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Characterization of microalgae native to Quebec for biofuel production

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Characterization of microalgae native to Quebec for biofuel production

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Résumé

La production de biodiésel par des microalgues est intéressante à plusieurs niveaux. Dans le premier chapitre, un éventail de pour et contres concernant l'utilisation de microalgues pour la production de biocarburant sont ici révisés. La culture d'algues peut s'effectuer en utilisant des terres non-arables, de l'eau non-potable et des nutriments de base. De plus, la biomasse produite par les algues est considérablement plus importante que celle de plantes vasculaires. Plusieurs espèces on le contenu lipidique en forme de triacylglycérols (TAGs), qui peut correspondre jusqu'à 30% - 40% du poids sec de la biomasse. Ces proportions sont considérablement plus élevées que celui des huiles contenues dans les graines actuellement utilisées pour le biodiésel de première génération. Par contre, une production pratique et peu couteuse de biocarburant par des microalgues requiert de surpasser plusieurs obstacles. Ceci inclut le développement de systèmes de culture efficace à faible coût, de techniques de récupération requérant peu d'énergie, et de méthodes d'extraction et de conversion de l'huile non-dommageables pour l'environnement et peu couteuses.

Le deuxième chapitre explore l'une des questions importantes soulevées dans le premier chapitre: la sélection d'une souche pour la culture. Une collection de souches de microalgues d'eau douce indigène au Québec a été établi et examiné au niveau de la diversité physiologique. Cette collection est composée de cent souches, que apparaissaient très hétérogènes en terme de croissance lorsque mises en culture à 10±2 °C ou 22±2 °C sur un effluent secondaire d'une usine municipale de traitement des eaux usées (EU), défini comme milieu Bold's Basal Medium (BBM). Des diagrammes de dispersion ont été utilisés pour étudier la diversité physiologique au sein de la collection, montrant plusieurs résultats intéressants. Il y avait une dispersion appréciable dans les taux de croissance selon les différents types de milieux et indépendamment de la température. De manière intéressante, en considérant que tous les isolats avaient initialement été enrichis sur milieu BBM, la distribution était plutôt symétrique autour de la ligne d'iso-croissance, suggérant que l'enrichissement sur BBM n'a pas semblé biaiser la croissance des souches sur ce milieu par rapport aux EU. Également, considérant que les isolats avaient d'abord été enrichis à 22°C, il

est assez surprenant que la distribution de taux de croissance spécifiques soit aussi symétrique autour de la ligne d'iso-croissance, avec grossièrement des nombres égaux d'isolats de part et d'autre. Ainsi, l'enrichissement à 22°C ne semble pas biaiser les cellules vers une croissance à cette température plutôt que vers 10°C. Les diagrammes de dispersion obtenus lorsque le pourcentage en lipides de cultures sur BBM ont été comparées à des cultures ayant poussé sur EU soit à 10°C ou 22°C rendent évident que la production de lipides est favorisée par la culture sur EU aux deux températures, et que la production lipidique ne semble pas particulièrement plus favorisée par l'une ou l'autre de ces températures. Lorsque la collection a été examinée pour y déceler des différences avec le site d'échantillonnage, une analyse statistique a montré grossièrement que le même degré de diversité physiologique était retrouvé dans les échantillons des deux différents sites.

Le troisième chapitre a poursuivi l'évaluation de la culture d'algues au Québec. L'utilisation de déchets industriels riches en nutriments minéraux et en sources de carbone pour augmenter la biomasse finale en microalgues et le produit lipidique à faible coût est une stratégie importante pour rendre viable la technologie des biocarburants par les algues. Par l'utilisation de souches de la collection de microalgues de l'Université de Montréal, ce rapport montre pour la première fois que des souches de microalgues peuvent pousser en présence de xylose, la source de carbone majoritairement retrouvée dans les eaux usées provenant des usines de pâte et papier, avec une hausse du taux de croissance de 2,8 fois par rapport à la croissance photoautotrophe, atteignant jusqu'à μ=1,1/jour. En présence de glycérol, les taux de croissance atteignaient des valeurs aussi élevées que μ=1,52/jour. La production lipidique augmentait jusqu'à 370% en présence de glycérol et 180% avec le xylose pour la souche LB1H10, démontrant que cette souche est appropriée pour le développement ultérieur de biocarburants en culture mixotrophe.

L'ajout de xylose en cultures d'algues a montré certains effets inattendus. Le quatrième chapitre de ce travail a porté à comprendre ces effets sur la croissance des microalgues et la production de lipides. Quatre souches sauvages indigènes ont été obersvées quotidiennement, avant et après l'ajout de xylose, par cytométrie en flux. Avec quelques souches de *Chlorella*, l'ajout de xylose induisait une hausse rapide de l'accumulation de lipide

(jusqu'à 3,3 fois) pendant les premières six à douze heures. Aux temps subséquents, les cellules montraient une diminution du contenu en chlorophylle, de leur taille et de leur nombre. Par contre, l'unique membre de la famille des *Scenedesmaceae* avait la capacité de profiter de la présence de cette source de carbone sous culture mixotrophe ou hétérotrophe sans effet négatif apparent. Ces résultats suggèrent que le xylose puisse être utilisé avant la récolte afin de stimuler l'augmentation du contenu lipidique de la culture d'algues, soit en système de culture continu ou à deux étapes, permettant la biorestauration des eaux usées provenant de l'industrie des pâtes et papiers.

Le cinquième chapitre aborde une autre déché industriel important: le dioxyde de carbone et les gaz à effet de serre. Plus de la moitié du dioxyde de carbone qui est émis dans l'atmosphère chaque jour est dégagé par un processus stationnaire, soit pour la production d'électricité ou pour la fabrication industrielle. La libération de CO_2 par ces sources pourrait être atténuée grâce à la biorestauration avec microalgues, une matière première putative pour les biocarburants. Néanmoins, toutes les cheminées dégagent un gaz différent, et la sélection des souches d'algues est vitale. Ainsi, ce travail propose l'utilisation d'un état de site particulier pour la bioprospection de souches d'algues pour être utilisé dans le processus de biorestauration. Les résultats montrent que l'utilisation d'un processus d'enrichissement simple lors de l'étape d'isolement peut sélectionner des souches qui étaient en moyenne 43,2% mieux performantes dans la production de biomasse que les souches isolées par des méthodes traditionnelles. Les souches isolées dans ce travail étaient capables d'assimiler le dioxyde de carbone à un taux supérieur à la moyenne, comparées à des résultats récents de la littérature.

Mots-clés : Les biocarburants, biodiesel des algues, photobioréacteur, l'extraction de pétrole, durable, d'algues, eaux usées, traitement des eaux usées, le biodiesel, les éléments nutritifs, la récolte, xylose, culture mixotrophe, culture hétérotrophe, biorestauration, les gaz de combustion, CO_2, capture du dioxyde de carbone.

Abstract

Biodiesel production using microalgae is attractive in a number of respects. Through the first chapter, a number of pros and cons for using microalgae for biofuels production are reviewed. Algal cultivation can be carried out using non-arable land and non-potable water with simple nutrient supply. The biomass productivity is much higher than those of vascular plants. Several species produce and store lipids in the form of triacylglycerols (TAGs), which can correspond from 30% to 40% of the biomass dry weight. The algal TAGs are very similar with those of oilseeds. On the other hand, practical, cost-effective production of biofuels from microalgae requires that a number of obstacles be overcome. These include the development of low-cost, environmental friendly and efficient growth systems, harvesting techniques, and methods for lipid.

The second chapter explores one of the important issues raised in chapter one: the selection of a strain for cultivation. A strain collection of freshwater microalgae native to Quebec was established and examined for physiological diversity. This collection consisted in 100 strains, which appeared very heterogeneous in terms of growth when they were cultured at 10±2 °C or 22±2 °C on the secondary effluent from a municipal wastewater treatment plant (WW) and defined BBM medium. Scatterplots were used to examine the diversity in physiology that might be present in the collection. These showed a number of interesting results. There was a fair amount of dispersion in growth rates by media type independent of temperature. Surprisingly considering that all the isolates had been initially enriched on BBM, the distribution was quite symmetrical around the iso-growth line, suggesting that enrichment on BBM did not seem to bias the cells for growth on this medium versus WW. As well, considering that all the isolates had been initially enriched at 22 °C, it is quite surprising that the distribution of specific growth rates was quite symmetrical around the iso-growth line with roughly equal numbers of isolates found on either side. Thus enrichment at 22 °C does not seem to bias the cells for growth at this temperature versus 10°C. The scatterplots obtained when the percentage lipid of cultures grown on BBM were compared with cultures grown on WW at either 10 °C or 22 °C made it apparent that lipid production was favored by growth on

WW at either temperature and that lipid production does not seem to be particularly favoured by one temperature over the other. When the collection was queried for differences with respect to sampling location, statistical analysis showed that roughly the same degree of physiological diversity was found with samples from the two different aggregate locations.

The third chapter continued the assessment of algal culture in Quebec. Some industrial wastes are rich in mineral nutrients and carbon sources, and can be used to increase the final microalgal biomass and lipid yield. This could be a low cost strategy to make algal biofuel technology viable. Using strains from the microalgal collection of the Université de Montréal, this chapter shows for the first time that microalgal strains can be grown on xylose, the major carbon source found in wastewater streams from pulp and paper industries, with an increase in growth rate of 2.8 fold in comparison to photoautotrophic growth, reaching up to μ=1.1/day. On glycerol, growth rates reached as high as μ=1.52/day. Lipid productivity increased up to 370% on glycerol and 180% on xylose for the strain LB1H10, showing the suitability of this strain for further development for biofuels production through mixotrophic cultivation.

The addition of xylose into algal cultures showed some unexpected effects. The fourth chapter of this work focused to understand these effects on microalgal growth and lipid production. Four wild-type indigenous strains were monitored daily, before and after xylose addition, using flow-cytometry. With some *Chlorella* strains xylose addition induced a rapid increase in lipid accumulation (up to 3.3 fold) during the first six to twelve hours. At later times cells showed a decrease in chlorophyll content, cell size and cell counts. On the other hand, the one member of the *Scenedesmaceae* family was able to profit from the presence of this carbon source during mixotrophic or heterotrophic cultivation without apparent negative effects. These results suggest that xylose could be used prior to harvesting to boost the lipid content of algal cultures in either continuous or two-stage systems growing and carrying out bio-remediation of wastewater from the pulp and paper industry.

The fifth chapter addresses another important industrial waste: carbon dioxide and flue-gases. More than half of the CO_2 that is emitted into the atmosphere every day is released by stationary process, either for generation of electricity or industrial manufacturing. The CO_2 exhaustion from theses sources could be attenuated through bioremediation with microalgae, a putative feedstock for biofuels. Nevertheless, every flue has a different gas, and algal strain selection is vital. This work proposes to use a particular site condition for bioprospection of the algal strain to be used in the bioremediation process. This work showed that using a simple enrichment process at the isolation stage can select strains that were in average 43.2% better performing in biomass production than the strains isolated through the traditional methods. The strains isolated in this work were capable of assimilating carbon dioxide at an above average rate, according to recent the results in the literature.

Keywords : Biofuels, algal biodiesel, photobioreactors, oil extraction, sustainability, algae, wastewater, wastewater treatment, biodiesel, nutrients, harvesting, xylose, mixotrophic cultivation, heterotrophic cultivation, bioremediation, flue gases CO_2 capture, carbon dioxide.

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List of Abbreviations

˚C	Degrees Celsius
ADP	Adenosine diphosphate
As	Arsenic
ATP	Adenosine triphosphate
BBM	Bold's Basal Medium
Be	Berylium
Ca	Calcium
CC	Culture Collection
CCM	Carbon Concentration Mechanism
Cd	Cadmium
cm	Centimeter
Co	Cobalt
CO_2	Carbon Dioxide
Cr	Chromium
Cu	Copper
DHA	Docosahexaenoic acid
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOE	Department of Energy (US)
EIA	Energy Information Administration (US)
En.SW	Enriched Sea Water
EU	eaux usées
FA	Fatty acid
FAEE	Fatty acid ethyl ester
FAMES	Fatty acid methyl ester
Fd	Ferredoxin

Fe	Iron
FNR	Ferredoxin-$NADP^+$ reductase
FQRNT	Fonds de recherche du Québec – Nature et technologies
FSC	Forward scatter
FW	Fresh Water
g	Grams
GHG	Green house gas
Gly	Glycerol
h	Hour
H^+	Oxidized Hydrogen
H_2O	Water
ha	Hectare
HRT	Hydraulic Retention Time
ISSN	International Standard Serial Number
ITIS	Integrated Taxonomic Information System
k	$1x10^3$
kg	Kilogram
L	Liter
LB	Lysogeny broth
LCA	Life cycle assessment
LED	Light emitting diodes
Li	Lithium
M	Molar
m	Meter
m^3	Cubic meter
Mg	Magnesium
mL	Mililiter
Mn	Manganese

Mo	Molybdenum
N	Nitrogen
N_2	Molecular Nitrogen
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP
NCBI	National Center for Biotechnology Information
NER	Net Energy Ratio
Ni	Nickel
NO_3	Nitrate
NO_x	Nitrous oxides
NRC	National Research Council (CA)
NS	Nova Scotia
O_2	Molecular Oxygen
OD	Optical Density
P	Phosphorus
PAR	Photosynthetic active radiation
Pb	Lead
PBR	Photobioreactor
pCO_2	Carbon Dioxide Partial Pressure
PCR	Polymerase chain reaction
pH	Power of hydrogen
Ph.Aut	Photoautotroph
Ph.D.	Doctor of Philosophy
PHB	Polyhydroxybutyrate
PO_4	Phosphate
PSI	Photosystem I
PSII	Photosystem II
PUFA	polyunsaturated fatty acid

QC	Quebec
RSM	Response surface methodology
RuBisCO	Ribulose-1,5-bisphosphate
Se	Selenium
SO_x	Sulphur Oxides
SSC	Side Scatter
SW	Salt Water
TAG	Triacylglycerol
UK	United Kingdom
US	United States
USD	US Dollar
V	Vanadium
VS	Volatile Solids
v/v	Volume per volume
W	Watt
Wt	Weight
WW	Waste Water
Xyl	Xylose
yr	Year
Zn	Zinc
µ	Growth rate
µL	Microliter
µm	Micrometer
µM	Micromolar

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A thesis is the acknowledgement of someone's dedication, hard word and persistence in his path toward science. When we read a scientific article, dissertation or thesis, it rarely crosses our minds how that work was put together. But this is not about countless hours of work; the crazy 18 hours shifts at the bench; or the nasty time-points that need to be taken during holidays, family dinners or birthdays. These, sometimes, we can even guess reading the results section. What doe not come to our mind is the hurdle that our family gladly accepts so we can pursue our dreams and become scientists. Thus, I take this space here to express that I am aware of the burden that is for a wife, daughter, family and friends to support someone going through the graduate school. Thank you very much for your patience and love during this long journey.

Finally, to my wife. There are no words to describe your courage and faith on me to accept this journey from a summer country to a glacial other, without knowing any of the official languages and no real perspective of work. You came, you succeeded and you supported me. I couldn't have done this without you. You are as much an author of this thesis as I am. Thank you.

"… to know, is not just to have the information inside books memorized, but to have your own ideas, which were generated from the information absorbed and transmuted by the mind that assimilated them.

Knowing is not having a closet full of information, but having the capacity to transform the digested acquisitions."

Rui Barbosa, 1920.

"...o saber não está na ciência alheia, que se absorve, mas, principalmente, nas idéias próprias, que se geram dos conhecimentos absorvidos, mediante a transmutação, por que passam, no espírito que os assimila.

Um sabedor não é armário de sabedoria armazenada, mas transformador reflexivo de aquisições digeridas..."

Rui Barbosa, 1920.

Chapter 1: Literature Review

Part of this literature review was based on one article review and two book chapters previously published by the author. The full reference is provided bellow. These works are annexed at the end of this thesis in their original form, as are the permissions from the publishers.

Leite G. B.; Abdelaziz A. E. M.; Hallenbeck P. C. (2013) Algal Biofuels; Challenges and Opportunities. Bioresource Technology. 145(0), pp.134–141: http://dx.doi.org/10.1016/j.biortech.2013.02.007

Leite, G.B. & Hallenbeck, P.C., (2013) Engineered Cyanobacteria: Research and Application in Bioenergy. In Bioenergy Research: Advances and Applications. Elsevier Science, pp. 389–406. http://www.sciencedirect.com/science/article/pii/B978044459561400022X

Leite, G. B.; Hallenbeck, P. C. (2012) Algae Oil. In Hallenbeck, P. C. (ed.), Microbial Technologies in Advanced Biofuels Production. New York : Springer. US; 2012. p. 231–59. http://link.springer.com/chapter/10.1007%2F978-1-4614-1208-3_13

Keywords: Biofuels; Sustainability; Algae; Wastewater; Wastewater treatment; Biodiesel; Nutrients; Harvesting; Oil Extraction

1. Scenario

The Transportation Sector plays a major role in the production of greenhouse gas (GHG) emissions. It is responsible for 28% of the total world primary energy consumption, mainly consisting of fossil fuels, and for 71% of the total crude oil used (Figure 1) (Administration 2014; Pienkos & Darzins 2009). Transportation fuels can be divided into three groups related to use: private vehicles (gasoline); commercial vehicles and stationary engines (diesel); or jet fuels. World consumption of diesel was nearly 3.83 trillion liters in 2011 (BP 2014) and fuel demand in the transportation sector is projected to increase by 40% over the period 2010 to 2040 (Mobil 2013). Most of this demand is driven by the commercial sector with heavy-duty vehicle fuel use increasing by 65%. Although the number of light-duty vehicles (cars) could double, the increased fuel demand might be largely offset by the increasing fuel efficiency and the advent of hybrid technologies (Mobil 2013).

Any plan to lower GHG emissions will require the substitution of at least part of the petroleum-based fuels used for transportation. Today we "*borrow land from the past*" (Wackernagel et al. 1993), by using carbon that was fixed in another era. Besides, even above U$ 100.00 a barrel (Figure 2), crude oil is a cheap, easily extractable and easy-to-use energy source. It just needs to be taken from its natural reservoir and distilled into products. However, its use reintroduces into the atmosphere carbon trapped millions of years ago. In addition to the role of fossil fuel combustion in climate change due to the increased concentration of CO_2 in the atmosphere, a well-established mathematical model used to calculate crude oil field reserves and production capabilities predicts peak oil within the next few decades (Figure 3).

After a hundred years of intensive use, mankind has become strongly dependent on fossil fuels. Now we are addicted to oil. The world's economy relies on the very efficient system of production, distribution and use that has been developed. Any transition to a new fuel will have to be "painless", using the technology and infrastructure of the existing system as much as possible.

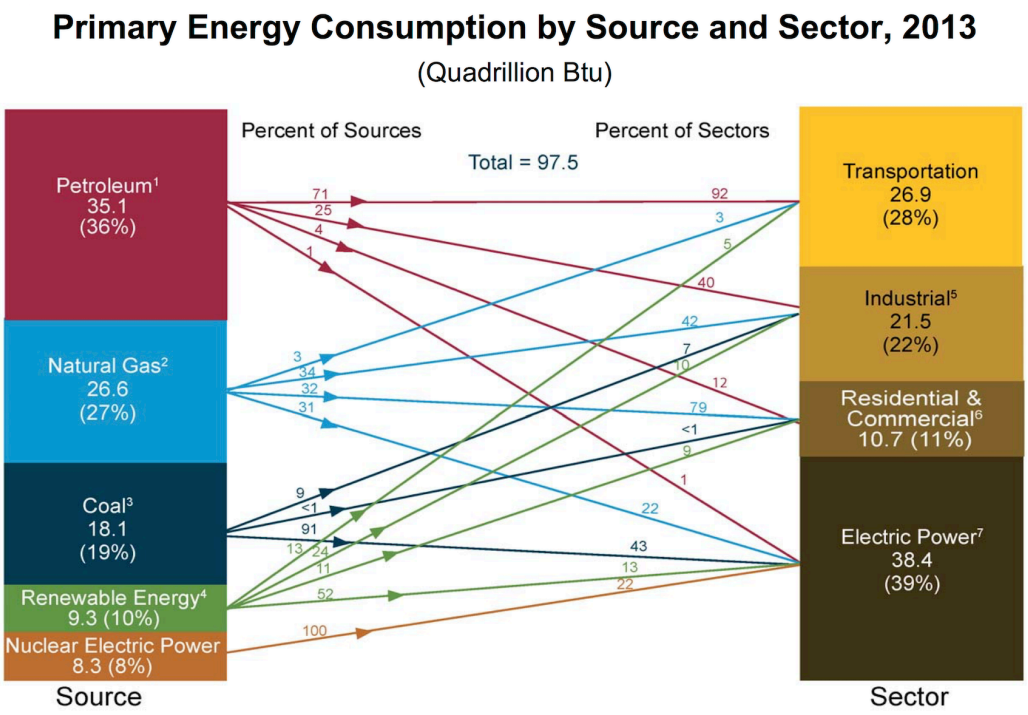

Figure 1: United States Primary Energy Consumption by source and sector. Adapted from U.S. Energy Information Administration (www.eia.gov) (Administration 2014)

The first-generation of biofuels fit this model as bioethanol and biodiesel require minimal or no adjustment of regular internal combustion engines, and can mostly be distributed, stored and pumped like conventional crude oil-derived fuels. The major drawback to the use of these alternative fuels is that the arable land is used to farm the corn, sugar cane or oil seed crops needed to produce these fuels. It would be impossible to produce the quantity of biofuels that would be necessary to meet present fuel demands using first-generation technology. In 2012, the United States consumed nearly 148 billion liters of diesel (Conti et al. 2014). To produce this volume of biodiesel using soybeans for example (average yield of 600 liters per hectare), would require 367 million hectares. In contrast, only 178 million hectares that is currently available for cropland and the 930 million hectares of total US land area (EIADOE 2012). In addition, the commodities used for first-generation biofuels production have other possible markets as sugar, animal feed or cooking oil. A farmer will negotiate the selling price of his product in order to profit as much as possible, enhancing even more the

competition between food and fuel and creating a complex fluctuation of food prices linked to fuel demand.

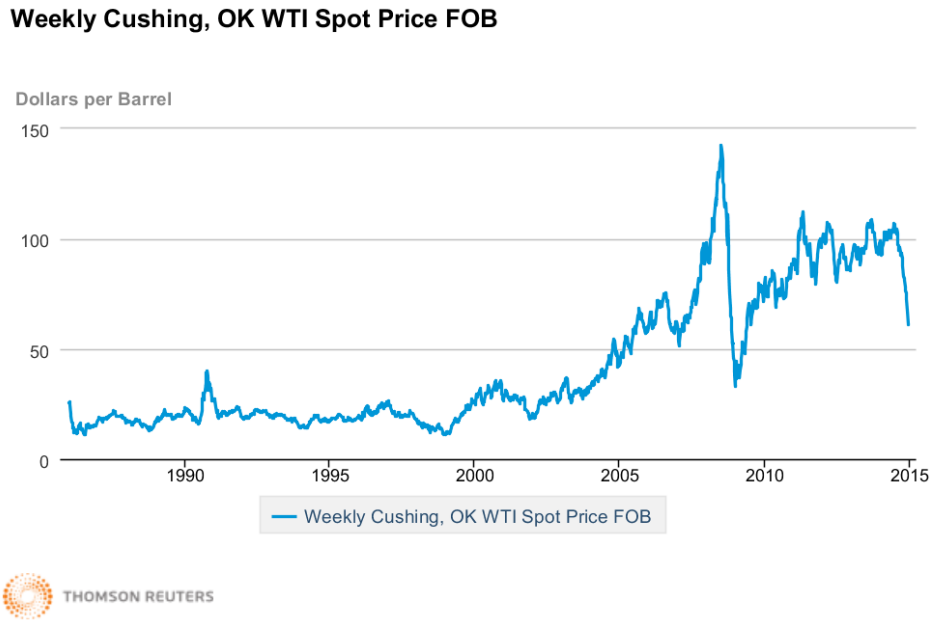

Figure 2: Weekly prices of the crude oil barrel. Adapted from U.S. Energy Information Administration (Energy 2014).

With actual world production of biofuels at 109 billion liters per year (86.6 billion liters bioethanol, 24.4 billion liters of biodiesel) (EIADOE 2012), there has been a great deal of speculation as to whether or not this is already happening. Thus, it is clear that although production of first-generation biofuels was an important step, it is however only a palliative solution and is untenable in the long term.

The call for advanced biofuels demands ‘‘drop in’’ fuels able to be used with the existing infrastructure for storage and distribution, from manufacture to the final customer, but with a production system able to be scaled up without competing with food crops for land. The second-generation biofuels come from an elegant idea: use lignocellulose waste from agriculture as feedstock. However, this technology still faces main challenges and is not likely to leave the bench scale soon (Gustafsson et al. 2014; Koppram et al. 2014; Cavka et al. 2014).

Considered the third-generation biofuel, microalgal biodiesel has been proposed as the most obvious choice.

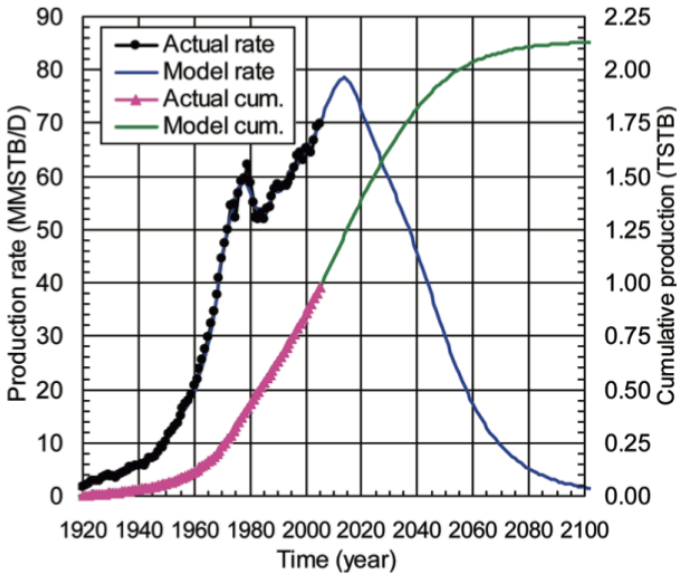

Figure 3 : Concatenation of the multicyclic Hubbert approach to the 47 largest crude oil production countries to forecast world production (Nashawi et al. 2010, *with permission*)

2. Microalgae

Microalgae form a broad and heterogeneous group with species spread among different phyla. Although there are many exceptions, they are commonly defined as oxygen-producing photosynthetic microorganisms containing a plastid with chlorophyll "a." They are mainly found as solitary cells, showing little or no cellular differentiation. Most species occur in aquatic habitats and can be isolated from fresh, brackish or saline waters, although some species can be found in the soil or rocks, in moist or even relatively dry environments. The simplest example of these organisms would be a single cell floating in the water column producing and storing its own sugar using sunlight and reproducing itself by simple binary cell division. This example would describe thousands of prokaryotic (cyanobacteria) and eukaryotic species that, being capable of using dissolved carbon dioxide as sole carbon source, they have a relatively simple nutritional demands (Andersen 2005). Although not considered

an algae, cyanobacteria are usually present in algal literature due to their intrinsic relationship (Graham et al. 2009).

Of course, the algal metabolic diversity is large, what is not surprising given the heterogeneity of distribution of these organisms in the tree of life and their long history of evolutionary adaptation. Although, obligatory heterotrophic species are known, these contain a defective plastid incapable of differentiating into a chloroplast and carry out photosynthesis. As a result, these cells are dependent on external carbon sources. In some cases, the obligatory heterotrophs live as parasites. However, many species are metabolically versatile and can either grow in autotrophic or heterotrophic conditions, depending upon the local environment (Graham et al. 2009).

2.1 Distribution and Phylogeny

Microalgae can be isolated from virtually any aquatic environment, from fresh to hyper saline waters. Some species are even found in non-aquatic environments such as rocks or soil. Many microalgae can survive in very dry or cold habitats, entering into a metabolically dormant state until enough moisture becomes available to resume metabolism (Graham et al. 2009). They are, together with the seaweeds and cyanobacteria, the only primary producers in the oceans, supporting directly and indirectly most of the life on 71% of the Earth's surface (Andersen 2005). In addition to the marine environment, they also play a crucial role in fresh or brackish water lakes, rivers, and soil, either supporting the food chain with their biomass or through nutrient recycle.

The term "algae" is an artificial attempt to group organisms with an incredible variety of morphologic and physiologic characteristic. There are over 30 thousand species already described, whereas some authors estimate that this number could easily reach a million (Bell & Hemsley 2000). Detailed phylogenetic analysis using ribosomal DNA data have shown that, many species derive from critical differentiation events, occurring prior to the common ancestor of plants (Cavalier-Smith & Chao 2006; Woese et al. 1990), Thus, they are now widespread in four kingdoms in the Domain Eukaryota (figure 4).

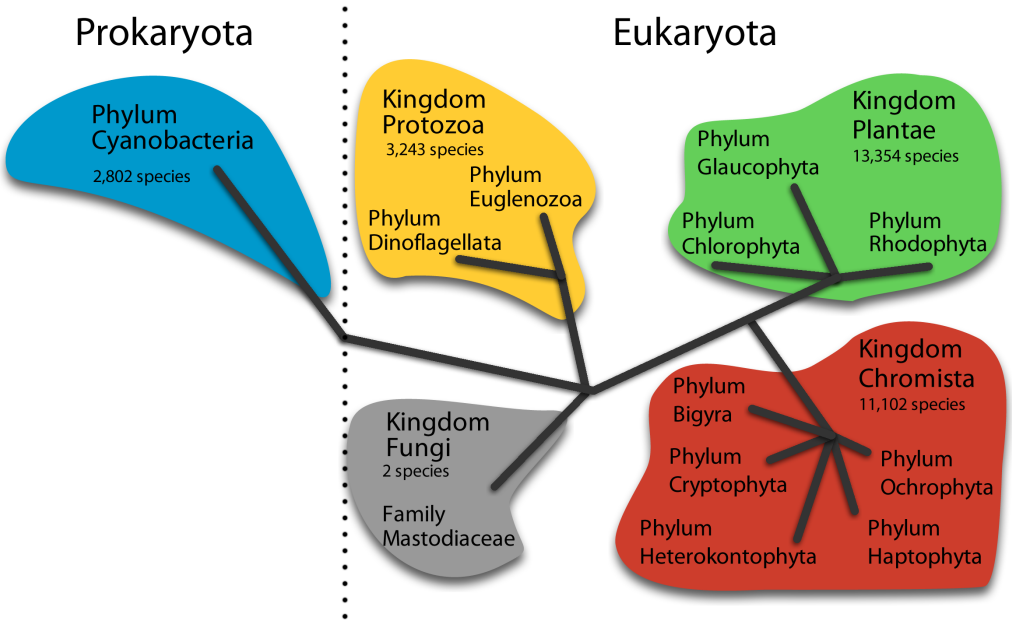

Figure 4 : Distribution of microalgae amongst groups in the Tree of Life as recognized by the ITIS and Species 2000 (www.itis.gov and www.catalogoflife.org) in 2011. The deep classification of algae is the subject of great debate and even the higher clades have been discussed and revised recently (Woese et al. 1990; Cavalier-Smith 2009). Image extracted from Leite and Hallenbeck (2012), *with permission.*

Of course, most of the species described are capable of autotrophic growth, using photosynthesis to provide the energy necessary for carbon fixation and the formation of sugars and other cellular components, including lipids. However, the strains that are capable of producing large amounts lipids as an energy reserve are not in the same phylogenetic clade. This phenotype is not taxon-specific, being present "randomly" in species of distant groups. Three kingdoms group most of the known lipid producers and will be described further: *Protozoa*, *Chromista*, and *Plantae*.

Protozoa: One species of *Dinoflagellate* is already being used for the industrial production of a nutritional supplement for infant formulas, a PUFA (polyunsaturated fatty acid) containing DHA (docosahexaenoic acid). However, this group is more likely to be associated with the production of high market value products than with biofuels.

Chromista: Among the organisms in this kingdom, the diatoms (*Bacillariophyceae*) are the most popular in studies of production of biodiesel. Among other characteristics, they have a fast growth rate and are likely to compete other species out in nutrient-rich and relatively cold systems. Some species were shown to accumulate large quantities of lipids. Some *haptophytes* have also proved to be good prospects for oil production, like *Pavlova lutheri*, which has a good balance between growth rate and lipid production per dry weight (Griffiths and Harisson, 2009).

Plantae: The green algae are the group where most efforts have been focused. Much work has been done with well-defined species. Molecular tools are already available, and some biotechnology companies claim that they were able to enhance the production through metabolic engineering, with, however, no data to this effect being shown yet. Organisms of this group can be found in moist soil and from fresh to saline water environments. Under optimal conditions strains of *Chlorella sorokiniana, Ankistrodesmus falcatus*, *Ettlia oleoabundans*, and *Botryococcus braunii* have shown very promising results.

2.2 Algal Growth Modes: Photoautotrophic, Heterotrophic and Mixotrophic

Most algal strains are photoautotrophs, using light energy to fuel CO_2 fixation and produce the necessary reduced carbon compounds (Figure 5). Heterotrophic metabolism is also common, and different organic molecules can be assimilated as carbon and energy source. The association of both trophic modes, in a so-called mixotrophic growth, is also found, but less frequent. Different studies reported what seems to be a shutdown of the light reactions apparatus when an organic carbon source is introduced into the photoautotrophic medium (K. C. Park et al. 2011b; Cerón-García et al. 2013; Kong et al. 2013). Thus, in principle, either growth modes could be used in a microalgae biodiesel production. However, as each algal species has its specific capacity and limitations for uptake and utilization of organic compounds, a rigorous strain selection needs to be taken into account when designing and operating a heterotrophic algal cultivation.

It could perhaps seem counterintuitive to use standard fermenters fed with plant-derived sugars to produce biodiesel with a commonly photosynthetic organism. However, this leads to a technologically simpler process, since either sugar production and fermentation

operation are well understood. There are no apparent technical barriers for producing biodiesel in this way and, although no detailed cost analyses are available, it should be feasible at moderate cost, probably only somewhat higher than producing ethanol from corn. Yields of conversion of glucose to lipid are in the range of 19 to 31%. The predicted energy efficiencies of glucose to biodiesel is 29% to 75%, not taking into account nutrient supply and the energy required for operations (O'Grady & Morgan 2010; Xu et al. 2006; Li et al. 2007). It should be pointed out however that heterotrophic production of algal biodiesel cuts off many of the proposed advantages of algal biofuels since the actual substrate is produced using traditional agricultural methods. In fact, this system would be classified as a first-generation biofuel, no better than biodiesel from soy or ethanol from corn.

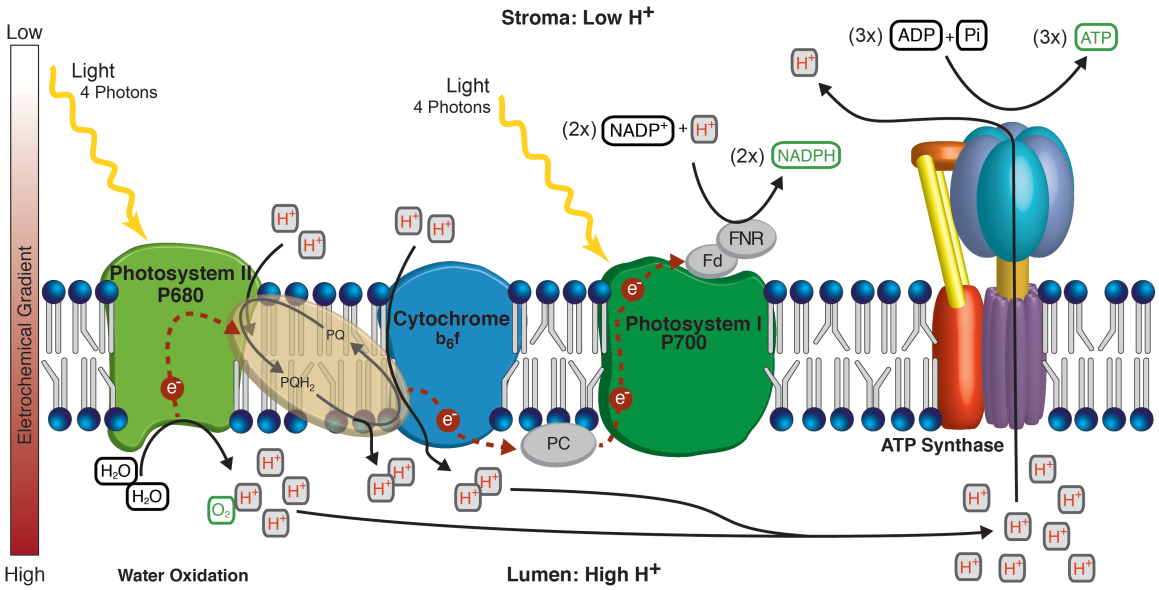

Figure 5: Scheme of light reactions in oxygenic photosynthesis. Photosystem II oxidizes a water molecule, harvesting the electron that will be used to synthesize NADPH, and producing an electrochemical gradient through the release of protons (H+) that will be used by ATP synthase to drive phosphorylation of ADP. The ATP and NADPH that are produced are used by the Calvin-Benson-Bassham cycle for CO_2 fixation in the light independent reactions (Leite and Hallenbeck 2013, *with permission*).

Nonetheless, most designs for algal biodiesel production are based on the ability of these organisms to capture sunlight and carry out photosynthesis with water as the substrate, using the metabolic energy that is generated to fix carbon dioxide (Figure 5). Of course, carbon fixation proceeds by the well-known Calvin-Benson-Bassham cycle using the key enzyme RuBisCO (Figure 6). This enzyme has a relatively low turnover rate, as well as a low affinity for CO_2. Consequently, synthesis of large amounts is necessary, making RuBisCO the most abundant protein on earth. In fact, the cellular content of this enzyme is so high that it is usually found in an almost crystalline form, often sequestered in special structures: carboxysome in prokaryotes (e.g. cyanobacteria), or pyrenoids in eukaryotic algae. The energy requirement for CO_2 fixation through this pathway is in the form of NADPH and ATP, supplied by light-dependent reactions of photosynthesis (Figure 5). Intermediate molecules containing three carbons (3-phosphoglycerate) are withdrawn and used to produce hexose sugars, or broken down to form TAGs (Figure 6). The energy requirement for the formation of a six carbons sugar is shown in the following formula, which represents six successive turns of this cycle.

$$6\ CO_2 + 18\ ATP + 12\ NADPH + 24H^+ \rightarrow \text{Hexose} + 18\ ADP + 16\ P_i + 12\ NADP^+$$

Equation 1 : Photosynthetic production of a glucose molecule

This is an energy intensive process. The light-dependent reaction provides the energy carriers ATP and NADPH. Two electrons are necessary to reduce one ferredoxin, which have been extracted from water and suffered an energy boost through both photosystem II (PSII) and photosystem I (PSI). They absorb energy from the incident photons to oxidise water and excite the harvested electrons. To generate the reducing power necessary to fix six CO_2 and create one hexose requires the capture of 48 photons (Taiz & Zeiger 2002). Each electron passing from PSII to PSI drives the translocation of 3 H^+. Current models of ATP synthase suggest that 12 H^+ are required for the synthesis of three ATP, so in total the passage of the 24 electrons involved in reducing the required amount of $NADP^+$ could generate the necessary ATP (3 x (72/12)). Thus, the fixation of enough carbon to form a six-carbon sugar requires

the capture of 48 photons or 8 photons per carbon fixed (Figure 5). This is one of the factors that helps to set an absolute limit to the maximum theoretical photosynthetic efficiency attainable (Taiz & Zeiger 2002). This quantum requirement is, of course, higher when biomass synthesis is considered since the biosynthesis of constituents like lipids, proteins and nucleic acids require additional energy. Thus, it can be estimated that the light requirement for the fixation of one CO_2 into biomass is more likely 10 or 12 photons.

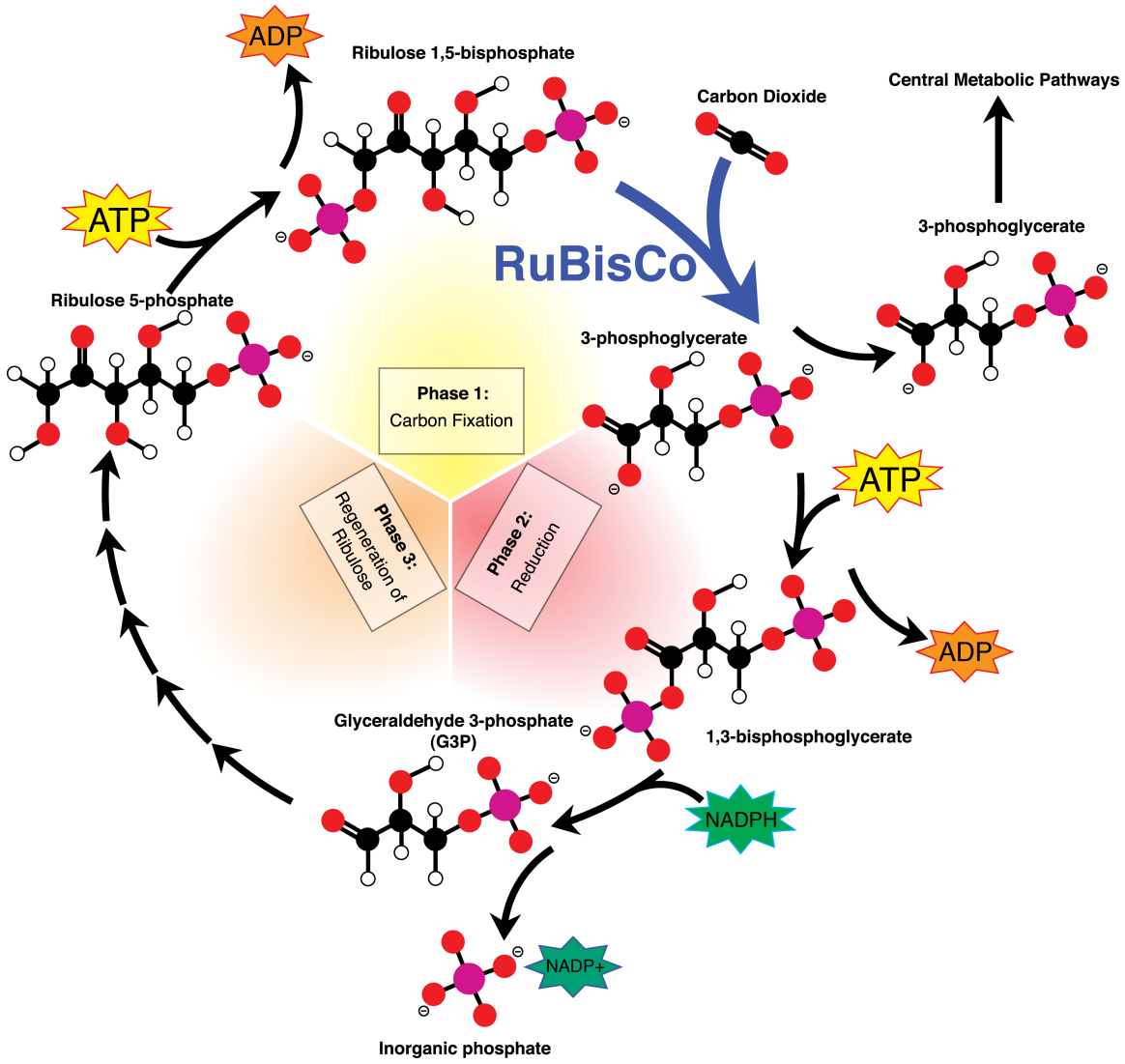

Figure 6: Calvin-Benson-Bassham cycle comprehend the light independent reactions of the photosynthesis. *Image credit: Mike Jones / CC-BY-SA-3.0*

To take advantage of the best characteristics of both, photoautotrophic and heterotrophic growth, a two-stage system has recently been proposed. In this, the microalgae are first grown photoautotrophically, expressing high levels of RuBisCO, fixing CO_2 and increasing cell biomass. At the end of log phase (120 hrs), the algal cells are allowed to settle overnight and are re-suspended in a nitrogen limited medium that supports heterotrophic growth and favors lipid production (45 g/l glucose, 2 g/l glycine) (Xiong et al. 2010). Somewhat surprisingly, these cells had a much (70%) higher lipid yield (0.3 g /g glucose) than cells that had been incubated solely under heterotrophic conditions (0.18 g /g glucose). Among possible explanations, the authors suggested that autotrophically pre-grown cells are more efficient since they retain RuBisCO and are able to re-fix the CO_2 given off during glucose breakdown (pyruvate decarboxylation). A process based on this concept was then patented: US 7,905,930 issued to Genifuel ("A process for production of biofuels from algae, comprising: a) cultivating an oil-producing algae by promoting sequential photoautotrophic and heterotrophic growth, b) producing oil by heterotrophic growth of algae wherein the heterotrophic algae growth is achieved by introducing a sugar feed to the oil-producing algae; and c) extracting an algal oil from the oil-producing algae.").

