Chlamydomonas* chloroplasts, due the advantages that offers as expression platform. Table 1 contains a list of therapeutic proteins produced in the chloroplast of this alga, the yield for each, the genetic elements used for expression and other relevant information are shown.

Concluding Remarks and Future Prospects

During the last two decades, the production of biopharming products has increased notably. A total of 222 products have been approved by regulatory agencies of the United States of America (USA) and European Union (EU) since 1995 to 2014. In this time, the market value of biopharmaceuticals has been increasing, reaching sales value of 140 billions of dollars for 2013 [2]. Projections suggest that this tendency for biopharmaceuticals products will continue rising.

Currently, in terms of popularity of expression system used to manufacture biopharmaceutical products, the mammalian Chinese Hamster Ovary (CHO) still predominates the market, follow by the bacteria *Escherichia coli* and the yeast *Saccharomyces cerevisiae* [2]. The plant-based system is winning major impact according as the economics and scale of production acquire more importance.

Today, there is a major interest in transplantomics strategies for the production of recombinant therapeutics proteins due to the advantages in terms of productivity and costs. Green algae like *Chlamydomonas* represent a suitable expression platform due they combine the desirable features of the land plants plus distinct advantageous features like homologous recombination, absence of silencing mechanism, easy scale-up, transgene multicopy, are microorganisms classified as GRAS, among others. Unfortunately, the yields yet are not enough for competing with others expression platforms for the commercial production of recombinant proteins.

Some factors that affect the transgene expression yield have been identified, as codon preferences of the tRNAs in chloroplasts, a protein that has toxicity or present detrimental effects on the growing cells, protease degradation of the nascent polypeptidic chains, transformation-associated genotypic modifications, reduced ribosome association that delay elongation of recombinant molecules [38, 61, 62]. For diminishing these effects transgenes and to maximizing the production of recombinant protein levels is necessary use nucleotide sequences codon optimized, control the activity of the proteases, analyze many transformants and to development an inducible system for transgene expression [63]. Recently, Ramundo and Rochaix (2015) developed a system that permits to control the expression of genes based on vitamin-regulated system. They used the promoter psbD 5´UTR which require the Nac2 gene expressed in the nucleus. Nac2 is regulated by the MetE promoter and Thi4 riboswitch, which can be controlled supplying vitamin B12 and thiamine to the growth medium [45].

Other works have been reported in order to increase the recombinant proteins production, Ferreira-Camargo et al. founded that when the culture medium was supplemented with selenocystamine (a small molecule capable of catalyzing the formation of disulfide bounds), increase the accumulation of recombinant proteins containing disulfide bonds [64].

Given the characteristics above mentioned, it is not difficult to think a near future where *Chlamydomonas* and other similar microalgae [65, 66], become a viable and commercially attractive platform for production of biopharmaceuticals products, and may yet make most impact in terms of economic, biosafety and scale production that actual platforms.