2.3 Photosynthetic Efficiencies

One of the fundamental insurmountable constraints on algal production of biodiesel is the maximum theoretical photosynthetic efficiency. Of course, this applies to the production of any biofuel from a resource that is ultimately derived from the solar driven biological fixation of CO_2. This sets an absolute upper limit to the amount of fuel that can be obtained per square meter of collector area per year. A series of physical and biological factors combine to reduce total possible energy recovery to only a small fraction of the incident solar radiation (Table 1). This issues are covered in great detail elsewhere (Tredici 2010) and summarized in this section.

First, only slightly less than half (45%) of the solar spectrum can be captured by the photosynthetic pigments of living organisms. An additional amount, estimated as 10%, is lost

through reflection from the surface of the reactor (or leaf). The reaction center, where the process of charge separation is initiated, leading to conversion of the light energy to chemical energy, is composed of a special chlorophyll a, P700, which absorbs at 700nm. This creates a downhill gradient for efficient transfer of the excitation energy captured by the antenna pigments which absorb light of shorter wavelengths, but this also means that this fraction of the energy of photons of shorter wavelength is lost (21%). The conversion of the energy which reaches the reaction center to the chemical energy in the fixed carbon compounds that are formed (glucose for example) is only 35% efficient. Some of the chemical energy that is made must be used for respiration to supply the necessary energy to support vital functions of the cell during darkness (20%). Finally, as much as 40% on the average of the light energy that is captured by the photosynthetic apparatus cannot be used by the cells since high light intensities saturate the process; photons are received faster than they can be used and the energy is wasted as heat or fluorescence. Thus, maximum photosynthetic efficiencies cannot be higher in theory than 5.5%, and in practice achieving efficiencies of 1 or 1.5% are exceptional.

Source of energy loss	% Loss	% Remaining
Radiation outside useable range (non-PAR)	55%	45%
Reflection	10%	41.5%
Transfer to reaction center	21%	32.8%
Conversion to chemical Energy	65%	11.5%
Respiration	20%	9.2%
Photosaturation and photoinhibition	40%	5.5%

Table 1: Photosynthetic efficiency train

2.4 Algal Oil

Any organism that depends on sunlight as its primary energy source needs to store energy-rich compounds to avoid starvation when light is not available. Vascular plants

synthesize a variety of energy rich molecules to save enough energy for a rainy day (or night). A Canadian example would be the maple tree and its phloem with high sugar content (Maple Syrup). A variety of plants also produce oil as energy and carbon sources for germination. To increase embryo viability, some plants accumulate part of the energy in the seed as TAGs (triacylglycerols), which are historically accessed by press extraction (e.g. olive oil). Different microalgal species are capable of the synthesis and accumulation of a variety of high-energy molecules, including fatty acids (FA), usually stored in TAGs, the main feedstock for biodiesel production (Figure 7). However, this phenotype is not specific to one taxon, and the amount or the proportion of lipids and other energy molecules (e.g. starch) to be stored in the cell is variable (Table 2). Therefore, there is a large disparity in quantity and quality of lipid content among algal strains.

The profile of the FAs (and consequently TAGs) produced by microalgae varies considerably between species, strains and may also vary according to specific culture conditions (Table 2) (Abou-Shanab et al. 2011). The FA composition has a large impact on the potential production of biodiesel since FA length and degree of saturation will significantly influence the resulting fuel properties. For example, the difference between various petroleum derived fuels is basically the length of the hydrocarbon chain. The gasoline is a mixture of saturated chains containing from 6 to 12 carbons, while diesel is mainly composed of molecules with chain lengths between 12 and 18 (Srivastava & Prasad 2000). Thus, the length and degree of saturation contained in the FA profile of the microalgae will directly affect the properties of the biodiesel.

Specie	Lipid Content (% dry wt)	Lauric acid C12:0	Palmitic acid C16:0	Stearic acid C18:0	Oleic acid C18:1	Y-Linolenic acid C18:3	Others
Scenedesmus obliquus	29%	11%	29%	17%	20%	23%	0%
Chlamydomonas pitschmannii	51%	10%	26%	20%	13%	23%	8%
Chlorella vulgaris	26%	5%	22%	5%	53%	8%	7%
Chlamydomonas mexicana	29%	34%	50%	6%	0%	0%	10%

Table 2: Fatty acids composition of some microalgal species (Abou-Shanab et al. 2011, *with permission*).

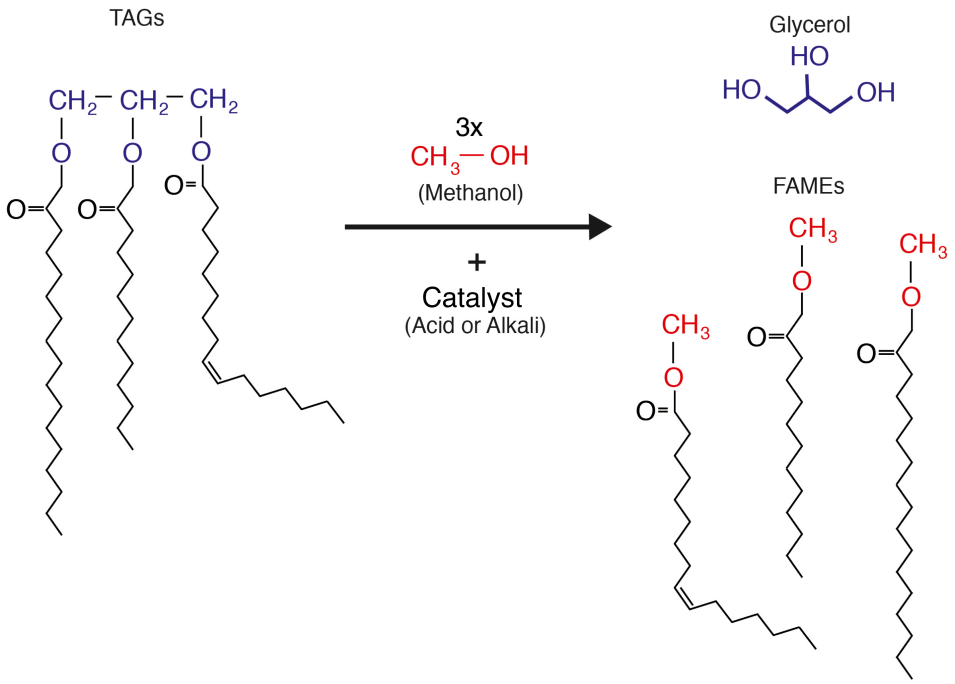

Figure 7: Example of triacylglycerol (TAG) structure and the products of a transesterification reactions. The fatty acids chains within a TAG molecule can vary in response to several variables.

Although not common, different algal strains were shown to have lipid content higher than 50%. This is one of the advantages of using microalgae instead of vascular plants for biodiesel production. Only the seeds of a vascular plant are used when making plant-derived biodiesel, with the rest of the biomass usually considered waste. Consequently, the aerial production yield of lipids from microalgae has the potential to be many times higher than that of the already developed technology of oil seed crops, with the advantage of not requiring arable land. Another key factor in choosing microalgae as a system for biodiesel production is their potentially low nutritional requirements. Microalgae can be grown in fresh or marine water, on marginal lands, and even in association with wastewater treatment plants or industrial parks where their cultivation offers the additional benefit of bioremediation. After the extraction of hydrocarbon for biodiesel production, the biomass can be processed in an anaerobic digester for methane production, a secondary source of energy. The digester effluent

could be fed back into the algae cultivation system as a source of nutrients. Even though production with such a system may not completely satisfy local fuel demands, it will evidently lower the importation of fuel, creating a decentralization of production, improving the local economy and helping the environment.

Photobioreactor	Issue	Open ponds
Easy	Control of culture conditions (pH, temp., dissolved CO_2.	Medium
Low	Susceptibility to culture contamination	High
Low	Water evaporation	High
High	Productivity per m^2	Medium
High	Energy input	Low
High	Structure cost	Low

Table 3: Photobioreactors and Open Ponds; Pros and Cons.

3. Cultivation

Achieving anywhere near realistic photosynthetic conversion efficiencies and productivities, depends critically on the geometry and physical properties of the cultivation system used. This is not as straight forward as one might naively think since the essential nutrient here, sunlight, is used differently with respect to dilution rate than a nutrient that is dissolved in the liquid phase. Thus, there is a disconnection between growth rate and productivity (Tredici 2010). Maximum specific growth rates (i.e. doubling time of cell biomass) are obtained under conditions of photosaturation, obtained only with very dilute cultures. In practice, mass algal cultures need to be run under conditions of photolimitation to maximize areal productivity. Under these conditions, the increased density of the culture ensures that all the impinging photons are captured, but consequently self-shading is increased with negative effects on growth rate. This effect becomes obvious when looking through a tubular photobioreactor with a late logarithmic phase algal growth. The dense culture is completely opaque within only a few centimeters (Figure 8). Two basic types of cultivation

systems have been proposed and studied: photobioreactors and open ponds. Each with their own advantages and disadvantages (Table 3). First, these are briefly reviewed, and then they are compared for use in biofuels production.

Figure 8: Horizontal tubular photobioreactor of 4000 liters capacity. (*Photo credit: IGV Biotech/CC-BY-SA-3.0*)

3.1 Photobioreactors

These are systems where the cultures are enclosed in some transparent recipient (Figure 8). Photobioreactors (PBRs) can have different sizes and shapes: plastic bags, flat panels, tubes, fermenter like and others. Vertical tubes are among the most popular system due to their relatively easy maintenance, low cost and high surface to volume ratio (Suali & Sarbatly 2012). Among the advantages of using photobioreactors are resistance to contamination by wild algae strains or herbivores, high productivity per unit area, and the

possibility of easily controlling various parameters (Table 3), including pH, temperature, and light intensity. The PBR can be placed indoors or outdoors, using sunlight, artificial light or a mixture of both. An interesting variation of a lighting system is the use of optical fibers to carry the outdoor sunlight into an indoor culture (C.-Y. Chen et al. 2008). Artificial light can be provided by any regular light source such as tungsten or fluorescent bulbs. The use of LEDs (light emitting diodes) is increasing due to their low heat generation, low power consumption and the specificity of the light wavelength emitted. This allows energy saving due to the restriction of the light emitted to PAR (photosynthetic active radiation), and the analysis of how different wavelengths and intensities impacts the metabolism of these microorganisms. A recent study showed that different wavelengths might have a significant influence on biomass and lipid productivity, as well as on the lipid profile. A locally isolated strain of *Nannochloropsis* showed a higher growth rate, lipid productivity and different lipid profile under blue light (470nm) when compared with growth under white, red (680nm) or green (550nm) (Das et al. 2011).

3.2 Open Ponds

Algal cultivation in open ponds is carried out in shallow basins open to the environment. The most common types are raceway, circular, inclined and unmixed (Figure 9). They are considered relatively inexpensive and easy to construct, as long as the area is relatively flat. Cultivation can be made directly over the soil, or some simple surface covering can be used to minimize water loss due to seepage. Other improvements can be done to increase solar energy capture, and decrease contamination issues. Mixing can be provided effectively by low cost and low energy consuming paddle wheels, which can be enough to maintain aeration and nutrient dispersion. Due to the small depth and large surface area, water loss through evaporation can become a major issue, limiting its operation to areas where low-cost water is available. Marine waters and wastewaters are good matches for this system, as environmental and sustainability issues would prevent large open pond cultivation using potable water.

Operation and maintenance costs are relatively small. Thus, this system is capable of generating biomass production at the best price. There is already some experience in large

scale production using these types of systems, either in pilot projects partially funded by the government, in wastewater treatment plants, where it is used in secondary or tertiary treatment of sewage, or in commercial-scale algal cultivation for the health food market. As a bioproduction system, its simplicity is a double-edged sword. The contamination risk level is high, and a strain with high lipid productivity can easily be overrun by a fast growing wild strain (J Sheehan 2003). Another dangerous type of contamination is herbivores. There is not much information available on how to deal with predation, but it is well known that they are capable of clearing a high-density pond in a matter of days.

Figure 9: Design of a raceway open pond. (*Image credit: Ivan Castilho/CC-BY-SA-3.0*)

3.3 Water and Nutrient Supply

Microalgae culture is water intensive. At large scale, the demands for nutrients and water are enormous. If the production is not supposed to compete with food and still be cost-effective, the major nutrients and feedstocks should be provided from cheap sources, especially nitrogen and phosphorous.

Theoretically, these conditions can be met by using suitable wastewater. Of course, wastewater streams vary widely in their composition. The nutrient removal (uptake) appears to be a complex function of a number of factors, including nutrient levels and algal strains (Cai et al. 2013). The use of algae for nutrient removal from municipal wastewater has been extensively investigated and in general this nutrient stream provides a good microalgal growth medium (Abdelaziz et al. 2013; Abdelaziz et al. 2014). Other waste streams promise to provide most of the nutrients for abundant microalgal growth as well (Cabanelas et al. 2013; Choi & S. Y. Lee 2013). Coupling biofuels production with wastewater treatment makes sense since it results in considerable energy savings, improving the NER (Net Energy Ratio) of the algal production process (Beal et al. 2012).

3.4 CO_2 Enrichment

One approach to raise productivity is to increase the concentration of CO_2 (J Sheehan 2003; Lin et al. 2012). In fact, the enzyme responsible for CO_2 fixation, RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase), has a low affinity for CO_2 and also functions as an oxidase of 1,5 bisphosphate, interacting with molecular oxygen. Therefore, O_2 is a competitive inhibitor with CO_2. Since the atmospheric concentration of CO_2 is much lower than that of O_2, the oxygen can cause a significant effect. Evolutionarily, this problem has been managed by the development of carbon concentration mechanisms (CCM). The cell locally increases the CO_2 concentration around the RuBisCO enzyme to ensure its function in CO_2 fixation (Giordano et al. 2005). Given the large degree of diversity in the microalgae, it is perhaps not surprising that there are a number of different CCMs used depending upon the species. A detailed discussion of this subject is available (Giordano et al. 2005). Regardless of the detailed mechanisms that differ in how substrate is delivered to RuBisCO, all CCMs depend upon active transport of either HCO^- or CO_2 into the cell. However, even though these organisms have been adapted to relatively low atmospheric CO_2 concentrations, growth is enhanced when CO_2 is supplied. Among the reasons, rapid inorganic carbon uptake will cause local depletion, and hence an increased carbon dioxide partial pressure (pCO_2) will help

maintain levels above those needed to saturate transporters. As well, at high levels CO_2 diffusion through the membrane could lessen the need of transporters and the expended energy in this process. The energy savings would, therefore, be translated into more energy available for cell growth and other processes, leading to growth enhancement. Thus, CO_2 enrichment is taken into consideration when projecting algae oil productivities and when planning growth facilities. This is perhaps one of the most important factors in practical algal culturing as any increase in productivity directly translates into a decrease in land footprint, water resource requirements, and operational costs and energy demands. Indeed, sparging CO_2 into the culture medium is known to increase its cell density. Two different approaches are frequently reported: the use of CO_2 to adjust the pH, and CO_2 enrichment as a way to bioremediate flue gases (Grobbelaar 2000; Rodolfi et al. 2009; Yoo et al. 2010; McGinn et al. 2011). Of course, any feedstock to be used in large-scale production will play a major role on the final price. CO_2 is not an exception, and if employed in a production system, it will probably be coupled with a bioremediation process.

3.4.1 CO_2 Mitigation?

Unfortunately, the algal CO_2 fixation has been turned by some into a selling point, claiming that algal cultures are able to carry out CO_2 mitigation, or even sequestration! A little reflection will show that this is a shady accounting practice, analogous to the deceptive repo101 used by the now defunct and discredited Enron. In that case, liabilities were removed from the books prior to issuing quarterly statements by selling them to dummy companies, and then repurchased once the glowing reports had been issued. In the algae case, it is true that the algae absorb CO_2 emitted by fossil fuel burning power plants, thus preventing immediate release into the atmosphere. However, if the algae are used to produce fuel, the residence time of the CO_2 in a fixed state will only be a matter of weeks or at most months before it is released by combustion. Thus, the CO_2 coming from flue gasses that are fixed by the algae cannot be taken off the books for enough time to make any difference. This reasoning has recently been recognized by the Advertising Standards Authority of the United Kingdom. They issued a judgment against ExxonMobil for an advertisement in which they had a scientist

claiming "In using algae to form biofuels, we're not competing with the food supply, and they absorb CO_2, so they help solve the greenhouse problem as well." (9 March 2011 ASA Adjudication on Exxon Mobil UK Ltd. - Advertising Standards Authority http://www.asa.org.uk/ASA-action/Adjudications/2011/3/)

3.5 PBRs *versus* Open Ponds

There is presently a great deal of discussion as to whether future microalgal biofuel facilities will consist of open ponds or closed photobioreactors (Table 3). One practical view of the open system versus photobioreactor debate is provided by a look at how industries that are currently producing microalgae and cyanobacteria for the nutraceutical market are cultivating their microorganisms. This is a relatively high value product compared to the value of algae grown for biofuel production; ~$5000/ton versus ~$875/ton. However, even at approximately ten times the anticipated value of algae for oil, presently operating plants (Cyanotech, Earthrise Nutritionals, etc.) are all invariably open pond systems.

This indicates the difficulty of making the economics work for photobioreactors in large-scale production of very low value products. “Anyone working on closed photobioreactors has got a problem,” says Benemann. “And there are dozens of these companies out there,” he says. “Just like in agriculture, you have to keep it as simple as possible and as cheap as possible. You cannot grow commodities in greenhouses, and you cannot grow algae in bioreactors.” (Waltz 2009). A number of companies are presently producing photobioreactors and touting them for use in making biodiesel. Unfortunately, some of these companies use their selling points productivity numbers that are so high that they are not even theoretically possible (Tredici 2010). There are serious obstacles to develop photobioreactors for use in biofuels production, problems that are ignored at as the following quote shows. “The old algae world has produced some old-timers who are negative. We are trying not to listen to them,” quoting Bob Metcalf from Polaris Venture Partners, investor in Greenfuel Technology (Waltz 2009). Greenfuel Technology, a photobioreactor provider, went bankrupt five months later.

3.6 Productivity *versus* Oil Content

Industrial production systems using microalgae will probably need to be specifically tailored on a case-by-case basis. Several variables play key roles in microalgal processes, and some will likely be project specific. For example, the geographical site and local climate, which directly affect annual variations in humidity, temperature and solar radiation; may change the optimum for certain variables. Other factors to be considered include desired products and/or by-products, outdoor or indoor culture, species to be cultivated, harvesting approach and others. Strict optimization may not be required for high-value products where the production scale is low. However, biodiesel is a high volume low value product with high demand, and under current carbon trading schemes (or the lack of them) the production cost must be low enough so it can compete with petro-diesel.

The best microalgal species to be cultivated in a given system strongly depends on those variables, thus selecting the proper strain might be a challenge in itself. Until now, there is no consensus about which group of algae would be the most appropriate for large scale / low-cost TAG (triacylglycerol) production. Considering known algal diversity, very few strains are currently under study for biodiesel production. Moreover, although a strain from a culture collection might be well characterized, favoring its laboratory study, it is very questionable if these strains could adapt to different local climates or would be able to compete with indigenous strains. Thus, although time consuming and labor intensive, bioprospecting for local microalgal species capable of high levels of lipid production might be advisable. Some of the properties considered desirable in an algal strain for mass culture are given in Table 4. A database containing the characteristic of local microalgal species would have extreme utility for different projects for algal biodiesel production, such as their use in tertiary treatment in municipal sewage treatment plants or for treatment and biofuels production from local industrial wastewater.

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Characteristics	Advantages
High growth rate	Higher biomass productivity, reduce area needed, overcome invader species
High lipid content	Higher value of biomass, higher productivity
High value by-products	Decrease the cost of production
Large cells, colonial or filamentous	Easier to harvest
Planktonic	Less growth attached to surfaces: easier to harvest and maintain
Tolerance of culture conditions	Require less control of pH, temperature and others
CO_2 uptake efficiency	Less cost required to supplement CO_2
Tolerance to contaminants	Potential growth on very eutrophic water or flue gases
Tolerance of shear force	Allow cheaper pumping and mixing methods to be used
No excretion of autoinhibitors	Higher cell density expected: higher biomass productivity.
Naturally competitive	Harder to be overcome by invader species

Table 4: Some of the desirable characteristics on an algal strain for a large-scale culture. Adapted from Griffiths & Harrison (2009)

High overall TAG productivity is obviously one of the major keys to the successful production of biodiesel from algae. Overall TAG production is the result of three interacting variables: growth rate, lipid content and metabolic yield. Obviously, for the strict photosynthetic production of TAGs, cellular metabolism is directly constrained by the availability solar radiation and the efficiency of its conversion. Restriction at this level limits the availability of fixed carbon, and the cell must prioritize its use according to current needs (e.g. “housekeeping”, secondary metabolite production, cell division, carbon reserves). Thus, fast growth (i.e. high cell division rates) doesn’t necessarily translate to high-level lipid

productivity. In fact, with respect to growth versus lipid content in a specific strain, three basic scenarios are expected:

A - Faster growth, but lower lipid content

B - Medium growth with medium lipid content

C - Slower growth with higher lipid content

Figure 10 exemplifies the different behavior of four species when grown in nutrient replete medium (Griffiths & Harrison 2009). *Chlorella sorokiniana* and *Chlorella calcitrans* show opposite metabolic strategies, while *Chlorella sorokiniana* invests heavily in growth rate, *Chlorella calcitrans* is "preoccupied" with energy storage. Both, *Pavlova lutheri* and *Chlorella vulgaris* showed average to slightly high growth rates and lipid content.

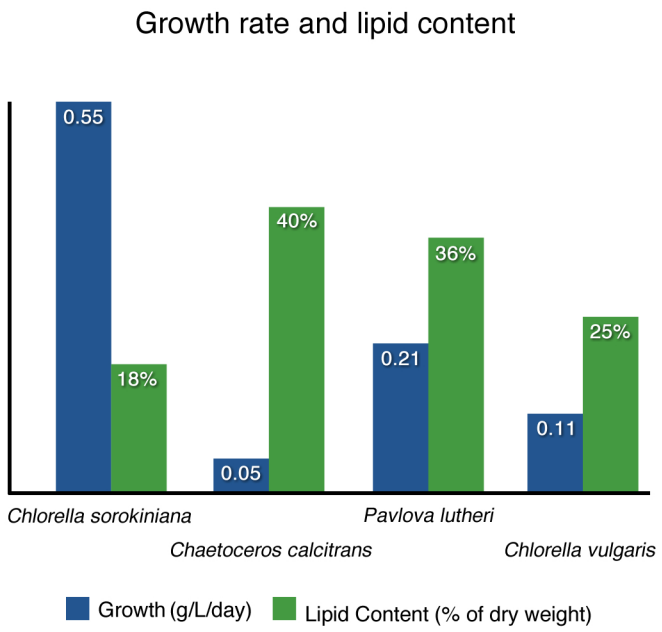

Figure 10: Growth rate and lipid content of four different species under optimal conditions. The best lipid productivity is not always found in the species with higher lipid content. Data extracted from Griffiths and Harisson (2009), *with permission*.

Thus, different species have their own metabolic particularities, and often their response may be different depending upon culture conditions. The three scenarios mentioned will have different set points for each strain which, therefore, be analyzed individually. Of course, lipid productivity is a function of both growth rate and lipid content, and the best strain may not be the one with the highest lipid content. For example, as shown in Figure 10, although having the highest lipid content, *C. calcitrans* was shown to have the lowest lipid productivity.

Although it is not possible to overcome the natural limitation on lipid productivity due to the inverse relationship between growth rate and lipid accumulation, several strategies can be used to improve lipid yields. Growth can be carried out in two stages with improved cellular oil content after the first stage of fast growth. The idea here is to use strains with natural rapid growth under nutrient-rich conditions until they reach the appropriate density, whereupon they are induced to accumulate lipids. Lipid induction has been achieved in many species through nitrogen deprivation (Table 5) and is thought to lower the costs of harvesting considerably.

Species	Nitrogen (+) Lipid content (% of dw)	Nitrogen (-) Lipid Content (% of dw)	Reference
Chlamydomonas applanata	18%	33%	(Shifrin & Chisholm 1981)
Chorella emersonii	29%	63%	(Illman et al. 2000)
Chorella minutissima	31%	57%	(Illman et al. 2000)
Chorella Vulgaris	18%	40%	(Illman et al. 2000)
Ettlia oleoabundans	36%	42%	(Gatenby et al. 2003)
Scenedesmus obliquos	12%	27%	(Ho et al. 2010)
Selenastrum gracile	21%	28%	(Shifrin & Chisholm 1981)

Table 5: Enhancement of lipid production achieved in different species through nitrogen starvation. Cellular lipid content is shown as a percentage of the dry weight on a medium containing Nitrogen (left) and after the nitrogen was depleted (right).

This is the simplest way to artificially induce the production of fatty acids. Many unnecessary secondary metabolites, at least from the point of view of biodiesel production, are normally made, and nitrogen deprivation shuts down their synthesis, driving metabolism towards the synthesis of fatty acids. Molecular tools for algae are being developed and it is thought that through manipulating cell signals and rerouting carbon flux it should be possible to enhance lipid production.

To make a significant impact on the use of fossil fuels, a massive production of biodiesel will be required. In 2010, the U.S. alone consumed 220 billions of liters of diesel (www.eia.gov). A production of 44 billion liters of biodiesel is necessary to satisfy the current blend limit of biodiesel in petrodiesel, 20%, while only 1.3 billion are currently produced annually. Of course it is impractical, if not impossible, to supply this quantity using biodiesel derived from oil seeds and waste oil, and attention is turning to oil from microalgae as a possible solution. One of the problems in this field is the highly exaggerated lipid productivity projections that are made sometimes. These are based on the dubious extrapolation of the best case scenario results obtained under highly controlled, optimized laboratory conditions and projected values as high as 137,000 L/ha/year (Chisti 2007). However, in reality practical yields for any kind of large scale outdoor production will be much lower. A number of relatively large-scale production studies under optimal conditions with raceway ponds indicate that biomass productivities of around 20-30g/m^2/day are probably achievable (Sheehan et al. 1998). If this could be sustained year round, 73 tons biomass/ha/year would be produced. If the microalgal biomass were 30% lipid, a high value considering that these productivities are obtained under nutrient sufficient conditions, only 20 tons of biodiesel/ha/year would be produced. While this is higher than oil crops, about 3 times that of palm oil (6 tons/ha/yr), it is a far cry from the numbers that originally sparked a “green gold rush”.

Confirmation of this more realistic view is given by a recent pilot-scale project, which used outdoor photobioreactors and achieved an extrapolated annual production of 20 tons of oil per hectare (Rodolfi et al. 2009). However, if this extrapolation can be confirmed in any future very large algae farm is quite uncertain. There are many known and unknown risks involved in massive algal cultures, and there is in reality no data available about large-scale

production at this level. At any rate, it is evident that the reproduction of laboratory results on such a macro scale is just not possible.

The algae oil productivity per hectare is still very attractive when compared to regular oil crops, but no data is available about the actual production cost. In fact, a thorough economic analysis is quite difficult at present given the many unknown variables in ultimate achievable biomass productivity, the scale of production that is feasible, and suitable technologies for harvesting and oil refining that have yet to be developed. Thus, many studies have tried to estimate the putative price of algal oil under different production circumstances, but the disparity between the values found, highlights the lack of data from large scale cultures. In general, realistic projected prices are too high to make biodiesel competitive with petrodiesel under current market conditions. For example, one study predicted the algal biodiesel at a projected price of between $25.00/gallon ($6.60/L) to $2.50/gallon ($0.66/L). In this study, the major factor driving the prices was the difference between low and high productivities (Pienkos & Darzins 2009). The challenges to be met in this respect can be seen by comparing pump prices for diesel then, $1.03/L, with crude oil, being sold at $95 per barrel (http://www.eia.gov/) with the market price of palm oil, $1.15/kg = $1.08/L. Thus despite the promise (sometimes overblown), developing practical systems for biodiesel production from algae faces many formidable challenges.

4. From Algal Biomass to Biodiesel

In a general sense, the production of microalgal biodiesel is very similar to the production of first-generation biodiesel. The biomass is produced, harvested; lipids are extracted and then processed through transesterification into FAMES (Fatty Acid Methyl Ester), which is used as biodiesel. However, unlike oilseed plants, harvesting microalgal cells can prove to be quite challenging. The tiny cells floating in the water cannot be accessed as easily as macroscopic plants, and consequently oil extraction gets more complicated than the centuries old press procedure traditionally used for oilseeds. Moreover, algal cultures are very dilute, usually around 1% for autotrophic growth and up to 10% for heterotrophic growth (Wu & Shi 2007; Gouveia & Oliveira 2008). A dewatering process is necessary prior to use the

biomass. Many standard techniques have been evaluated for use in mass algal cultivation. They are reviewed bellow and explained in detail elsewhere (Molina Grima et al. 2003; Mata et al. 2010; Zhu & Ketola 2012).

5. Harvesting

The harvesting process can be done at once or divided into different steps, each one varying depending upon the desired final total solids concentration. Usually, the first step produces nothing more than green slurry, and further drying may be necessary. Of course, the choice of harvest method will vary depending on the ultimate use of the biomass. Nutraceutical products may require physical processes for harvesting to avoid chemical contamination and maintain the product's natural characteristics. In this case, the high value of the product will compensate for the high cost and energy intensity of the method. Continuous centrifugation is the preferred method when the algal culture will be used for fish feeding purposes, due to the extended shelf life. This method is the most widely used due to its efficiency and its well-documented techniques (Heasman et al. 2000; Molina Grima et al. 2003). However, it is among the low value high demand products, such as biodiesel, that harvesting and dewatering methodologies play a crucial role. The use of energy intensive process for harvesting, such as centrifugation and tangential filtration, can represent 20% to 57% of the final biomass cost (Molina Grima et al. 2003; Van Den Hende et al. 2011), and compromise the overall net energy ratio (Sander & Murthy 2010).

5.1 Possible Promising Harvesting Technologies

Thus, one major hurdle in developing a viable biodiesel from microalgae production process is how to harvest the biomass effectively in a cost-effective manner (Uduman et al. 2010). A variety of methods are potentially available, including; centrifugation, flocculation, filtration, sedimentation, and mat formation. As reviewed below, a number of recent studies provide some hope for the near-term development of a cost-effective harvesting technology. Of course, how effective many of these are can sometimes be species dependent. Thus,

acceptable harvesting procedures can be highly dependent on the cultivation method. Although, as discussed above, open pond systems are preferred for biofuel production for a number of reasons. These are likely to produce mixed cultures, or at the very least, monocultures whose composition differs according to location specific conditions. Thus, techniques that rely on characteristics of specific species can probably only be successfully used with cultures grown on photobioreactors where, at least in principle, some species control is possible.

5.2 Centrifugation

As noted above, centrifugation has been the method of choice for small scale studies since it is highly effective and capable of harvesting all but the most fragile species. Yet, it has been argued that this method is too energy intensive for application to what is essentially a low value product and where there is a need to keep the NER (Net Energy Ratio) as high as possible. This is undoubtedly true if high levels of removal are sought. However, it has recently been argued that interesting harvest costs can be obtained by increasing the flow (i.e. volumetric throughput) and accepting lower capture efficiency (Dassey & Theegala 2013). These authors found that energy consumption could be decreased by 82% when only 28% of the algal biomass was collected resulting in a harvesting cost that they estimated to be $0.864/L oil.

5.3 Flocculation

Flocculation is a well-known process that has been used for years to remove algae and other suspended particles from the water during treatment to produce potable water. In this process externally added compounds cause the suspended algae to form flocks, that will freely sediment. The flock formation is a physicochemical process, and the resulting particle size is a function of mixing speed (L. W. Hallenbeck 1943). Due to the negative charge of microalgal cell walls, they tend remain dispersed in solution. Flocculation agents can neutralize this charge, causing the cells to aggregate and settle, which facilitates the harvest process.

Chemical flocculation methods and agents that can be used in microalgal cultures have been systematically investigated (Molina Grima et al. 2003; Uduman et al. 2010; Beach et al. 2012; Riaño et al. 2012). A desirable flocculant should be non-toxic, recyclable, inexpensive, and efficient at low concentrations. Due to the massive scale predicted for production of biodiesel, any chemical needed for the biomass cultivation or processing will have a significant impact on the market price. Thus, recycling the compounds used for algal cultivation and processing is both an economic and a sustainability issue.

Various chemical flocculants, such as alum (hydrated aluminum potassium sulfate) or alkali are traditionally used, but cannot be considered for use in harvesting microalgae for biofuel production because, in addition to cost considerations, their toxic nature precludes further use of the algal biomass, for example for animal feed, after lipid extraction. However, this process might be adapted to make a cost-effective harvesting technology for biofuel production from microalgae if the right compound could be found. Moreover, a recent study suggests that previously projected costs might be too high as it was found that the amount of flocculant required varied with the logarithm of cell density instead of linearly. One widely accepted theory of flocculation is that it works through charge neutralization; the compound added (an alkali normally), neutralizes the negative charges on the surface of the algal cell thus allowing aggregation. Thus, this theory might be thought to predict a requirement for flocculant that increases linearly with cell number. Contrary to this, highly dense cultures were found to require substantially less flocculant, thus potentially substantially reducing costs (Schlesinger et al. 2012). That study proposed that cost effective flocculation using a mixture of calcium and magnesium hydroxides, with a cost of less than $10.00 per ton of algal biomass, could be achieved, due to the low concentration of flocculating agents required (< 12μM) and the high density of the algal culture used (6 × 107 cell/ml). However, flocculant demand will probably also be a function of the particular algal species since coagulation properties are dependent upon a complex set of characteristics including cell size and extracellular polysaccharide production (Eldridge et al. 2012). In an interesting recent development, it has been shown up to 99% of the biomass can be effectively recovered using ammonia (L. Chen et al. 2012), which can be recycled into the culture as a source of nitrogen

after neutralization of the pH. It is not know if this procedure can be applied to a broad range of algal species.

Bio-flocculation is a promising and poorly explored alternative. Some algal strains have a natural ability to auto-flocculate under some specific conditions (Olguín 2012), while others can be flocculated by the addition of a bacterial culture (Kim et al. 2011). This suggests that novel compounds might be found that could be used as flocculants and that would avoid at least some of the disadvantages of presently-used chemical flocculants. One example is the newly described flocculant excreted by cultures of *Solibacillus silvestris,* which has been shown to efficiently flocculate cultures of the marine microalgae *Nannochloropsis oceanica* and which can be reused (Wan et al. 2013). Likewise, a bioflocculant has been isolated from an autofloculating *Scenedesmus* (Guo et al. 2013). Of course, it is desirable that any flocculant be of use with a wide variety of species.

5.4 Filtration

By definition, filtration relies on specific cell or colony sizes and it is more efficiently applied to large species or on those who grows in filaments or colonies. In laboratory scale, filtration can be a very effective harvesting method. For large scale it implies that the desired species be maintained as a nearly homogenous monoculture. Most microalgae are too small to be effectively harvested this way since their small size and extracellular material quickly clog filters that have been tested. The membrane replacement and pumping represent the major drawbacks of this technology in terms of costs and energy demands (0.3-2kW/m^3), bringing down the NER (Gouveia 2011). Commercial utilization of this technique includes the cultivation of *Spirulina sp.* (filamentous), *Coelastrum sp*., and *Micractinium sp.*, species that can be found grouped in colonies over 70µm (Brennan & Owende 2010; A. K. Lee et al. 2008). Tangential or Cross-Flow Filtration can be used to reduce clogging. In this case, the continuous flow of the algal culture helps removing particles that would plug the membranes pores. Alternatively, special membranes coated with polyvinyl alcohol polymer were shown to attenuate fouling formatting (Hwang et al. 2013). Tangential flow filtration is a method that causes low physical stress, conserving the integrity of the cells and maintaining its natural

proprieties. For high value products or in the cases where the algal biomass will not be immediately processed, this could represent an important feature (Gouveia 2011). It was shown to harvest effectively up to 89% of the algal biomass in a solution, but still has to be scaled up (Petrusevski et al. 1995; Rossignol et al. 1999).

No harvest technique is perfect by itself and a combination of methods in a harvesting procedure composed of different steps might be the solution. Flocculation and filtration, for instance, could be used as serial treatments. Micro-straining filtration was shown to be feasible when used after a flocculation step, which solved the clogging issue (Molina Grima et al. 2003). Filtration through a micro-strainer is a low cost method; units are readily available in large size; are easily operated; and have low energy demands. It is ineffective when faced with a solution containing microscopic particles, but very efficient when used after the flocculation step (Molina Grima et al. 2003).

5.5 Sedimentation

If not mixed and given enough time, many algal strains in a dense culture will precipitate. This will allow the recovery of a large fraction of the water that can be readily recycled. The logic of the process is simple, the gravity separation of liquids or solids in other liquids. The algal sedimentation velocity is about 0.1-2.6 cm/h, depending on the particle density. This, of course, can only be used in moderation and associated with another harvesting method, since it can be time and space consuming and is not likely to produce a biomass with low water content (Milledge & Heaven 2012). The theoretical settling speed can be calculated considering the particles density and radius and, of course, the medium's density (Stokes' Law). Considering that fresh water density at 20˚C is approximately 0.998g/mL (salt water is only 0.027g/mL higher); and that a typical algal cell used in microalgal cultivation is 1.070g/mL (*Chlorella vulgaris*), the settling speed for this species would be around 4.1 cm/h. Now, unlike the photosynthesis theoretical efficiency, the particle sedimentation can be optimized through different methodologies, among them is culture methodologies, ponds/photobioreactor design or specific settling tanks. One approach related to culture methodology is to increase speed in detriment of efficiency. Recovering only 60% of the

biomass in a solution, the particle sedimentation speed was shown to reach 149cm/h (Ras et al. 2011), where that the rest of the water solution containing 40% of the biomass can be re-grown and even be used as a culture seed. Changing the design of the settling tanks, Smith and Davis (2013) used a diluted culture of *Chlorella sp.* of 0.7g/L claimed to be able to harvest the biomass with a solid concentration of 59g/L through simple sedimentation (Heasman et al. 2000). In their design, they changed the angle of the regular settling tanks from $\theta=55^{\circ}$ to a narrow $\theta=8^{\circ}$. Besides, instead of continuous operation, they opted for semi-continuous recovery together with a continuous culture flow, allowing the sedimentation of a volume several times higher than the tank capacity before collecting the precipitate.

Like any other technique, sedimentation is under development, and different variables are being played with to increase performance. Gravimetric sedimentation demands a very low energy input ($0.1 kWh/m^3$) and delivers a product free from contaminants (Uduman et al. 2010). On the other hand, the water content is still likely to be high and as a time consuming process, cellular components might be altered during the waiting period, when cells are likely to be under stress and in the dark.

5.6 Biofilm Formation

Species that readily form biofilms have been little studied for biofuel production since it is of course difficult to maintain them in a homogenous suspension in the cultivation medium. However, several recent studies, with two different systems, have shown that this kind of growth mode can offer the ease of simple mechanical harvesting, leading to slurries with a dry weight content of 9-16 %. In one case, algae were grown on a rotating drum, in what was otherwise an open pond system, and simple mechanical harvesting was achieved by simply unspooling and scraping the cotton “rope” fiber that was used (Christenson & Sims 2012). In another approach, the algae were grown on a flat surface which was drip-watered. At the end of the growth period the algae were recovered by simple mechanical scraping (Ozkan et al. 2012). Not only was harvesting greatly simplified in both cases, both protocols achieved high rates of biomass production at respectable light conversion efficiencies.

6. Lipid Extraction and Transesterification

The majority of the lipids produced by microalgae are usually between 12 and 22 carbons long and can be saturated or unsaturated (Medina et al. 1998). These can be directed to membrane synthesis (polar lipids) or stored as carbon reserve (neutral lipids). For biodiesel production, saturated fatty acids between 12 and 16 carbons are desirable (Srivastava & Prasad 2000), with a different ideal proportion of chain length/unsaturation degree depending upon the climate of the region where the fuel is going to be used (Dunn & Bagby 1995). A biodiesel produced to be used in Colombia, Cuba, or Mexico could become semi-solid or pasty at the low temperatures of the Canadian winter. The parameter that makes reference to this property is the cloud point: the temperature where tiny crystals are formed giving a cloudy appearance to the fuel.

The classical lipid extraction procedures use dried algal biomass as feedstock and organic solvents to dissolve and extract the neutral lipids from the cell. However, the permeability of the algal cells to these solvents varies according to the strain, and limits extraction yield. In line with the efforts to find a solution for low cost harvesting of microalgal biomass, a great deal of research on lipid extraction is examining wet extraction methods since the harvest process can be simpler and cheaper if biomass with a very low water content is not required. A promising recent development is the demonstration of a wet lipid extraction procedure (Sathish & Sims 2012). In this process, the harvested algae do not require complete drying prior to extraction, close to 80% of the lipids susceptible to transesterification could be recovered from wet algal biomass (84% moisture content). Other technologies aim at increasing extraction yields through some form of cell disruption, facilitating solvent access. Pulsed field electroporation seems particularly promising in this regard due to its relatively low energy demand (de Boer et al. 2012). Another technology that requires low dewatering degree is the hydrothermal liquefaction, where the algal sludge is "cooked" under high temperature and pressure. In this method, the lipids are converted to oil together with carbohydrates, proteins and several others molecules, as chlorophyll and carotenoids (Biller & Ross 2011). The reaction product consists of a gaseous fraction, an aqueous fraction and the bio-crude fraction. This bio-crude still has to be refined into biofuels through the same process used for fossil crude oil. The conversion of other cellular components into oil was shown to

increase yield by only 10% to 15% in comparison to the cell lipid content, suggesting that there is room for improvement (Ross et al. 2010; Brown et al. 2010; Sawayama et al. 1999).

When just a simple extraction is used, the fraction, primarily TAGs, must be transesterified to produce molecules, acyl-esters of the free fatty acids. This involves the substitution of an alcohol for the glycerol found in the TAG, with either methanol or ethanol being used, producing FAMEs (fatty acid methyl esters) or FAEEs (fatty acid ethyl esters). This reaction requires a catalyst, either an acid or a base, to occur at reasonable rates and relatively low temperatures and pressures. This reaction employs the same reagents commonly used in production of biodiesel from oil seeds: methanol (which is cheaper than ethanol but produced from fossil fuel); sodium (or potassium) hydroxide; or sodium methoxide.

Direct or wet transesterification, is merely the omission of the extraction step, using the whole biomass as feedstock for the reaction. Surprisingly, the exclusion of the extraction step was found to increase efficiency, raising the lipid yield per gram of biomass (Griffiths et al. 2010). The major drawbacks of this method are the variation of efficiency when applied to different strains, and the use of volatile solvents, a dangerous pollutant.

A more ecological option would be extraction using switchable solvents. These can be either a polar or a non-polar, and can be switched between the two by bubbling N_2 or CO_2 respectively (Jessop et al. 2005). In a polar configuration, they are highly miscible with water, facilitating entry into the cell and contact with the neutral lipids. Once switched back to a non-polar state, they will extract the lipids out of the cells and out of the aqueous phase. Recovery of the lipids and solvent can be performed by switching to polar and then back to nonpolar, avoiding the distillation process commonly used for volatile solvents and increasing the recovery rate. This green chemistry has been tested with vegetable oil (Phan et al. 2009), yeast, and microalgal biomass (Young et al. 2010; Boyd et al. 2012). It was suggested that lipid extraction of diluted algal cultures might be also feasible (Samorì et al. 2010).

A variety of different novel extraction and conversion procedures are under active investigation with the goal of obtaining high biodiesel yields in an energy-efficient manner that doesn't require extensive use of toxic solvents. As pointed out above, the use of organic solvents, the traditional method for oil extraction from oil seeds, should be avoided both from

the perspective of eliminating possible toxic pollutants, but also from an energetic point of view given the energy intensive processes required for solvent recovery. One elegant way around this impasse is the use of switchable solvents (Boyd et al. 2012). Since they can be interconverted between having a polar and a nonpolar character simply by using CO_2, solvent recovery through distillation is not required. Moreover, relatively environmentally benign solvents can be used.

7. Valuable Co-Products?

Microalgae culture, depending upon the species being cultivated, can produce a large number of high and medium value products. Among these are food supplements as polyunsaturated fatty acids (omega-3), possible pharmaceutical agents, various pigments (e.g. chlorophyll and carotenoids), and can also be used as protein rich livestock feed. When faced with the severe challenges and dismal economics of large scale production of a high volume, low value product such as a biofuel, many propose improving the economics by introducing a co-product generating scheme (Singh & Gu 2010). In this type of scenario, often called a biorefinery, biofuel production is essentially subsidized by the revenue derived from the sale of a much higher valued product.

However, the problem with this approach is that, at the production scale needed to generate a significant amount of biofuel, so much of the co-product would be produced that its price would sink. A now classic example of this is the glycerol produced as a side product of biodiesel manufacture. In the early days of biodiesel production, the glycerol was a value added product. As biodiesel production has grown significantly, a glut on the glycerol market was created and the bottom has dropped out of the market with the price of glycerol falling over ten-fold. Biodiesel manufacturers are now basically forced to burn it as it has changed from a valuable byproduct to a nuisance hazardous waste. Of course, the petrochemical industry survives through the numerous revenue streams generated by its refineries, but in this case hundreds, even thousands, of different medium and high value products are generated which help the economics of the production of relatively low value fuels. Thus, if the biorefinery concept is to work, multiple products must be made, something more difficult

given the chemical composition of microalgae than the panoply of compounds available in crude oil.