References

1. Govea-Alonso DO, Rybicki E, Rosales-Mendoza S (2014) Plant-based vaccines as a global vaccination approach: current perspectives. In Genetically engineered plants as a source of vaccines against wide spread diseases (1stedn), Springer. New York.
2. Walsh G1 (2014) Biopharmaceutical benchmarks 2014. See comment in PubMed Commons below Nat Biotechnol 32: 992-1000.
3. Walsh G (2010) Biopharmaceutical benchmarks 2010. See comment in PubMed Commons below Nat Biotechnol 28: 917-924.
4. Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. See comment in PubMed Commons below Biotechnol Adv 27: 297-306.
5. Schillberg S, Fischer R, Emans N (2003) 'Molecular farming' of antibodies in plants. See comment in PubMed Commons below Naturwissenschaften 90: 145-155.
6. Dove A (2002) Uncorking the biomanufacturing bottleneck. See comment in PubMed Commons below Nat Biotechnol 20: 777-779.
7. Staub JM1, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, et al. (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. See comment in PubMed Commons below Nat Biotechnol 18: 333-338.
8. Rosales-Mendoza S (2014) Genetically engineered plants as a source of vaccines against wide spread diseases (1stedn), Springer. New York.
9. Saalbach I, Giersberg M, Conrad U (2001) High-level expression of a single-chain Fv fragment (scFv) antibody in transgenic pea seeds. J Plant Physiol 158:529–533.
10. Daniell H (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. See comment in PubMed Commons below Biotechnol J 1: 1071-1079.
11. Cardi T, Lenzi P, Maliga P (2010) Chloroplasts as expression platforms for plant-produced vaccines. See comment in PubMed Commons below Expert Rev Vaccines 9: 893-911.
12. Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. See comment in PubMed Commons below Appl Microbiol Biotechnol 65: 635-648.
13. Dreesen IA, Charpin-El Hamri G, Fussenegger M (2010) Heat-stable oral alga-based vaccine protects mice from Staphylococcus aureus infection. See comment in PubMed Commons below J Biotechnol 145: 273-280.
14. Eibl R, Loffelholz C and Eibl D (2011). Single-Use Bioreactors: An OverviewSingle-Use Techology in Biopharmaceutical Manufacture, John Wiley & Sons, Newark .
15. Doran PM (2000) Foreign protein production in plant tissue cultures. See comment in PubMed Commons below Curr Opin Biotechnol 11: 199-204.
16. Agrawal V and Bal M (2012) Strategies for Rapid Production of Therapeutic Proteins in Mammalian Cells. BioProcess International. 10(4): 32-48.
17. Morrow J (2002) Economics of antibody production. Genet. Eng. News 22:1-39.
18. Maul JE1, Lilly JW, Cui L, dePamphilis CW, Miller W, et al. (2002) The Chlamydomonas reinhardtii plastid chromosome: islands of genes in a sea of repeats. See comment in PubMed Commons below Plant Cell 14: 2659-2679.
19. Yang Z, Li Y, Chen F, Li D, Zhang Z, et al. (2006) Expression of human soluble TRAIL in Chlamydomonas reinhardtii chloroplast. Chin Sci Bull. 51 (14): 1703-1709.
20. Purton S (2007) Tools and Techniques for chloroplast transformation of Chlamydomonas. In Transgenic Microalgae as Green Cell Factories (1stedn), Landes Biociencie and Springer science + business media. London.
21. Boynton EJ, Gillham WN, Harris H, Hosler PJ, Johnson MA, et al. (1988) Chloroplast Transformation in Chlamydomonas with High Velocity Microprojectiles. Reports 240: 1534-1538.
22. Barnes D, Franklin S, Schultz J, Henry R, Brown E, et al. (2005) Contribution of 5'- and 3'-untranslated regions of plastid mRNAs to the expression of Chlamydomonas reinhardtii chloroplast genes. See comment in PubMed Commons below Mol Genet Genomics 274: 625-636.
23. Mayfield SP, Franklin SE (2005) Expression of human antibodies in eukaryotic micro-algae. See comment in PubMed Commons below Vaccine 23: 1828-1832.

24. Rosenberg JN, Oyler GA, Wilkinson L, Betenbaugh MJ (2008) A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. See comment in PubMed Commons below Curr Opin Biotechnol 19: 430-436.

25. Bock R (2014) Genetic engineering of the chloroplast: novel tools and new applications. See comment in PubMed Commons below Curr Opin Biotechnol 26: 7-13.

26. Newman SM, Boynton JE, Gillham NW, Randolph-Anderson BL, Johnson AM, et al. (1990) Transformation of chloroplast ribosomal RNA genes in Chlamydomonas: molecular and genetic characterization of integration events. See comment in PubMed Commons below Genetics 126: 875-888.

27. Adam Z. (2007) Protein stability and degradation in plastids. Top. Curr. Genet. 19, 315–338.

28. Fletcher PS, Muto M, Mayfied PS (2007) Optimization of recombinant protein expression in the chloroplasts of green algae: Transgenic Microalgae as Green Cell Factories. (1stedn), Landes Bioscience and Springer science. New York.

29. Mathieu-Rivet E, Kiefer-Meyer MCM, Vanier G, Ovide C, Burel C, et al. (2014) Protein N-glycosylation in eukaryotic microalgae and its impact on the production of nuclear expressed biopharmaceuticals. Front. Plant Sci 5: 1-13.

30. Gustafsson C, Govindarajan S, Minshull J (2004) Codon bias and heterologous protein expression. See comment in PubMed Commons below Trends Biotechnol 22: 346-353.