8. Economic Analysis

Of course, before moving to large-scale microalgal biofuels cultivation, the production system needs to be subjected to both a detailed LCA (life cycle assessment); to determine possible environmental impacts and NER (net energy ratio); and an economic analysis. However, to do this in a meaningful way requires specific inputs on system components, and since many of the outstanding questions raised here; cultivation method (open ponds versus photobioreactors), harvesting technologies, and even extraction and transesterification reactions, remain to be answered, this cannot really be done in a meaningful way at present.

Moreover, an economic analysis which compares the price at the pump of a biofuel with that of a fossil fuel, is wrong in its in essence. For biofuels, a metaeconomic analysis that takes into account indirect costs associated with fossil fuel production and use is necessary. A quick overview suggests that there are in fact many hidden costs to fossil fuel use and that the "real" cost of gasoline or diesel is significantly higher than the price paid by the consumer at the pump (NRC 2011). The additional costs must be paid either now or later in other ways, typically through a higher tax burden. There are of course direct subsidies to the fossil fuel industry, estimated at about $50 billion (USD) over the next ten years in the US alone (EESI 2011).

Although this number is significant, the more hidden costs of fossil fuel use are much higher. Damages from external effects, such as impacts on the health system, but not including those related to climate change, ecosystems, infrastructure and security were estimated at $120 billion for the US in 2005 alone (NRC 2011). To this of course must be added the costs of climate change due to fossil fuel use. One way to estimate the damage is to look at the cost of adapting to climate change, although this does not provide the actual full costs incurred since this represents less than full mitigation. An initial international study estimated these costs at $49 to 171 billion (USD) per year (UNFCCC 2007), and it has been argued that this is in fact

an underestimate (Parry et al. 2009). Of course, these estimates are highly dependent on the accumulated of atmospheric CO_2 burden over time as well as a great deal of uncertainty as to actual impacts. Thus, determining what the competitive cost of a biofuel really should be will require detailed economic analysis. In addition, as mentioned above, detailed costing is not possible given the many uncertainties in the design specifics of a practical algal biodiesel plant. Thus, a realistic cost analysis is impossible at present.

9. Challenges for the Development of Practical Systems

In one LCA (life cycle analysis) study, based on projecting current laboratory observations and current practices in the first-generation biodiesel production industry, open raceway ponds were conceptualized for the cultivation facility, photobioreactors were considered too expensive even considering the possible increased productivity (Lardon et al. 2009). Even using optimistic assumptions, it was concluded that only wet extraction of low-N grown microalgae had a positive energy balance, a reflection of the preponderance of total energy consumption taken up by lipid extraction (90% dry extraction, 70% wet). It was concluded that development of a sustainable, net energy producing system will require minimizing the energetic demands of the production, harvesting and extraction steps, minimizing nitrogen fertilizer use, and extraction of the energy and recycling of the minerals in the oil cakes through efficient anaerobic digestion.

One of the potentially cost intensive inputs to an algal cultivation system is the supply of macro and micronutrients. Lowering the cost and increasing the sustainability of such a process requires that cheap, or even "free" sources of fixed nitrogen and phosphate be found. An obvious solution that can partially satisfy the appreciable water requirement, is to use some kind of waste stream, probably domestic wastewater (Figure 11). In essence, if operated as a pond, which in all likelihood it would be, this would be a high rate algal treatment pond operated for biofuel production (Craggs et al. 2011; K. C. Park et al. 2011b; Pittman et al. 2011; Rawat et al. 2011). This would appear to be a much more environmentally sound and economically attractive option than a dedicated algal biofuels production unit using large amounts of freshwater with addition of fertilizers.

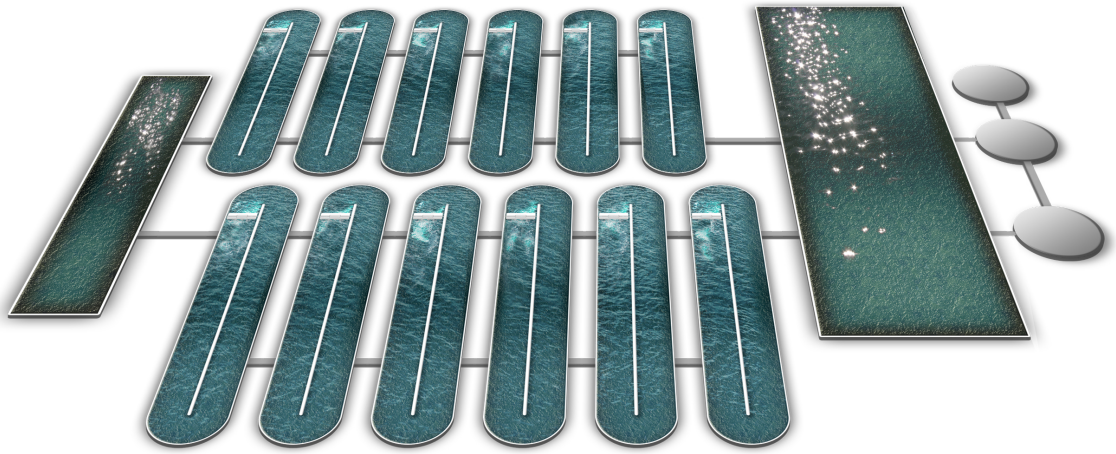

Figure 11: Example of a design of a microalgal cultivation coupled with a domestic wastewater treatment (Leite & P. C. Hallenbeck 2011, *with permission*). Two series of algal ponds in parallel (middle), are fed by the effluent of the wastewater secondary treatment (right). The algal biomass is then settled and harvested in the settling tank (left).

However, a number of challenges would have to be met; provision of CO_2 for maximum productivity, control of the species which are grown to assure high lipid content and suitability for downstream processing, control of grazers, and suitable harvesting strategies, possibly bioflocculation (J. B. K. Park et al. 2011a). Some at least partial solutions to these potential limiting factors are on the table. As noted above, CO_2 could potentially be supplied through the use of flue gas, or alternatively, from the CO_2 remaining after use of the biogas stream coming from anaerobic digestion of the residual algal biomass.

Stable operation of high rate treatment ponds for biofuel production would require the establishment of a regime capable of maintaining the desired strain (one with naturally high lipid content) as the dominant species over a reasonably long period of time, i.e. several months at least. However, at present the factors that enable one species to dominate are not well understood and are probably a combination of a wide range of environmental (temperature, light, water quality), operational (pH, HRT) and biological (pre-adaptation,

resistance to predators, etc.) variables. Attempts to grow introduced species invariably fail due to the cultures becoming overrun by native algae or being decimated by zooplankton. This is the major challenge for effective use of high-rate treatment ponds, or indeed any open pond system, for biofuels production. One possible strategy would be to use some form of biomass recycle where a fraction of the desired algae are collected and reintroduced into the system, thus effectively increasing their apparent growth rate over that of other species. Of course for this to work the desired species has to have some easily used specific characteristic, for example, filamentous species could be selectively retained over unicellular forms by screening with nylon mesh and a fraction reintroduced (Weissman & Benemann 1979).

One of the greatest challenges in producing biodiesel from microalgae is the need to develop low cost, effective harvesting. Most of the microalgae so far known showing promise for either biodiesel production or wastewater treatment are small, highly negatively charged (self repelling) and have a similar buoyant density, making their harvesting problematic. Both centrifugation and chemical flocculation are highly effective but too energy or cost intensive to be used in any large scale practical process. One promising avenue that requires further research to determine if a practical application is possible is to select strains which, under proper conditions, are capable of auto- or bio-floculation. Cells capable of forming large aggregates could then be harvested by gravity sedimentation and final dewatering could potentially use centrifugation, cost effective if the solids concentration obtained through gravity sedimentation is high enough and therefore only small volumes need to be treated.

Although we are perhaps a long way from large-scale deployment of combined waste treatment and biodiesel production processes, some initial laboratory scale research has given promising results. Cultivation of a freshwater alga, *Chlorella ellipsoidea* on actual effluent from several different secondary treatment processes has shown that high biomass yields are possible (425 mg/L in the secondary effluent with the highest phosphate concentration) with high levels of lipid accumulation (35-40%) in stationary phase while at the same time removal of nitrogen and phosphorous was above 95% (Yang et al. 2011).

The biomass residue after oil extraction could be used as a feedstock for a "biorefinery". But as noted above, the potential for deriving value by making high cost byproducts is limited, and the use of residual material as animal feed is questionable given the

need to ship it, a costly option considering its value. Nevertheless, something needs to be done with the residual material as otherwise it becomes an immense waste disposal problem. Probably the best option is to develop anaerobic digestion methods suitable for converting much of the mass into biogas. The produced methane could be used to power plant operations, and, at the same time, this would allow recovery of some of the fixed nitrogen, phosphate, and trace elements necessary to continue algal growth operations. Some initial studies have shown the feasibility of this approach, with yields around 0.2 to 0.3m^3/kg VS (Volatile Solids), on the lower end of the range for what is typical of standard anaerobic digestors (Ehimen et al. 2011; Wiley et al. 2011).

10. Objectives of the Present Study

This work evaluates the potential of the microalgae native to Québec to be exploited in microalgal cultures for biofuel and bioremediation purposes. The initial effort was concentrated on the isolation of microalgae from a variety of environments and geographical locations in Quebec. This goal required the development of high-throughput methodology to allow the efficient physiological characterization of an elevated number of microalgae strains.

After the isolated algae had been characterized by their growth and lipid content, selected strains showing the good performance were analyzed evaluated for their capability to grow using different organic carbon sources. Productivity is still a bottleneck in microalgal oil cultivation, and some companies investing in high-value products are switching to the heterotrophic cultivation of microalgae. Several algal strains can grow heterotrophically, and the addition of an organic carbon source into the medium is known to increase productivity. However, this also increases the production cost, and decrease the final energy conversion efficiency, since this carbon being used is usually directly derived from energy crops (e.g. corn and sugarcane). An opportunity to be explored is the industrial waste, a cheap source of different nutrients, including carbon sources. The third chapter of this thesis evaluated the feasibility of algal cultivation in the main component of the waste produced by the biodiesel refineries (glycerol), and by the pulp industry (xylose). Growth and lipid production was

analysed in heterotrophic and mixotrophic cultivation. These were compared to the photoautotrophic growth to evaluate the performance and quality of the biodiesel produced.

The work described in chapter three revealed for the first time the assimilation of xylose by a microalgal strain to support growth. None the less, this sugar showed some intriguing inhibitory effect. The chapter four aimed to evaluate phenotypes using single cell analysis technology, through a flow cytometer. Here, the focus was to understand the effects caused by xylose on growth, lipid storage, cell size and cell count. The response to xylose addition into a grown and healthy culture was evaluated using short time-points, which provided the dynamics of two metabolic patterns in different strains.

In the fifth chapter, the conventional methodology for microalgal bioprospection was re-evaluated. The hypothesis is that enriching a water sample could lead to the isolation of different strains, more adapted to the conditions of the enrichment. The objective was to compare the strains isolated from the same water sample but submitted to different methodologies: the conventional and the proposed enrichment. In this case, the isolation method was focused on mitigation of the CO_2 emission from a theoretical industry. The project aimed to verify if both isolation processes would yield the same strains and if it would be a productivity improvement in the strains from the proposed method.

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Chapter 2: Exploring the Diversity of Microalgal Physiology for Applications in Wastewater Treatment and Biofuels Production

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Author contributions: Always under the guidance of Dr. Patrick C. Hallenbeck, the author designed the strategy for sampling, handling and isolation of strains and built the culture collection used in this and other works. A.E.M.A did most of the preliminary identification. The author also designed and, with help from A.E.M.A, adapted the methods, performed the experiments and contributed for the data analyses.

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Exploring the Diversity of Microalgal Physiology for Applications in Wastewater Treatment and Biofuels Production

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Abstract

A recently established strain collection of freshwater microalgae native to Quebec was examined for physiological diversity. The 100 strains appeared very heterogeneous in terms of growth when they were cultured at 10±2 °C or 22±2 °C on the secondary effluent from a municipal wastewater treatment plant (WW) and defined BBM medium. Scatterplots were used to examine the diversity in physiology that might be present in the collection. These showed a number of interesting results. There was a fair amount of dispersion in growth rates by media type independent of temperature. Surprisingly considering that all the isolates had been initially enriched on BBM, the distribution was quite symmetrical around the iso-growth line, suggesting that enrichment on BBM did not seem to bias the cells for growth on this medium versus WW. As well, considering that all the isolates had been initially enriched at 22 °C, it is quite surprising that the distribution of specific growth rates was quite symmetrical around the iso-growth line with roughly equal numbers of isolates found on either side. Thus enrichment at 22 °C does not seem to bias the cells for growth at this temperature versus 10°C. The scatterplots obtained when the percentage lipid of cultures grown on BBM were compared with cultures grown on WW at either 10 °C or 22 °C made it apparent that lipid production was favored by growth on WW at either temperature and that lipid production does not seem to be particularly favored by one temperature over the other. When the collection was queried for differences with respect to sampling location, statistical analysis showed that roughly the same degree of physiological diversity was found with samples from the two different aggregate locations.

1. Introduction

There is a great deal of interest at present at both the research and development levels in microalgal biofuels production systems. A number of very significant challenges remain to be overcome before the dream of sustainable algal biofuels production becomes a reality (Georgianna & Mayfield 2012; Leite & Hallenbeck 2011; Larkum et al. 2012; Abdelaziz, Leite & Hallenbeck 2013a; Abdelaziz, Leite & Hallenbeck 2013b; Leite et al. 2013; Work et al. 2013). Among the many challenges, some may be solved by technological advances, e.g. harvesting and effective lipid extraction and conversion to biodiesel, whereas others, including: fast growth rates, high levels of lipid production, competitiveness, tolerance to variation in local conditions, are a function of the biology of the system. These latter goals then can be addressed through strain selection, with native, indigenous strains more likely to be competitive and hardy under local conditions.

Thus, a number of studies involving the isolation of strains in different locales have been undertaken with an eye to biofuels production since the late 1970's over a number of continents and climatic zones (Sheehan et al. 1998; Nascimento et al. 2012; Griffiths & Harrison 2009; Mutanda et al. 2011; Rodolfi et al. 2009; Elliott et al. 2012; Do Nascimento et al. 2012; Abdelaziz, Leite, et al. 2014b; Araujo et al. 2011; Zhou et al. 2011). Almost invariably these bioprospecting efforts have had a narrow, immediate focus on very specific attributes, such as high lipid productivity, and usually go on to examine in detail the characteristics of only a few strains. Recently, we established a collection of over 100 native freshwater microalgae indigenous to Quebec (Abdelaziz, Leite, et al. 2014b). We have shown that some of the strains have interesting characteristics in terms of growth and lipid production and have gone on to carry out a RSM (response surface methodology) analysis of lipid production and growth, showing that one of the strains in the collection, PCH90 could grow well at both 22 and 10 °C on secondary wastewater effluent, producing up to 36% lipid (Abdelaziz, Ghosh, et al. 2014a). A more in depth look at some of the other strains indicated that there were a significant number that might be of potential interest for biofuels production.

However, such a collection also has the potential of being a valuable resource as a long term genetic resource or in examining local diversity. Algal diversity has long fascinated

and in fact was the subject of the first book devoted to scientific photographs (Atkins 1843). Thus, from the very beginning discussions of algal diversity has been dominated by morphology. In general biodiversity has traditionally been described through taxonomy and taxonomic concepts, and since the phenome used to describe species is usually morphology, taxonomically defined biodiversity of microalgae has been dominated by microscopic examination. Even from this view point microalgal diversity has been regarded as enormous with an estimate of hundreds of thousands of undiscovered species being given more than two decades ago (Andersen 1992).

Traditional taxonomy, arguably the oldest recognized scientific profession (Genesis 2:19) (Boero 2010), may be at a cross-roads for a number of reasons; the number of described species is increasing exponentially, but so are the numbers of taxonomists, with no end in sight (Boero 2010; Joppa et al. 2011; Bacher 2012). Additionally, the naming of species present is not enough to describe the diversity present at a deep level due to: "hidden" diversity, the absence of observation of rare species; "cryptic" species, organisms with significant differences below the morphological level (Hopkins 2005; Bickford et al. 2007); and even the recently demonstrated existence of "ecotypes" in marine microalgae (Rodríguez et al. 2005). Thus, another descriptive process that looks at diversity on a different level is some type of DNA analysis, with a particularly useful approach called DNA barcoding where using sequence analysis of multiple genetic markers; mitochondrial cytochrome oxidase (COX), LSU rDNA, and Rubisco (rbcL), can identify new species and uncover cryptic species (Le Gall & Saunders 2010). This has of course greatly increased the information available about microalgal taxonomic diversity, but at a cost. As the number of sequences accumulates with time, there are fewer and fewer associated with taxonomically recognized names (<20 % in 2010), suggesting that taxonomy is on the road to a future without names (Clerck et al. 2013)!

Obviously, each approach has its benefits and should be matched to the specific goal at hand. However, ultimately and on many levels, including ecological and biotechnological (bioprospecting), the most interesting question is, what is the functional diversity that is present. Nevertheless, this is seldom addressed. While it is known that the functional diversity of large culture collections is vast(Lang et al. 2011), little is known about physiological

diversity, i.e. physiological and metabolic robustness, within small, region specific collections. Since we had established a collection of one hundred different strains collected from the local waters of Quebec, we were interested in assessing the functional diversity present within this collection. The collection was queried for specific growth rate and lipid productivity on two different media at two different temperatures. The observed diversity was surprisingly large, suggesting that in general desirable microalgal phenotypes can readily be discovered in a restrained geographical search.

2. Materials and Methods

2.1 Establishment of the Microalgal Culture Collection

Water samples were collected from five different locations; three freshwater lakes (Lac Croche (45° 59' 24.37" N 74° 0' 21.01" W) and Lac Pilon (46° 0' 14.02" N 74° 1' 7.09" W), Lac Triton (45° 59' 17.11" N 74° 0' 20.55" W)), situated in the Laurentian region north of Montreal, Canada; and two on each side of the Saint Lawrence river, situated approximately 10 km downstream from the confluence with the Ottawa river, where the water of both rivers are not yet totally mixed (45° 25' 39.12" N 73° 49' 15.78" W and 45° 21' 23.36" N 73° 48' 49.96" W). The detailed sampling protocol and initial isolation were described previously (Abdelaziz, Leite, et al. 2014b). No enrichment was used other than the use of a filtration step to concentrate the samples (Abdelaziz, Leite, et al. 2014b). The isolation procedure involved BBM agar plates (Abdelaziz, Leite, et al. 2014b) and incubation in a light chamber at 22 ± 2 ºC with atmospheric CO_2 for a period of four to six weeks. Light was provided by warm white fluorescent bulbs at 25 W/m^2 operated on a 12/12 hour light/dark cycle. Individual strains were stored in dim light in 50 ml tubes.

2.2 Growth and Lipid Production

One hundred isolates were grown at 10±2 °C or 22±2 °C on the secondary effluent from a municipal wastewater treatment plant (La Praire, QC, Canada) and on BBM medium. The nitrate and phosphate content of the wastewater was determined as previously described

(Abdelaziz, Leite, et al. 2014b), giving an estimated N:P ratio of 37:1 with a phosphate concentration of 3 $mg \cdot l^{-1}$ (Table 1). Strains were inoculated (1% v/v of OD_{600} value 1.0) in un-treated 12 well flat bottom plates containing either 4 ml sterile municipal wastewater or BBM medium and incubated for 14 days in a photoincubator at 10±2 °C or 22±2 °C at a light intensity of 40 $W \cdot m^{-2}$ and a 12:12 h light/dark cycle. This method has some variability and an analysis of data obtained in this way indicates that variation between biological replicates done at different times is $\pm$25%. As well, although given the large number of strains it was not possible to carryout replicate samples at the same time, analysis of six duplicates that were included showed that the variation was +25%. Growth was quantified daily by measuring the optical density (OD_{600}) using a microplate reader (Biotek EL800) as previously described (Abdelaziz, Leite, et al. 2014b). Specific growth rates were calculated using the periodic OD measurements and choosing the exponential growth phase. The cellular content of lipid was determined by Nile Red as described previously(Abdelaziz, Leite, et al. 2014b) and given in Supplementary Materials. A Varian Vista MPX ICP-OES spectrophotometer was used to measure the partial elemental composition of the wastewater. Scatterplots were generated using Microsoft Excel and data analysis was made using intrinsic Excel tools or the Regression and Megabase addins.

3. Results and Discussion

The present article reanalyzes, from a different perspective, an existing data set, one that is too large to completely interpret in a single article. Previously, this collection was used to screen for a few selective strains that either were adept at wastewater treatment or that gave high lipid production under specific conditions. Here we have re-examined this collection (Table S1) in order to determine the extent of functional diversity present. The strains were tentatively identified by microscopic examination (Table S1). The identity of ten strains picked randomly was checked by sequence analysis of the 18S RNA and in every case agreed with that determined microscopically.

3.1 Culture Collection and Growth Curves

As described previously, we have established a culture collection of microalgae native to freshwaters of Quebec and, using a high throughput 12 well plate procedure, were able to select a few strains showing interesting growth properties, or an apparent capacity for high lipid production (Abdelaziz, Leite, et al. 2014b). We grew the close to 100 strains at 10±2 °C or 22±2 °C on the secondary effluent from a municipal wastewater treatment plant (La Prairie, QC, Canada) (the chemical composition is given Table 1) and BBM medium. It was apparent from the growth patterns that the collection was very heterogeneous in terms of metabolic properties under these conditions (Fig. S1A, B and Fig. S2A, B). A number of interesting questions arose in terms of the diversity in physiology that might be present. Although the strains were originally enriched with synthetic BBM medium, how did their patterns of growth on this medium compare with that on wastewater (WW)? Equally intriguing was the question of the effect of temperature on growth. All strains were originally enriched by cultivation at 22±2 °C, but what were their patterns of growth at 10±2 °C compared to the original 22±2 °C? In order to visualize these questions, we used scatterplots to assess the strain by strain covariance with the different parameters.

3.2 Patterns of Growth on BBM *versus* WW

Since two different temperatures were used, two separate plots are required to visualize specific growth rate (μ day^{-1}) differences by media type. The results are in fact quite instructive (Fig. 1 A & B). A comparison of Fig. 1A and 1B shows a number of interesting points. One, independent of the medium and temperature, there are a wide variety of growth rates, ranging from <0.25 day^{-1} to 1.5 day^{-1}. Second, there is a fair amount of dispersion in growth rates by media type independent of temperature. This can be seen by examination of either Fig. 1A or 1B. In these figures the dotted line represents the iso-growth line, that is the position of strains that grow equally well in either medium (line through the origin with a slope of 1). A large proportion of the isolates are found close to this line, with a fair number in the space between this line and the X-favored or Y-favored solid lines which define the slope where the specific growth rate on BBM is twice that on WW (X-favored) or where the

specific growth rate on WW is twice that on BBM (Y-favored). Outside of these solid lines, in the space closer to either X or Y axes, are found strains with a very strong predilection for one medium over the other. Independent of the temperature, there appears to be roughly an equal number that strongly prefer WW or BBM; at 10°C, 6 for WW versus 7 for BBM and at 22 °C, 11 for WW versus 9 for BBM. Interestingly, the individual strains forming these "outliers" were different at the two different temperatures. Finally, somewhat surprisingly considering that all the isolates had been initially enriched on BBM, the distribution was quite symmetrical around the iso-growth line with roughly equal numbers of isolates found on either side. Thus enrichment on BBM did not seem to bias the cells for growth on this medium versus WW.

Some of these observations can be quantified by using linear regression analysis (Table 2). Fitting the specific growth rates on BBM versus WW at 22 °C to a straight line through the origin gives a slope of 1.019, indicating that the specific growth rates of different strains are distributed equally on either side of the iso-growth line. Similarly, specific growth rates of BBM versus WW at 10 °C gives a slope of 0.894, suggesting a roughly equal distribution of specific growth rates with specific growth rates in BBM being in general slightly higher at 10 °C. The correlation coefficient (R^2) can be interpreted as an indication of the dispersion in values from the iso-growth line. Using this index, specific growth rates on either BBM or WW are clustered to iso-growth at 22 °C (R^2=0.818) and at 10 °C (R^2=0.800) (Table 2).

3.3 Patterns of Growth at 10 °C *versus* 22 °C

Since two different media were used, two separate plots are required to visualize specific growth rate (μ day^{-1}) differences by temperature. The results are in fact quite interesting and surprising (Fig. 2 A & B). A comparison of Fig. 2A and 2B shows a number of interesting points. One, independent of the medium and temperature, there are a wide variety of growth rates, ranging from ~ 0.25 day^{-1} to ~1.5 day^{-1}. Second, there is a fair amount of dispersion in growth rates by temperature independent of media type. This can be seen by examination of either Fig. 2A or 2B where again the dotted line represents the iso-growth line, that is the position of strains that grow equally well at either temperature. A large proportion

of the isolates are found close to this line, with a fair number in the space between this line and the X-favored or Y-favored solid lines which define the slope where the specific growth rate at 10 °C is twice that at 22 °C (X-favored) or where the specific growth rate at 22 °C is twice that at 10 °C (Y-favored). Outside of these solid lines, in the space closer to either X or Y axes, are found strains with a strong predilection for one temperature over the other. There was a great deal more divergence with temperature for cultures grown on wastewater (Fig. 2B WW) than for cultures grown on BBM (Fig. 2A BBM). There are a significant number of these "outliers", with higher numbers associated with growth at 22 °C than that at 10 °C. Finally, considering that all the isolates had been initially enriched at 22 °C, it is quite surprising that the distribution of specific growth rates was quite symmetrical around the iso-growth line with roughly equal numbers of isolates found on either side. Thus enrichment at 22 °C does not seem to bias the cells for growth at this temperature versus 10°C.

Again, these observations can be quantified by using linear regression analysis (Table 2). Fitting the specific growth rates on WW at 10 °C versus 22 °C to a straight line through 0,0 gives a slope of 1.118, indicating that the specific growth rates of different strains are distributed equally on either side of the iso-growth line. Similarly, specific growth rates on BBM at 10 °C versus 22 °C gives a slope of 0.964, suggesting again that the specific growth rates of different strains are distributed equally on either side of the iso-growth line. The correlation coefficient (R^2) can be interpreted as an indication of the dispersion in values from the linear regression line. Since the iso-growth line (slope = 1) is approximately the same as the linear regression lines (slopes of 1.118 and 0.964). Therefore, using this index, specific growth rates at either 10 °C or 22 °C are again roughly equally dispersed around the iso-growth line whether the medium is BBM (R^2=0.818) or WW (R^2=0.791).

3.4 Patterns of Lipid Production on BBM *versus* WW

It was also of interest to examine the capacity for lipid production and accumulation among the strains isolated and to analyze the differences brought about by growth on different media and at different temperatures. It should be noted that no special culture conditions to enhance lipid accumulation were used, thus the results shown here are certainly not the

maximum lipid production that might be expected. Nevertheless, this analysis might provide some additional insight into the diversity of physiology represented in the culture collection.

The scatterplots obtained when the percentage lipid (at the end of 14 days of incubation) of cultures grown on BBM were compared with cultures grown on WW at either 10 °C or 22 °C are quite striking (Fig. 3A and B). It is readily apparent that lipid production was favored by growth on WW at either temperature. This is perhaps not surprising as the macronutrient content of WW (NO_3 110 ppm, PO_4 3.0 ppm) is appreciably lower than BBM (NO_3 183 ppm, PO_4 163 ppm). At the end of the growth period at least some of the cultures were probably experiencing some degree of nutrient limitation, a known trigger in many microalgae for increased lipid production. These observations can be quantified by using linear regression analysis (Table 2) which gives best fit lines with slopes of 0.408 (10 °C) and 0.396 (22 °C). Not only are these slopes nearly identical, showing a high degree of quantitative similarity in this difference regardless of the temperature, these are half of the iso-lipid line with a slope of 1, indicating that on the average, the percentage lipid content of a given strain is twice that when grown on WW than when grown on BBM. This is borne out by a simple calculation of the average lipid content of all the strains under the four different conditions. The average lipid content on WW was 13.8% at 10 °C and 12.4% at 22 °C whereas on BBM it was 5.6% at 10 °C and 6.3% at 22 °C. It is apparent that independent of the temperature (10 °C or 22 °C), there were some high-performing strains producing over 30% lipid by weight when grown on WW (7 at 10 °C and 6 at 22 °C), see Table 3. It is interesting to note that in each case, with the exception of one strain (HA1B1*), the strains were different, again indicating the diversity in physiological capacities represented in the culture collection. On the other hand, the two highest producers when cultures were grown on BBM at 22 °C, PCH03 (39.4%) and MA2H1 (27.6%), also produced appreciable amounts of lipids when grown on BBM at 10 °C, PCH03 (15.7%) and MA2H1 (31.4%). (Results not shown).

We examined the potential correlation between nitrogen depletion and high lipid production in the best performers (highest lipid producers) on WW at both 10 °C and 22 °C (Table 3). Contrary to what might have been expected, there appears to be very little relationship between nitrate removal and lipid content. The one strain that showed high lipid

production at both temperatures, HA1B1*, did so even though at both temperatures more than 70% of the initial nitrate remained. Four of the top producing strains at 22 °C (AH2, PCH16, HA1B3, PCH36) showed nearly equal capacity for nitrate removal at both temperatures, with in fact higher removal at 10 °C. Thus, lipid production in these strains may be temperature dependent with higher rates at higher temperatures. For unknown reasons, nitrate removal was low for all the best performers at 10 °C when they were grown at 22 °C. Two of the top producers at 10 °C (PCH20 and LB2G5) had high levels (> 30%) of lipids only at the lower temperature even though a large amount (>60%) of the nitrate remained. Here again, high lipid production seems to be primarily related to temperature, but in this case it seems to be increased at colder temperatures. Finally, out of all the top producers, only five (PCH37, PCH41, LB2H5, AH30, and MA1A3) appeared to have a lipid production response that fits the classical notion of this response being driven by nitrogen deprivation (Table 3).

3.5 Patterns of Lipid Production at 10 °C *versus* 22 °C

It was also instructive to examine any potential temperature effect on lipid production for the culture collection as a whole by using scatterplots to compare the lipid contents of the strains grown either on BBM or WW at the two different temperatures (Fig. 4). A number of conclusions, corroborating what was seen when lipid production was compared between media types (Section 3.4), are immediately apparent. One, there is a great diversity seen in lipid production with the two different media types at the two different temperatures. Secondly, lipid production with WW is in general greater than that seen with BBM. Thirdly, lipid production does not seem to be particularly favored by one temperature over the other, with a significant number of strains growing on wastewater producing higher amounts of lipids at either the higher temperature (22 °C) or the lower temperature (10 °C). However, there were a larger number of strains that showed a strong temperature effect at 10 °C as compared to 22 °C. Thus, 12 strains showed two-fold or greater lipid production at 22 °C and 21 strains showed two-fold or greater lipid production at 10 °C. These strains occupy the region on or above the line with a slope = 2, or on or below the line with a slope = 0.5 (Fig. 4B).

3.6 Is the Observed Physiological Diversity Locale Dependent?

The above considerations show that this collection of microalgae isolated from a relatively few number of freshwater sources in Quebec appears to have a remarkable diversity in terms of growth on synthetic medium (BBM) versus wastewater (WW), and growth at 10 °C versus 22 °C. This diversity appears to extend to lipid production in response to environmental conditions and medium. Therefore the question arose as to whether or not the fact that the samples were obtained at different locales, three lakes and two rivers, had at least some contribution to the observed diversity in physiological responses. This question was addressed by binning the data by location and then examining lipid accumulation and specific growth rates under different conditions. The analysis was simplified and rendered more robust by grouping the strains in two different categories; St. Lawrence River, or the three Laurentian Lakes combined, which covered 55 % and 42 % of the total strain collection respectively.

First, the dispersion in growth rates on both BBM and WW was examined as a function of sampling location. An examination of scatterplots shows that both locations provided a great diversity in growth rates when specific growth rates of individual strains at 22 °C was compared to that obtained at 10 °C (Fig. 5 and Fig. 6). Independent of sampling location, the variation and diversity in specific growth rates at the two temperatures showed the same relationship to media type as before, growth rates appeared to be more clustered on BBM medium (Fig. 5) than on WW (Fig. 6). Once again, growth rates of strains from the two aggregate locations were equally distributed about the isogrowth line, as indicated by the slopes of linear regression fits, which were close to 1 in all cases (Table 4). As expected, the correlation coefficients for growth of the samples on WW were somewhat lower than on BBM, with relatively little difference with respect to sampling location.

As well, the ability to grow well at a particular temperature does not seem to correlate very well with the temperature of the body of water from which the samples were obtained. For example, of the five strains that grew much better on WW at 10 °C than at 22 °C (LB2H2, PCH13, NCID4, PCH10, and LB1H9), two were from 2 °C water, one from 15.2 °C water and two from relatively high temperature waters, 25.3 °C and 24.7 °C (Table S1). Conversely, of

the six strains showing a strong preference for growth on WW at 22 °C (LB1H11, MA1A14, PCH34, PCH28, LB2H6, and PCH98) three were isolated from 2 °C water, one from 12 °C water and only two from higher temperature waters, 23.5 °C and 24.7 °C (Table S1). Similarly, of the six strains that grew much better on BBM at 22 °C than at 10 °C (LB2H4, PCH06, PCH02, PCH38, HA1A3, and MA1A3), three were isolated from 2 °C waters, two from 12 °C water and only one from 25.3 °C water. Of course, the basis for this adaptive plasticity is unknown, but certainly demonstrates the underlying diversity present and might reflect a heterogeneous population of strains able to individually respond to the wide seasonal temperature variations encountered in these locations.

Next, the dispersion in the ability to accumulate lipid on both BBM and WW was examined as a function of sampling location (Fig. 7 and Fig. 8). Independent of sampling location, and as expected from the previous results, when growth on BBM was examined, most of the strains were clustered at the low end of percent lipid accumulation and appeared to be evenly distributed around the isolipid line (Fig. 7) with slopes of linear regression fits of approximately 1 (Table 5). Similarly, when percent lipid accumulation after growth on WW at 10 °C and 22 °C was examined as a function of sampling location, the variation appeared to be independent of where the samples had been collected (Fig. 8). As noted before, for both locations, lipid accumulation was greater at 10 °C than at 22 °C, most easily seen from the slopes of the linear regression fits, which were 0.736 (Laurentian Lakes) and 0.749 (St. Lawrence River) (Table 5). Again, the correlation coefficients, an indication of the dispersion in values around the linear regression line, were nearly the same, indicating the same degree of physiological diversity in samples from the two different aggregate locations. Additionally, when this question was examined in another manner, using PCA (principal component analysis), none of the principal components identified correlated with sampling location (Figs. S3).

A large number of the strains were identified as *Chlorella sp.* based on microscopic morphology and eight of those thus identified were also shown to be highly related to *Chlorella* by 18S RNA analysis. However, there were small sequence differences, and, as shown here, functional differences as well. There appeared to be no strong correlation between species identification and the properties examined here. For example, in Table 3,

four out of the five best performers at 22 °C and five out of the six best performers at 10 °C were all identified microscopically as strains of *Chlorella*.

4. Conclusion

An examination of specific growth rates and lipid accumulation by the collection of one hundred local microalgae indigenous to Quebec as a function of both medium type and temperature, showed that there was remarkable physiological diversity present within the collection. Indeed, the full spectrum of responses, strongly favoring one condition over the other, or relatively indifferent, appeared to be present. Within the collection were some strains of potential biotechnological interest in terms of wastewater treatment or lipid production. Of fundamental interest are strains showing good growth at low temperatures. Here a convenient multiwall plate format was used in order to collect data from all 100 isolates for this study. Further studies involving fewer strains will be done with a greater volume and higher degree of replication. When this relatively geographically constrained collection was further divided into sampling locales, the scatterplots obtained suggested that wide diversity was nevertheless retained. It has relatively recently been shown that phytoplankton species richness increase with area follows a power law (Smith et al. 2005). If this holds true for functional diversity, which is likely to be the case, than functional diversity at megascale must certainly be enormous.

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Tables

Table 1: Wastewater Chemical Composition

Macronutrients		**Micronutrients**	
Ion	***Conc (ppm)***	***Element***	***Conc (ppm)***
		As	0.034
NO_3^-	110	Be	0.00059
PO_4^+	3.0	Ca	32
		Cd	0.00081
		Co	0.0034
		Cr	0.0048
		Cu	0.085
		Fe	0.0086
		Li	0.055
		Mg	17
		Mn	0.0027
		Mo	0.011
		Ni	0.015
		Pb	0.019
		Se	0.080
		V	0.010
		Zn	0.051

Table 2: Linear Regression Analysis of Scatterplots Comparing Specific Growth Rates and Percent Lipid Production in Different Media and at Different Temperatures

Comparison		Slope	Correlation (R^2)
Specific growth rates	BBM vs WW 10 °C	0.894	0.800
	BBM vs WW 22 °C	1.019	0.818
	BBM 10 °C vs 22 °C	0.964	0.818
	WW 10 °C vs 22 °C	1.118	0.791
% lipid	BBM vs WW 10 °C	0.408	0.600
	BBM vs WW 22 °C	0.396	0.611
	BBM 10 °C vs 22 °C	1.028	0.747
	WW 10 °C vs 22 °C	0.749	0.653

Table 3: Percent Lipid Content and Percent Nitrate Removal for Best Performing Strains on WW at 10 ° C and 22 °C

Isolate	10 °C		22 °C	
	% Lipid	% NO_3 removed	% Lipid	% NO_3 removed
Best performers 22 °C				
AH2	12.1	96.7	34.9	92.3
PCH16	18.0	97.6	43.8	97.6
HA1B3	17.9	97.6	41.4	94.8
PCH36	23.6	97.6	35.7	85.9
PCH37	17.5	23.5	37.1	95.5
Best, both 10 °C and 22 °C				
HA1B1*	39.6	22.5	35.3	29.7
Best performers 10 °C				
PCH41	39.4	88.5	25.9	25.2
PCH20	32.3	27.0	13.5	14.3
LB2H5	33.3	97.6	12.9	19.7
AH30	34.5	88.1	22.6	32.0
MA1A3	45.5	97.6	9.39	22.3
LB2G5	31.5	34.3	11.1	27.0

Table 4: Population Covariance and Correlation of Growth Rates as a Function of Sampling Location

Medium and Location	Population covariance[a]	Slope	Correlation coefficient
BBM Laurentian Lakes 10 vs 22	0.0078	1.0723	0.818
BBM St. Lawrence 10 vs 22	0.01811	0.9125	0.826
WW Laurentian Lakes 10 vs 22	0.0055	1.0985	0.773
WW St. Lawrence 10 vs 22	0.01013	1.133	0.804

[a] The average of the product of the deviations of each data pair in two data sets

Table 5: Population Covariance and Correlation of Lipid Production by as a Function of Sampling Location

Medium and Location	Population covariance[a]	Slope	Correlation coefficient
BBM Laurentian Lakes 10 vs 22	14.6	1.014	0.871
BBM St Lawrence 10 vs 22	18.4	1.018	0.676
WW Laurentian Lakes 10 vs 22	36.1	0.736	0.637
WW St Lawrence 10 vs 22	30.4	0.749	0.664

[a]The average of the product of the deviations of each data pair in two data sets

Table S1: Pattern of Growth and Lipid Content

Growth rate and lipid content under of the strains cited in this work under different conditions (%L = % lipids / dry weight; WW = waste water; BBM = Bold's Basal Medium).

#	STRAIN	Tentative Identification	Date[a]	°C site	μ WW 10°C	μ WW 22°C	μ BBM 10°C	μ BBM 22°C	% L WW 10°C	% L WW 22°C	% L BBM 10°C	% L BBM 22°C
74	AH2	*Chlorella sp.*	4-20	2	0.60	0.77	0.54	0.41	12.2	40.2	3.56	5.71
70	AH30	*Chlorella sp.*	4-20	12	0.41	0.41	0.43	0.72	34.5	23.7	4.58	6.71
75	AH31	*Pseudochlorella sp.*	4-20	2	0.63	1.01	0.44	0.54	9.64	11.2	6.39	11.6
69	AH34	*Pseudochlorella sp.*	4-20	2	0.41	0.74	0.40	0.55	7.87	9.54	4.89	5.95
99	HA1A1*	*Chroococcus sp.*	5-20	12	1.11	0.90	0.96	0.72	2.82	9.11	3.32	1.70
48	HA1A3*	*Chlorella sp.*	5-20	12	0.51	0.64	0.40	0.90	4.05	3.67	5.94	4.22
49	HA1A5*	*Pseudokirchneriella sp.*	5-20	12	1.06	0.71	0.47	0.43	5.47	4.89	6.02	4.47
50	HA1A7	*Kirchneriella sp.*	5-20	12	0.52	0.65	0.35	0.34	6.10	7.42	3.47	6.66
51	HA1A7*	*Cylindrocystis sp.*	5-20	12	0.55	0.91	0.34	0.48	3.94	5.93	3.48	3.19
52	HA1A8	*Desmodesmus sp.*	5-20	12	0.71	1.01	0.63	0.24	8.30	18.2	5.68	5.29
44	HA1B1	*Scenedesmus sp.*	5-20	12	0.33	0.83	0.42	0.42	8.55	7.35	2.75	2.36
45	HA1B1*	*Scenedesmus sp.*	5-20	12	0.49	0.36	0.90	1.17	39.6	25.7	16.9	20.8
46	HA1B3	*Kirchneriella sp.*	5-20	12	0.61	0.76	0.53	0.43	13.2	24.4	5.34	7.77
47	HA1B4*	*Chlamydomonas sp.*	5-20	12	0.63	0.88	0.38	0.63	3.76	4.05	3.16	3.03
53	LA1H13	*Chlorella sp.*	6-09	23.2	0.55	0.81	0.46	0.75	7.64	10.4	6.71	7.44
85	LB1H01	*Oocystis sp.*	6-09	24.7	0.41	0.83	0.40	0.31	17.6	5.27	2.50	1.84
87	LB1H02	*Pseudochlorella sp.*	6-09	24.7	0.49	0.62	0.34	0.57	19.4	6.40	2.17	2.88
86	LB1H03	*Chlorella sp.*	6-09	24.7	0.63	1.03	0.80	0.42	8.63	3.25	2.81	2.27
40	LB1H06	*Chlorella sp.*	6-09	25.3	0.50	0.77	0.34	0.47	5.76	2.86	4.13	2.22
41	LB1H07	*Chlorella sp.*	6-09	25.3	0.45	0.62	0.54	0.51	18.9	25.4	5.80	5.04
77	LB1H09	*Pseudochlorella sp.*	6-09	24.7	0.43	0.39	0.52	0.60	23.4	23.5	16.7	14.3
42	LB1H10	*Chlorella sp.*	6-09	25.3	0.47	0.75	0.42	0.52	6.77	11.8	2.87	3.15
61	LB1H11	*Anabaena sp.*	6-09	24.7	0.03	1.31	0.33	0.06	17.9	2.92	11.0	14.3
43	LB1H12	*Chlorella sp.*	6-09	25.3	0.50	0.44	0.40	0.70	8.24	5.03	3.68	2.62
78	LB1H13	*Chroomonas sp.*	6-09	24.7	0.75	0.84	0.71	0.54	6.23	2.90	5.35	9.25
80	LB2F2	*Anabaena sp.*	7-05	25.3	0.72	0.51	0.69	0.80	6.02	11.2	12.6	10.0
55	LB2G5	*Chlorella sp.*	7-05	25.3	0.57	0.74	0.76	0.47	31.5	11.2	4.26	11.1
83	LB2H1	*Nitzschia sp.*	7-05	25.3	0.38	0.65	0.20	0.73	13.3	4.70	5.98	2.05
66	LB2H4	*Chlorella sp.*	7-05	25.3	1.32	0.55	0.46	0.99	14.9	14.5	1.99	2.21

54	LB2H5	*Anabaena sp.*	7-05	25.3	0.74	0.69	0.48	0.79	33.3	12.3	2.48	5.14
62	LB2H6	*Anabaena sp.*	7-05	25.3	0.29	0.94	0.21	0.53	9.79	4.68	9.46	4.37
56	LC1H2	*Oocystis sp.*	6-09	24.4	0.67	0.69	0.56	0.45	23.6	9.12	5.41	7.57
35	MA1A02	*Chlorella sp.*	5-20	12	0.39	0.77	0.40	0.43	15.5	15.2	4.26	6.09
88	MA1A03	*Chlorella sp.*	5-20	12	0.74	0.98	0.38	0.81	45.5	9.16	4.56	7.16
81	MA1A04	*Chlorella sp.*	5-20	12	0.39	0.43	0.39	0.51	8.17	13.9	3.11	5.08
67	MA1A05	*Desmodesmus sp*	5-20	12	0.45	0.69	0.79	0.76	5.79	7.99	3.18	7.32
36	MA1A09	*Chlorella sp.*	5-20	12	0.67	0.80	0.25	0.71	6.76	14.6	8.89	2.41
65	MA1A11	*Chlorella sp.*	5-20	12	0.25	1.48	0.32	0.69	14.9	8.13	4.39	4.58
37	MA1A13	*Chlorella sp.*	5-20	12	0.52	0.55	0.52	0.51	5.87	4.79	3.18	2.79
68	MA1A14	*Chlorella sp.*	5-20	12	0.25	0.35	0.75	0.45	4.00	4.89	3.75	4.44
38	MA1A20	*Closterium sp.*	6-19	12	0.17	0.16	0.33	0.31	12.8	12.0	4.63	3.56
39	MA1A22	*Closterium sp.*	6-19	12	0.53	0.33	0.19	0.48	4.31	11.9	6.55	4.02
31	MA2H1	*Chlorella sp.*	6-19	18	0.54	0.70	1.65	0.93	27.5	15.6	31.4	27.6
32	MA2H3	*Chlorella sp.*	6-19	18	0.62	0.80	0.69	0.46	6.34	4.64	3.54	3.34
60	MA2H4	*Chlorella sp.*	6-19	17.5	0.87	0.92	0.81	0.84	8.83	4.88	3.26	3.12
33	MA2H6	*Chlorella sp.*	6-19	18	0.80	1.14	0.65	0.59	7.37	5.14	2.35	6.81
34	MA2H7	*Chlorella sp.*	6-19	18	0.40	0.72	0.65	0.42	7.09	6.91	5.11	2.51
64	NBID4	*Chlorococcum sp.*	6-06	15.2	0.14	0.30	0.20	0.24	16.7	14.2	8.12	9.01
63	NCID4	*Chlorococcum sp.*	6-06	15.2	0.62	0.16	0.64	0.38	15.9	16.9	4.62	7.80
82	PAD	*Anabaena sp.*	6-19	12	0.62	0.89	0.31	0.93	12.1	3.33	6.13	2.85
98	PCH01	*Chlorella sp.*	4-20	2	0.63	0.65	0.61	0.87	8.38	28.3	11.6	6.92
1	PCH02	*Chlorella sp.*	4-20	2	0.49	0.40	0.55	1.19	28.0	11.8	4.47	4.01
97	PCH03	*Chlorella sp.*	4-20	2	0.24	0.33	1.06	0.59	28.6	13.8	15.7	39.4
2	PCH04	*Chlorella sp.*	4-20	2	0.66	0.61	1.10	1.03	7.68	4.77	4.41	3.54
96	PCH05	*Chlorella sp.*	4-20	2	0.56	0.45	0.44	0.34	21.6	7.06	5.65	7.25
3	PCH06	*Chlorella sp.*	4-20	2	0.59	0.75	0.54	1.04	11.7	19.1	4.47	4.90
4	PCH07	*Chlorella sp.*	4-20	2	0.85	0.45	0.65	0.93	5.40	6.95	5.27	4.08
6	PCH09	*Chlorella sp.*	4-20	2	0.57	0.31	0.46	0.22	9.45	9.31	7.96	11.9
7	PCH10	*Chlorella sp.*	4-20	2	0.59	0.06	0.95	1.03	7.87	18.6	2.03	20.1
8	PCH11	*Chlorella sp.*	4-20	2	0.57	0.69	0.54	0.46	8.24	11.2	2.50	2.84
92	PCH12	*Chlorella sp.*	4-20	2	0.32	0.29	0.54	0.73	21.7	14.0	5.79	6.96
9	PCH13	*Chlorella sp.*	4-20	2	0.91	0.10	0.73	0.58	9.28	16.7	6.02	8.62
10	PCH14	*Pseudochlorella sp.*	4-20	2	0.34	0.82	0.27	0.76	18.2	9.89	9.77	1.03
11	PCH15	*Pseudochlorella sp.*	4-20	2	0.56	0.30	0.51	0.43	13.2	13.8	14.9	6.72

12	PCH16	*Chlorella sp.*	4-20	2	0.73	0.82	0.45	0.70	18.0	42.8	1.97	1.87
13	PCH17	*Chlorella sp.*	4-20	2	0.60	0.49	0.49	0.42	6.80	5.68	2.84	2.07
79	PCH18	*Closterium sp.*	4-20	2	0.57	0.58	0.35	0.42	13.7	5.38	4.51	5.10
93	PCH19	*Chlorella sp.*	4-20	2	0.71	0.62	0.85	0.56	3.51	3.76	6.72	5.19
14	PCH20	*Chlorella sp.*	4-20	2	0.39	0.68	0.57	0.75	32.3	14.5	2.83	3.73
15	PCH21	*Scenedesmus sp.*	4-20	2	0.77	0.65	0.42	0.57	6.16	2.89	2.50	2.63
16	PCH22	*Botrydiopsis sp.*	4-20	2	0.50	0.80	1.12	0.82	7.53	12.4	4.54	4.45
17	PCH23	*Chlorella sp.*	4-20	2	0.79	1.26	0.34	0.73	21.8	8.87	2.65	2.47
18	PCH24	*Chlorella sp.*	4-20	2	0.53	0.85	0.46	0.37	4.54	9.71	4.83	1.98
19	PCH28	*Chlorella sp.*	4-20	2	0.14	1.01	0.67	0.34	18.9	13.1	1.78	2.53
20	PCH29	*Chlorella sp.*	4-20	2	0.50	0.42	0.56	0.41	17.1	9.42	3.14	2.22
21	PCH30	*Scenedesmus sp.*	4-20	2	0.44	0.61	0.51	0.51	7.26	6.47	3.74	2.90
22	PCH31	*Chlorella sp.*	4-20	2	0.47	0.49	0.69	0.34	20.9	21.6	4.47	2.56
95	PCH32	*Scenedesmus sp.*	4-20	2	0.77	0.92	0.48	0.42	7.23	7.15	2.23	4.62
23	PCH34	*Chlorella sp.*	4-20	2	0.71	1.59	0.75	0.55	20.0	23.1	4.88	1.40
24	PCH36	*Chlorella sp.*	4-20	2	0.74	0.78	0.70	0.71	23.7	34.8	9.32	11.6
25	PCH37	*Chlorella sp.*	4-20	2	0.74	0.94	0.92	0.55	17.5	37.8	3.36	4.95
26	PCH38	*Chlorella sp.*	4-20	2	0.69	0.92	0.41	1.00	9.07	11.3	5.73	4.80
76	PCH41	*Chlorella sp.*	4-20	12	0.73	0.53	0.43	0.62	39.36	28.1	15.2	10.3
27	PCH43	*Chlorella sp.*	4-20	2	0.58	0.91	0.57	0.78	7.80	12.3	4.77	4.63
28	PCH44	*Chlorella sp.*	4-20	2	0.65	0.61	0.77	0.49	10.8	5.55	2.61	3.90
29	PCH46	*Chlorella sp.*	4-20	2	0.66	0.69	0.54	0.45	17.4	15.3	2.62	9.27
30	PCH49	*Chlorella sp.*	4-20	2	0.55	0.90	0.76	0.65	8.19	3.00	3.42	4.98
73	PCH88	*Chlorella sp.*	4-20	2	0.58	0.79	0.41	0.52	10.6	16.5	3.53	9.21
71	PCH90	*Chlorella sp.*	4-20	2	0.71	0.97	0.53	0.46	28.2	21.4	4.81	15.5
72	PCH98	*Chlorella sp.*	4-20	2	0.46	0.96	0.30	0.44	13.6	16.6	5.78	7.47

Figures

Figure 1: Effects of Medium on Specific Growth Rates; Comparison of WW and BBM at 10 °C (A) and 22 °C (B)

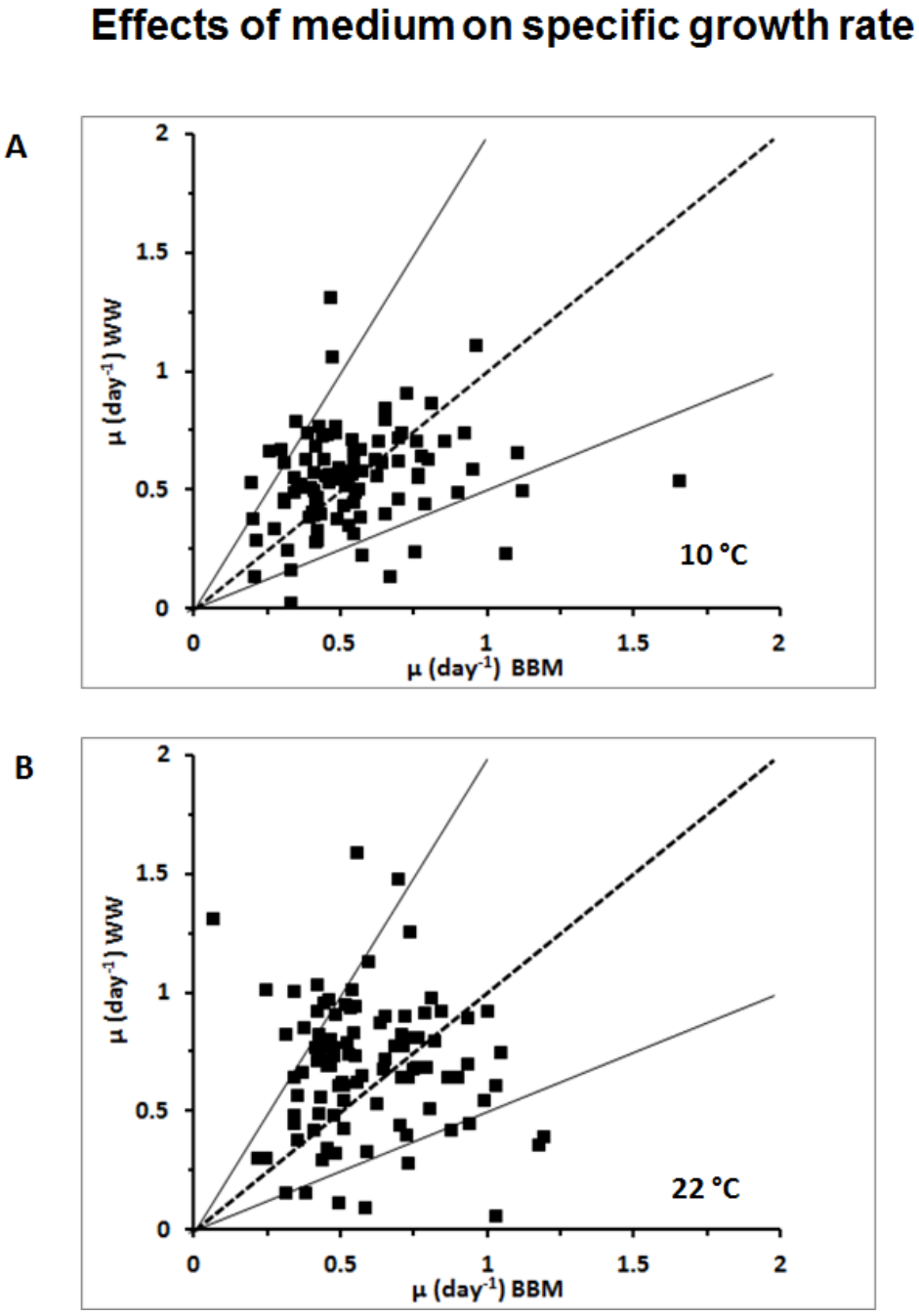


Figure 2: Effects of Temperature on Specific Growth Rates; Comparison of 10 °C and 22 °C Growth in BBM (A) and WW (B)

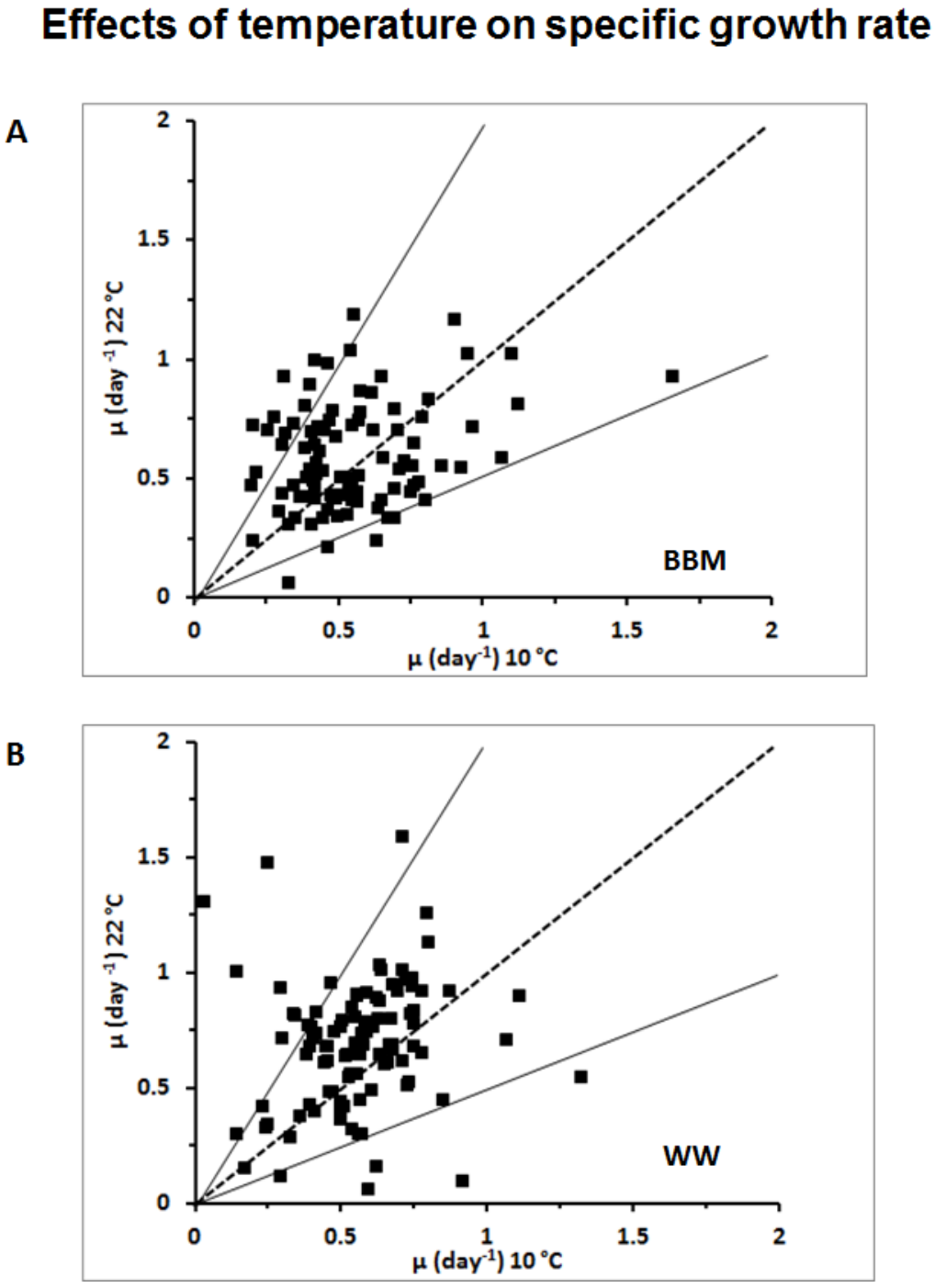


Figure: 3 Effects of Medium on Lipid Accumulation; Comparison of WW and BBM at 10 °C (A) and 22 °C (B)

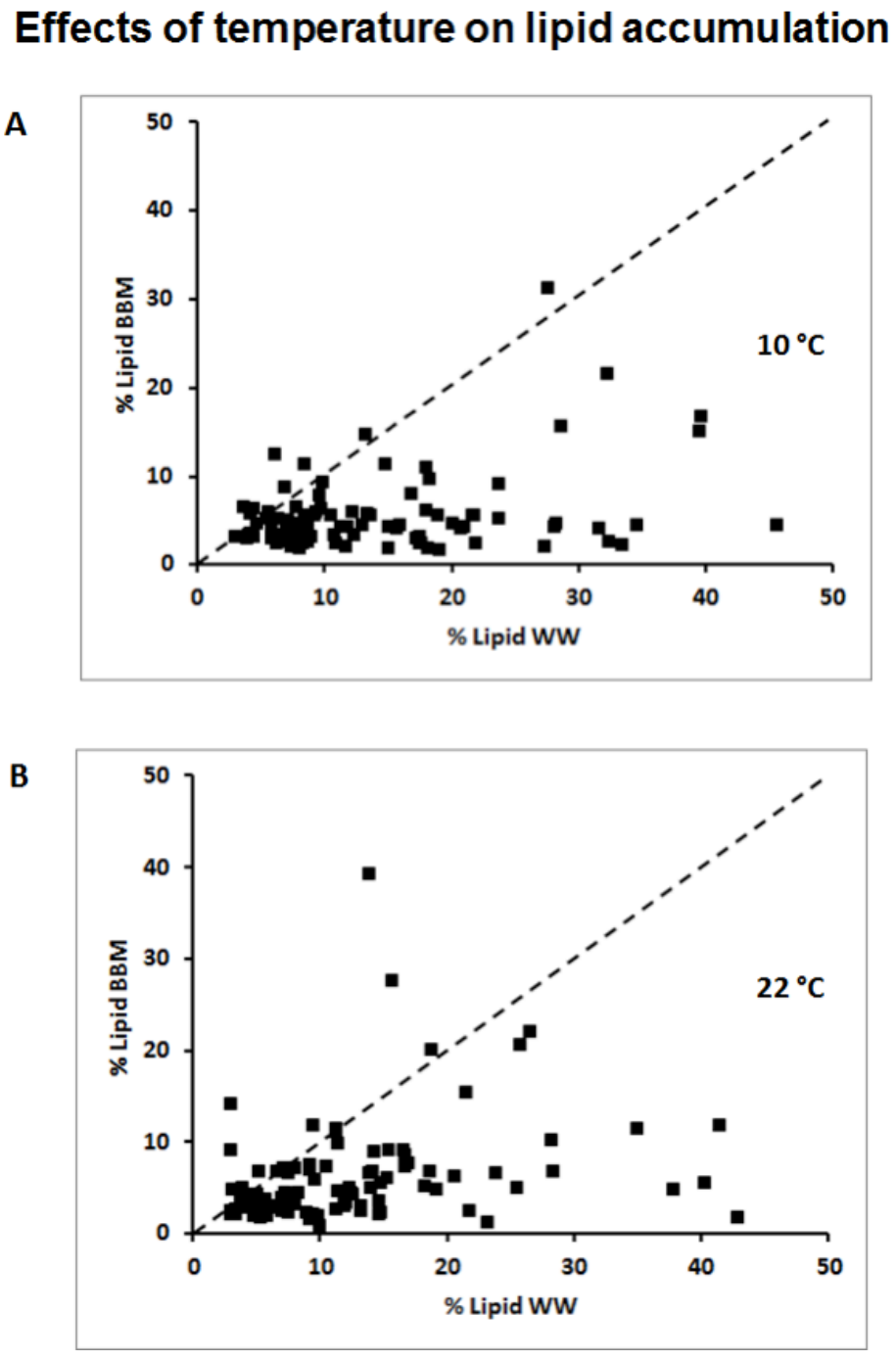


Figure: 4 Effects of Temperature on Lipid Accumulation; Comparison of 10 °C and 22 °C Growth Conditions in BBM (A) and WW (B)

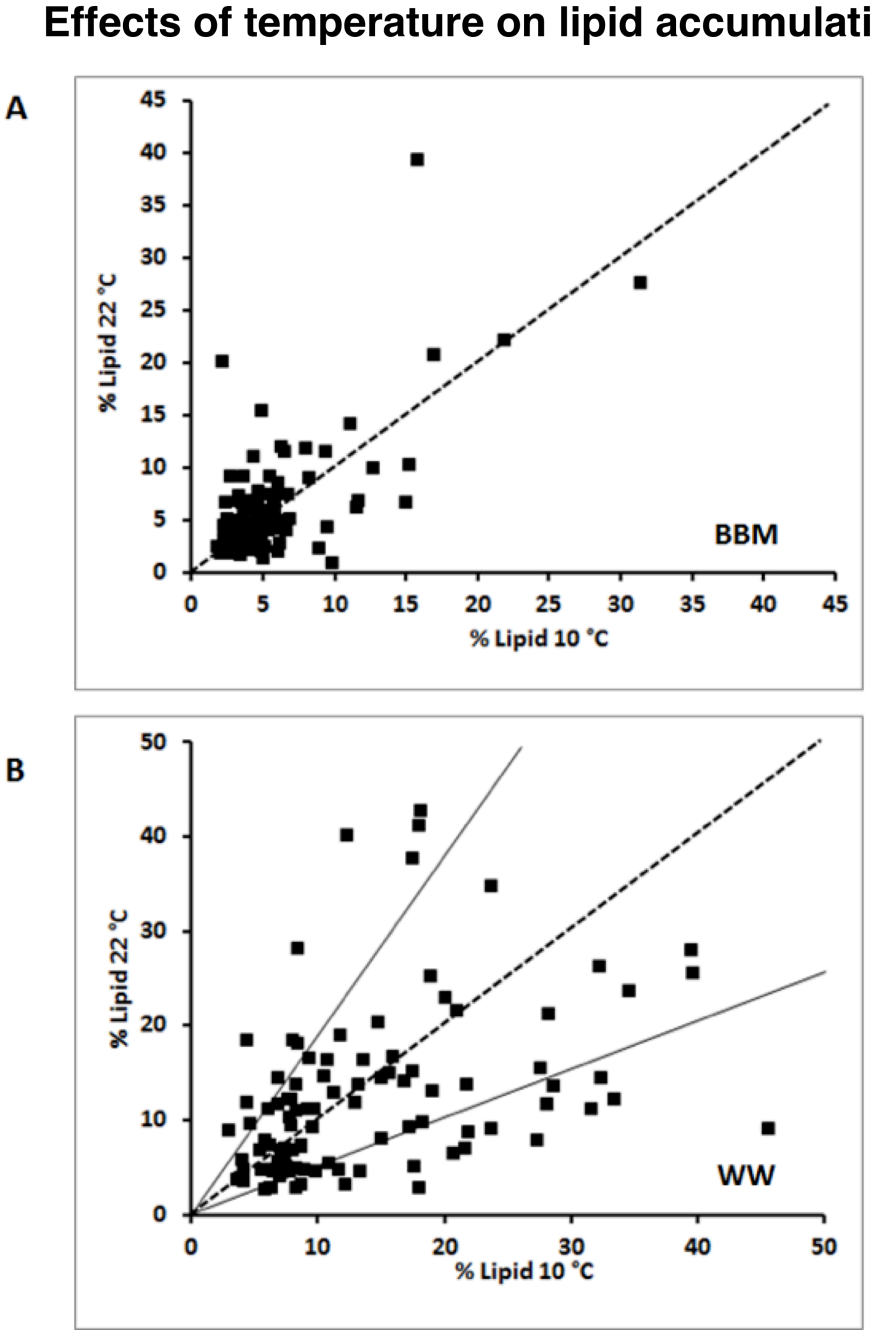


Figure: 5 Effect of Sampling Location on Specific Growth Rates in BBM
Cultures from the St Lawrence (A) and the Laurentian Lakes (B) were grown on BBM at 10 °C and 22 °C

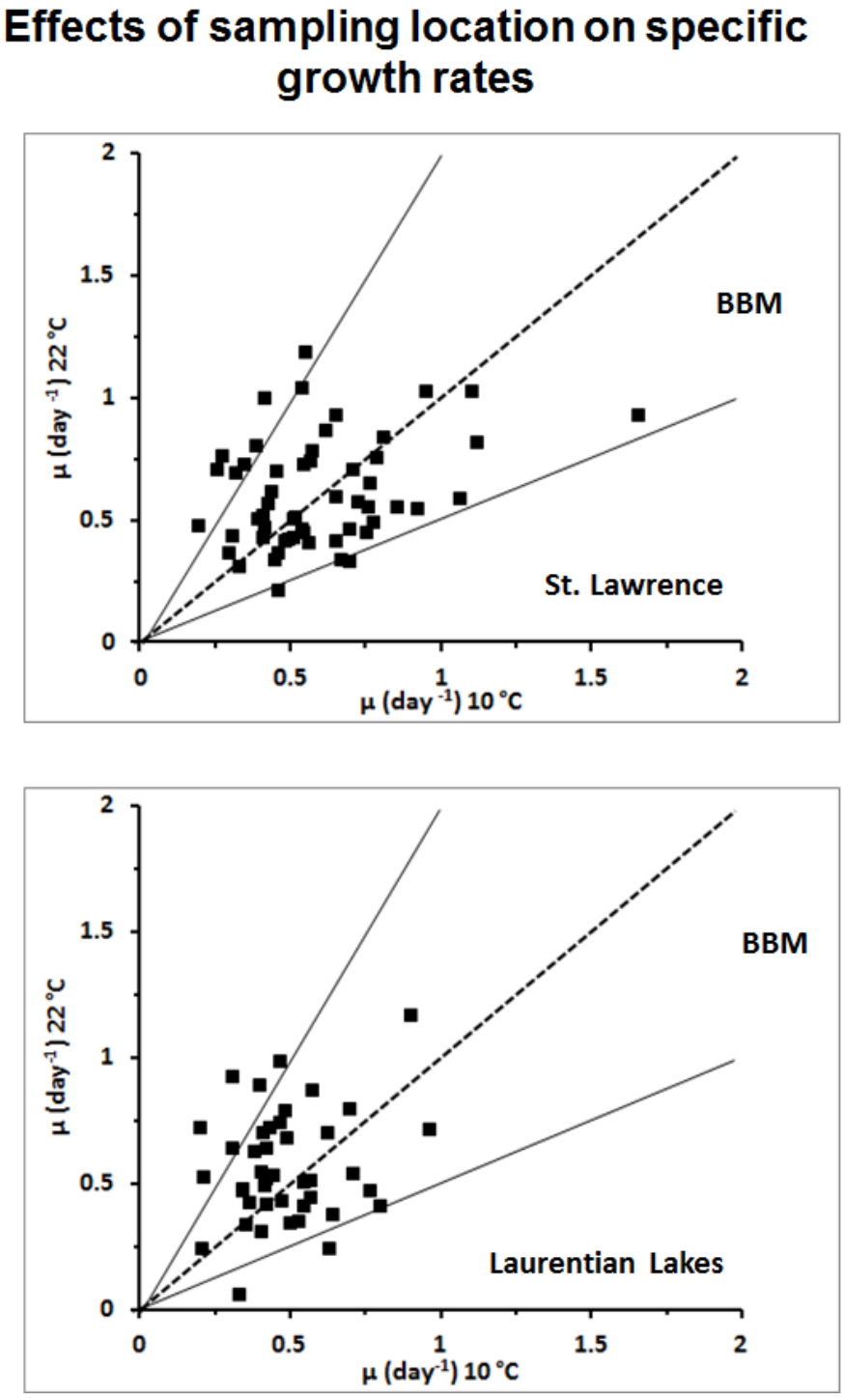


Figure 6: Effect of Sampling Location on Specific Growth Rates in WW
Cultures from the St Lawrence (A) and the Laurentian Lakes (B) were grown on WW at 10 °C and 22 °C

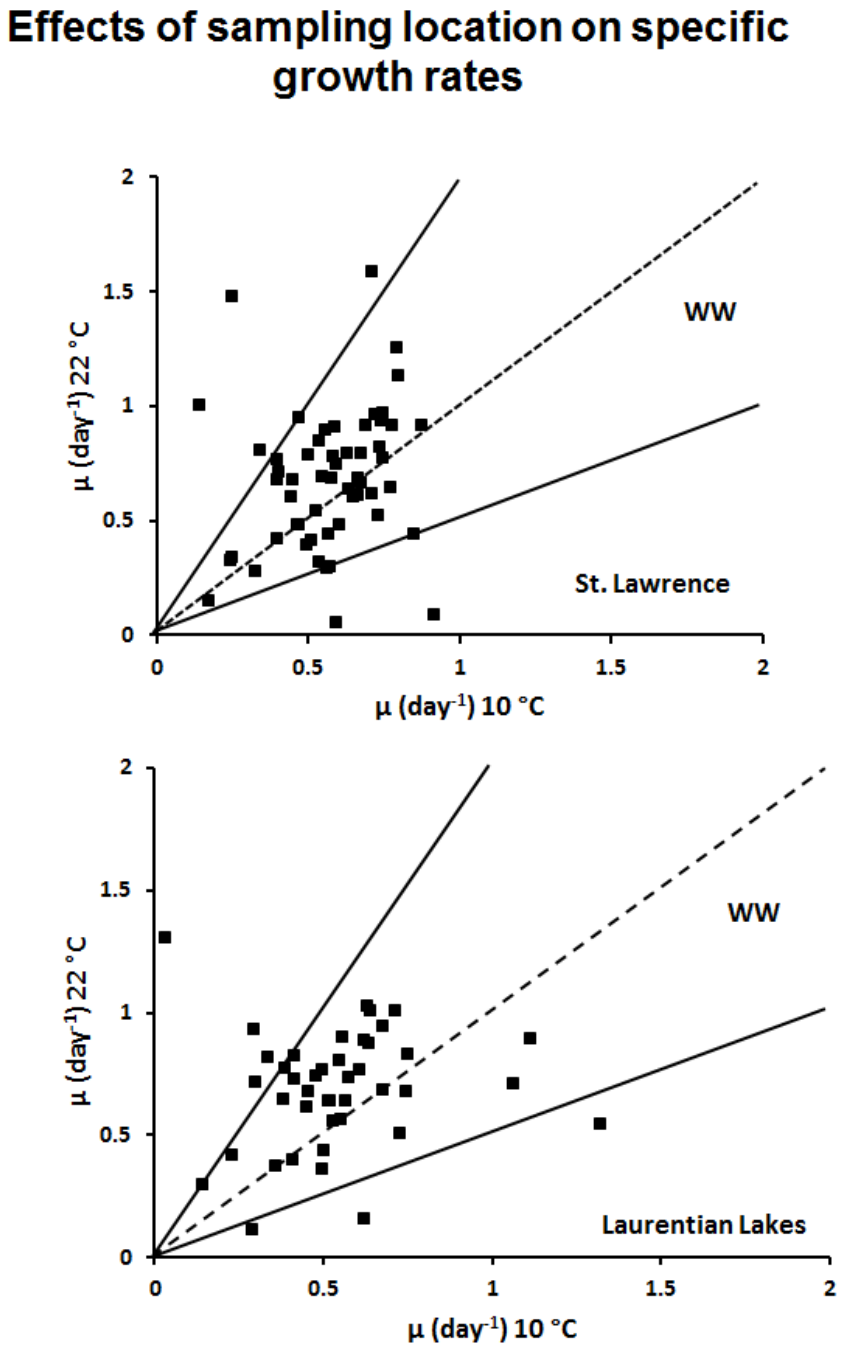


Figure 7: Effect of Sampling Location on Lipid Accumulation in BBM
Cultures from the St Lawrence (A) and the Laurentian Lakes (B) were grown on BBM at 10 °C and 22 °C

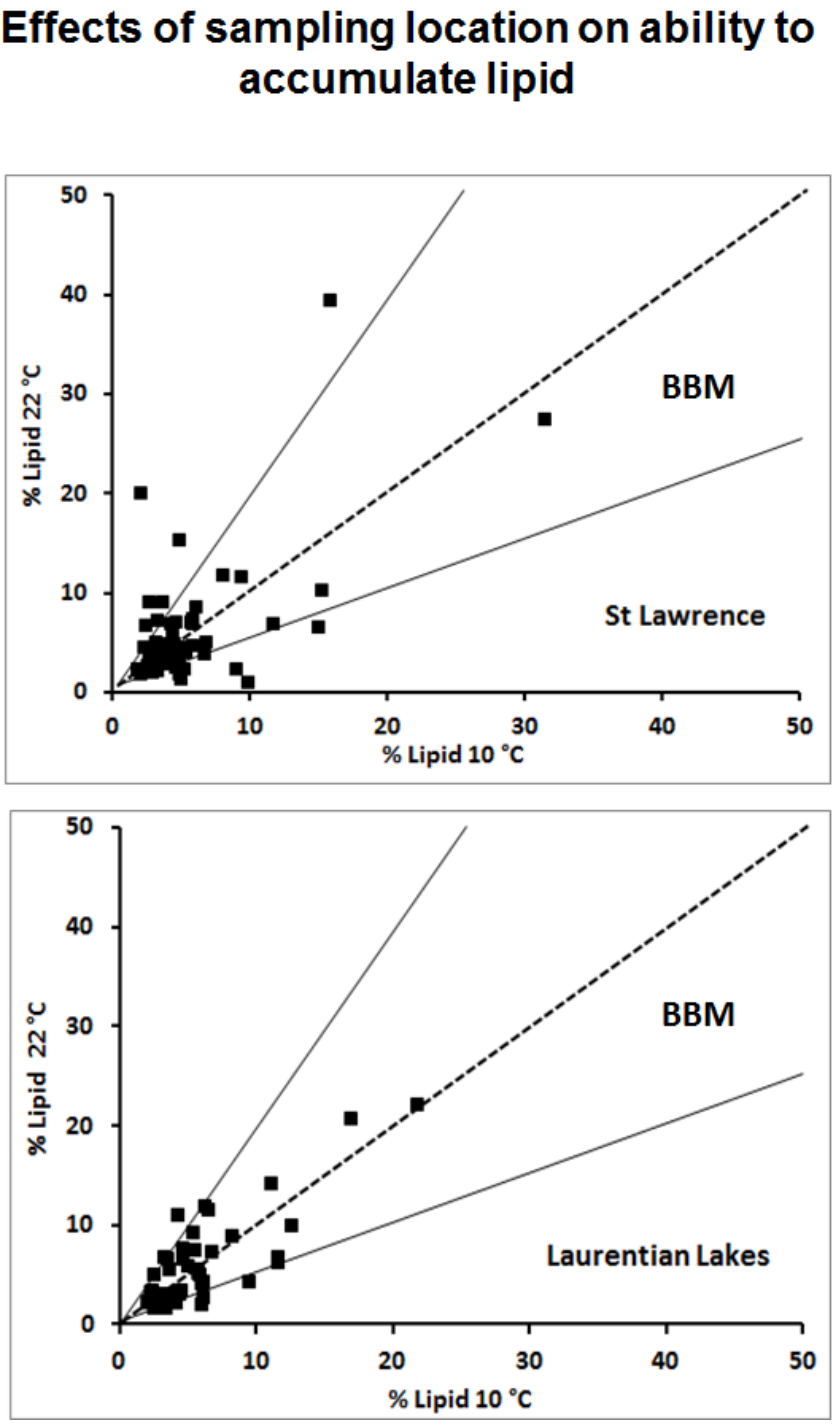


Figure: 8 Effect of Sampling Location on Lipid Accumulation in WW
Cultures from the St Lawrence (A) and the Laurentian Lakes (B) were grown on WW at 10 °C and 22 °C

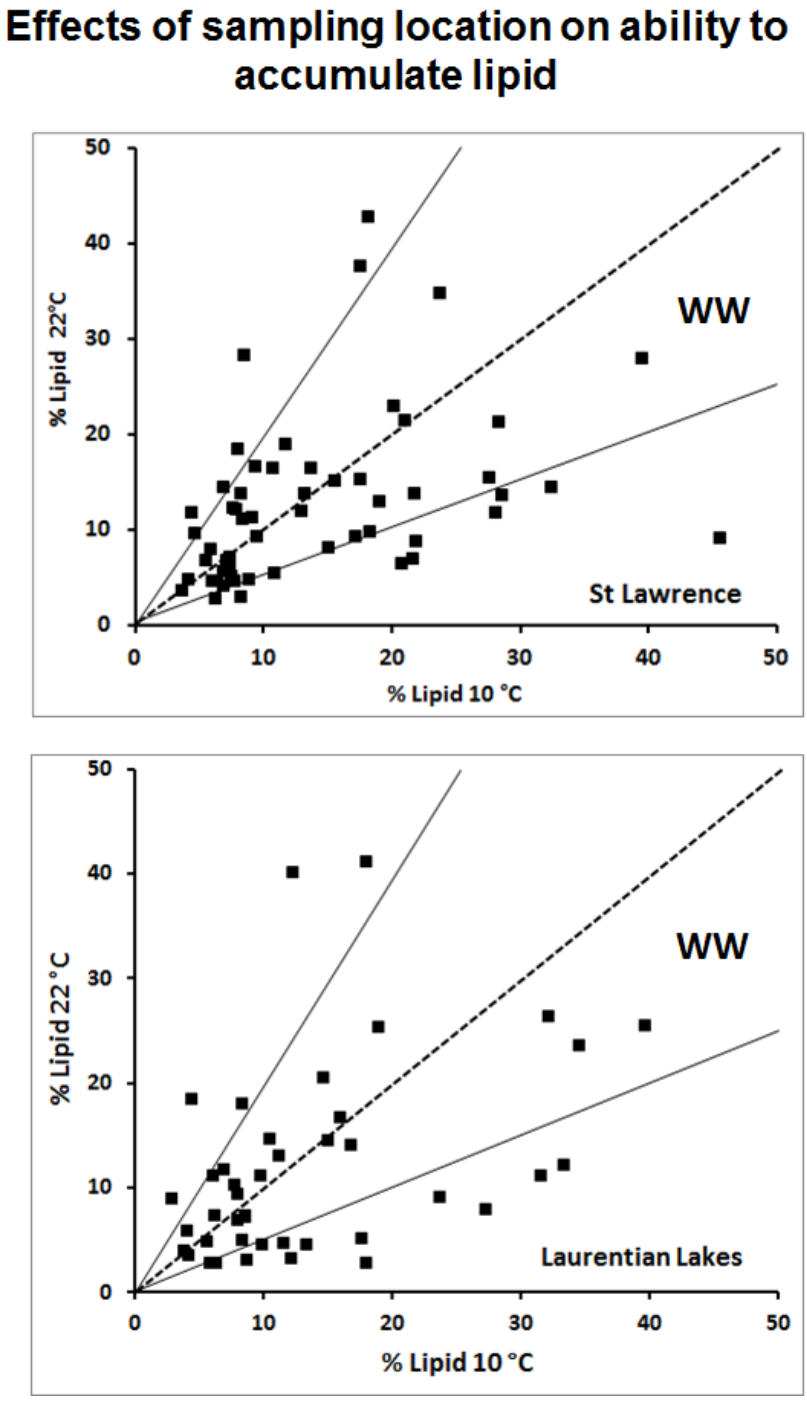
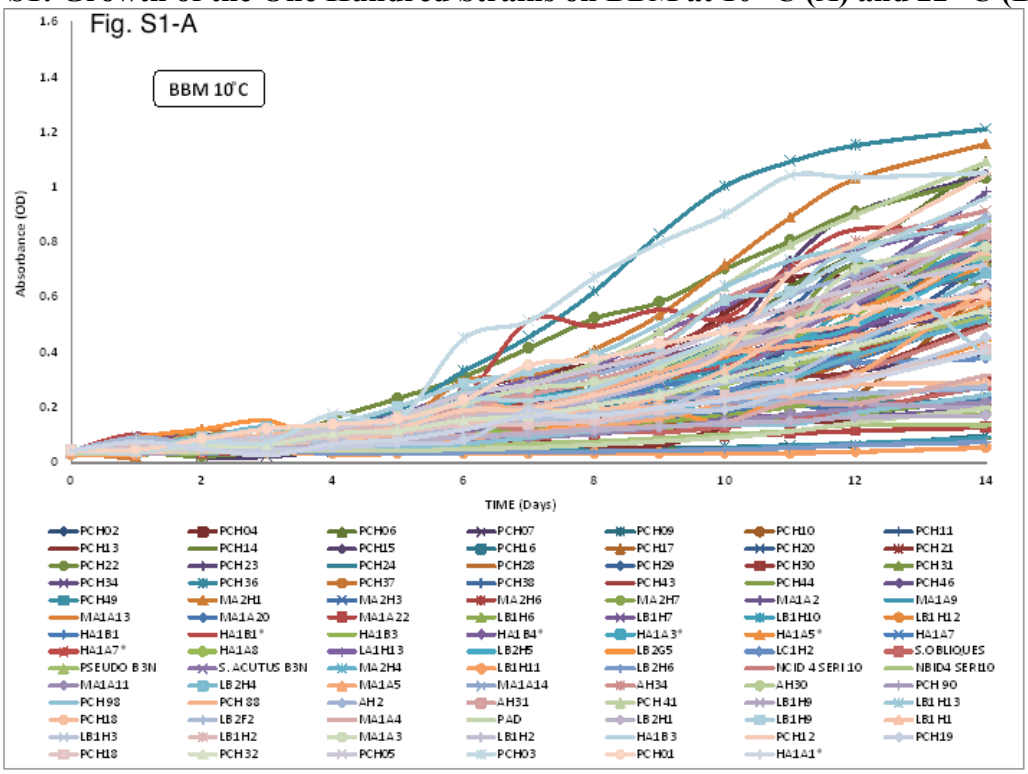


Figure S1: Growth of the One Hundred Strains on BBM at 10 °C (A) and 22 °C (B)



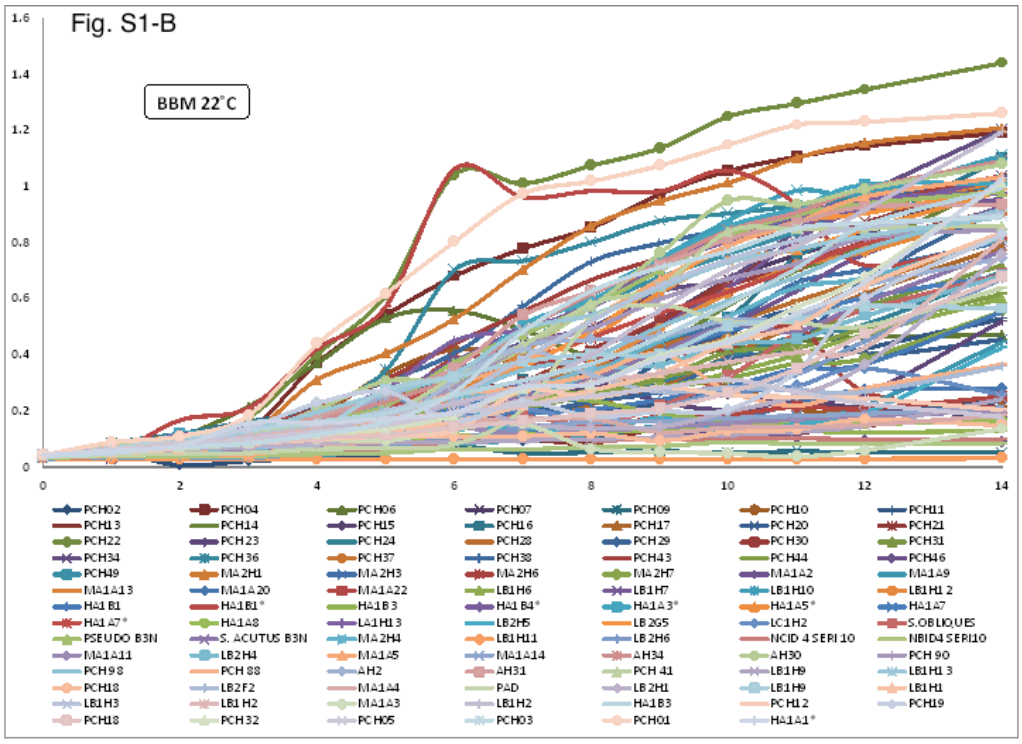
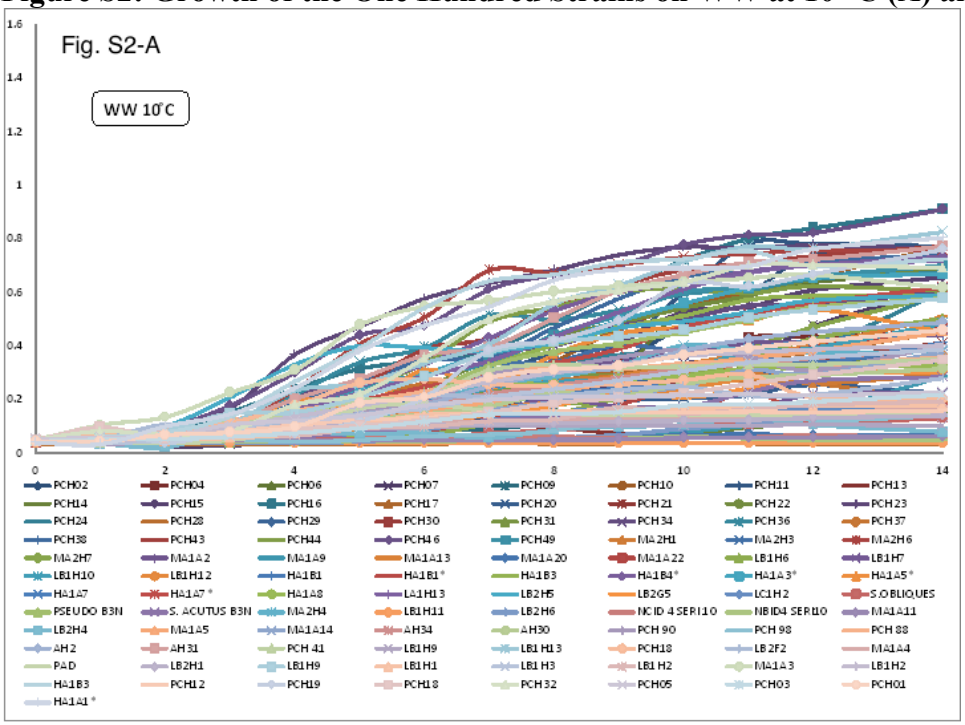