31. Moriyama EN, Powel JR (1997) Codon usage bias and tRNA abundance in Drosophila. J Mol Evol. 45, 514– 523.

32. Duret L (2000) tRNA gene number and codon usage in the C. elegans genome are co-adapted for optimal translation of highly expressed genes. See comment in PubMed Commons below Trends Genet 16: 287-289.

33. Lavner Y, Kotlar D (2005) Codon bias as a factor in regulating expression via translation rate in the human genome. See comment in PubMed Commons below Gene 345: 127-138.

34. Nakamura Y, Gojobori T, Ikemura T (1999) Codon usage tabulated from the international DNA sequence databases; its status 1999. See comment in PubMed Commons below Nucleic Acids Res 27: 292.

35. Franklin S, Ngo B, Efuet E, Mayfield SP (2002) Development of a GFP reporter gene for Chlamydomonas reinhardtii chloroplast. See comment in PubMed Commons below Plant J 30: 733-744.

36. Mayfield SP, Schultz J (2004) Development of a luciferase reporter gene, luxCt, for Chlamydomonas reinhardtii chloroplast. See comment in PubMed Commons below Plant J 37: 449-458.

37. Beligni MV, Yamaguchi K, Mayfield SP (2004) The translational apparatus of Chlamydomonas reinhardtii chloroplast. See comment in PubMed Commons below Photosynth Res 82: 315-325.

38. Surzycki R, Greenham K, Kitayama K, Dibal F, Wagner R, et al. (2009) Factors effecting expression of vaccines in microalgae. See comment in PubMed Commons below Biologicals 37: 133-138.

39. Kindle KL (1990) High-frequency nuclear transformation of Chlamydomonas reinhardtii. See comment in PubMed Commons below Proc Natl Acad Sci U S A 87: 1228-1232.

40. Remacle C, Cardol P, Coosemans N, Gaisne M, Bonnefoy N, et al. (2006) High-efficiency biolistic transformation of Chlamydomonas mitochondria can be used to insert mutations in complex I genes. See comment in PubMed Commons below Proc Natl Acad Sci U S A 103: 4771-4776.

41. Economou C, Wannathong T, Szaub J and Purton S, et al. (2014) A simple, low-cost method for chloroplast transformation of the green alga Chlamydomonas reinhardtii. In Chloroplast Biotechnology Methods and Protocols. Methods in Molecular Biology 1132. Springer Protocols. New York. USA.

42. Kindle KL, Richards KL, Stern DB (1991) Engineering the chloroplast genome: techniques and capabilities for chloroplast transformation in Chlamydomonas reinhardtii. See comment in PubMed Commons below Proc Natl Acad Sci U S A 88: 1721-1725.

43. Barrera D, Gimpel J, Mayfield S (2014) Rapid Screening for the robust expression of recombinants proteins in algal plastids. In Chloroplast Biotechnology Methods and Protocols. Methods in Molecular Biology 1132. Springer Protocols. New York. USA.

44. Puigbò P, Guzmán E, Romeu A, Garcia-Vallvé S (2007) OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. See comment in PubMed Commons below Nucleic Acids Res 35: W126-131.

45. Ramundo S, Rochaix JD (2015) Controlling expression of genes in the unicellular alga Chlamydomonas reinhardtii with a vitamin-repressible riboswitch. See comment in PubMed Commons below Methods Enzymol 550: 267-281.

46. Fischer R1, Emans N (2000) Molecular farming of pharmaceutical proteins. See comment in PubMed Commons below Transgenic Res 9: 279-299.

47. Almaraz-Delgado AL, Flores-Uribe J, Pérez-España VH, Salgado-Manjarrez E, Badillo-Corona JA, et al. (2014) Production of therapeutic proteins in the chloroplast of Chlamydomonas reinhardtii. See comment in PubMed Commons below AMB Express 4: 57.

48. Mayfield SP, Franklin SE, Lerner RA (2003) Expression and assembly of a fully active antibody in algae. See comment in PubMed Commons below Proc Natl Acad Sci U S A 100: 438-442.

49. Sun M, Qian K, Su N, Chang H, Liu J, et al. (2003) Food-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in Chlamydomonas reinhardtii chloroplast. Biotechnol. Lett 25: 1087-1092.

50. Manuell AL, Beligni MV, Elder JH, Siefker DT, Tran M, et al. (2007) Robust expression of a bioactive mammalian protein in Chlamydomonas chloroplast. See comment in PubMed Commons below Plant Biotechnol J 5: 402-412.