Figure S2: Growth of the One Hundred Strains on WW at 10 °C (A) and 22 °C (B)



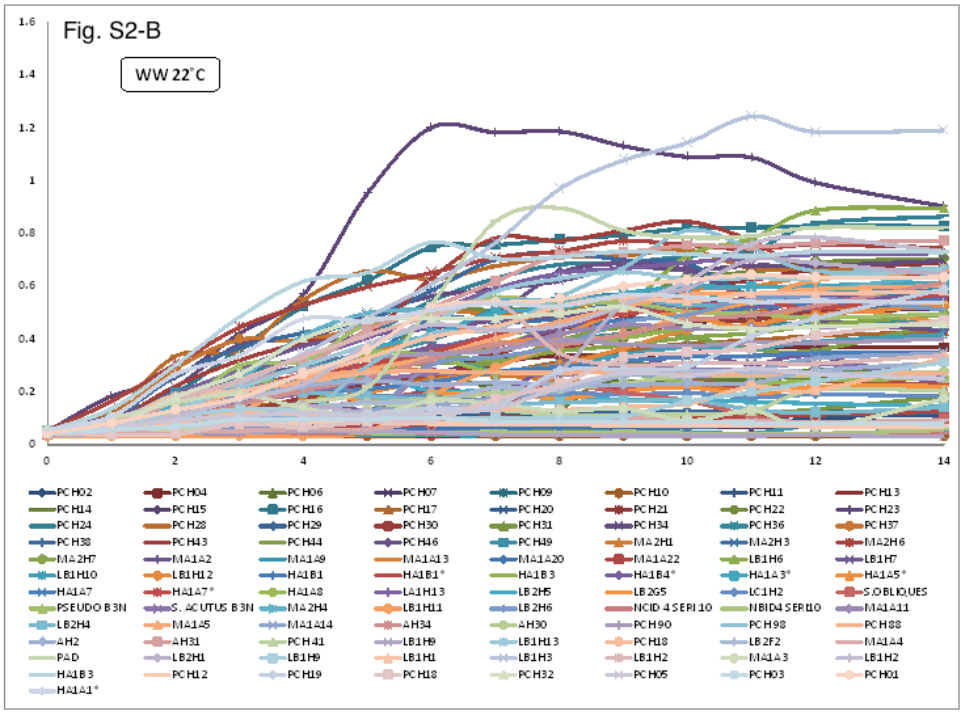
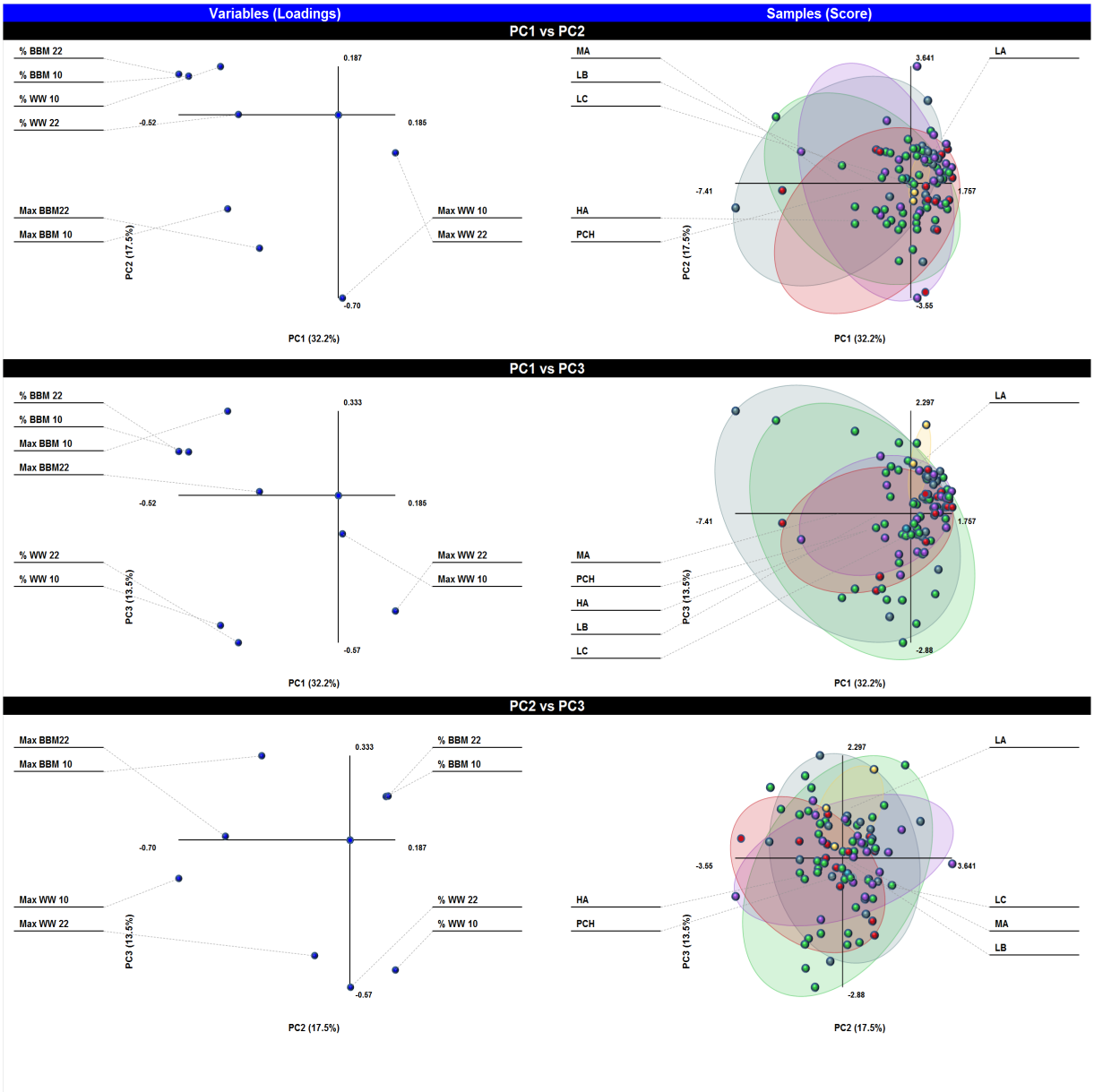


Figure S3: Principal Component Analysis of Variation in Physiological Properties with Sampling Location

The plots on the left show the loadings that lead to the particular principal components examined here. Principal components are combinations of the variables that lead to high correlations. Variables that are close are correlated. Thus PC1 is very closely related to maximum growth on WW at both 10 °C and 22 °C, PC2 is closely related to the percent lipid formed on WW at both 10 °C and 22 °C and PC3 is closely related with maximum growth rate on BBM at 22 °C.



Chapter 3: Utilization of Industrial Waste-Derived Glycerol or Xylose for Increased Growth and Lipid Production by Indigenous Microalgae.

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Author contributions: The author was responsible for the project concept design; experiments and analysis, always under the supervision and guidance of Dr. Patrick C. Hallenbeck. K.P. performed some of the biochemical analysis, under supervision of the author. The manuscript was written by the author and revised by Dr. Patrick C. Hallenbeck

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Utilization of Biodiesel-Derived Glycerol or Xylose for Increased Growth and Lipid Production by Indigenous Microalgae

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[redacted]

Abstract

The use of industrial wastes rich in mineral nutrients and carbon sources to increase the final microalgal biomass and lipid yield at a low cost is an important strategy to make algal biofuel technology viable due to its impact on overall production costs. Using strains from the microalgal collection of the Université de Montréal, this report shows for the first time that microalgal strains can be grown on xylose, the major carbon source found in wastewater streams from pulp and paper industries, with an increase in growth rate of 2.8 fold in comparison to photoautotrophic growth, reaching up to μ=1.1/day. On glycerol, growth rates reached as high as μ=1.52/day. Lipid productivity increased up to 370% on glycerol and 180% on xylose for the strain LB1H10, showing the suitability of this strain for further development for biofuels production through mixotrophic cultivation.

1. Introduction

After the industrial revolution, the development of the new social system required a mobile, cheap and easy to use energy source. Petroleum was the most obvious choice, fueling the exponential growth of the world population from one billion in the eighteen hundreds to more than seven billion people in 2014. However, the versatility of crude oil has proved to be a double-edged sword, creating a society dependent upon oil as the main source of energy and the most important feedstock for the chemical industry. Today, the full consequences of an abrupt interruption in crude oil supplies are unimaginable, but the era of cheap and abundant oil is already gone. The ever growing demand, with ramped-up prices over the last few decades, and the perspective of a decline in oil production rates (peak oil) in the near future, indicate that we are reaching a critical point (Nashawi et al. 2010). Moreover, the direct and indirect toll on the environment and health systems is beginning to change the cost-benefit calculus of using this black gold. The transportation sector alone consumes more than 70% of the crude oil produced, making the development of biofuels an essential element in any strategy to decrease fossil fuel dependency (Abdelaziz et al. 2013).

Deriving biofuels from microalgae is an interesting proposition since they can be grown using sustainable cultivation systems, which do not require arable land and therefore don't displace food crops. In general, microalgae have faster growth rates and higher lipid yields than traditional oil crops, producing drop-in fuels which do not require modification of the present storage and distribution systems, and little to no modification of current internal combustion engines. Many algal species produce considerable amounts of triacylglycerol (TAG), easily converted into biodiesel, as an energy reserve (Cerón-García et al. 2013). However, like more traditional agriculture, microalgal productivity is of course limited by photosynthetic efficiency, a fact very relevant to geographical locations with a low annual solar irradiation.

Many algal species are capable of assimilating different carbon sources while harvesting light energy (Ukeles & Rose 1976). Mixotrophic cultivation could represent an important strategy for microalgal production in many situations, including locations in high latitudes. However, the addition of organic carbon to the culture medium could significantly

impact the operational cost, constituting between 35% and 80% of the medium price depending on the choice of carbon source (Cheng et al. 2009; X. Li et al. 2007). In addition, supplementation with organic fixed carbon can have a negative impact on the sustainability footprint if its production competes with that of food crops. Thus, carbon sources to be used in algal medium should preferably be industrial wastes. Among the possible options, the most obvious one is probably glycerol, an important biofuel waste since it represents about 10% of the products of TAG transesterification. With the vast amount of biodiesel presently made from oil crops, more than a billion kilograms of crude glycerol are produced annually, with a corresponding drastic decline in its value. The availability of crude (or technical) glycerol as a cheap carbon source has led to many studies evaluating the use of crude glycerol as feedstock for the biological production of valuable chemicals as dihydroxyacetone, citric acid, vancomicin, cephalosporine and others (Dobson et al. 2012; Liu et al. 2013; Morgunov et al. 2013; Shin et al. 2011; Zeng et al. 2013). However, if these conversion processes are successful, the value of their products, produced at enormous levels, would also fall precipitously. The only product for which there is a nearly insatiable market is some type of fuel. Indeed, the heterotrophic production of ethanol, butanol and hydrogen using fungi or bacteria has also been reported (Dobson et al. 2012; Ghosh et al. 2012; Sabourin-Provost & Hallenbeck 2009). Heterotrophic cultivation of the alga *Chlorella protothecoides* showed equivalent growth on pure or crude glycerol (Y.-H. Chen & Walker 2011). Recently, mixotrophic cultivation of *Chlorella vulgaris* on glycerol and glucose was shown to give higher yields (Kong et al. 2013), while another study with *Chlorella pyrenoidosa* showed a 20 fold increase in lipid productivity under mixotrophic conditions (Rai et al. 2013).

Another abundant waste that is available worldwide comes from the pulp and paper industry. The waste stream from pulp production is rich in xylose, turning it into a putative cheap source of carbon with high chemical energy content (Pérez et al. 2002). One suggested strategy is the chemical transformation of this waste into valuable products such as furfural and carboxylic acids (Xing et al. 2011), or directly into fuels (Xing et al. 2010). However, these processes are highly dependent on the purity of the waste stream and face several challenges before being scaled up. Biological approaches being developed include production of PHB (polyhydroxybutyrate), a feedstock for the production of bioplastics (Garcez Lopes et

al. 2011); and biofuels, such as ethanol, isobutanol and triacylglycerol (TAG) (Brat & Boles 2013; Kurosawa et al. 2013; Q. Li et al. 2008). Nevertheless, all current proposed biological approaches use either prokaryotes or fungi only and until now the utilization of xylose by algae has not been reported.

The use of these types of substrates can therefore not only provide cheap fixed carbon for potentially augmenting algal growth and lipid production, but can also serve a valuable function in waste treatment. Here, the photoautotrophic, heterotrophic and mixotrophic growth and lipid productivity performance of ten strains indigenous to Québec (Abdelaziz et al. 2014) was assessed using glycerol or xylose as alternative carbon sources.

2. Methods

2.1 Strains and Cultivation

The strains used in this work are part of the collection of the Département de microbiologie, infectiologie et immunology of the Université de Montréal. These are indigenous strains of mainly *Chlorella sp.* isolated in the region of Québec, Canada, and were previously described (Abdelaziz et al. 2014). The medium used for photoautotrophic cultivation was the Bold's Basal Medium (BBM) described by Andersen (Andersen 2005). For the mixotrophic cultivation, BBM was supplemented with 20 mM xylose or glycerol.

The pre-inoculum was grown using photoautotrophic conditions (BBM medium only) in 12-well plates until mid-log phase. The cultures were diluted to an optical density at 630nm (OD_{630}) of 0.1 and used for inoculum (5% v/v). Three biological replicates of each algal strain were grown for 17 days in 12 well microtiter plates containing 3.5mL of culture medium, and illuminated under a light/dark cycle of 12 hours using day-light LED boards at an incident light intensity of 40W/m^2 (approximately 190 μE/m^2/s) Each strain and medium condition was also carried out in continuous darkness (biological triplicates) to provide a comparison under heterotrophic conditions. Growth was measured by reading the optical density at 630nm using a microtiter plate reader (Biotek EL800). Growth rates were calculated according to equation 1 (eq.1) using the optical density data (OD_{630}) between days 1 and 4 of cultivation.

Eq.1 $\mu\ d^{-1} = (\ln OD_{630}^{f} - \ln OD_{630}^{i}) / t^{f} - t^{i}$

Eq.1. Growth rate formula used, where $\mu\ d^{-1}$ = growth rate per day; $\ln OD_{630}^{f}$ = final optical density; $\ln OD_{630}^{i}$ = initial optical density; t^{f} = final time in days, t^{i} = initial time in days

2.2 Spectrophotometric Determination of Nitrate, Glycerol and Xylose

The amounts of residual nitrate, glycerol or xylose were assessed in analytical triplicates. Established colorimetric methods were adapted for use in 96 well plate format and were performed for each of the biological replicates. The results are shown as the mean of the nine values obtained for each strain. At the end of the growth period the samples were centrifuged at 2000g for 8 minutes and the supernatant used for analysis. The classic colorimetric assay for quantification of reducing sugars using 3,5-dinitrosalicylic acid was adapted for measurement of xylose (Miller 1959). 90µL of DNS solution (10g/L dinitrosalicylic acid; 10g/L sodium hydroxide; 0.5g/L sodium sulphite) was mixed with 90µL of the sample or standard and incubated for 15 minutes in a water bath at 90°C; immediately cooled down in an ice bath, and then 30µL of 40% potassium sodium tartrate solution was added to stop the reaction. The optical density at 630nm (Biotek EL800 microtiter plate reader) was compared to a standard curve obtained under the same conditions.

Glycerol was quantified using the colorimetric method described by Bondioli and Bella (Bondioli & Bella 2005), a two steps process with the periodate oxidation of glycerol followed by the formation of formaldehyde through Hantzsch's reaction. Here, 100µL of samples or standards were placed in a 96 well plate, mixed with 60µL of the sodium periodate solution followed by 60µL of acetylacetone solution, mixed and incubated in a water bath at 70°C for one minute, cooled down immediately in a water bath to room temperature and the optical density at 530nm was then read in a Biotek EL800 microplate reader. Both these solutions were prepared daily as previously described (Bondioli & Bella 2005).

The residual nitrate in the culture broth after 17 days of cultivation was detected using the method described by Bartzatt et. al (Bartzatt & Donigan 2004). Here, 20µL of sample was placed in a 96 well plate and mixed with 90µL of diphenylamine solution (3.34g of

diphenylamine in 14.4M H_2SO_4) and 85µL of pure H_2SO_4 was then added. The microplates were shaken for 10 minutes and the read (OD_{630}) using the Biotek EL800 microplate reader .

2.3 Assessment of Bacterial Contamination

Since the strains used were not necessarily axenic, it was important to determine the degree. At the end of the cultivation period, an aliquot of each culture was diluted to reach a concentration of 500 +/- algal cells per mL and 100µL was plated on LB agar. Colonies were counted after 48h incubation at 37˚C. An estimate of the potential contribution of bacterial biomass to the optical density readings was made using a culture of *Escherichia coli* DH5α together with plating and optical density readings as noted above. This analysis shows that on the average, bacterial contamination probably contributed no more that 2.2% of the final biomass, and in the worst case no more than 14%.

2.4 Lipid Quantification

The algal lipid content was quantified using Nile Red fluorescence measurements. Nile Red is considered an efficient dye for algal neutral lipids (Bertozzini et al. 2011; W. Chen et al. 2009; Elsey et al. 2007; Huang et al. 2009; Kou et al. 2013; Lee 1998). In this work we adapted the method previously described by (Abdelaziz et al. 2014). After 17 days of incubation, 50µL of the algal culture sample was stained with Nile Red at 0.5µg/mL final concentration, using dimethylsulfoxide (DMSO) at 25% as carrier. The assay was brought to a final volume of 200µL and was incubated for 15 minutes with agitation using a microtiter plate shaker (DSG Titertek Flow Laboratories, Meckenheim, Germany). The fluorescence was then read in a Synergy NEO HTS Microplate Reader with excitation set to 520nm and emission captured at 570nm. The fluorescence output was then compared to a standard curve made using extra virgin olive oil. The values are indicated as the average of three analytical readings of each biological replicate.

For the lipid profile analysis, triacylglycerols (TAGs) were extracted and transesterified in a single step, following methods already described (Cao et al. 2013). Approximately 33 mg of dried biomass was placed in 2mL screw cap microcentrifuge tube, 500 µL of a methanol solution acidified with 10% sulphuric acid were added, it was heated to 90°C for 90 minutes (mixed in a vortex mixer every ten minutes) and allowed to cool to room temperature. Then, 1mL of n-Hexane was added and vortexed for 1 minute. Cell debris was pelleted by centrifugation and the supernatant saved in a glass test tube. The hexane step was then repeated 4 more times, adding the supernatant to the same glass tube, which was then heated to 90°C for 20 minutes, allowed to cool down to room temperature and the proper phase was collected and stored at -20°C. The lipid profile was analyzed in an Agilent 7890A gas chromatograph (GC) equipped with the column Omegawax 250. An internal control (50 µL of C19:0) was added to each 250 µL of fatty acid methyl ester (FAME).

2.5 Statistical Analysis

A parametric, paired, two-tailed t-test was performed using Prism 6.0D software (GraphPad) to determine if differences in biomass productivity or growth curves were influenced by exposition to light.

3. Results and Discussion

Ten microalgal strains indigenous to Québec were examined for biomass and lipid productivity under different growth modes: photoautotrophic, mixotrophic (light), and heterotrophic (dark) using CO_2 and/or glycerol or xylose. Glycerol and xylose in particular were considered since a large quantity of "waste" glycerol is currently available as a side-product of biodiesel manufacture (1 kg glycerol per ten kg of biodiesel produced), and xylose is abundant in hemicellulose waste-streams of the pulp and paper industry. The strains were tentatively identified as *Chlorella sp.* based on morphological characteristics as determined by light microscopy.

3.1 Maximal Growth Rates Under the Different Conditions

One important aspect is the influence of glycerol and xylose on growth rates and yields under the different conditions. These were calculated by choosing a period of time that would include the active growth period of all the strains under the different kinds of treatments, yet being restricted to the growth phase as much as possible. It is important to note that, while this method provides an important tool for comparing the performance of these strains under different conditions, it is also likely to underestimate the performance of some strains. Thus, the reported growth rates are conservative.

The different strains were quite varied in their responses. The variation in patterns of growth can be seen in Figure 1 for selected strains. However a number of generalizations can be made (Table 1). First, it is notable that all the strains examined were capable of some degree of heterotrophic (in the dark) growth on the two different organic carbon substrates. As far as we are aware, this is the first report of the utilization of xylose by microalgae. The majority of the strains (7/10) showed an enhancement in growth rate, up to 2.8-fold (PCH44), whereas three were relatively unaffected by its presence. In contrast, in all but two strains, LB1H10 and PCH44, the presence of xylose decreased the growth rate in the light. In the dark, not surprisingly, the controls (CO_2 only) did not show any growth (not shown). However, half the strains (LB1H09, LB1H10, MA2H01, PCH03, and PCH44) had a growth rate that was higher, up to 2.6-fold (PCH44) than when incubated under photoautotrophic conditions. The others showed a decreased growth rate under these conditions, with PCH90 having only 40% of its photoautotrophic growth rate. In stark contrast to glycerol, xylose in general decreased the growth rate when added to cultures incubated in the light. The only exceptions were LB1H10 and PCH44 whose growth rate was moderately stimulated in the presence of xylose. This trend was also seen in the cultures containing xylose and incubated in the dark, where the majority (7/10) were also inhibited by xylose, when compared to their growth rate under photoautotrophic conditions. Averaging the results of all the strains together gives a growth rate under photoautotrophic conditions of 0.98 μ/d, less than with

glycerol in the light, 1.52 μ/d, or in the dark, 1.42 μ/d. Average growth on xylose was not as good, 1.096 μ/d (light) and 0.881 μ/d (dark).

3.2 The Influence of Glycerol on Final Biomass and Lipid Yields

As discussed above, all the strains showed the capacity for heterotrophic growth (in the dark) using glycerol as sole carbon source and the cultures seemed to be capable of metabolizing this compound in the light as under both conditions as assays showed that the glycerol in the medium had been completely consumed by the end of the cultivation period. As well, there was no detectable nitrate left in any of the cultures at this point in cultivation. In general, as was noted above for the growth rates, growth with glycerol in the light gave higher biomass yields than growth in the dark (Figure 2). However, strikingly, final biomass yields in the light were, with only one exception (LB1H10), lower in the presence of glycerol than in its absence in spite of what were generally higher maximal growth rates. An examination of the growth curves (some examples are shown in Figure 1) shows that this is the case because the two different types of culture show different growth patterns. Cultures with glycerol added show little to no lag phase and quickly reach maximal growth rates as opposed to photoautotrophic cultures which have an appreciable lag phase, reach a lower maximal growth rate, but are able to maintain this over a longer period of time, thus reaching a higher final cell density. Eight of the ten strains had higher final biomass production when grown with glycerol in the light cycle compared to the dark, with the other two (LB1H09 and PCH44) showing no significant difference between the two conditions (Figure 2).

While the addition of glycerol did not lead to an increase in the production of biomass above that found under photoautotrophic conditions, with the exception of strain LB1H10, lipid production was higher for eight of the ten strains when cultivated under these conditions (Figure 3). Once again, the highest increase was for strain LB1H10, which showed a 370% increase in lipids compared to photoautotrophic conditions. The increase in lipids represented only 10% of the total extra produced biomass, what can probably be further optimized. Strain LB1H12 tripled its lipid productivity, and three other strains doubled it (Figure 3, Table S1). That lipid productivity increased in spite of the overall decreased biomass yields was a

reflection of the very large increase in some cases in the cellular lipid content (Figure 4). The lipid content of most cultures increased more than two-fold, with two, LB1H09 and PCH03 showing a nearly five-fold increase and LB1H12 giving a remarkable more than seven-fold increase in lipid content. In spite of the large increase in the productivity of most strains, the best natural lipid producer under photoautotrophic conditions, PCH90, in fact suffered a slight decrease in productivity in medium with glycerol. This strain showed a similar pattern of growth under photoautotrophic and mixotrophic conditions, and further optimization would be necessary to increase yields with added organic carbon. Nevertheless, lipid productivities shown here during mixotrophic cultivation with glycerol are several fold higher than previously reported (Y.-H. Chen and Walker, 2011; Kong et al., 2013; Rai et al., 2013).

In contrast to the augmentation in lipid production in the light when glycerol is added to the medium, even though the strains can all grow in the dark at the expense of glycerol, lipid production was low (Figure 3), a reflection of both the lower biomass yields under these conditions and the relatively low cellular lipid content (Figure 4). The remarkable increase in lipid content with some strains in the presence of glycerol in the light suggests that further research, in particular to increase biomass yields under these conditions, could lead to very significant increases in overall lipid productivity.

3.3 The Influence of Xylose on Final Biomass and Lipid Yields

Xylose is a major carbon source found in wastewater discharged by the paper/pulp industry. Little work has previously been done on the utilization of xylose by microalgae, and if strains capable of utilizing xylose were found and suitable bioprocess strategies developed, it might be possible to simultaneously treat these wastes and produce fuel or fuel precursors. Therefore, the performance of the same isolates when grown in the presence of xylose was assessed. Only one strain, LB1H10, showed a significant enhancement in growth rate when incubated in the light in the presence of xylose (Table 1). Strain PCH44 grew only slightly faster in with xylose, and the growth of all the other strains was significantly decreased by the presence of this substrate. Interestingly, all the strains were capable of some heterotrophic

growth on xylose, and growth rates under these conditions were similar to those observed under photoautotrophic conditions.

In terms of growth yields, xylose appears to be a poor candidate for alternative carbon source. The biomass yield was low, and three out of ten strains (LB1H09; PCH03; PCH44) had negligible growth under either condition: light or dark (Figure 2, Table S1). All strains showed a lower biomass production on xylose than in the other conditions examined; photoautotrophic, and cultures with added glycerol, light and dark. In fact, growth is so drastically reduced that it suggests a direct or indirect growth inhibition by this substrate. Interestingly, the biomass productivity was consistently higher in dark than in the light ($P=0.018$), with the sole exception of LB1H10 (Figure 2, Table S1), implying that light has a negative influence when xylose is present.

With the exception of LB1H10, lipid productivity in the presence of xylose was poor, in general much less than that seen under photoautotrophic conditions (Figure 3). Although the cellular lipid content in some strains significantly increased when incubated with xylose in the light (strains PCH05, PCH06, PCH44, and PCH90) (Figure 4), this was more than offset by the drastic decrease in biomass under these conditions (Figure 2). As with glycerol, little or no enhancement in lipid content was seen when the cultures were incubated with xylose in the dark, with the sole exception of LB1H10 (discussed in detail below).

3.4 Unique Characteristics of LB1H10 as a Promising Biofuels Producer

Thus, with both carbon sources, the performance of strain LB1H10 stands apart from the other strains tested. This strain appears to possess the ability of actively assimilate glycerol in the light, producing both higher levels of biomass and increasing its lipid productivity. Moreover, under mixotrophic conditions, this strain produces nearly four-fold higher amounts of lipid (Figure 3). In addition, LB1H10 shows significant in terms of the conversion of xylose to biomass and lipid. This strain accumulated significant amounts of biomass from xylose both in the light (76% of photoautotrophic) and in the dark (54% of photoautotrophic). Since lipid content is augmented with xylose both in the light and the dark

(Figure 4), this means that this culture shows important increases in lipid productivity with xylose, from more than three-fold in the light to almost two-fold in the dark. The neutral lipid profile was examined after conversion to FAMES (fatty acid methyl esters) (Figure 5). This analysis showed that the molecules of interest for biodiesel production C14, C16, C18 saturated or mono-saturated FAs, account for at least 58% of the total lipids under photoautotrophic conditions with even higher percentages when grown in the presence of fixed carbon; 67% with xylose and 63% with glycerol (Figure 5A). There was little variation of the FAME profile with growth conditions. Interestingly, the quantites of PUFAs (poly-unsaturated fatty acids) were also relativel high: 5% photoautotrophic, 4.2% with xylose, and 7.3 % with glycerol (Figure 5B). Thus, development of a bioprocess involving this strain might hold promise for dual pulp and paper mill wastewater treatment and biofuel production.

4. Conclusions

In the initial screening of ten strains reported here, a number were shown to possess interesting characteristics in terms of fixed carbon utilization. Yields of lipids and biomass are likely to be improved in future optimization studies. Nevertheless some of the strains showed lipid productivity during mixotrophic cultivation with glycerol that was several fold higher than previously reported. One strain showed efficient use of both carbon sources in the light, gaining increased biomass: 39% more with xylose and 96% more with glycerol. To our knowledge, this is the first report of xylose utilization by a microalga.

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Tables

Table 1: Growth Rates

Photoautotrophic growth (Ph.Aut) and mixotrophic growth on glycerol (Gly/L) or xylose (Xyl/L) or heterotrophic growth (Gly/D or Xyl/D). Refer to Material and Methods (section 2.1) for more details.

	Growth rate per day (μ/day)				
Strain	Ph.Aut	Gly / L	Gly / D	Xyl / L	Xyl / D
LB1H09	0.83	1.32	1.24	0.57	0.79
LB1H10	0.65	0.95	1.11	1.10	0.85
LB1H12	0.88	0.77	0.52	0.45	0.51
MA2H01	0.94	1.28	1.25	0.74	0.65
PCH03	0.66	1.52	1.42	0.56	0.88
PCH05	0.90	0.90	0.33	0.68	0.65
PCH06	0.98	1.29	0.59	0.73	0.64
PCH36	0.84	1.16	0.42	0.60	0.79
PCH44	0.44	1.22	1.15	0.52	0.48
PCH90	0.95	0.81	0.38	0.74	0.63

Table S1: Biomass and Lipid Production

Photoautotrophic growth (Ph.Aut) and mixotrophic growth on glycerol (Gly/L) or xylose (Xyl/L) or heterotrophic growth (Gly/D or Xyl/D)

	Biomass Production mg/L					Lipid production mg/L				
Strain	Ph.Aut	Gly / L	Gly / D	Xyl / L	Xyl / D	Ph.Aut	Gly / L	Gly / D	Xyl / L	Xyl / D
LB1H09	1170	390	430	38	110	42	64	8	4	6
LB1H10	580	900	462	438	314	8	40	12	24	36
LB1H12	11400	570	456	298	418	14	46	8	22	20
MA2H01	950	450	334	214	422	62	52	10	10	8
PCH03	1020	436	348	44	136	20	40	8	2	4
PCH05	1100	732	240	232	320	26	54	8	16	10
PCH06	1200	874	344	212	404	30	38	8	32	24
PCH36	1100	596	256	212	456	44	46	12	14	10
PCH44	500	426	432	50	54	18	40	14	10	4
PCH90	960	812	612	340	472	130	108	8	70	22

Figures

Figure 1: Biomass Accumulation

Photoautotrophic growth (Ph.Aut) and mixotrophic growth on glycerol (Gly/L) or xylose (Xyl/L) or heterotrophic growth (Gly/D or Xyl/D).

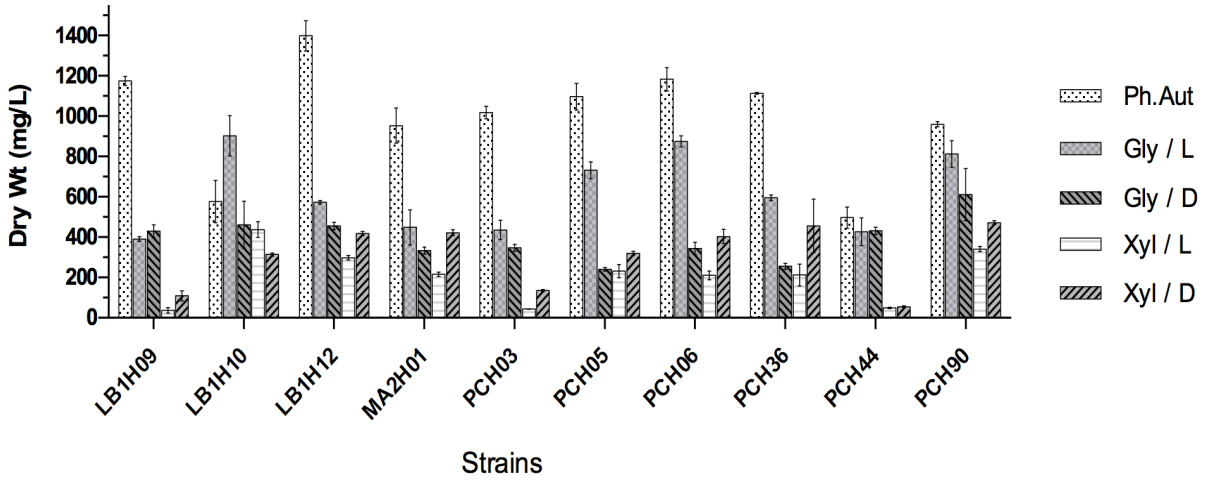


Figure 2: Growth Curves Patterns

Photoautotrophic growth (Photo) and mixotrophic growth on glycerol (Gly/L) or xylose (Xyl/L) or heterotrophic growth (Gly/D or Xyl/D). These are representative cases of the patterns found through out this work. Standard deviation (in average 9%) was omitted to improve clarity of the figure.

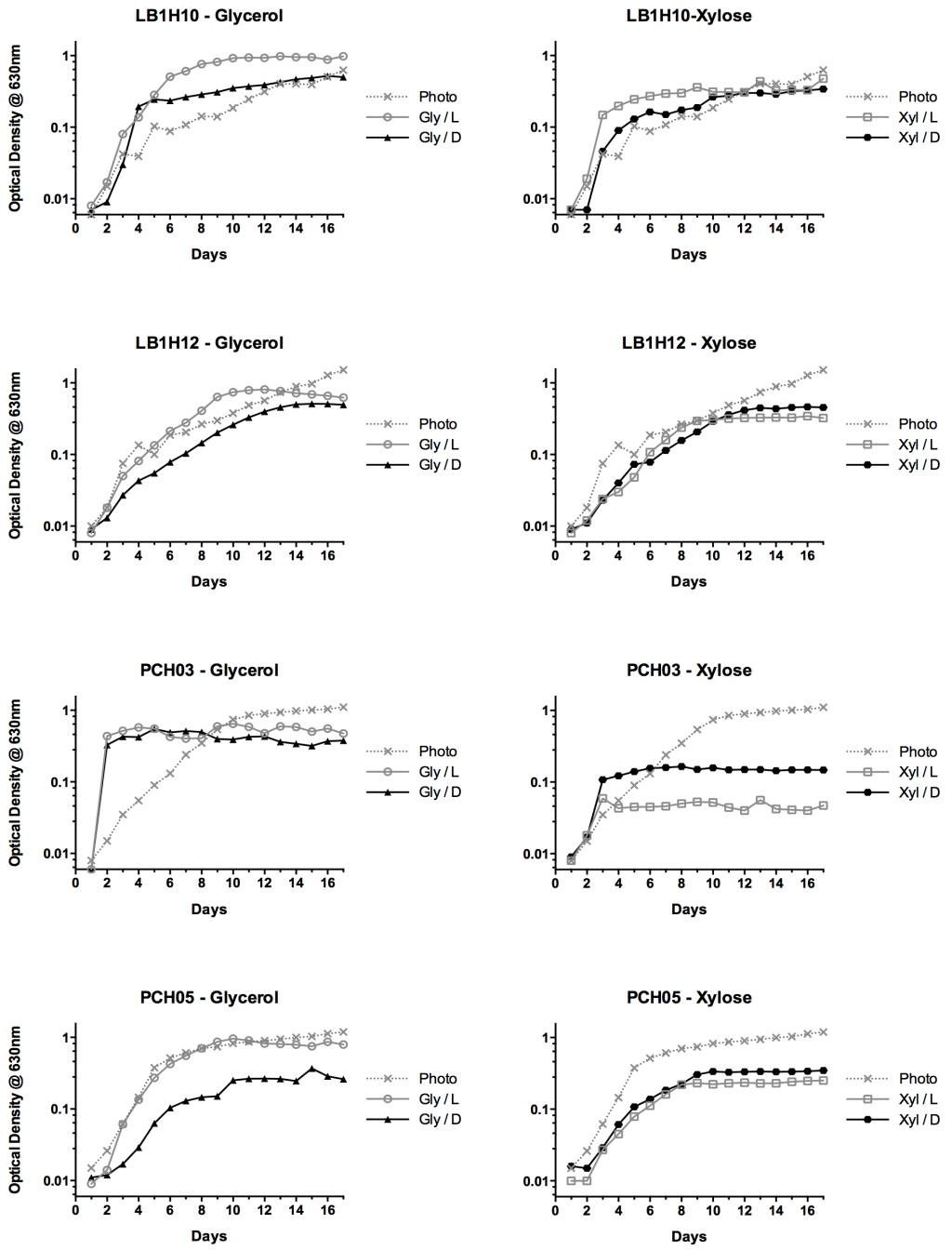


Figure 3: Comparison of the Lipid Production Performances.

Values are represented as percentage of gain or loss of the mixotrophic (Light) or heterotrophic (Dark) cultivation against the photoautotrophic results.

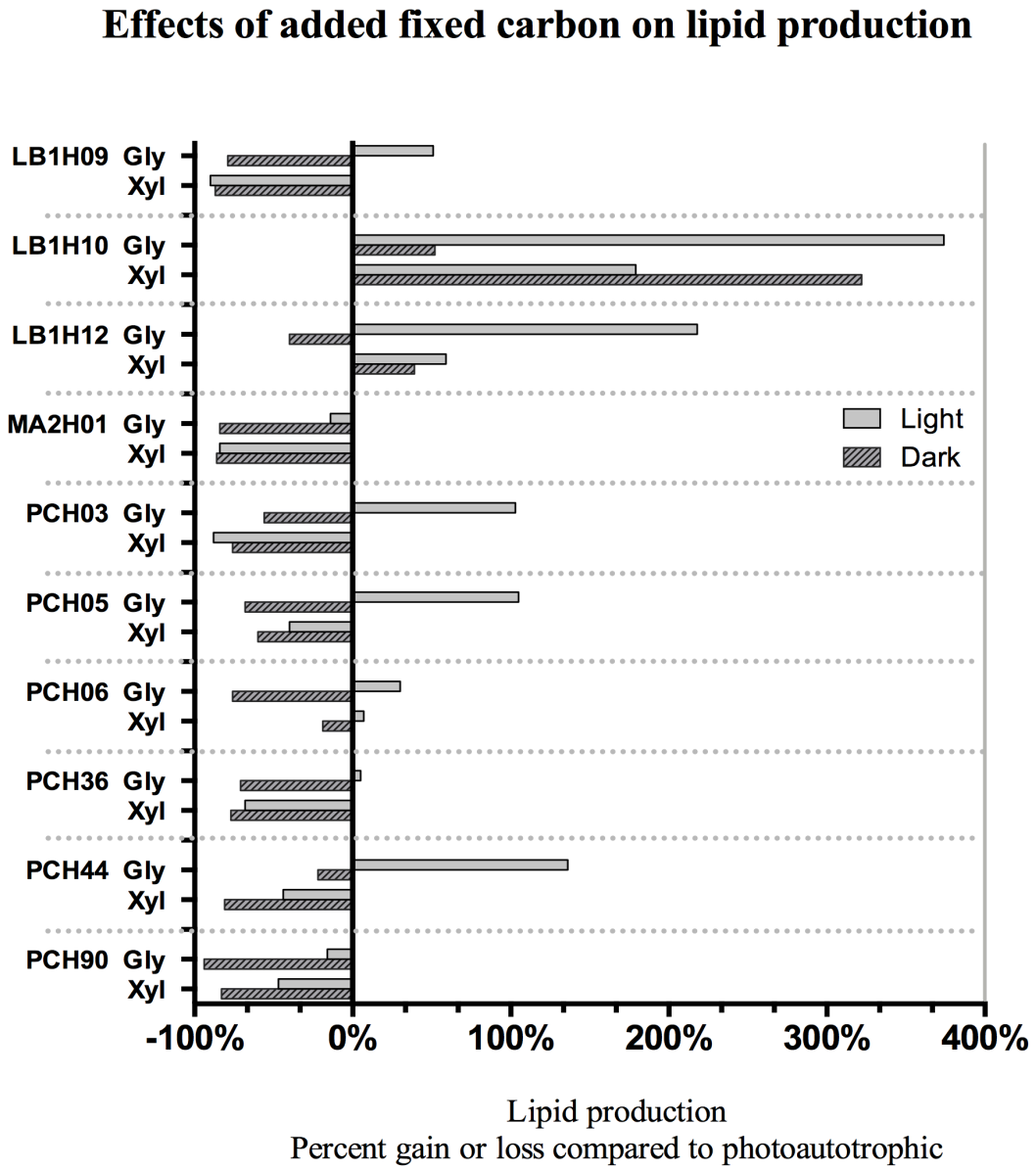


Figure 4: Lipid Content

Photoautotrophic growth (Ph.Aut) and mixotrophic growth on glycerol (Gly/L) or xylose (Xyl/L) or heterotrophic growth (Gly/D or Xyl/D).

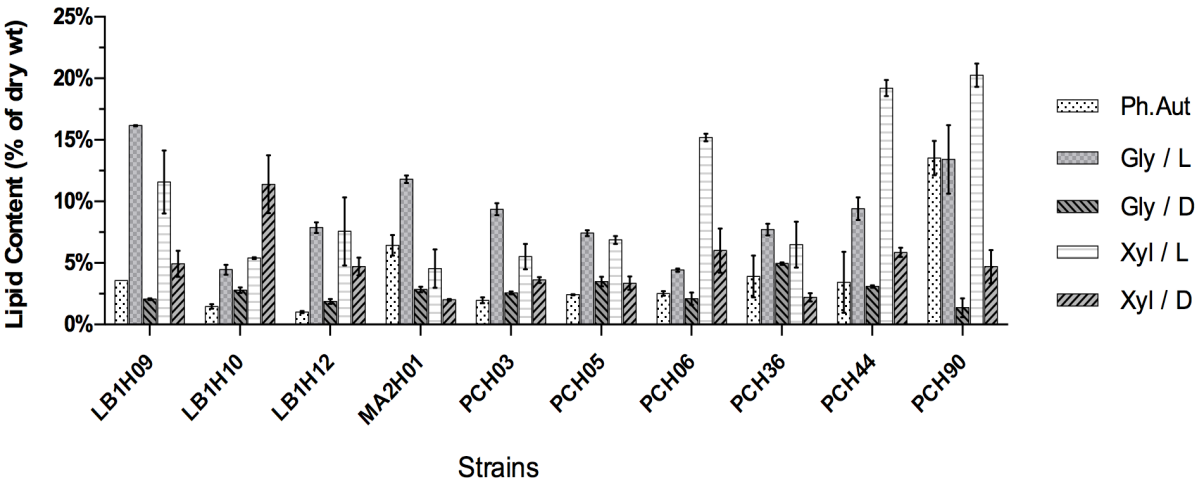
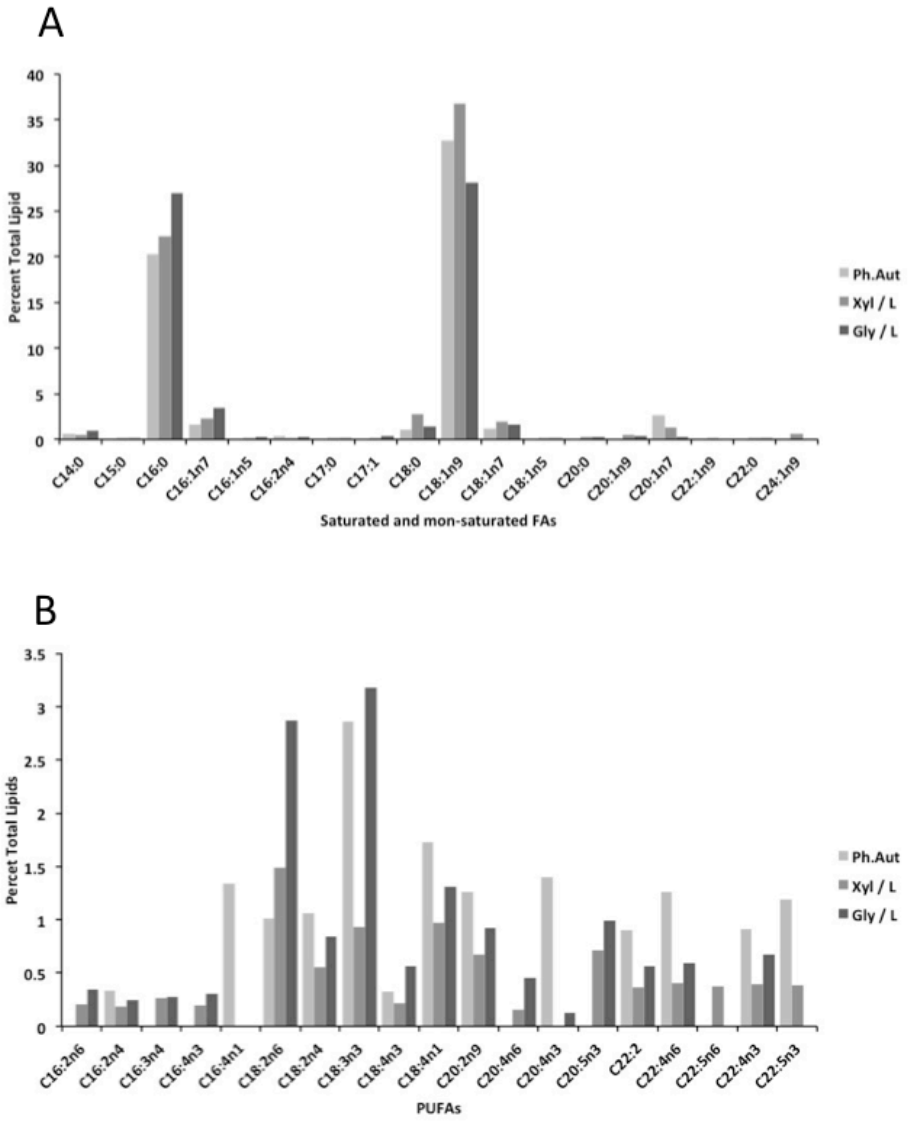


Figure 5: FAME Profile of LB1H10 Under the Different Growth Conditions.