51. He DM, Qian KX, Shen GF, Zhang ZF, Li YN, et al. (2007) Recombination and expression of classical swine fever virus (CSFV) structural protein E2 gene in Chlamydomonas reinhardtii chroloplasts. See comment in PubMed Commons below Colloids Surf B Biointerfaces 55: 26-30.

52. Wang X, Brandsma M, Tremblay R, Maxwell D, Jevnikar AM, et al. (2008) A novel expression platform for the production of diabetes-associated autoantigen human glutamic acid decarboxylase (hGAD65). BMC Biotechnol 8: 1-13.

53. Tran M, Zhou B, Pettersson PL, Gonzalez MJ, Mayfield SP, et al. (2009) Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. See comment in PubMed Commons below Biotechnol Bioeng 104: 663-673. Rasala BA, Muto M, Lee PA, Jager M, Cardoso MFR, et al. (2010) Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chlororplast of Chlamydomonas reinhardtii. Plant Biotechnol J 8:719-733.

54. Michelet L, Lefebvre-Legendre L, Burr SE, Rochaix JD, Goldschmidt-Clermont M, et al. (2011) Enhanced chloroplast transgene expression in a nuclear mutant of Chlamydomonas. See comment in PubMed Commons below Plant Biotechnol J 9: 565-574.

55. Yoon SM, Kim SY, Li KF, Yoon BH, Choe S, et al. (2011) Transgenic microalgae expressing Escherichia coli AppA phytase as feed additive to reduce phytate excretion in the manure of young broiler chicks. See comment in PubMed Commons below Appl Microbiol Biotechnol 91: 553-563.

56. Gregory JA, Li F, Tomosada LM, Cox CJ, Topol AB, et al. (2012) Algae-produced Pfs25 elicits antibodies that inhibit malaria transmission. See comment in PubMed Commons below PLoS One 7: e37179.

57. Tran 1, Van C, Barrera DJ, Pettersson PL, Peinado CD, et al. (2013) Production of unique immunotoxin cancer therapeutics in algal chloroplasts. See comment in PubMed Commons below Proc Natl Acad Sci U S A 110: E15-22.

58. Gregory JA, Topol AB, Doerner DZ, Mayfield S (2013) Alga-produced cholera toxin-Pfs25 fusion proteins as oral vaccines. See comment in PubMed Commons below Appl Environ Microbiol 79: 3917-3925.

59. Tran M, Henry RE, Siefker D, Van C, Newkirk G, et al. (2013) Production of anti-cancer immunotoxins in algae: ribosome inactivating proteins as fusion partners. See comment in PubMed Commons below Biotechnol Bioeng 110: 2826-2835.

60. Preiss S, Schrader S, Johanningmeier U (2001) Rapid, ATP-dependent degradation of a truncated D1 protein in the chloroplast. See comment in PubMed Commons below Eur J Biochem 268: 4562-4569.

61. Coragliotti AT, Beligni MV, Franklin SE, Mayfield SP (2011) Molecular factors affecting the accumulation of recombinant proteins in the Chlamydomonas reinhardtii chloroplast. See comment in PubMed Commons below Mol Biotechnol 48: 60-75.

62. Potvin G, Zhang Z (2010) Strategies for high-level recombinant protein expression in transgenic microalgae: a review. See comment in PubMed Commons below Biotechnol Adv 28: 910-918.

63. Ferreira-Camargo LS, Tran M, Beld J, Burkart MD, Mayfield SP (2015) Selenocystamine improves protein accumulation in chloroplasts of eukaryotic green algae. See comment in PubMed Commons below AMB Express 5: 126.

64. Vanier G, Hempel F, Chan P, Rodamer M, Vaudry D, et al. (2015) Biochemical Characterization of Human Anti-Hepatitis B Monoclonal Antibody Produced in the Microalgae Phaeodactylum tricornutum. See comment in PubMed Commons below PLoS One 10: e0139282.

65. Hempel F, Lau J, Klingl A, Maier UG (2011) Algae as Protein factories: Expression of a Human antibody and the respective antigen in the diatom Phaeodactylum tricornutum. PLoS ONE 6: e28424. Doi:10.1371/journal.pone.0028424.