(A) Saturated and mono-saturated FAMES are shown as a percentage of total material detected. (B) PUFAs (poly-unsaturated fatty acids) are shown as a percentage of total material detected.



Chapter 4: The Short-Term Lipid Booster Effect of Xylose on Cultures of *Chlorellaceae* and *Scenedesmaceae* Wild Strains

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Author contributions: The author was responsible for the project concept design; experiments and analysis, always under the supervision and guidance of Dr. Patrick C. Hallenbeck. K.P. performed the PCRs and designed the phylogenetic tree under the supervision of the author. The manuscript was written by the author and revised by Dr. Patrick C. Hallenbeck

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The Breakfast of the Champions: The Short-Term Lipid Booster Effect of Xylose on Cultures of *Chlorellaceae* and *Scenedesmaceae* Wild Strains

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Abstract

To understand the effects that the addition of xylose has on microalgal growth and lipid production, four wild-type indigenous strains were monitored daily, before and after xylose addition, using flow-cytometry. With some *Chlorella* strains xylose addition induced a rapid increase in lipid accumulation (up to 3.3 fold) during the first six to twelve hours. At later times cells showed a decrease in chlorophyll content, cell size and cell counts. On the other hand, the one member of the *Scenedesmaceae* family was able to gain from the presence of this carbon source during mixotrophic or heterotrophic cultivation without apparent negative effects. These results suggest that xylose could be used prior to harvesting to boost the lipid content of algal cultures in either continuous or two-stage systems growing and carrying out bio-remediation of wastewater from the pulp and paper industry.

1. Introduction

Petroleum represents 36% of all primary energy sources. The transportation sector alone consumes 71% of this resource, which on the other hand, relies on crude oil for 92% of its energy demand in the U.S.. The two main forces driving biofuel development are energy security and sustainability. Crude oil is a versatile source of energy and chemical feedstock. However, despite new technologies for extraction (e.g. fracking, tar sands and deep sea exploration), it is by definition a limited resource, and the production peak is expected to be achieved within the next few decades (Malik et al., 2014; Nashawi et al., 2010). Different strategies are currently being investigated to substitute, or at least decrease the crude oil dependency, and it is becoming apparent that no technology alone will be able to displace fossil fuels. Alternatively, the future of transportation will probably hold vehicles using different energy carriers (e.g. butanol, methane, hydrogen, ethanol, biodiesel and electric batteries). These would be chosen accordingly to the particular duty, local resource availability, sustainability or customer preferences. For example, the green concept of electric vehicles is increasing in popularity as a sustainable city car. Controversially, quite often these cars are recharged through an electrical grid fed by a coal-powered power plant.

A different alternative is to replace gasoline and diesel fuel by another liquid fuel that can be stored, distributed and used with no or little modification of the actual system. These so-called drop-in fuels come with the promises of small investments in the current infrastructure and a sustainable production (Leite and Hallenbeck, 2011). The first generation biofuels produced from sugarcane/corn (ethanol) or oilseeds (biodiesel) requires arable land for cultivation, limiting the production capacity due to a possible competition with food crops (G. Chen et al., 2015; Zhang et al., 2014). To avoid this problem, a second generation of biofuels proposed to use agricultural waste (lignocellulose) as feedstock, but this technology still have to overcome significant challenges. The third generation is the algal biofuels, claiming to be a sustainable production strategy for drop-in fuels. Microalgae is an artificial group of microorganisms from different kingdoms containing a plastid (functional or not) and chlorophyll a (Andersen, 2005; Leite and Hallenbeck, 2011). These are versatile organisms, colonizing almost any niche containing some humidity, from fresh to salt water or even relatively dry environments (Graham et al., 2009). They were the original feedstock used by

nature to form petroleum and the source of essential fatty acids as the omega 3 in the fish oil (Wackernagel et al., 1993). As any aquaculture, it does not require arable land. It can be cultivated in closed systems or open ponds, on marginal land or even urban areas (Leite and Hallenbeck, 2011; Lim et al., 2013); using fresh, seawater or wastewater (Cabanelas et al., 2013; McGinn et al., 2011; Park et al., 2011). Many strains accumulate a large amount of lipids in the form of triacylglycerol (TAG) as reserve material. This molecule is the same feedstock extracted from oil crops to be further converted into fatty-acid-methyl-ester (FAME) through transesterification and used as biodiesel. The promising characteristics of this technology led to intense investigation on each step of the production chain, to improve yields and lower the production costs. Among the approaches to increase lipid productivity are: the optimization of open or closed cultivation systems and the exploitation of physiological conditions that leads to an increase of cell lipid content (e.g. nitrogen starvation).

However, if algal cultivation does not compete with food crops for land, a production at the scale required for the needs of the transportation sector would impact the fertilizers availability and prices. Therefore, it is important to recycle the nutrients within the algal culture and find other sources of nutrients. A possible solution being currently investigated is the use of domestic or industrial wastewater. In wastewater treatment plants, microalgae are commonly employed in the secondary treatment to remove nutrients and avoid eutrophication of lakes and local hydrographic basin (Pittman et al., 2011). The bioremediation of wastewater coupled with algal biomass production is an elegant solution: combining treatment of the large volume of water and nutrients available in wastewater, the decentralization of the fuel production, and generating one of the few side products that can lower the cultivation costs and will not flood the market: clean water. Different set-ups have been tried and biomass yields range from 20 to 350 mg/L/day depending on wastewater nature, strains, temperature, etc. (Abdelaziz et al., 2013; Gentili, 2014). Considering the microalgae potential to grow mixotrophically, the organic carbon present in some industrial wastewater could boost lipid biomass/production. Glycerol is an important waste product from the transesterification of triacylglycerol (TAG) into biodiesel, and mixotrophic exploitation of this chemical in algal cultivation has been reported, (Kong et al., 2013; Leite et al., 2014; Rai et al., 2013). Xylose is a waste product released by pulp and paper industry, and its feasibility for algal biomass

production was just recently reported (Leite et al., 2014; Yang et al., 2014). However, these studies showed a putative negative effect that the xylose could cause on growth, drastically increasing the lag phase, decreasing the biomass produced and for some strains, or hindering heterotrophic growth. This work analyzed the short-term effects of the addition of xylose into a photoautotrophic culture of microalgae, in terms of: biomass and lipid production; lipid and chlorophyll content; and cell size.

2. Methods

2.1 Strains and Cultivation

Four strains native to Quebec and maintained at the Université de Montréal Algal Culture Collection were used (Hallenbeck et al., 2014). A preliminary identification was previously made using optical microscopy (Leite et al, 2014), and this was confirmed by DNA sequence analysis (see section 2.5). Experiments were performed using Bold's Basal Medium (BBM), (Andersen, 2005), modified by the addition of 50 mM MES pH 7.4. The pre-inoculum and experimental cultures were grown under the same conditions: 125 mL Erlenmeyer flasks containing 50 mL of medium; with agitation (100 rpm) and a light intensity of 35 W/m^2 (approximately 166$\mu E/m^2/s$); with a light-dark cycle of 12 hours unless otherwise noted. Pre-inocula were grown until mid-log phase, diluted with medium to an optical density of 0.5 at 630nm (OD_{630}) and used as inoculum (10% (v/v)). The time points in this study make reference to the day when xylose was added into the cultures. Cells were initially allowed to grow for three days before samples were collected for data acquisition. Cultures were then followed for two days (data points: t=-48h and t=-24h), after which xylose was added (t=0h) to a final concentration of 30mM. At this stage, one set of biological triplicates was exposed to the light regime described above, and another set of triplicates was placed in the dark. Two other sets of triplicates were kept under photoautotrophic conditions: one was exposed to a 12h light dark cycle as above, and the other was kept in the dark.

2.2 Flow Cytometry Analysis

The natural chlorophyll fluorescence and cellular lipid content were analyzed using a BD FACSort Flow Cytometer. For lipid content analysis, the samples were stained with BODIPY® 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4adiaza-s-indacene) to a final concentration of 0.5µM and 5% DMSO; as described elsewhere (Brennan et al., 2012; Govender et al., 2012). The flow cytometer used in this study uses a laser of 488nm (excitation); three emission detectors: FL1 (515-545nm); FL2 (562-607nm) and FL3 (>630nm); a forward scatter detector (FSC); and a side scatter detector (SSC). The mean fluorescence of the FL1 and FL3 channels, representing the fluorescence output of BODIPY 505/515® (neutral lipid dye, see section 2.3 for details) and the natural chlorophyll fluorescence respectively, were recorded at different time points over a 6 day period.

2.3 Lipid Quantification

The flow cytometry analysis gives, by definition, the mean lipid content per cell in arbitrary fluorescence units (A.U.). Thus, it is necessary to make a correlation between these values and an independent measurement in order to make the FL1 results quantitative. The Nile Red technique was used to standardize the FL1 output in productivity (per volume) and content (per dry weight). Nile red is an efficient dye for quantification of neutral lipids (Bertozzini et al., 2011; W. Chen et al., 2009; Elsey et al., 2007), and its property of changing the fluorescence emission according to the solvent is a useful property for lipid quantification in multi-well plates. Here, the procedures used elsewhere (Leite et al., 2014) were used with slight modifications. At the different time points, 50µL of the algal culture were incubated with Nile Red (final concentration 0.5µg/mL) and dimethylsulfoxide (DMSO) (25%) for fifteen minutes under agitation using a microtiter plate shaker (DSG Titertek Flow Laboratories, Meckenheim, Germany). The plates were read using a Synergy NEO HTS Microplate Reader set for excitation at 520nm and emission detection at 570nm. A unique standard curve was made for each each 96 wells plate using extra-virgin olive oil. All standard points and sample readings were made in analytical triplicates. The fluorescence output (FL1)

determined by flow cytometry was then compared to the standard for the determination of lipid concentration for each sample. The indicated values are the average of the analytical triplicate readings of each biological replicate.

2.4 Spectrophotometric Determination of Xylose

A method previously described for xylose quantification in small volume samples (Leite et al., 2014) was used with slight modifications. This method is a scaled down version of a classical assay described by Miller (Miller, 1959). Here, 90µL of the culture supernatant is mixed and incubated for 15 minutes in a water bath at 90˚C with 90µL of a DNS solution (10g/L 3,5-dinitrosalicylic acid; 10g/L sodium hydroxide; 0.5g/L sodium sulphate). After the incubation, the 96 wells plates were cooled down immediately using an ice bath and the color development stabilized by adding 30µL of 40% potassium sodium tartrate solution. The optical density at 630nm was recorded using a Biotek EL800 microtiter plate reader and compared to a standard curve made up in the same multi-well plate.

2.5 Molecular Identification

To confirm the previous morphological identification (Hallenbeck et al., 2014), different analyses were performed using DNA sequences of the internal region of the 18S ribosomal DNA: (i) samples were compared to the NCBI database for simple alignment comparison; (ii) the DNA sequences were aligned with known strains from established culture collections, where similarities and divergences are expressed in the form of a tree of life. The targeted DNA was amplified and sequenced using the primers 18S-int-forward 5'-GTGGTAACGGGTGACGG-3' and 18S-int-reverse 5'-GTGCGGCCCAGAACATC-3'. The PCR products were purified and sequenced using the Sanger method at IRIC Genomics Platform (https://genomique.iric.ca). The chromatogram data were analyzed with the software CLC Main Workbench (version 6.8.4), and the phylogenetic tree was made using the MEGA

(version 5.2.2) software, through the neighbour joining method and Jukes-Cantor model at 1000 bootstrap.

2.6 Statistical Analyses

Statistical analyses were made using the Prism 6.0D (GraphPad) software. To determine the statistical significance between the photoautotrophic, mixotrophic and heterotrophic groups, the two-tailed t-test, with the confidence interval of 95% or two-way ANOVA were used according to their intrinsic suitability.

3. Results

In previous reports on mixotrophic and heterotrophic algal cultivation using xylose as alternative carbon source, cultures were exposed to xylose throughout growth (Leite et al., 2014; Yang et al., 2014). These studies reported long lag phases and substantial pigment loss by the algal cells, both suggesting that the cells were under significant physiological stress. Here, the molecular identification and phylogenetic clade of the four most relevant strains previously shown under xylose cultivation is presented (Leite et al., 2014). Moreover, this work explored the capabilities of a flow cytometer to analyze the short-term effects caused by xylose addition on biomass, lipid and chlorophyll content in growing algal cultures, which showed a marked boost in lipid accumulation boost over a very short (12 h) initial period. The use of flow cytometry allows the monitoring of the size, neutral lipids, chlorophyll and accessory pigments content, potentially giving information about stress and different cell adaptations within a short time period. Finally, this report suggests a different mixotrophic cultivation approach, exposing the culture to xylose only after a previous photoautotrophic growth, saving on feedstock costs and increasing productivity.

3.1 Strain Identification

To identify the strains used in this work, the internal part of the 18S rDNA was amplified, sequenced, compaired against the NCBI database, aligned and compared to other known species for a precise placement of their phylogenetic clade. A comparison of the sequences obtained with the ones present in the NCBI database revealed that three of the strains (PCH03, PCH90, LB1H09) were within the *Chlorella* genus with a fourth strain (LB1H10) belonging to the family *Scenedesmaceae*. To place these strains into a taxonomic group requires the construction of a phylogenetic tree based on the alignment of the 18S rDNA sequences of established culture collection strains (figure 1). The tree was rooted only at the phylum level (*Chlorophyta*) revealing the distance between LB1H10 (*Chlorophyceae*) and the other three strains in this study, all of them associated with the class *Trebuxiophyceae*. Four different species of the genus *Chara* were used to root the four strains in this study under the kingdom Plantae. The *Chara* genus is organized under the phylum *Charophyta*, one clade above the divergence of LB1H10 and the other strains. The *Chlamydomonadales* order served as an outgroup for LB1H10, grouping it inside the *Scenedesmaceae* family, probably within the genus *Scenedesmus* or *Acutodesmus* (figure 1). The three other strains: LB1H09, PCH03, PCH90 were classified as members of the genus *Chlorella*. The genus *Prasiola* and *Lobosphaera* differs from *Chlorella* at the order level (orders *Prasiolales* and *Trebuxiales* respectively) . The genus *Micractinum* is related to *Chlorella vulgaris* at the family level and was used as to root the genus, and *Chlorella sorokiniana* confirmed LB1H09, PCH03 and PCH90 as different strains of *Chlorella vulgaris*. Although the level of similarity was too high to resolve the exact positions of the strains PCH03 and PCH90 with respect to A1-65 (accession number KF661335.1), CCAP 211/21A (accession number KJ756823.1) and D2 (accession number JX185298.1); the strain LB1H09, although also a strain of *Chlorella vulgaris,* was consistently separated from these (figure 1).

3.2 Effects of Xylose on Growth

The strains used in this work were previously shown to be capable of heterotrophic growth using xylose as a carbon source (Leite et al., 2014). Here, their performance under a two stage cultivation protocol and the short term effects of the addition of xylose as well as the time required for metabolic adaptation were examined (figure 2).

The two groups switched to darkness after xylose addition (heterotrophic and dark photoautotrophic) showed a slight decrease in biomass (OD) in the first twelve to twenty-four hours. Thereafter, the heterotrophic group resumed growth, indicating a lag phase of twelve hours for three of the strains (PCH03, PCH90, and LB1H10) and twenty-four hours for LB1H09. The photoautotrophic culture kept in darkness did not show any increase in optical density. After the lag phase, the heterotrophic growth rate was similar for three out of four strains (Table 1). The exception, PCH03, showed growth rate that was two fold higher, reaching μ=1.11 day^{-1}. Interestingly, the growth curves of this strain were similar for both heterotrophic and mixotrophic conditions (figure 2).

The addition of xylose to previously photoautotrophically grown cultures revealed a potential inhibitory effect of this sugar regardless of whether or not the cultures were subsequently exposed to light. Two days additional incubation were sufficient to see a significant bleaching effect and three of the strains tested (PCH03, PCH90 and LB1H09, all *C. vulgaris* strains) were virtually completely bleached by the fourth day. The only exception was LB1H10, for which both mixotrophic (xylose) and photoautotrophic cultures had a healthy appearance (figure S1). The pH of the mixotrophic and photoautotrophic cultures was followed in order to determine whether any of the effects seen could be caused by pH stress. Despite the addition of MES buffer, the pH of these cultures varied depending upon the species and treatment (figure 3). As expected, the pH of the photoautotrophic group increased over time and at the end of the experiment was essentially the same for all strains (pH= 10.93 $\pm$ 0.15). For the treated group, the addition of xylose induced an acidification whose level and timing varied for each of three strains. The only exception was strain PCH90 which, when cultivated in the light after the addition of xylose, maintained a relatively stable pH throughout the experiment (pH = 7.74 $\pm$ 0.07). Nevertheless, when strain PCH90 was cultivated under the same conditions but in a medium without buffer, the pH decreased as well (data not shown).

Two strains, PCH03, and LB1H09, showed a similar pH acidification when growing on xylose (figure 3). Both reached a pH under six on the fourth day after xylose addition. Interestingly, strain PCH03 was completely bleached by the fourth day, while the two other strains negatively affected by xylose addition (LB1H09 and PCH90), still showed the presence of carotenoids and other accessory pigments (figure S1). These results suggest that the bleaching effect seen in cultures with xylose is (i) species specific; (ii) not due to acidification of the medium, since the medium pH of strains PCH90 and PCH03 remained in the neutral for the first 72 hours after the addition of xylose; (iii) xylose toxicity is not likely to be due to photo-oxidative stress, as the cultures kept in the dark showed similar or worse bleaching than cultures incubated in the dark in the absence of xylose (see section 3.3 for details).

Despite the bleaching observed over the long term, all strains tested showed some biomass gain in the first 72 hours after the addition of the xylose, whether cultivated in the dark or in the light. During this initial period the group exposed to light accumulated more biomass than the dark heterotrophic group (figure 2). However, only one strain, strain LB1H10, showed increased biomass production under mixotrophic conditions (58% more) compared to photoautotrophic conditions. Of course, cultures of all strains showed no or negative biomass increase when incubated in the dark in the absence of xylose.

The size of algal cells is usually found within a specific narrow range during growth, increasing when the source of carbon (CO_2) and energy (light) is still available, but some other nutrient or factor limits cell division. Cell size was analyzed in this experiment through the forward scatter channel (FSC) of the flow cytometer. The data were recorded as the mean scattered light in arbitrary units per event. As expected, since all of them belong to the same genus, strains LB1H09, PCH03, and PCH90 presented a similar size range throughout the experiment. Strain LB1H10, as a member of *Scenedesmaceae* family, can be found as solitary cells or as coenobia of two to four cells. This property causes variation in the data obtained from the FSC channel, where coenobia would show as a single large event. Indeed, strain LB1H10 showed a considerable variation in the signal between the -48h and -24h time points (figure 3), increasing by four fold. However, the O.D. data for this strain over the same period was stable, strongly suggesting that the variation seen in the FSC channel (four fold increase),

could be due to a change in cellular arrangement, going from a single cell to the classical coenobium of four cells side-by-side.

As expected, the *Chlorella* strains incubated in the dark without xylose showed a small decrease in cell size (figure 3). When the same strains where cultivated heterotrophically, the FSC signal shrank to one-third of in only 72 hours. As expected, photoautotrophically incubated cultures showed some increase in FSC, but mixotrophically incubated cells showed a notable decrease in FSC, suggesting that a decrease in overall size is occuring (figure 3), an event affecting the entire cell population as evidenced by the histogram of the FSC channel (figure S2). In flow cytometry, the cell size does not interfere with the fluorescence readings (e.g. lipids or chlorophyll), and no correlation between the evolution of cell size and lipid content are to be expected (channels FSC and FL1 respectively). Among the strains tested, there was no apparent relationship between size (Figure 3 - right) and lipid content (Figure 4 - left) over time. In addition to the decrease in FSC, a decrease in cell count of the *Chlorella* strains was also observable 72 hours after the addition of xylose (data not shown).

3.3 Effects of Xylose Addition on Chlorophyll Fluorescence and Lipid Content

The FL3 channel of the flow cytometer used is equipped with a 630nm long pass filter (LP), allowing the analysis of chlorophyll fluorescence without interference of the BODIPY fluorescent dye. The previous reports of the mixotrophic and heterotrophic utilization of xylose by microalgae mentioned the pale white characteristics of the culture when this sugar was present (Yang et al., 2014). This striking phenotype could be due to several factors, from metabolic shifts to toxicity (e.g. photo oxidative stress). The use of flow cytometry permitted the observation of chlorophyll fluorescence before and after the addition of xylose. This data provides reliable information on cellular chlorophyll content and therefore can be used to follow the maintenance of chlorophyll levels under these conditions.

Samples were analyzed starting from when the cultures reached an OD630 of 0.1 (t=-48h). During the next two days (from time point -48h to 0h), the amount of chlorophyll per

cell increased and then stabilized (figure 4). As expected, after the 0h time point, all strains incubated in the dark in the absence of xylose sustained a slow decrease in chlorophyll content. In contrast, under heterotrophic conditions (xylose in the dark), all strains showed a strong decline in chlorophyll content, although to different levels (figure 4). The difference between the absence and presence of xylose was statistically significant ($P < 0.05$) for three of the four strains, the exception was PCH90 ($P=0.1171$). This exception agrees with the results previously described here (section 3.2) and elsewhere (Leite et al., 2014), suggesting that this strain is not as efficient as the other strains in using either xylose or glycerol to support mixotrophic or heterotrophic growth.

For the group incubated in the light, the photoautotrophic cultures were rather stable in terms of chlorophyll fluorescence. The small decrease seen between time points 12h and 24h is likely due to the periodicity of the light cycle as this period corresponds to the cycle dark period. The addition of xylose quickly affected the levels of chlorophyll with fluorescence fading over time for three of the strains (PCH03, Pch 90, and LB1H09) tested ($P < 0.01$). The exception was strain LB1H10, which despite the variation seen over the different time points and within the replicates (figure 4), showed a stable chlorophyll content with time, as evidenced by mean FL3 flourescence. The cellular content of chlorophyll under these conditions was comparable to photoautotrophic cultures. This data is in contrast to the cultures incubated in the dark, where cultures without xylose showed a slow steady decrease in chlorophyll, and samples to which xylose had been added were characterized by a steep decrease soon after xylose had been added. These results strongly suggest that the influence of xylose on cellular chlorophyll content is not light-dependent.

The fluorescent dye BODIPY 505/515 and flow cytometry can be used to analyze lipid content in algal cells in a sensitive and reliable manner (Cooper et al., 2010; Govender et al., 2012). This presents several advantages over Nile Red including the wavelength of emitted fluorescence which, with BOBIPY 505/515 is far from the natural fluorescence of an algal cell, and can detected using a different channel (FL1) than that used either for chlorophyll (FL3) or for phycobiliproteins (FL2). A second advantage is increased cellular permeability, giving this method more reliability (Mutanda et al., 2011).

The use of flow cytometry to analyze short-term effects revealed a fast boost in algal cellular lipid content. The cultures kept in the dark showed a slight increase in lipid content, not at all comparable with is seen for cultures exposed to light cycle which showed very hgh increases; up to a 3.3 fold increase in 12 hours for strain LB1H09 or 2.4 fold increase in 6 hours for strain PCH03. This increase in cellular lipid content does not seem to be linked to any change in cell morphology, as ascertained using flow cytometry channels FSC and SSC, pH, or disassembly of the photosynthetic apparatus (channel FL3). Nevertheless, after xylose addition cell viability started to drop (see section 3.2), suggesting that the xylose toxicity observed could be related to metabolite accumulation (e.g. xylitol, d-xylulose) or some type of regulatory interference on metabolism. In mammals, an intermediate of xylose assimilation, xylulose-5-phosphate, is directly involved in the regulation of lipogenesis genes (Iizuka and Horikawa, 2008). Thus, the regulatory role of xylose or the metabolites involved in its assimilation in microalgae would not be a complete surprise.

As for xylose consumption, strain LB1H10 consumed 19% of this carbon source in 48 hours, the time point for maximum lipid content for this strain (Table 2). This was approximately an eleven fold gain in lipid content compared to the photoautotrophic culture at the same time point (Table 2). Strain LB1H09 reached peak lipid levelst at 12 hours at this which time 12% of the initial xylose had been depleted. This represented a 12 fold gain in lipid content over the photoautotrophic culture at the same time point.

4. Conclusions

Productivity levels and production costs of algal biodiesel still is one of the significant bottlenecks to the practical application of this technology. Xylose is an abundant molecule in nature, and an important constituent of many primary producers. It could be naturally available, although in a very dilute form, in water bodies through the decomposition of plants. On the other hand, it also represents a cheap and readily available alternative carbon source. Here the effects of xylose on a growing algal cultures were analyzed using flow cytometry and three indigenous strains identified as *Chlorella vulgaris* strain and one from the *Scenedesmaceae* family. The addition of xylose produced a similar effect on the three

Chlorella strains, inducing a fast lipid accumulation, doubling or tripling the lipid content within 6 to 12 hours under mixotrophic conditions. These results suggest that xylose could be used in relatively small amounts to boost lipid production, aiding in the practical development of the algal biofuels. After twenty-four hours, the cells started losing chlorophyll, size and viability. The *Scenedesmaceae* strain was shown to be tolerant to the presence of xylose and to assimilate it under mixotrophic and heterotrophic growth conditions. Thus, this strain might be useful for systems where the xylose removal is a priority.

5. Acknowledgments

This research was supported by a Grant from FQRNT (Le Fonds Québécois de la recherche sur la nature et les technologies), programme de recherche en partenariat contribuant à la séquestration des gaz à effet de serre (2011-GZ-141307) to P.C.H. We thank Patrick McGinn and Shabana Bhatti (Halifax, NS), NRC Institute for Marine Biosciences, for advice and training at the Ketch Harbour Research Center and for the FAME analysis.

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Tables

Table 1: Growth Rates and Biomass Production

Growth rate and biomass production after time=0h, representing only the biomass gained after the point when xylose was added. Since there was no growth related to dark cultivation without xylose (dark control), the data was omitted to simplify the visualization table. Abbreviations in the table: L = cultivation on light; D = cultivation on dark; -xyl = no xylose added; +xyl = xylose added.

	μ/day after t=0			Biomass gain mg/L		
Samples	**L -xyl**	**L +xyl**	**D +xyl**	**L -xyl**	**L +xyl**	**D +xyl**
LB1H09	0.35 (± 0.04	0.44 (± 0.02)	0.52 (± 0.06)	301 (±65)	234 (±75)	158 (±30)
LB1H10	0.18 (± 0.07)	0.37 (± 0.01)	0.51 (± 0.07)	176 (±42)	299 (±87)	310 (±1)
PCH03	0.31 (± 0.01)	0.55 (± 0.12	1.17 (± 0.21)	297 (±46)	386 (±30)	263 (±114)
PCH90	0.19 (± 0.02)	0.25 (± 0.06)	0.75 (± 0.01)	205 (±58)	180 (±40)	217 (±39)

Table 2 : Effect of Xylose on Productivity

Data relative to the lipid content peak as showed in flow cytometer channel FL1 (cultivation days). Strains were all grown for 5 days from the inoculum to the addition of xylose. Biomass, lipid and xylose data was calculated in relationship to the cultivation days: e.g. xylose consumption of LB1H09 after 12 hours of exposition to this carbon source. At t=0, xylose was added to a final concentration of 4,500mg/L (30mM).

(-) = not applicable.

Productivity in function of the peak lipid - induced by the addition of xylose								
Strain	**Cultiv. days**	**Peak**	**Biomass mg/L**	**Biomass mg/L/d**	**Lipids mg/L**	**Lipids mg/L/d**	**Lipid content**	**Xylose depleted mg/L**
LB1H09	5.5	—	341 (±36.4)	61.9	30 (±7.7)	5.4	8.7%	—
LB1H09 + Xylose	5.5	t=12h	207 (±30.6)	37.7	74 (±17.6)	13.3	35.4%	540 (±90)
LB1H10	7	—	359 (±24)	51.3	94 (±13.9)	13.5	26.2%	—
LB1H10 + Xylose	7	t=48h	505 (±48.3)	72.2	107 (±11.6)	15.2	21.1%	871 (±45)
PCH03	5.25	—	279 (±37.4)	53.2	28 (±10.4)	5.35	9.85%	—
PCH03 + Xylose	5.25	t=6h	163 (±44.2)	31.2	65 (±15.6)	12.36	39.7%	195 (±165)
PCH90	5.5	—	330 (±13.1)	60	28 (±9.6)	5.1	8.51%	—
PCH90 + Xylose	5.5	t=6h	238 (±31)	43.2	93 (±13.9)	16.9	39.2%	135 (±165)

Figures

Figure 1: Phylogenetic Tree

Neighbour joining phylogenetic tree based on the alignment of the internal region of the 18S rDNA. The strain LB1H10 was placed within the class *Chlorophyceae* while the others strains were classified as different strains of Chlorella vulgaris (class *Trebuxiophyceae*).

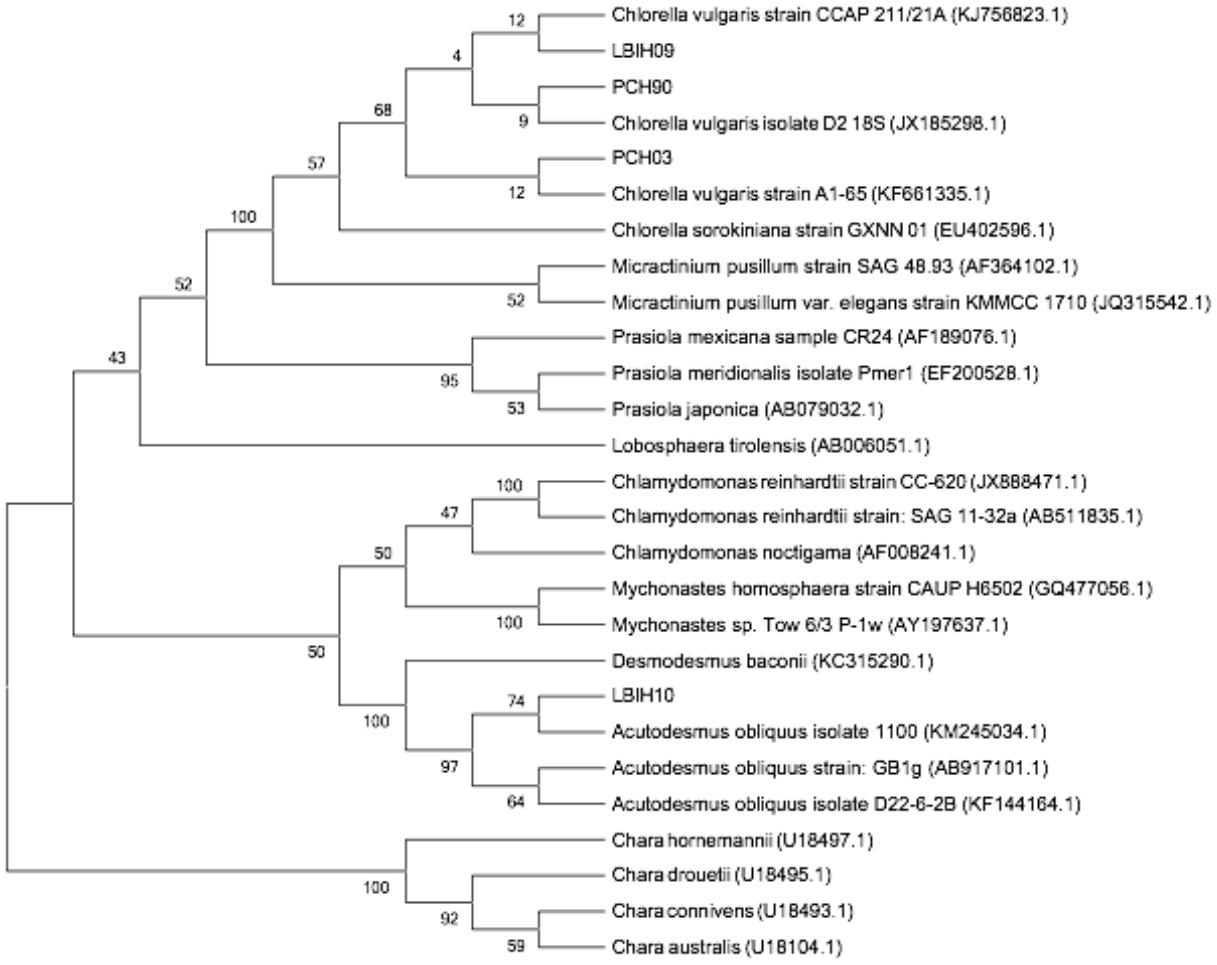

Figure 2: Evolution of pH

Medium pH of the photoautotrophic (triangles) and mixotrophic cultures (circles) at different time points. The pH was measured prior the inoculum and daily after the addition of xylose (t=0).

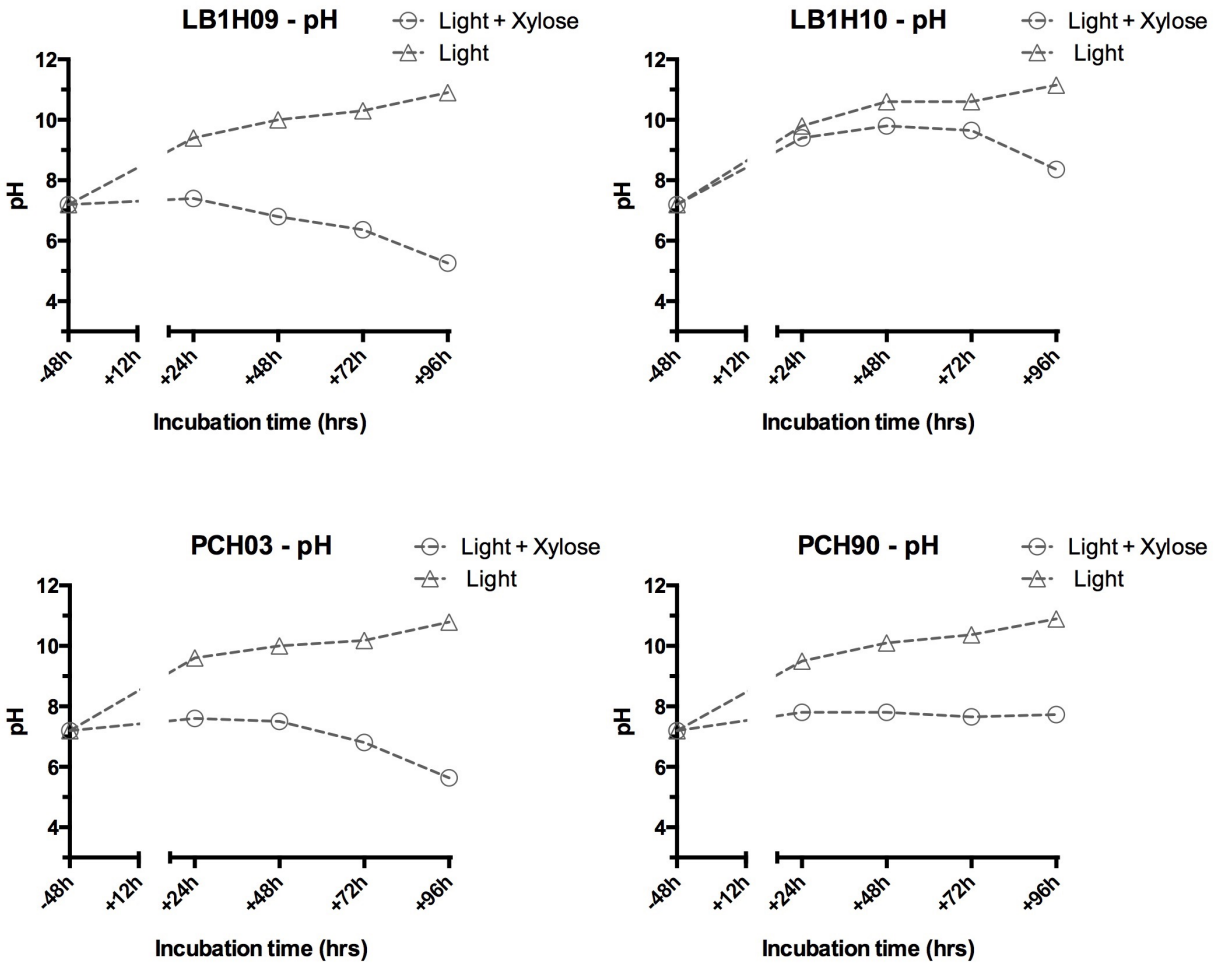

Figure 3: Evolution of OD and FSC Output

Optical density (left) and Forward Scatter (FSC - right) recorded over time. Vertical dotted line represent the moment when the xylose was added into the treated groups (time = 0). Circles represent the treated group and the triangles represent the control groups (clear=light; black=dark).

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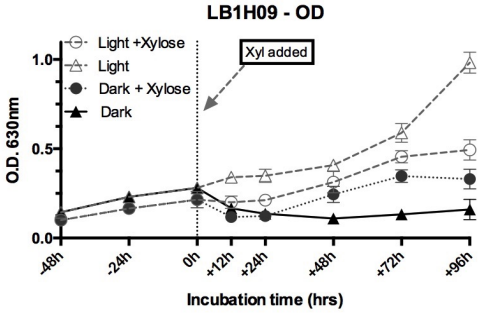
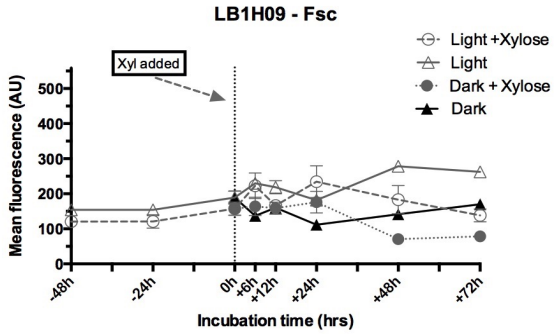
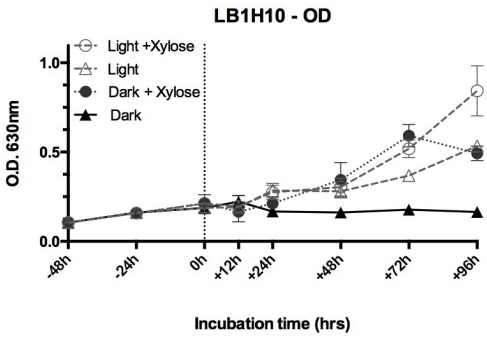
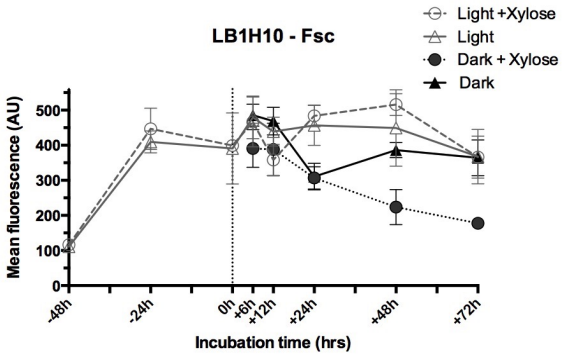
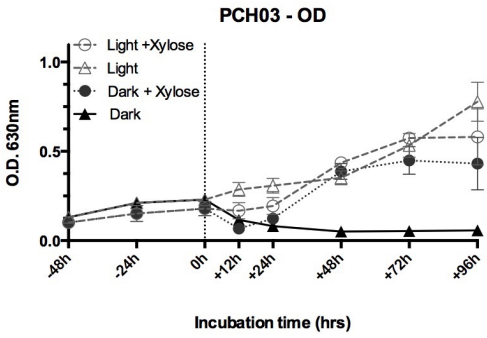
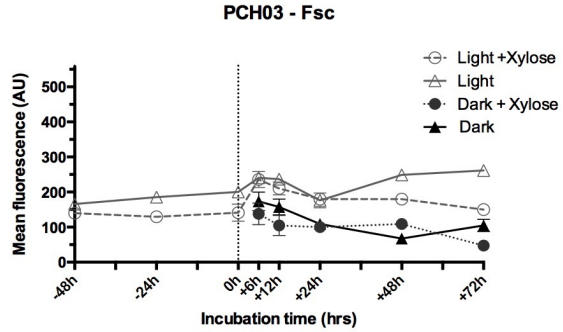

LB1H09 - OD
Light +Xylose
Light
Dark + Xylose
Dark
Xyl added
O.D. 630nm
1.0
0.5
0.0
-48h
-24h
0h
+12h
+24h
+48h
+72h
+96h
Incubation time (hrs)

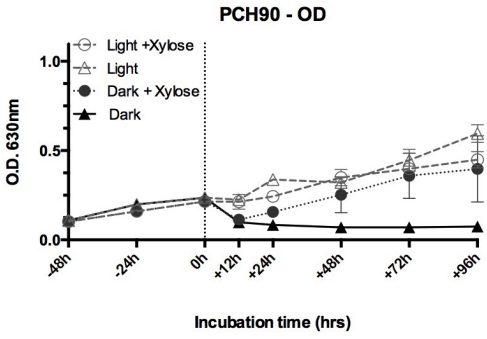

LB1H09 - Fsc
Light +Xylose
Light
Dark + Xylose
Dark
Xyl added
Mean fluorescence (AU)
500
400
300
200
100
0
-48h
-24h
0h
+6h
+12h
+24h
+48h
+72h
Incubation time (hrs)


LB1H10 - OD
Light +Xylose
Light
Dark + Xylose
Dark
O.D. 630nm
1.0
0.5
0.0
-48h
-24h
0h
+12h
+24h
+48h
+72h
+96h
Incubation time (hrs)


LB1H10 - Fsc
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
500
400
300
200
100
0
-48h
-24h
0h
+6h
+12h
+24h
+48h
+72h
Incubation time (hrs)


PCH03 - OD
Light +Xylose
Light
Dark + Xylose
Dark
O.D. 630nm
1.0
0.5
0.0
-48h
-24h
0h
+12h
+24h
+48h
+72h
+96h
Incubation time (hrs)


PCH03 - Fsc
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
500
400
300
200
100
0
-48h
-24h
0h
+6h
+12h
+24h
+48h
+72h
Incubation time (hrs)


PCH90 - OD
Light +Xylose
Light
Dark + Xylose
Dark
O.D. 630nm
1.0
0.5
0.0
-48h
-24h
0h
+12h
+24h
+48h
+72h
+96h
Incubation time (hrs)

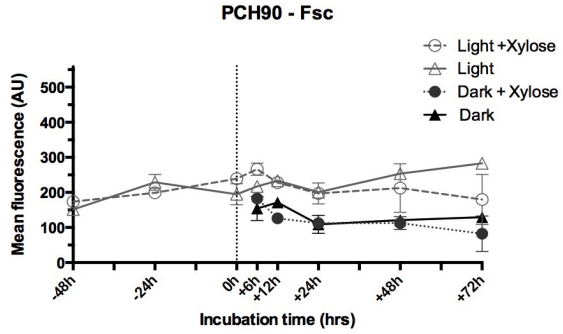

PCH90 - Fsc
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
500
400
300
200
100
0
-48h
-24h
0h
+6h
+12h
+24h
+48h
+72h
Incubation time (hrs)

Figure 4: Evolution of Lipid and Chlorophyll Content

Overtime flow cytometer mean fluorescence measurements of the channels FL1 (lipids) and FL3 (chlorophyll). Vertical dotted line represents the moment when the xylose was added into the treated groups (time = 0). For three of the strains, a fast accumulation of lipids can be seen on the first twelve hours after the addition of xylose, followed by a continuous decrease of the chlorophyll content. Circles represent the treated group and the triangles represent the control groups (clear=light; black=dark)

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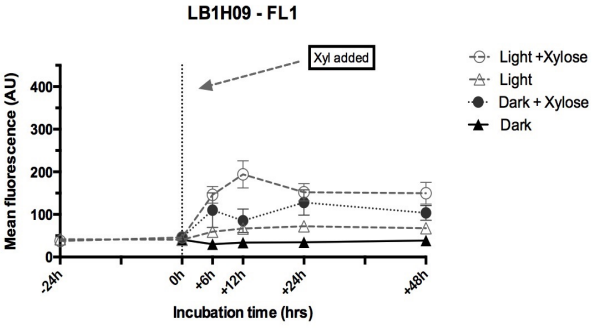
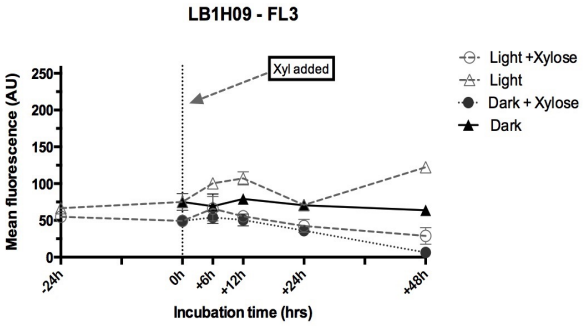
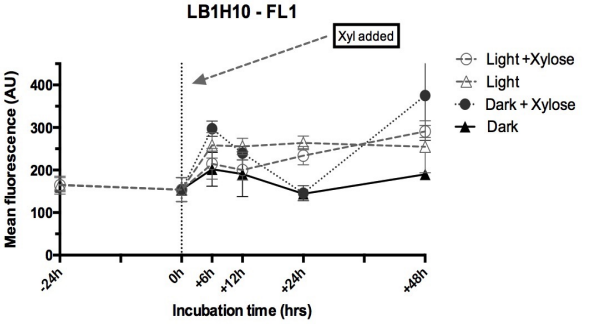
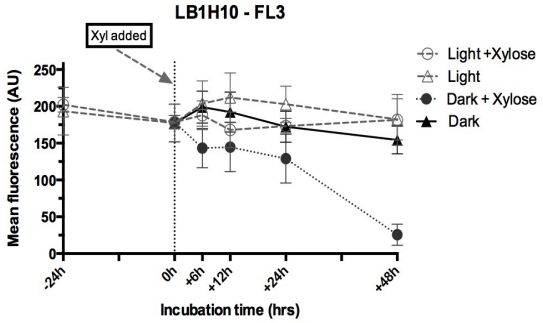
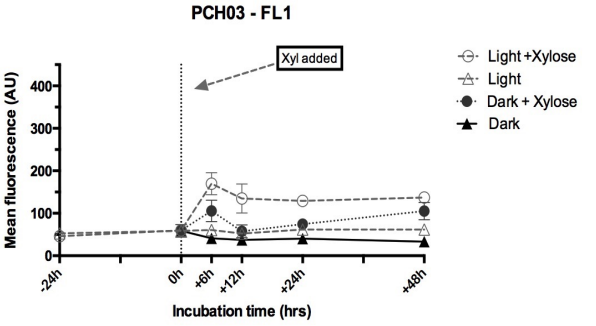
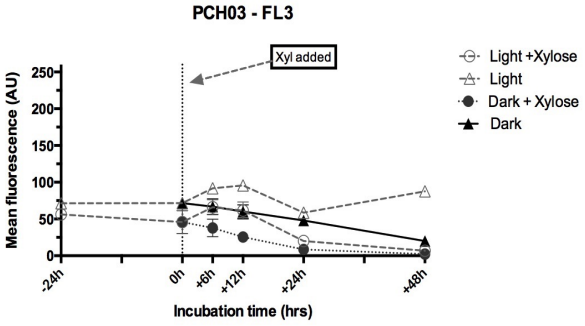

LB1H09 - FL1
Xyl added
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
0
100
200
300
400
-24h
0h
+6h
+12h
+24h
+48h
Incubation time (hrs)


LB1H09 - FL3
Xyl added
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
0
50
100
150
200
250
-24h
0h
+6h
+12h
+24h
+48h
Incubation time (hrs)


LB1H10 - FL1
Xyl added
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
0
100
200
300
400
-24h
0h
+6h
+12h
+24h
+48h
Incubation time (hrs)


LB1H10 - FL3
Xyl added
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
0
50
100
150
200
250
-24h
0h
+6h
+12h
+24h
+48h
Incubation time (hrs)


PCH03 - FL1
Xyl added
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
0
100
200
300
400
-24h
0h
+6h
+12h
+24h
+48h
Incubation time (hrs)


PCH03 - FL3
Xyl added
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
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150
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250
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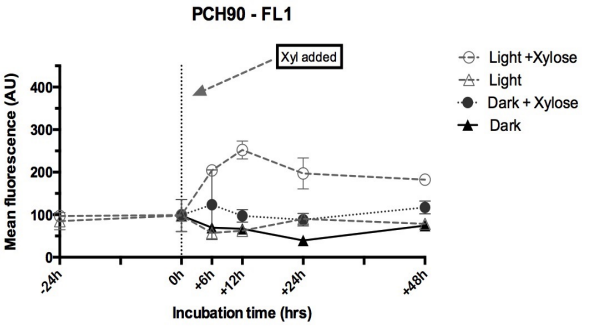
0h
+6h
+12h
+24h
+48h
Incubation time (hrs)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Light
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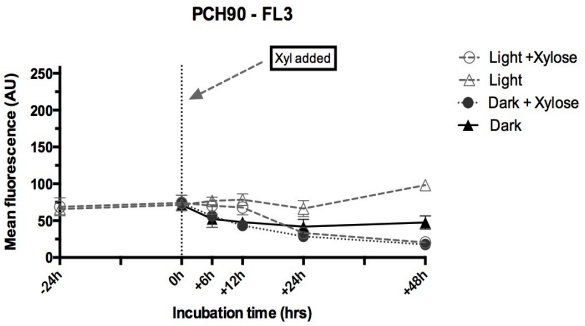
Incubation time (hrs)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Xyl added
Light +Xylose
Light
Dark + Xylose
Dark
Mean fluorescence (AU)
0
50
100
150
200
250
-24h
0h
+6h
+12h
+24h
+48h
Incubation time (hrs)

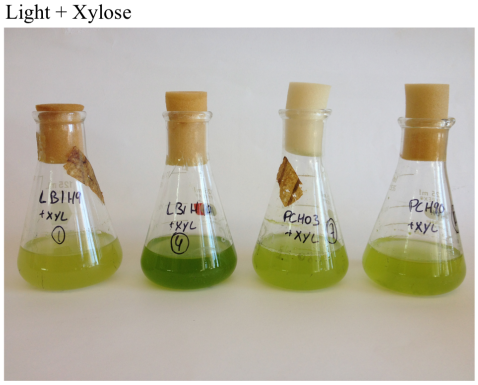
Figure S1: Color Evolution

Photos of the Erlenmeyer flasks at different time points. Change in color begun to be noticeable 48 hours after the xylose was added.

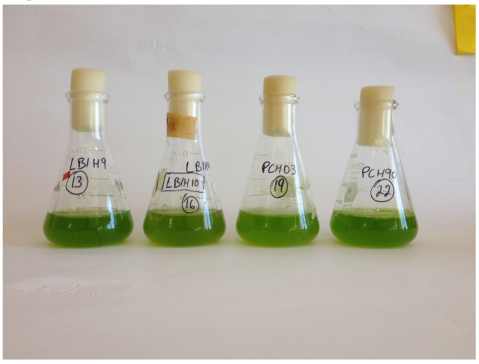
Cultures pictures:

26-sept-2014 @ 13:00 (T=48hrs)

Light + Xylose

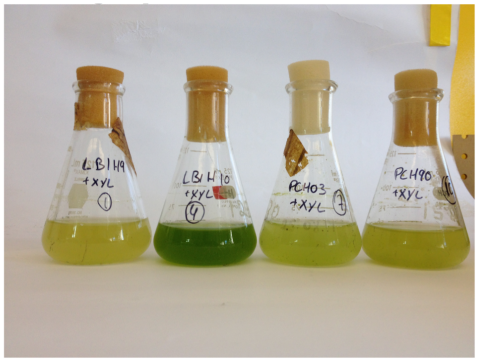


Light

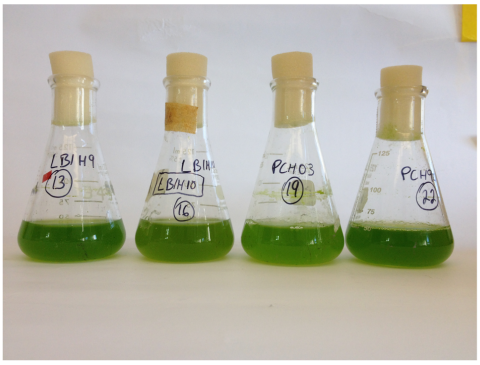


27-sept-2014 @ 11:10 (t=72hrs)

Light + Xylose

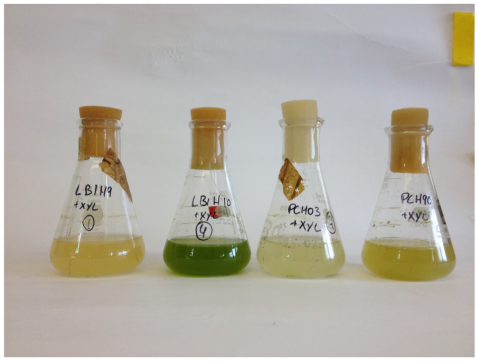


Light

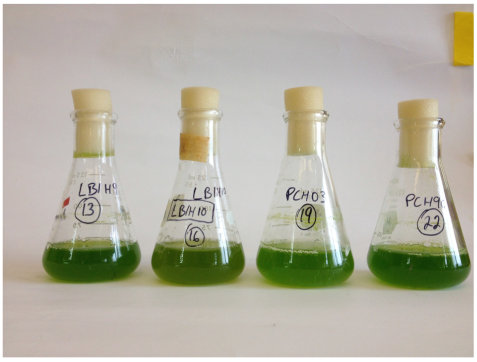


28 sept 2014 (t=96hrs)

Light + Xylose



Light



Chapter 5: Using CO_2 Enrichment to Isolate Algal Strains for Flue Gas Bioremediation and Algal Biomass Production.

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Author contributions: The author was responsible for the project concept design; experiments and analysis, always under the supervision and guidance of Dr. Patrick C. Hallenbeck. T.M.K. prepared the phylogenetic tree constructs. K.P. and A.B. performed preliminary experiments. The manuscript was written by the author and revised by Dr. Patrick C. Hallenbeck

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Using CO_2 Enrichment to Isolate Algal Strains for Flue Gas Bioremediation and Algal Biomass Production.

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[redacted]

Abstract

More than half of the carbon dioxide that is emitted into the atmosphere every day is released by stationary processes, either for generation of electricity or industrial manufacturing. The CO_2 exhausted from these sources could be attenuated through mitigation with microalgae, a putative feedstock for biofuels. Nevertheless, every flue has a different gas composition. An algal cultivation system using the CO_2 enrichment provided by these flue gases could turn the medium into a harsh environment. One option to find an algal strain capable to thrive in these conditions is the bioprospection. Here it is proposed the use of the particular site conditions on the bioprospecting for algal strains flue gas mitigation. It is shown that using a simple enrichment process at the isolation stage can select for strains that are, on average, 43.2% better performing in biomass production than the strains isolated through the traditional methods. The strains isolated in this work were capable of assimilating carbon dioxide at an above average rate, according to recent the results in the literature.

1. Introduction

The anthropogenic emission of carbon dioxide on earth is probably among the most evident mark of the development of our species (www.epa.gov). Human activities have disrupted the natural carbon circulation on the planet, between oceans, the atmosphere, soil and biome (carbon cycle). By flushing enormous amounts of CO_2 from natural storages into the atmosphere (e.g. crude oil, natural gas and coal) and disrupting natural sinks, like forests, humankind is raising the CO_2 levels in the atmosphere. The U.S. carbon dioxide emissions accounted for 82% of all greenhouses gas (GHG) generated in 2002, followed by methane (9%) and nitrous oxide (6%) (US EPA 2014). 52% of carbon dioxide and most of the nitrous oxide emissions comes from stationary sources, being 38% related to electricity generation and 14% to the industrial sector (US EPA 2014). These sources are good candidates for bioremediation processes, capturing the exhausted gases and transforming them into biomass to be used as feedstock for biodiesel production or other purposes (Huntley & Redalje 2006).

Bioremediation is the use of organisms to assimilate or degrade a pollutant. It is based on the capability of certain microorganisms and plants to absorb, degrade or transform pollutants that are toxic to other organisms (Wang et al. 2015). This biological clean-up procedure received considerable attention from the scientific community, and several research works have been published identifying the capabilities of different species of algae and plants to remove pollutants from water or soil through bioaccumulation or biodegradation (Teixeira et al. 2014). This approach is being actively used at various sites in Europe (Majone et al. 2015), and is usually based on the isolation of organisms from the polluted site or using this pollutant as an enrichment for the isolation of resistant microorganisms (Perruchon et al. 2015; Dellagnezze et al. 2014; Wang et al. 2015). In some cases, bioremediation processes can produce feedstocks for other industries, as bioplastics (Berezina et al. 2015) or biomass for biofuel (Aravantinou et al. 2013) .

Domestic and industrial wastewater requires treatment before being released into the water supply. While the primary treatment is basically a screening process, the secondary treatment is a kind of bioremediation, using microorganisms to consume the organic carbon present in the stream. The tertiary treatment employs photosynthetic microorganisms to reduce

the levels of nutrients (e.g. nitrates, phosphates and micronutrients) still present in the secondary treatment effluent (Abdelaziz et al. 2013). This treatment was shown to be feasible in tropical, and cold climate regions (Abdelaziz et al. 2013; Grönlund & Fröling 2014). The last decade saw an increasing interest in algal biofuels, and microalgal biomass that could be turned into a feedstock for the biodiesel industry in the near future (Leite et al. 2013). The association of wastewater treatment and production of algal biofuel is a natural path for coupling bioremediation with bioenergy generation (Grant et al. 2012; Cabanelas et al. 2013; Aravantinou et al. 2013; Abdelaziz et al. 2014).

Using the same idea as the bioremediation approach, the strategy used by many researchers to couple wastewater treatment with microalgal biofuel production starts with the isolation and selection of native strains occurring at the specific treatment site (Aravantinou et al. 2013; T.-Y. Zhang et al. 2013; Doria et al. 2011; Ji et al. 2013). In fact, studies comparing the performance of culture collection strains to local isolates found that an equivalent or higher productivity can be achieved with native species (Arbib et al. 2014; Ji et al. 2013). Biomass accumulation in these systems usually in the range of 250 to 400 mg/L (Table 1). A biomass of 920 mg/L was demonstrated in a concentrated secondary treatment effluent (Li et al. 2011), at a productivity rate of 65.7 mg/L/day (Table 1). Nutrient supplementation could be a strategy in systems where biomass production is prioritized over wastewater remediation. To understand the nutritional limitations of wastewater (WW) for biomass production, Zhang et al. (2013) used a Design of Experiment methodology (DoE) and found that supplying with 4.41 mg/L of phosphate and 3.8 mg/L of $FeCl_3$ it was possible to increase the biomass accumulation up to 1,460 mg/L (C. Zhang et al. 2014a). Despite the productivity of these locally isolated microalgae, they are likely to be more robust in the long term production.

Among the major challenges to be overcome by the biofuel technology using microalgae, is the productivity and costs. The use of wastewater usually decreases the costs and environmental impact of algal biomass production, but at the expense of productivity. The bioremediation of flue gases could help the productivity aspect. Flue gas is a mixture usually containing different proportions of N_2, O_2, CO_2 NOx, SOx and water vapor. The CO_2 availability is a significant issue for any photoautotrophs. To work around the low affinity of the enzyme for carbon fixation (RuBisCO), some authors has tried to bypass the

photorespiration pathway (Shih et al. 2014), or introduce a carbonic anhydrase, increasing the dissolved inorganic carbon availability (Chen et al. 2012). A different approach is just to provide carbon dioxide from flue gases. Increasing the CO_2 atmospheric concentration changes the growth rate, biomass accumulation and lipid and fatty acids profile. Tang et al. found that 10% of CO_2 was the optimal concentration for biomass production and carbon dioxide capture (Tang et al. 2011), achieving 1840 mg/L biomass DW using the nitrogen rich medium BG11 (Table 1). As it happens with wastewater, the nature of the flue gases is very specific to each site, and the proportion of the gases could be more or less toxic (Cuellar-Bermudez et al. 2014). The NOx component of the flue gas can be toxic, but also can be used as a nitrogen source (Shihady 2014). Nitrate levels tolerated by an algal strain was shown to reach up to 88mM (X. Zhang et al. 2014b). In a large-scale experiment using raceway open-ponds up to 8000L, *Nannochloropsis sp*. from a culture collection was shown to thrive in a culture enriched with treated flue gas from a coal-fired power plant, reaching up to 340mg/L DW biomass (Zhu et al. 2014). However, all the approaches proposed to CO_2 capture and bioremediation of flue gases use strains from culture collections and the bioprospection based on CO_2 enriched samples was not addressed so far.

This work simulates a driven bioprospection process to compare the traditional isolation method to the strategy commonly used in microbial bioremediation. An artificial condition is used to enrich natural samples. This would allow the selection process to favour the isolation of the most suitable strains for the task, in this case, mitigation of CO_2 and biomass production.

2. Methods

2.1 Sampling and Isolation

A water sample taken from Lake Saint-Louis, a widened area of the Saint Laurence river on the south shore of the Montreal Island, Quebec, Canada (45°25'42.1"N 73°49'04.4"W), was filtered through a 30µm mesh membrane (to avoid zooplankton

contamination) and taken to the laboratory for algal isolation. This sample was submitted to two different isolation procedures (Figure 1). Approximately one liter of water sample was filtered through a series of membrane filters with a mesh size of 0.45µm. Using a sterile swab, the particles retained by the membrane filter were streaked on several agar plates made with Bold's Basal Medium (BBM), (Andersen 2005), modified by the addition of 50mM MES pH 7.4. These plates were incubated at 22˚C with a light intensity of 24 W/m^2. When green colonies were visible, they were re-streaked on separate agar plates to ensure a proper isolation, and were transferred to a liquid modified BBM medium and cultivated in the same conditions (22˚C and 24 W/m^2) under agitation. These cultures were then used to seed experimental and culture collection vials.

In the second isolation procedure, the water sampled was enriched with the modified BBM to 50% (v/v) and incubated in sealed vials containing 50% CO_2 (Figure 1). These bottles were incubated at 22˚C with a light intensity of 24 W/m^2 for ten days, when an aliquot was taken with a sterile syringe and streaked on different BBM agar plates. These plates were incubated at 22˚C, with a light intensity of 24 W/m^2 and 10% CO_2. When green colonies were visible, they were re-streaked on separated agar plates to ensure proper isolation. When colonies were separated, and the plates appeared homogenous the isolated strains were transferred to a liquid BBM medium and cultivated under agitation (22˚C and 24 W/m^2). These cultures were then used to seed experimental and culture collection vials.

2.2 DNA Extraction and Amplification Conditions

Initially, all samples were submitted to direct-PCR, where intact cells were used as DNA template for polymerase chain reaction. The rate of success for direct-PCR was 81%. The strains which did not yield PCR results were submitted to DNA extraction, and purification, by an adaptation of the method described by Fawley et al. (2004). In a microcentrifuge tube, an aliquot of 1.5mL of a grown culture was centrifuged at 16,000g for 1 minute, the pellet was resuspended in 200µL extraction buffer (1M NaCl, 70mM Tris, 30mM Na_2EDTA, pH 8.6). This step was repeated once, followed by the addition of enough glass

beads fill the conical part of the microcentrifuge tube (G-8772, Sigma Chemical Co., St. Louis, MO, USA), 25µL of 10% (v/v) DTAB and 200µL of chloroform. At this point, the samples were kept in ice. Then, the tubes were submitted to three rounds of 40 seconds agitation on a bench top vortex, hitting the side of the microcentrifuge tube. The tubes were then centrifuged at 2,000g for 2 minutes for phases separation. At this point, a successful extraction can be evaluated by the presence of chlorophyll in the organic phase (bottom). The upper phase was collected, and a DNA purification step was followed, using a standard DNA spin-column purification kit based on silica-DNA binding.

The detection and amplification of the genes, to be used on phylogenetic analysis and molecular identification, were made using universal primers for 18S rDNA and for the gene *rbcL* (Table 2). Amplification was made with a Phusion DNA polymerase (Fermentas), using a 4 minutes primary denaturation step at 98˚C, followed by 35 cycles of the following temperatures and times for detection of the complete 18S rDNA: 98˚C for 30”; 60˚C for 30”; 72˚C for 40”. The amplification of the rbcL gene used the same conditions, except for the annealing, temperature, set at 58˚C. An extra pair of primers targeting an internal section of the 18S rDNA was designed for sequencing purposes (Table 2).

2.3 Strain Identification and Phylogenetic Analysis

The identification of the strains isolated in this work was based on the comparison of the 18S rDNA sequences with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) and phylogenetic relationship to other strains from typed culture collections. These comparisons were made using a 1.6Kb segment of the 18S rDNA. To increase the definition capabilities of the phylogenetic analysis, a section of 900 bases of the gene encoding the large sub-unit of the RuBisCO enzyme (annotated as *rbcL*) was also sequenced. A consensus strand was built using the sequence generated by the forward and reverse primers for each gene. Additionally, due to the length of the 18S rDNA amplicon, an internal primer was used to unite both strands and to serve as a third segment to build the contigs (Table 2). The sequencing reactions were performed by Bio Basic Canada Inc.

Chromatograms were analyzed using the CLC Main Workbench software (version 6.8.4), and the phylogenetic tree was constructed using the MEGA software (Version 5.2.2), using the neighbor-joining method and Jukes-Cantor model set to 1000 bootstraps.

2.4 Cultivation and Growth Measurements

The experiments, pre-inoculum and strain maintenance were performed using BBM Medium, (Andersen 2005), modified by the addition of 50mM MES pH 7.4. Pre-inoculum was grown under agitation in 125mL Erlenmeyer flasks at 22˚C and 24 W/m^2 and atmospheric CO_2. The experiments were carried out in 12 wells plates containing 3.5mL of modified BBM. The plates were incubated at 22˚C and exposed to light at 40 W/m^2, in ligth dark cycle of 12 hours light by 12 hours dark, provided by high intensity, cool white LEDs, under atmospheric air or 10% CO_2, according to the treatment. Growth was measured by the optical density, read daily at 630nm using a Biotek EL800 microtiter plate reader. The plates were shaken for five minutes prior to the measurements using a PMS-1000 microtiter plate shaker (Boekel). Maximum growth rate (μmax) was measured according to Equation 1, where the initial and final times were chosen as the period giving the highest μmax with at least two days apart. The maximum theoretical productivity was calculated as the optimal day for harvest in terms of biomass yield. All cultures and measurements were carried in biological and analytical triplicates

Equation 1: $$\mu_{max} = \frac{(LnOD_2) - (LnOD_1)}{T_2 - T_1}$$

2.5 Statistical Analysis

The statistical significance between to treatments were determined using the software Prism 6.0D (GraphPad) with a confidence interval of 95%.

3. Results and Discussion

This work evaluated the successful rate of two strategies for the isolation of microorganisms for dioxide capture and biomass production. The classical methodology, isolating different organisms from the environment, was compared to an adaptation of the method used in the bioremediation process, where usually the sampling site is already submitting the local microbiome to a selective pressure. Here, in contrast to the classical isolation procedure, the same water sample was subjected to CO_2 enrichment and a half strength BBM. This medium contains a particularly low proportion of nitrates in comparison to phosphates, which is closer to the ratio found in some secondary wastewater effluents (Abdelaziz et al. 2013).

3.1 Isolation and Identification

Any environmental isolation procedure has a particular risk of redundant selection, where the same strain is "picked" more than one time. The methods tried in this work were no exception, and a crucial point is whether the CO_2 and artificial medium enrichment would promote a different selection of strains or just provide an adaptation to the same strains isolated in the classical selection procedure. Thus, before analyzing growth, carbon dioxide capture capabilities and biomass productivities, the strains were identified and compared. The DNA sequence of the 18S rDNA of all strains was aligned, trimmed and compared. Surprisingly, the primary analysis showed that there were no identical sequences between the two treatments, and the only strains redundancy found was within an isolation procedure. However, some of the strains show little or no sequence divergence with others from their isolation groups. From the conventional isolation procedure, the strains A03, A04 and A29; A27 and A30 where found to be identical. Regarding the enriched isolation, the strains C12 and C16 were grouped as the same. From the thirty-three colonies initially selected, three strains were identified as cyanobacteria and removed from the study (Table 3). The other

isolates showing no base pair divergence in Figure 2 were confirmed as different strains after further analysis using longer alignment of the 18S rDNA and *rbcL* gene.

On the contrary to what was expected, the enrichment did not produce many redundant strains. Of course, this could be associated with extra care when choosing colonies to be subcultured. Nevertheless, the divergence among the strains in Figure 2 indicates that classical isolation would provide strains with higher diversity than the enriched method. This was a possibility expected and was corroborated by the phylogenetic analysis (Figure 2). The major part of the strains isolated after the enrichment procedure is found under the *Scenedesmaceae* or *Chlorellaceae* families, two of the most commons groups in freshwater environments and popular in microalgae biomass production projects and studies. The classic method produced a collection widespread in the phylogenetic tree (Figure 2).

3.2 Growth and Biomass Productivity

As the two isolation procedures successfully generated different strains in terms of molecular background, these lineages were evaluated for growth, biomass accumulation and productivity in two distinct conditions. The thirty strains were cultivated in biological triplicates under atmospheric air and 10% CO_2, and their growth was measured by optical density. To evaluate the different isolation strategies, the growths of the two groups were concatenated as the average value for each data point. Under 10% CO_2, the enriched isolation group showed in average 43% higher productivity, 11.25% higher growth rate and 27.9% higher biomass accumulation at the optimal harvest set point (Table 3). On average, the enriched group exited exponential growth on the fifth day. This short growth period could be explained by the low initial concentration of nitrate available in the culture medium (2.98mM NO_3) or another nutrient that became unavailable. Both groups showed approximately the same maximum biomass accumulation, but while the enriched group took on average only five days to start leaving exponential, and 8 days to reach the maximum productivity, the traditional group needed eleven days to reach the same position (Figure 3). Concatenating the daily optical density of the two groups, enabled the generation of a growth curve showing the

average performance in growth on both conditions for both groups. The difference in the growth of the two groups was statistically significant for both conditions: 10% CO_2 and atmospheric air.

4. Conclusions

The same water sample was submitted to two different treatments for isolation of microalgal cells: (i) Traditional filtration/concentration followed by agar plating, and (ii) a preliminary step of enrichment of CO_2 and half strength BBM medium, followed by agar plating. The two methods yielded different strains with no overlap among the isolates, the traditional method produced a larger phylogenetic variety while the enriched method yield strains concentrated in two known families of fast growing algae: *Scenedesmaceae* and *Chlorellaceae*. This group showed an average 43.2% higher biomass production with 11.25% higher growth rate. The difference in performance between both groups was statistically significant. The strains isolated after enrichment showed competitive performance of biomass production and are candidates for further optimization of culture conditions.

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Tables

Table 1: Productivity of Different Strains

Comparison of the performance between strains in different reports. The strains with higher yields are strains isolated locally. Source[a]: CC = Culture Collection, EnSW = Enriched, FW = Fresh Water, WW = Wastewater, μ_{max}= maximum growth rate, (-) = data not available.

Microalgal species	Source[a]	Medium	CO_2 %	Light (cycle)	Days	Biomass mg/L	μ_{max}/ day	Biomass mg/L/day	References
Chlorella. vulgaris	WW	WW	15%	(16:8)	7	290	1.37	41	(Ji et al 2013)
Ourococcus. multisporus	WW	WW	15%	(16:8)	7	310	1.00	44	(Ji et al. 2013)
Scenedesmus. obliquus	CC	WW	15%	(16:8)	7	310	1.14	44	(Ji et al. 2013)
Chlorella sp.	FW	WW	ATM	(24:0)	14	920	0.677	66	(Li et al. 2011)
Botrycoccus. braunii	CC	WW	1%	(24:0)	10	340	--	34	(Sawayama et al. 1992)
Nannochloropsis sp.	CC	WW	15%	(24:0)	7	212	--	30	(Jiang et al. 2011)
Scenedesmus. obliquus	FW	DM	10%	(24:0)	12	3500	1.19	293	(Ho et al 2011)
Scenedesmus. obliquus	CC	BG11	10%	(-)	(-)	1840	0.89	155	(Tang et al. 2011)
Chlorela. pyrenoidosa	CC	BG11	10%	(-)	(-)	1550	0.99	144	(Tang et al. 2011)
Nannochloropsis sp.	CC	En.SW	13%	(12:12)	16	316	0.066	3	(Zhu et al. 2014)
Acutodesmus. obliquus	FW	BBM	10%	(12:12)	4	946	0.9	236	This work
Acutosedmus. obliquus	FW	BBM	10%	(12:12)	4	935	1.03	234	This work

Table 2: Primers Used for DNA Amplification

Primer	Sequence	Reference
18S-Ext-F	CTGGTTGATYCTGCCAGT	(Harding et al. 2011)
18S-Ext-R	TGATCCTTCTGCAGGTTCACCTAC	(Harding et al. 2011)
18S-Int-F	GTGGTAACGGGTGACGG	This work
18S-Int-R	GTGCGGCCCAGAACATC	This work
Rubi-F	CTCCTCAACCAGGTGTTCC	This work
Rubi-R	CTGGCATGTGCCATACGTG	This work

Table 3 Average Results for Traditional and Enriched Isolation
From the 33 strains initially isolated, seven isolates were eliminated from this study, two from the enrichment process and five from the traditional (see section 3.1).

Strategy	Number of Isolated	Different Strains	Biomass mg/L	$Prod_{max}$ $_{m}$g/L/day	μ_{max}/day	Biomass at harvest mg/L
Enriched	15	13	1062	189	0.89	848
Traditional	18	13	1056	132	0.80	663

Table 4: Identification, Growth and Biomass Production Information of the Strains in this Work.

Identity, growth rate and biomass production of the 26 different strains in this work. From the 33 strains initially isolated, 7 were eliminated for being redundant or from a different group of microorganism (see section 3.1).

(Next Page)

Strain	Name	% ID	µ/day	Max biomass accumulation mg/L	Days to reach max	Biomass productivity mg/L/day	Max biomass prod. mg/L/day	Days to reach max productivity	Biomass at harvest mg/L
A01	Acutodesmus obliquus	99.52%	0.85	1140	11	104	179	5	895
A02	Acutodesmus obliquus	99.82%	0.92	999	9	111	179.8	4	719
A04	Acutodesmus obliquus	100.00%	1.00	972	11	88	191.3	4	765
A05	Acutodesmus obliquus	99.88%	1.00	1048	11	95	164	4	656
A06	Micractinium sp.	99.45%	0.68	996	15	66	148.7	5	469
A19	Monoraphidium sp.	98.20%	0.75	1007	11	92	67.5	4	952
A20	Coronastrum ellipsoideum	99.82%	0.62	926	15	62	68.1	6	333
A21	Coronastrum ellipsoideum	99.82%	0.71	999	17	59	81.5	4	326
A22	Desmodesmus armatus	99.07%	0.92	1281	18	71	165	4	661
A23	Micractinium sp.	99.45%	0.87	1150	16	72	136.6	5	683
A25	Desmodesmus armatus	100.00%	0.84	1282	16	80	154	5	771
A26	Chlorodium saccharophilum	100.00%	0.64	1155	18	64	101.1	8	809
A27	Ankistrodesmus gracilis	100.00%	0.56	772	18	43	76.2	4	586
		Average	0.80	1055.77	14	77.45	131.75	4.77	663.40
C07	Acutodesmus obliquus	99.88%	0.90	957	5	191	236	4	947
C08	Acutodesmus obliquus	100.00%	0.99	973	11	88	215	4	860
C10	Acutodesmus obliquus	100.00%	0.97	981	9	109	218	4	874
C11	Scenedesmus deserticula	99.77%	0.96	1024	11	93	182	4	730
C12	Acutodesmus sp.	99.94%	0.82	1248	9	139	204	4	817
C13	Acutodesmus obliquus	100.00%	0.88	1221	8	153	210	4	840
C14	Acutodesmus obliquus	100.00%	0.84	963	8	120	183.5	5	917
C15	Acutodesmus obliquus	99.85%	0.78	996	8	124	184.8	5	924
C17	Acutodesmus obliquus	99.88%	0.92	860	9	96	159.1	4	637
C18	Scenedesmus bajacalifornicus	97.16%	0.89	986	8	123	151.9	5	759
C31	Micractinium sp.	99.70%	0.82	1182	11	107	125.9	8	1007
C32	Acutodesmus obliquus	100.00%	1.03	1172	9	130	234	4	935
C33	Micractinium sp.	99.70%	0.76	1240	11	113	154	5	771
		Average	0.89	1062	9	122	189	4.62	848

CO_2 Enrichment Atmospheric CO_2

Figures

Figure 1: Isolation Procedure

Isolation procedures compared in this work. The same water sample was used in two different strategies, a classical procedure and a driven isolation process, where the water sample was enriched using the conditions of a theoretical cultivation system.

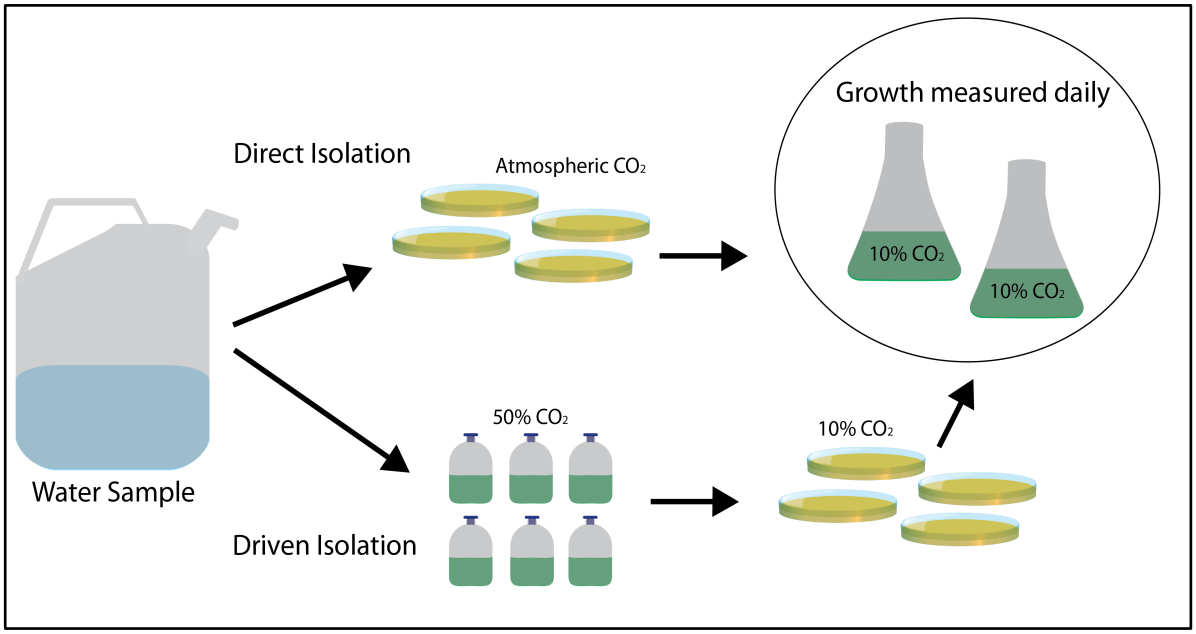


Figure 2: Phylogenetic Analysis of the Strains Isolated in this Work.

The strains isolated through the classical procedure are notated with an "A", while the samples isolated after CO_2 enrichment are notated with the letter "C" before the sample number.

(Next page)

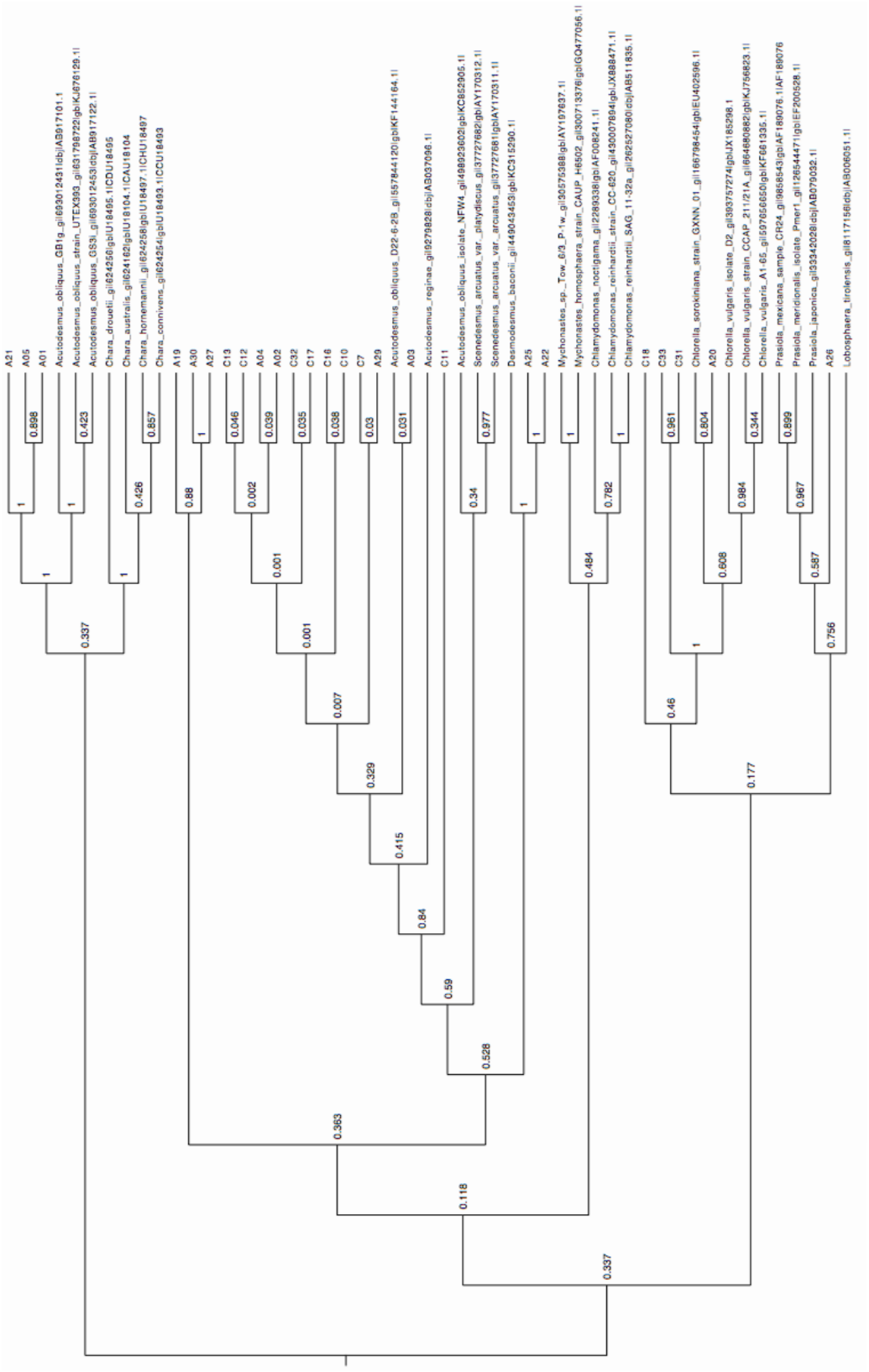
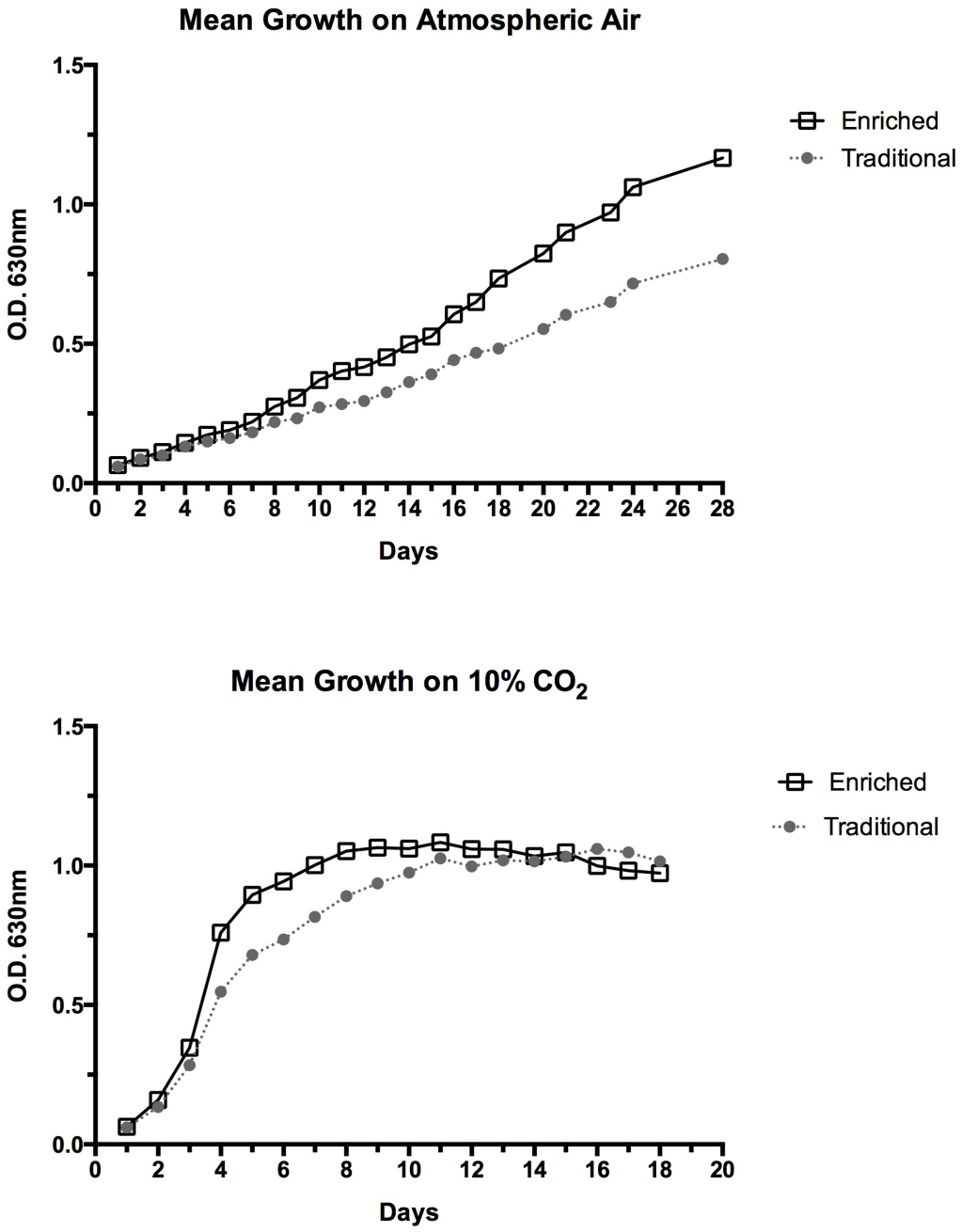

A21
A05
A01
Acutodesmus_obliquus_GB1g_gi|693012431|db|AB917101.1
Acutodesmus_obliquus_strain_UTEX393_gi|631798722|gb|KJ876129.1|
Acutodesmus_obliquus_GS3i_gi|693012453|db|AB917122.1|
Chara_drouetii_gi|624256|gb|U18495.1|CDU18495
Chara_australis_gi|624162|gb|U18104.1|CAU18104
Chara_hornemannii_gi|624258|gb|U18497.1|CHU18497
Chara_connivens_gi|624254|gb|U18493.1|CCU18493
A19
A30
A27
C13
C12
A04
A02
C32
C17
C18
C10
C7
A29
Acutodesmus_obliquus_D22-6-2B_gi|557844120|gb|KF144164.1|
A03
Acutodesmus_reginae_gi|9279828|db|AB037096.1|
C11
Acutodesmus_obliquus_isolate_NFW4_gi|498923602|gb|KC852905.1|
Scenedesmus_arcuatus_var._platydiscus_gi|377276821|gb|AY170312.1|
Scenedesmus_arcuatus_var._arcuatus_gi|377276811|gb|AY170311.1|
Desmodesmus_baconii_gi|449043453|gb|KC315290.1|
A25
A22
Mychonastes_sp._Tow_6i3_P-1w_gi|305753681|gb|AY197637.1|
Mychonastes_homosphaera_strain_CAUP_H6502_gi|300713376|gb|GQ477056.1|
Chlamydomonas_noctigama_gi|32893381|gb|AF008241.1|
Chlamydomonas_reinhardtii_strain_CC-620_gi|430007694|gb|JX888471.1|
Chlamydomonas_reinhardtii_SAG_11-32a_gi|262527080|db|AB511835.1|
C33
C31
Chlorella_sorokiniana_strain_GXNN_01_gi|166798454|gb|EU402596.1|
A20
Chlorella_vulgaris_isolate_D2_gi|383757274|gb|JX185298.1
Chlorella_vulgaris_strain_CCAP_211/21A_gi|656480682|gb|KJ756823.1|
Chlorella_vulgaris_A1-6S_gi|597656650|gb|KF661935.1|
Prasiola_mexicana_sample_CR24_gi|98558543|gb|AF189076.1|AF189076
Prasiola_meridionalis_isolate_Pmer1_gi|126544471|gb|EF200528.1|
Prasiola_japonica_gi|53342028|db|AB079032.1|
A26
Lobosphaera_tirolensis_gi|81171561|db|AB006051.1|
1
0.898
1
0.423
1
0.337
1
0.857
0.426
1
0.88
1
0.046
0.039
0.002
0.035
0.001
0.038
0.001
0.03
0.007
0.031
0.329
0.415
0.84
0.59
0.34
0.977
0.528
1
1
1
0.484
0.782
1
0.961
0.804
1
0.608
0.984
0.344
0.899
0.967
0.587
0.756
0.46
0.177
0.363
0.118
0.337

Figure 3: Curve of the Average Growth.

Curve of the mean growth of the two isolation procedures under atmospheric air (top) and 10% CO_2 (bottom). Each point represent the optical density average of all strains of that group.



Chapter 6: Discussion

6.1 Current State of the Art and Challenges to the Practical Development of Algal Biofuels

Over the past ten years or so there has been a significant interest in the development of microalgae as a novel and renewable feedstock for biodiesel. This subject has captured the public interest, with frequent headlines seeming to suggest that cheap renewable "green" oil is just around the corner, as well as attracting significant interest in the investment world. Well over one billion dollars of venture and big oil capital (Mascarelli, 2009) has been invested in more than one hundred algal biofuels start-ups (Waltz, 2009). Unfortunately, an uncritical listing of the proposed advantages of microalgae for biodiesel production, with encouraging numbers extrapolated from laboratory experiments carried out under specific, idealized conditions, has sometimes led to extravagant claims. This hype has also been fuelled by uncertainties towards the current high dependency on crude oil. This non-sustainable energy source is raising concerns related to environmental, political and economic security (Pienkos and Darzins, 2009)

The production of biofuels using microalgae is promising since of all photosynthetic organisms they have the highest growth rates, and they can be cultivated using non-arable land with wastewater as a source of nutrients. However, much research is still needed before the practical production of biofuels from microalgae become a reality. Very little is known about algal physiology, the regulation of lipid synthesis and general metabolism. Moreover, there are several uncertainties as to cultivation strategies, lack of effective low-cost harvesting methodologies, and the need for an oil extraction and biodiesel conversion technology adapted to algal biomass. Recent advances in some of these areas are encouraging, and the next decade will probably see the successful demonstration of algal cultivation for biodiesel production on a pilot scale or larger, probably at low latitude regions with high solar incidence.

To some degree algal cultivation shares some characteristics with traditional agricultural operations. For example, productivity in cold climates is a natural challenge. As for food crops, algal strains adapted to the conditions found at high latitudes will be probably

necessary. Besides adaptation, isolation of native strains already suited to the local climate could prove to be a fruitful methodology. Indigenous algal species are more likely to show the required robustness to thrive under local conditions and outgrow possible contaminants (Powell et al., 2008).

6.2 Physiological Diversity of Microalgae Strains Native to Quebec and Development of a High Throughput Cultivation Method

Chapter two addresses the hypothesis that bioprospecting can identify native algal strains that would be more robust for local cultivation then those commercially available in culture collections. To perform the isolation and the characterization of a vast number of strains, it was necessary to develop a high-throughput screening method aiming to select strains suitable for cultivation in cold climates and wastewater secondary effluents. Primarily, this method was used to combine nutrient removal and production of algal neutral lipid for potential use as a biofuel feedstock. The high-throughput method developed was successfully applied, allowing the characterization of growth, nutrient removal, biomass and lipid production of one hundred strains under four conditions and with three biological replicates. Using conventional methodologies (Mutanda et al., 2011), the same experiments would demand an enormous amount of resources, approximately 1200 Erlenmeyer flasks, almost one hundred liters of culture medium and 8 months or more of daily measurements. This would probably be impractical, demonstrating the importance of the developed methodology to access the physiological features of the Quebec's algal microbiome. This system consisted in the use of twelve well- microtiter plates for cultivation, construction of a suitable lighting system using LEDs, and the adaptation of all the colorimetric and fluorometric analytical assays. These methods were downscaled to be used in 96 well- microtiter plates, since in the original methodsthe volume of sample required was considerably large. For example, the method to quantify the lipid content demanded 10 to 15mg of wet-cell weight (Akoto et. al, 2005). This would require individual cultivations of 100mL or more per replicate. After the adaptation, only 50µL of the culture is used.

Using these- techniques, this work identified highly efficient strains able to carry out

the nearly complete removal of nitrate and phosphorus from municipal wastewaters, and selected wastewater-grown strains had a neutral lipid content and productivity as high as 45% and 29mg/1/d. A number of strains showed good growth at low temperature (10 °C) and might be useful in a waste-to-biofuel process combining wastewater treatment and lipid production (Abdelaziz et al., 2014).

The analysis of the lipid accumulation and growth rates of the collection as whole, and as a function of culture medium, temperature and sampling location, showed a great variety of physiological responses to the environment conditions. To visualize this diversity, several scatter plots charts were made (pages 81 to 88). The growth rate as a function of the medium (wastewater or BBM) showed a well-dispersed pattern at 10˚C and 22˚C. Although there was a slightly improvement when wastewater was used, some strains showed almost no development when cultivated in wastewater and an above average growth in BBM. Surprisingly, several strains were plotted close to the straight marked at 45˚, where neither conditions favors the other. In terms of temperature (page 82), a slight shift towards 22˚C was observed, but in general this difference was only expressed more in wastewater. The dashed line in the chart represents where neither condition-is favored over the other (45˚) while the straight line (22.5˚ and 67.5˚) represents the point where one variable yielded twice that of the other. In both media, most of the strains are constrained inside these two lines. The occurrence of strains strongly favoring one temperature over the other is more evident on BBM. Here, several strains showed growth rates at 22˚C more than two fold higher than at 10˚C. Eliminating these strains and looking within the two continuous straight lines, the distribution was rather homogenous. In contradiction, the occurrence of strains with slight improvement in growth rates at room temperature was more evident. The characterization of strains expressing small variations of growth rate as a function of temperature or even strongly favoring low temperatures with impressive growth rates under this condition indicates the importance of bioprospecting in unraveling natural adaptations that could improve the development of bioprocesses in cold climate regions.

For lipid accumulation, the medium was shown to be an important variable, while the temperature did not cause a general shift in the cellular neutral lipid content (page 83). The influence of the medium is likely due to the different availability of nutrients. Nitrogen

starvation is known to induce lipid metabolism, and an earlier time point of depletion of nitrates in the wastewater treatment would induce lipid accumulation. Of course other nutrients, as well as other factors, could also have played a critical role in the observed phenotype. However, unfortunately very little is known about algal lipid metabolism, and thus it is not possible to explain with certainty the cause of this effect. With each medium (page 84), there was no trend of lipid accumulation as a function of cultivation temperatures, and this phenotype seemed to be evenly distributed. The lipid content of most of the strains tested on BBM was in accord with the results observed for growth rates in terms of dispersion, although the majority of the strains were concentrated in the lower end of the chart. A similar dispersion was found for wastewater, but as already mentioned, with higher accumulation of lipids than BBM.

Apparently, the sampling location did not have any effect on growth rates or lipid accumulation (pages 85 and 86). Samples from both locations (Laurentian lakes and San Laurence river) yielded strains equally distributed for specific growth rates and lipid accumulation capabilities.

6.3 Lipid and Biomass Productivity During Photoautotrophic, Heterotrophic and Mixotrophic Cultivation

To increase lipid yields during microalgal cultivation, some companies have turned to heterotrophic systems, feeding sugar from sugarcane or any other relatively cheap available sugar (e.g., Solazyme and Algenol). This thesis examined the advantages of mixotrophic cultivation over heterotrophic and photoautotrophic. Ten selected strain were cultivated under three conditions using glycerol or xylose as organic carbon sources. Biomass, lipid production and organic carbon depletion were measured. In terms of biomass production, the organic carbon addition tended to decrease yields in comparison to photoautotrophic growth. Glycerol tended to be preferred over xylose, and the biomass production under mixotrophic conditions was often higher than under heterotrophic conditions, but lower then under photoautotrophic conditions. However, lipid productivity increased by more than two fold in comparison to the photoautotrophic growth. This shows that the toll on biomass productivity was more than paid

back by the lipid production. In general, lipid content during photoautotrophic growth is low until the stationary phase. Interestingly, if the addition of glycerol somehow decreases the cell division rate, it induced accumulation of lipids to a point where the final lipid productivity was higher for eight of the ten strains and more than doubled in four of them. In fact, one of the strains showed more than 200% improvement while another, the only one with a biomass increase as well, showed almost 400% improvement when compared to photoautotrophic numbers. A variety of responses was observed in terms of the growth curve. The strain showing the highest biomass and lipid productivity, LB1H10, clearly showed improved growth and lipid production when either organic carbon source was available. This strain was unique in terms of its versatility. With glycerol, the growth rate was slightly higher under heterotrophic then under mixotrophic growth coditions, but with a final biomass accumulation and lipid productivity higher than for mixotrophic growth. In fact, while heterotrophically grown cultures showed a 35% improvement in lipid productivity, the mixotrophically grown cultures showed nearly a 400% improvement, the highest achieved (page 115). This makes it a putative strain for mixotrophic culture optimization. Besides, the difference in the physiological responses of this strain in comparison with the others suggests that it could be an interesting candidate for deeper fundamental research around the interference caused by assimilation of organic carbon to the overall photosynthesis driven metabolism. Xylose proved to be a more complex carbon source to be efficiently assimilated. In fact, this is the first report showing its assimilation by microalgae in heterotrophic cultures. The strain LB1H10 appeared to have been able to assimilate xylose to a point where the growth rate, biomass and lipid productivity were significantly improved compared to the results obtained under photoautotrophic conditions. Most of the other strains were more productive when using photosynthesis, and xylose seemed somehow to be toxic or inhibitory. In fact, only two strains showed a considerable improvement in lipid productivity when xylose was available, and another two showed only some improvement. Examination of the growth curves suggests that heterotrophic cultivation gave better growth and development than when the cultures exposed to light, suggesting a possible negative effect of light when coupled with xylose assimilation. To verify if the addition of xylose could lead to a damaging photo-oxidative process, another set of experiments were performed, as is discussed in the next section.

6.4 The Effects of Xylose on Algal Cell Fitness and Lipid Accumulation

To analyze in more detail the impact of xylose on algal cells, four strains previously shown to exhibit different phenotypes caused by the addition of xylose were selected. These four strains had been previously identified in a preliminary manner by optical microscopy. This type of identification has its limitations and for this thesis a molecular procedure of identification was developed, based on the sequence of the 18S rDNA and used. . The comparison of the obtained sequences revealed that three of the strains, (PCH03, PCH90 and LB1H09) were members of the genus *Chlorella,* and the last strain grouped within the *Scenedesmaceae* family. A phylogenetic tree was constructed with the alignment of the 18S rDNA to confirm the identification and to reveal the taxonomic rlatedness of the strains (chapter 4, fig. 1, page 139). The tree was rooted only at the phylum level (*Chlorophyta*), revealing the distance between LB1H10 (*Chlorophyceae*) and the other three strains in this study, all of them associated with the class *Trebuxiophyceae*. Four different species of the genus *Chara* were used to root the four strains in this study under the kingdom Plantae. The *Chara* genus is organized under the phylum *Charophyta*, one clade above the divergence of LB1H10 and the other strains. The *Chlamydomonadales* order served as an outgroup for LB1H10, grouping it inside the *Scenedesmaceae* family, probably within the genus *Scenedesmus* or *Acutodesmus*. The three other strains: LB1H09, PCH03, PCH90 were classified as members of the genus *Chlorella*. The genus *Prasiola* and *Lobosphaera* differ from *Chlorella* at the order level (orders *Prasiolales* and *Trebuxiales* respectively). The genus *Micractinum* is related to *Chlorella vulgaris* at the family level and was used to root this genus, and *Chlorella sorokiniana* confirmed LB1H09, PCH03 and PCH90 as different strains of *Chlorella vulgaris*. Although the degree of similarity was too high to resolve the exact positions of the strains PCH03 and PCH90 with respect to A1-65 (accession number KF661335.1), CCAP 211/21A (accession number KJ756823.1) and D2 (accession number JX185298.1); the strain LB1H09, although also a strain of *Chlorella vulgaris,* was consistently separated from these.

An experiment was carried out with photoautotrophic growth until the late log phase, followed by the addition of xylose to a moderate concentration (20mM). Every strain was incubated under four different conditions (+/- sugar; +/- light). As expected, a photoautotrophic culture maintained in the dark during the cultivation experiment did not show any increase in biomass. This was the basis for the evaluation of the heterotrophic cultures, where a lag phase of only 12 to 24 hours showed that assimilation of xylose started rapidly after it was available. Three of the strains kept the same growth rates, except for PCH03 which achieved μ=1.11/d, twice that of the others. Interestingly, this strain showed similar growth curves for both heterotrophic and mixotrophic cultures as if heterotrophic metabolism was favored through a shutdown of the light-dependent reactions. . Indeed, this strain showed the most severe bleaching effect (see below), but there is no data to support this suggestion, and as algal metabolism is largely unknown (including the assimilation of xylose), further experiments would be necessary to establish a relationship between organic carbon uptake and light dependent reactions.

The addition of xylose into the pre-grown cultures seemed to have shown an inhibitory effect (and maybe even toxic), decreasing the density in the first 12 hours. Within two days, a bleaching effect was already evident, with the exception of LB1H10, which seemed remain a healthy culture throughout the whole experiment, and no color difference could be discerned between the photoautotrophic and mixotrophic replicates. This is in agreement with the data from the previous chapter, where this strain showed the best performance on either glycerol or xylose. Indeed, this strain shows a good versatility and thus is a putative candidate for further studies and cultivation optimization.

It could be argued that a drastic change in pH could be lead to this bleaching effect. To examine this possibility, the pH was recorded daily (chapter 4, fig. 2, page 140). Even with a buffered medium, the heterotrophic culture tended to acidify the medium while the pH of the phototrophic cultivation increased. However, this is unlikely to have caused the bleaching effect as the timing was not synchronized. The pH was still within an acceptable range when the cultures started losing color, suggesting it could be a second effect induced by xylose, instead of the cause. With the exception of LB1H10, all others showed a decrease in optical density during the first twenty-four hours after the addition of xylose. The short-term effect on

cell size varied among the strains and over time (recorded by the FSC channel of the flow cytometer). After three days, the cell size tended to be smaller for all strains except LB1H10. Also, the cell density measured by the flow cytometer started to decline rapidly after three days, data that agrees with the optical density readings. This could indicate that xylose might not exert a severe toxicity or cause inhibition by itself; it did not induce photo-oxidative stress; this effect is species specific.

The effects on lipid and chlorophyll content were also tracked using flow cytometry. Yang et al. reported that *Scenedesmus* strains cultivated in a medium containing xylose tended to produce white biomass during growth (Yang et al. 2014). Although this study was not able to demonstrate the assimilation of this sugar, the bleaching effect was noted. The use of a flow cytometer permitted analysis over time of the decrease of chlorophyll content in all strains of the genus *Chlorella.* The content of photosynthetic pigment decreased continuously in a similar manner for heterotrophic and mixotrophic conditions; with a faster decrease under both conditions then with the photoautotrophic culture kept in the dark. This is another indication of the influence caused by xylose on cell fitness. In terms of lipid accumulation, the cultures kept in the dark showed a slight increase in lipid content, not at all comparable to what is seen in cultures exposed to the light cycle. This increase in cellular lipid content does not seem to be linked to any change in cell morphology, as ascertained using the FSC and SSC flow cytometry channels. Nevertheless, after xylose addition cell viability started to drop, suggesting that the xylose toxicity observed could be related to metabolite accumulation (e.g. xylitol, d-xylulose) or some type of regulatory interference on metabolism. In mammals, an intermediate of xylose assimilation, xylulose-5-phosphate, is directly involved in the regulation of lipogenesis genes (Iizuka and Horikawa, 2008). Thus, the regulatory role of xylose or the metabolites involved in its assimilation in microalgae would not be a complete surprise.

6.5 Using CO_2 Enrichment to Isolate Microalgae Strains for Flue Gas Bioremediation and Algal Biomass Production.

The hypothesis for this part of the thesis was that an enriching an environmental sample would favor the isolation of strains with higher chances to thrive under the conditions used for enrichment. After the isolation procedure, it was necessary to compare the identity of the strains originating from both methods. This would allow to ascertain whether the different processes yielded different strains or if any phenotypic differences were merely due to adaptation. Thus, to evaluate the the enrichment process the strains isolated using the conventional strategy and the proposed process were sequenced, identified and compared using the 18S rDNA and rbcL (RuBisCO large subunit) genes. The 18S rDNA, being essential, is highly conserved between separate distant groups. The amplified gene had 1.6Kb, enough for the resolution needed. The Sanger sequencing method was used, and due to limitations of the technique, a maximum of only 1Kb can be obtained, but still with high risks of mismatches at the beginning and end of the fragments. To have a fragment of this size sequenced with a low rate of mismatches, it was essential to use three primers: two externals and one internal. Thus, for each strain, three sequencing reactions were made. The two external primers would have only a small part of complementation, which is not ideal since both would be at the end of the fragment, a section more susceptible for reading errors. The third primer was designed to connect these two sections and correct the mismatches. These reads were assembled in a contig, and the consensus strand used for identification and construction of the phylogenetic tree. To differentiate closer groups, however, as is the case for strains of the same species or very related species, the differences of the 18S rDNA could not be enough to define relationships. Therefore, the variability found in the RuBisCO gene was explored through the comparison of the sequences of the gene *rbcL*. The sequenced region was of about 900 bases, and two reactions (both directions) were made for each strain.

The redundancy of isolates, meaning the strains that were picked more than one time through the isolation process, was small. In one case, three isolates were shown to be the same strain, in two other cases, two isolates were reduced to one strain. Thus, seven isolates were

reduced to three. The redundancy happened only inside a specific treatment and was more frequent in the conventional isolation procedure. Of course, this cannot be just attributed to the conventional method, as both methodologies counted on manual isolation and subjective interpretation of colony morphology. The proposed method undergoes an enrichment process, and it was expected to produce higher redundancy. However, the opposite was found. This could be due to an extra carefulness while choosing colonies, driven by the knowledge that these were previously enriched. Nevertheless, the main purpose of this step was to verify if the same strains were being isolated in the conventional and proposed method. The absence of redundant strains between the two procedures was surprising and showed that a simple enrichment is capable to shift the selection of different strains.

The second part of this chapter compared the biomass production and specific growth rates of the strains isolated in both methods. Here, all the strains isolated were cultivated under atmospheric CO_2 and with an enrichment of 10% to verify if the proposed process would lead to the isolation of strains with better performance.

Of course, the patterns of the strains of both groups were varied, and in both groups there were good and poor performers. One way to evaluate the proposed method is comparing their results as a whole, using the average of the biomass productivities, growth rate, and the average of the optical density data at each time point treatment. The last generated a concatenated growth curve, measured as the mean value for each data point. In the average, both groups achieved about the same biomass accumulation when cultivated under 10% CO_2. This was expected since the usual limiting factor for biomass accumulation in a given medium is the availability of nutrients, and the average capability to assimilate nutrients of both groups should not divert significantly. As for growth performance, the strains isolated through the proposed method tended to perform better under both conditions..

Under atmospheric CO_2, the growth curve was closer to a linear pattern, a regular characteristic of the algal growth. Nevertheless, the group isolated by the proposed method showed higher growth rate and higher biomass accumulation. Under 10% CO_2, the growth curve pattern resembled a logarithmic curve. The enriched isolation group showed on the average 43% higher productivity, 11.25% higher growth rate and 27.9% higher biomass accumulation at the optimal harvest point. This point is calculated as the maximum biomass

production rate, dividing biomass concentration per days of culture.

The average performance of the group isolated from the enrichment process showed it was more likely to reveal strains with higher productivity in this environment. They were able to achieve the stationary in less than half of the time needed for the group isolated using the conventional method. This is a significant factor for cultivation, resulting in higher productivity, faster harvesting time and less risk of culture crash, as it would be exposed for a shorter period of time.

Chapter 7: Conclusion and Perspectives

7.1 Conclusions

The collection built during this study is a unique representation of the microalgal diversity in the south of Quebec, including one hundred strains from different aquatic niches. The characterization of the individuals in this collection revealed strains for possible application in nutrient removal and lipid production even in relatively low temperature (10 °C). Exploring this collection in terms of specific growth rates and lipid accumulation as a function of optimal temperature and medium type showed that there was remarkable physiological diversity. Indeed, the full spectrum of responses, strongly favoring one condition over the other, or relatively indifferent, appeared to be present. Within the collection were some strains of potential biotechnological interest in terms of wastewater treatment or lipid production. Of fundamental interest are strains showing good growth at low temperatures. Other physiological characteristics could be of putative interest for industrial exploitation.

Mixotrophic cultivation could represent an opportunity to increase algal biomass productivity and be a solution for bioremediation of several different industrial wastes. Glycerol is an important biodiesel waste while xylose represents more than 50% of the hemicellulose discharged by the pulp industry. In this chapter, ten strains native to Québec were shown to successfully assimilate glycerol or xylose to improve lipid production. The lipid productivity in mixotrophic growth with glycerol was improved several folds. One strain was found to be highly versatile, increasing biomass and lipids when either, glycerol or xylose was available.

During the experiments carried out in chapter three, it was observed that xylose was causing curious effects on algal cultures. In chapter four these effects were studied in more detail. Here, the short-term impact of the addition of this pentose was analyzed using a flow cytometer. Surprisingly, xylose was shown to induce fast lipid accumulation, with the cellular lipid content increasing more than four fold in only six to twelve hours. On the other hand, this phenotype was followed by chlorophyll degradation and possible subsequent cell lysis, as observed by the natural chlorophyll fluorescence and cell counts. One strain was not

negatively affected by xylose. The assimilation of this sugar was evidenced by the increase in biomass and depletion of the pentose in the medium, even if the cell lipid content was shown to remain approximately constant. These results indicate the potential use of xylose as a short-term booster and of the possible application of the strain LB1H10 for use of bioremediation. Besides, several fundamental questions regarding the xylose metabolism and the effects of its precursors were raised and answering them could reveal new pathways and insights on the regulation of algal lipid metabolism.

The use of bioprospecting for the isolation of strains for commercial use is highly debated. While some believe that there are already enough algal strains available in culture collections, others believe that specific challenges would be better faced by locally isolated strains. In chapter five this subject was explored in a new direction, using the conditions related to a theoretical cultivation coupled with bioremediation. These conditions were used to enrich environmental water samples before the isolation process. When compared to the conventional isolation procedure, the proposed enrichment method yielded strains that were not genetically related. This was verified through the alignment of the sequenced 18S rDNA together with an internal fragment of the *rbcL* gene. Thus, the enrichment process was more likely to yield different strains instead of working as a pre-adaptation process. These new strains were more likely to have a better performance for biomass accumulation then the strains isolated through the traditional method.

7.2 Perspectives

Algal biofuels were advertised as the solution to the energy shortage. Microalgal lipids can be readily converted to biodiesel, and its yields are higher than vascular plants. Nevertheless, the hype around the algal technology is fated to fade soon. There are several constraints at many of the links in the production chain. The lack of fundamental research on microalgae is among the factors slowing the development of this technology. At the cultivation link, much research is still needed to improve productivity and ease harvesting. From the bioprospecting point of view, every site of production has environmental particularities, and different sorts of driven isolation could be adopted. Also, co-cultivation

could represent an extra protection against contamination and easy harvest through bio-flocculation, but little has been done in this direction. Exploring bioremediation processes for microalgal cultivation is also a promising direction. Industrial and domestic wastes can be used for the simple production of biomass or specific molecules. Microalgal production of nutraceutical products is already a reality but on a very small scale. The production of medium and high-value molecules is more likely to achieve economic feasibility. Microalgae naturally produce several molecules of interest for these industries, including essential fatty acids and anti-oxidants. Addressing these markets could help finance the applied algal research for biomass production, harvest, and extraction technologies.

In another direction, the development of molecular tools for genetic manipulation of microalgae is also an open venue. There have been just a few methods proposed and, in general, when the manipulation is doable, stability is still a concern. The development of molecular tools would also certainly help in increasing our understanding of metabolic regulation in algae and open new perspectives for microalgal production.

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Annexe I: Review Articles and Book Chapters Partially Used for the Chapter 1 (Literature Review)

The literature review presented in the chapter 1, contain parts of one article review and two book chapters. These were published during the course of my PhD training; they are annexed here on its whole form and are also available in the online at the addresses bellow.

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Algal biofuels: Challenges and opportunities

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HIGHLIGHTS

- Algae are promising for biofuels production.
- Higher productivity and lipid content than plants.
- Open ponds are better than PBRs for biofuels.
- Technical hurdles include harvesting and oil extraction.

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ABSTRACT

Biodiesel production using microalgae is attractive in a number of respects. Here a number of pros and cons to using microalgae for biofuels production are reviewed. Algal cultivation can be carried out using non-arable land and non-potable water with simple nutrient supply. In addition, algal biomass productivities are much higher than those of vascular plants and the extractable content of lipids that can be usefully converted to biodiesel, triacylglycerols (TAGs) can be much higher than that of the oil seeds now used for first generation biodiesel. On the other hand, practical, cost-effective production of biofuels from microalgae requires that a number of obstacles be overcome. These include the development of low-cost, effective growth systems, efficient and energy saving harvesting techniques, and methods for oil extraction and conversion that are environmentally benign and cost-effective. Promising recent advances in these areas are highlighted.

1. Introduction

The transportation sector plays a major role in the production of greenhouse gas (GHG) emissions, as well as being responsible for 28% of total world primary energy consumption, mainly consisting of fossil fuels, and for 71% of the total crude oil used (Energy, 2004; Pienkos and Darzins, 2009). Transportation fuels can be divided into three groups related to use: private vehicles (gasoline); commercial vehicles and stationary engines (diesel); or jet fuels (kerosene). World consumption of diesel was nearly 1460 trillion liters in 2011 (OPEC). Fuel demand in the transportation sector is projected to increase by 40% over the period 2010–2040 (ExxonMobil, 2013). Most of this demand is driven by the commercial sector with heavy duty vehicle (diesel) fuel use increasing by 65%. Although the number of light-duty vehicles (cars) could double, the increased fuel demand might be largely offset by increased fuel efficiency and the switch to hybrid technologies (ExxonMobil, 2013).

Any plan to lower GHG emissions will require the substitution of at least part of the petroleum-based fuels used for transportation. Today we "*borrow land from the past*" (Wackernagel and Yount, 1998), by using carbon which was fixed in another era. Even at present prices, crude oil is cheap, easily extracted and easy to use since it just needs to be taken from its natural reservoir and distilled into products. However, its use reintroduces into the atmosphere carbon trapped millions of years ago. In addition to the role of fossil fuel combustion in climate change due to the increased concentration of CO_2 in the atmosphere, a well established mathematical model used to calculate crude oil field reserves and production capabilities predicts peak oil within the next few decades (Nashawi et al., 2009).

After a hundred years of intensive use, humanity has become strongly dependent on fossil fuels, we are addicted to oil. The world's economy relies on the very efficient system of production, distribution and use that has been developed. Any transition to a new fuel will have to be "painless", using the technology and infrastructure of the existing system as much as possible. The first generation of biofuels fit this model as bioethanol and biodiesel require minimal or no adjustment of regular internal combustion engines, and can generally be distributed, stored and pumped like conventional crude oil-derived fuels. The major drawback to the use of these alternative fuels is that arable land is used to farm the corn, sugar cane or oil seed

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crops needed to produce these fuels. In addition, it would be impossible to produce the quantity of biofuels that would be necessary to meet present fuel demands using first generation technology. In 2010, the US consumed nearly 220 trillion liters of diesel (Energy Information Administration, 2012). To produce this volume of fuel using soybeans for example (average yield of 600 liter per hectare), would require 367 million hectares, in contrast with the only 178 million hectares that is currently available for cropland and the 930 million hectares of total US land area (EIA, 2012). In addition, the commodities used for first generation biofuels production have other possible markets as sugar, animal feed or cooking oil. A farmer will negotiate the selling price of his product in order to profit as much as possible, enhancing even more the competition between food and fuel and creating a complex fluctuation of food prices linked to fuel demand. With actual world production of biofuels at 109 trillion liters per year (86.6 trillion liters bioethanol, 24.4 trillion liters of biodiesel) (EIA, 2012), there has been a great deal of speculation as to whether or not this is already happening. Thus it is clear that although production of first generation biofuels was an important step, it is however only a palliative solution and is untenable in the long term.

2. Microalgae

The call for advanced biofuels demands "drop in" fuels able to be used with the existing infrastructure for storage and distribution, from manufacture to the final customer, but with a production system able to be scaled up without competing with food crops for land. Microalgal biodiesel has been proposed as the most obvious choice. Microalgae are oxygen producing microorganisms containing chlorophyll "a", mostly autotrophs, using atmospheric CO_2 as primary carbon source whereas some can grow mixotrophically, facultatively using an organic source of carbon in addition to CO_2, or even heterotrophically, using only previously fixed carbon as a carbon source. Some are obligate heterotrophs, unable to perform photosynthesis due to a defective plastid. Thus, microalgae can be pictured as single or associated cells floating in oceans, rivers or lakes and using sunlight to produce and store fixed carbon. Thousands of prokaryotic (cyanobacterial) and eukaryotic species match this description; they are the primary producers in oceans, supporting three-fourths of the planetary food chain. The ancestors of microalgae go all the way back to the origin of life and have been directly linked to past events of climate change, transforming the composition of the Earth's atmosphere by the production of O_2, and mitigating CO_2 by sinking fixed carbon deep in the ocean (Buesseler, 2012).

By the above definition, the term "algae" is an artificial way to group tens of thousands species which are in fact taxonomically distributed over several kingdoms; Protista, Chromista, and Plantae (Woese et al., 1990; Cavalier-Smith, 2004; Guiry and Guiry, 2012). These organisms inhabit the most divergent environments, with some species colonizing the Earth's poles and others causing blooms in the tropics (de Morais and Costa, 2007; Cellamare et al., 2010; Mutanda et al., 2011; Pereira et al., 2011). They are found in hyper-saline to fresh water environments, over a broad range of pHs, and even relatively dry environments such as soil and rocks. Microalgae are adapted to inhabit almost any place with enough humidity, and many are also able to enter into a dormant state until there is enough moisture to resume metabolism. They are taxonomically rooted with the ancestor of land plants, an organism formed by the endosymbiosis between a heterotrophic eukaryotic host cell and a cyanobacterium, which formed the plastid. This event is thought to have happened 1.5 billion years ago (Yoon, 2004) and subsequent differentiation and further endosymbiotic events gave rise to branches such as the green algae and the red algae.

Table 1
Conventional diesel cost as of August 2012 (retail price US-$1.05/L) {EIAUSEnergyInfo:tm}.

Diesel fuel cost	Share (%)	Value (US-$)
Taxes	12	0.126
Distribution and marketing	14	0.147
Refining	14	0.147
Crude oil	60	0.630

3. Algal biofuels

Any organism dependent on sunlight as its primary energy source needs to store energy-rich compounds to avoid starvation when light is not available. Vascular plants synthesize a variety of energy rich molecules to save enough energy from the sunlight period for a rainy day (or night). A Canadian example would be the maple tree and the phloem with its high sugar content (Maple Syrup). Vascular plants often produce oil as a carbon reserve for germination. To increase embryo viability, some plants accumulate part of the energy in the seed as TAGs (triacylglycerols), which is historically accessed by press extraction (e.g. olive oil). Microalgae are capable of the synthesis and accumulation of a variety of high energy molecules, including fatty acids (FA) and TAGs, the major feedstock for biodiesel production.

However, species with a high lipid content are not phylogenetically related, occurring in different kingdoms, Protista (e.g. Dinoflagellates), Chromista (e.g. Diatoms) and Plantae (e.g. Chlorophytes). TAG content varies among strains of the same species in quantity and quality (Leite and Hallenbeck, 2012). Nevertheless, lipid content higher than 50% is frequently described in many species, which represents one of the advantages of using microalgae instead of vascular plants for biodiesel production. Only the seeds of a vascular plant are used when making plant-derived biodiesel, with the rest of the biomass usually considered waste. Consequently, the aerial production yield of lipids from microalgae has the potential to be many times higher than that of the already developed technology of oil seed crops, with the advantage of not requiring arable land. Another key factor for choosing microalgae as a system for biodiesel production is their potentially minimal nutritional requirements. Microalgae can be grown on fresh or marine water, on marginal lands, and even in association with wastewater treatment plants or industrial parks where their cultivation offers the additional benefit of bioremediation. After the extraction of hydrocarbon for biodiesel production, the biomass can be processed in an anaerobic digester for methane production, a secondary source of energy, with the digester effluent fed back into the algae cultivation system as a source of nutrients. Even though production with such a system may not completely satisfy local fuel demands, it will evidently lower the importation of fuel, creating a decentralization of production (Table 1), improving the local economy and helping the environment.

4. Cultivation

Two basic alternatives for microalgae cultivation exist and their relative merits are the basis of ongoing debate. Some of the factors involved are listed in Table 2.

4.1. Photobioreactors

These are systems where the cultures are enclosed in some transparent recipient. Photobioreactors (PBR) can have different sizes and shapes: plastic bags, flat panels, tubes, fermenter like and others. Vertical tubes are among the most popular system

Table 2
Photobioreactors and open ponds; pros and cons.

Photobioreactor	Issue	Open ponds
Easy	Control of of culture conditions pH, temp., dissolved CO_2	Medium
Low	Susceptibility to culture contamination	High
Low	Water evaporation	High
High	Productivity per m^2	Medium
High	Energy input	Low
High	Structure cost	Low

Table 3
Photosynthetic efficiency train.[a]

Minimum energy loss	(%)	Percentage remaining (%)
Radiation outside useable range (non-PAR)%	55	45
Reflection	10	41.5
Transfer to reaction center	21	32.8
Conversion to chemical energy	65	11.5
Respiration	20	9.2
Photosaturation and photoinhibition	40	5.5

[a] Taken from Leite and Hallenbeck, (2012).

due to their realtively easy maintenance, low cost and high surface to volume ratio (Suali and Sarbatly, 2012). Among the advantages of using photobioreactors are resistance to contamination by wild algae strains or herbivores, high productivity per unit area, and the possibility of easily controlling various parameters (Table 2), including pH, temperature, and light intensity. The PBR can be placed indoors or outdoors, using sunlight, artificial light or a mixture of both. An interesting variation of a lighting system is the use of optical fibers to carry the outdoor sunlight into an indoor culture (Chen et al., 2008). Artificial light can be provided by any regular light source such as tungsten or fluorescent bulbs. The use of LEDs (light emitting diodes) is increasing due to their low heat generation, lower power consumption and the specificity of the wavelength of emitted light, allowing the restriction of light to PAR (photosynthetic active radiation) and even the study of the influence of different wavelengths and intensities on these microorganisms. A recent study showed that different wavelengths may have a significant influence on biomass and lipid productivity, as well as on the lipid profile. A locally isolated strain of *Nannochloropsis* showed a higher growth rate, lipid productivity and different lipid profile under blue light (470 nm) when compared with growth under white, red (680 nm) or green (550 nm) (Das et al., 2011b).

4.2. Open ponds

Open pond cultivation is carried out in shallow basins open to the environment. The most common types are raceway, circular, inclined and unmixed. They are considered relatively inexpensive and easy to construct, as long as the area is relatively flat. Cultivation can be made directly over the soil or some simple surface covering can be used to minimize water loss due to seepage, and other improvements can be made to increase solar energy capture, and decrease contamination issues. Mixing can be provided effectively with low cost and low energy consuming paddle wheels, which can be enough to maintain aeration and nutrient dispersion. Due to the low depth and large surface area, water loss through evaporation can become a major issue, limiting its operation to areas where low cost water is available. Marine waters and wastewaters are good matches for this system, as environmental and sustainability issues would prevent large open pond cultivation using potable water.

Operation and maintenance costs are relatively low. Thus, this system is capable of generating biomass production at the best price. There is already some experience on large scale production using these types of systems, either in pilot projects partially funded by the government, in wastewater treatment plants, where it is used in secondary or tertiary treatment of sewage, or in commercial scale algal cultivation for the health food market. As a bioproduction system, its simplicity is a double-edged sword. The contamination risk level is high and a strain with high lipid productivity can easily be overrun by a fast growing wild strain (Sheehan et al., 2003). Another dangerous type of contamination is herbivores. There is not much information available on how to deal with predation, but it is well known that they are capable of clearing a high density pond in a matter of days.

4.3. Productivity

The purpose of the mass algal culture and local weather conditions may make the choice of system obvious. However, excluding these special needs and conditions, the main comparison between the two systems is principally cost and productivity. Regarding the productivity per unit area or volume, PBR are said to outperform open ponds. PBR structures can be made vertically, creating a high density cell culture in three dimensions. Open ponds require larger and more level cultivation areas to achieve the same productivity. The low mixing rate of open ponds intensifies the self-shading effect due to cell concentration (Table 3), and the physical structure of open ponds prevents proper aeration, causing a low medium CO_2 partial pressure. These effects limit the productivity rate per unit of area and volume, requiring a larger area to achieve the productivity of a PBR.

Of course PBRs have several advantages over open ponds as a cultivation system. However, an open pond is considerably cheaper. The total cost can be analyzed as infrastructure costs (CapEx), maintenance costs and operational costs (OpEx). All are in favor of open ponds. The installation and maintenance costs of PBRs may prove prohibitive for the production of low cost compounds, but acceptable for the nutraceutical industry. Carotenoids and some poly-unsaturated fatty acids (PUFAs), such as omega-3 and linoleic acid, are some of the high value products that can be produced in a microalgal system, where closed cultivation is more easily justified. Nevertheless, the development of PBRs is being pushed by research on microalgal biodiesel. Different PBR designs are being tested and some studies showed high productivities using systems requiring only relatively simple operation and maintenance. Using innovative 110L flat green wall photobioreactors, a production of 204 mg L^{-1} d^{-1} was reached (Rodolfi et al., 2009). Thus, open ponds offer a cheaper operation, but at the expense of productivity. Long term studies with outdoor open ponds have reported productivities ranging from 20 to 50 mg L^{-1} day^{-1} (Das et al., 2011a; Moazami et al., 2012).

4.4. CO_2 enrichment

One approach to raising productivity is to increase the concentration of CO_2 (Sheehan et al., 2003; Lin et al., 2012). In fact, the enzyme responsible for CO_2 fixation, Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase), has a low affinity for CO_2 and also functions as an oxidase of 1,5-bisphosphate, interacting with O_2. Therefore, O_2 is a competitive inhibitor with CO_2 and since to the atmospheric concentration of CO_2 is much lower than that of O_2, oxygen can have a significant effect. Evolutionarily this problem has been managed by the development of carbon concentration mechanisms (CCM), where the cell locally increases the CO_2 concentration around the Rubisco enzyme to ensure its function

in CO_2 fixation (Giordano et al., 2005). This mechanism is wide spread amongst the algae and illustrates the advantages of raising the CO_2 concentration in mass cultures. Indeed, sparging CO_2 into the culture medium is known to increase its cellular concentration and two different approaches are frequently reported, the use of CO_2 to adjust the pH, and CO_2 enrichment as a way to mitigate flue gases (Grobbelaar, 2000; Rodolfi et al., 2009; Yoo et al., 2010; McGinn et al., 2011). Of course any feedstock used in large scale production will play an important role on the final price and CO_2 is not an exception. Thus, this type production should optimally be coupled to a bioremediation process.

5. From biomass to biodiesel

5.1. Harvesting

In a general sense, the production of microalgal biodiesel is very similar to the production of first generation biodiesel. The biomass is produced, harvested; lipids are extracted and then processed through transesterification into FAMES (Fatty Acid Methyl Ester), commonly called biodiesel. However, unlike oil seed plants, harvesting microalgal cells can prove to be quite challenging. The tiny cells floating in water cannot be accessed as easily as macroscopic plants, and consequently oil extraction gets more complicated than the centuries old press procedure traditionally used for oil seeds. Moreover, algal cultures are very dilute, usually around 1% for autotrophic growth up to 10% for heterotrophic growth (Wu and Shi, 2007; Gouveia and Oliveira, 2008), and dewatering is necessary prior to biomass use. Many standard techniques have been evaluated for use in mass algal cultivation and their limitations are reviewed in detail elsewhere (Molina Grima et al., 2003; Mata et al., 2010; Zhu and Ketola, 2012).

Thus, harvesting can be done at once or divided into different steps, each one varying depending upon the desired final total solids concentration. Usually, the first step produces nothing more than a green slurry, and further drying may be necessary. Of course, the choice of harvest method will vary depending on the ultimate use of the biomass. Nutraceutical products may require physical processes for harvesting, thus avoiding chemical contamination, and maintaining the product's natural characteristics. In this case, the high value of the product will compensate for the high cost and energy intensity of the method. Continuous centrifugation is the preferred method when the algal culture will be used for fish feeding purposes, due to a longer shelf-life. This method is very effective and it is still the most widely used due to its efficiency and well documented techniques (Heasman et al., 2000; Molina Grima et al., 2003). However, it is among the low value high demand products, such as biodiesel, that harvesting and dewatering methodologies play a key role and, the use of energy intensive process for harvesting, such as centrifugation and tangential filtration, can represent 20–57% of the final biomass cost (Molina Grima et al., 2003; Van Den Hende et al., 2011) and compromise the overall net energy ratio (Sander and Murthy, 2010).

5.1.1. Possible promising harvesting technologies

Thus, one major hurdle in developing a viable biodiesel from microalgae production process is how to effectively harvest the biomass in a cost-effective manner (Uduman et al., 2010). A variety of methods are potentially available, including; centrifugation, flocculation, filtration, sedimentation, and mat formation, and, as reviewed below, a number of recent studies provide some hope for the near-term development of a cost-effective harvesting technology. Of course, how effective many of these are can sometimes be species dependent. Thus, acceptable harvesting procedures can be highly dependent on the cultivation method. Although, as discussed above, open pond systems are to be preferred for biofuels production for a number of reasons, these are likely to produce mixed cultures, or at the very least, monocultures whose composition differs according to location specific conditions. Thus, techniques that rely on species specific characteristics can probably only be successfully used with cultures grown on photobioreactors where, at least in principle, some sort of species control is possible.

5.1.2. Centrifugation

As noted above, centrifugation has been the method of choice in small scale studies since it is highly effective and capable of harvesting all but the most fragile species. Yet, it has been argued that this method is too energy intensive for application to what is essentially a low value product where there is a need to keep as high a NER (Net Energy Ratio) as possible. This is undoubtedly true if high levels of removal are sought. However, it has recently been argued that acceptable costs can be obtained by increasing the flow (i.e. volumetric throughput) and accepting a lower capture efficiency (Dassey and Theegala, 2013). These authors found that energy consumption could be decreased by 82% when only 28% of the algal biomass was collected resulting in a harvesting cost that they estimated to be $0.864/L oil.

5.1.3. Flocculation

Flocculation is a well-known process that has been used for years to remove algae and other suspended particles from water during treatment to produce potable water. In this process externally added compound causes the suspended algae to form flocs, which if of the correct size, will freely sediment. In fact, floc formation is a physic-chemical process and the resulting particle size is a function of mixing speed (Hallenbeck, 1943). Due to the negative charge of microalgal cell walls, they tend remain dispersed in solution. Flocculation agents can neutralize this charge, causing the cells to aggregate and settle, which facilitates the harvest process. Chemical flocculation methods and agents that can be used in microalgal cultures have been systematically investigated (Molina Grima et al., 2003; Uduman et al., 2010; Beach et al., 2012; Riaño et al., 2012). A desirable flocculant should be non-toxic, recyclable, inexpensive, and efficient at low concentrations. Due to the massive scale predicted for production of biodiesel, any chemical needed for the biomass cultivation or processing will have a significant impact on market price. Thus, recycling the compounds used for algal cultivation and processing is both an economic and a sustainability issue.

Various chemical flocculants can be applied, alum (hydrated aluminum potassium sulfate) or alkali are traditionally used, but cannot be considered for application in harvesting microalgae for biofuels production because, in addition to cost considerations, their toxic nature precludes further use of the algal biomass, for example for animal feed, after lipid extraction. However, this process might be adapted to make a cost-effective harvesting technology for biofuels production from microalgae if the right compound could be found. Moreover, a recent study suggests that previously projected costs might be too high as it was found that the amount of flocculant required varied with the logarithm of cell density instead of linearly. One widely accepted theory of flocculation is that it works through charge neutralization; the compound added (an alkali normally), neutralizes the negative charges on the surface of the algal cell thus allowing aggregation. Thus, this theory might be thought to predict a requirement for flocculant that increases linearly with cell number. Contrary to this, highly dense cultures were found to require substantially less flocculant, thus potentially substantially reducing costs (Schlesinger et al., 2012). That study proposed that cost effective flocculation using a mixture of calcium and magnesium hydroxides, with a cost of less than $10.00 per ton of algal biomass, could be achieved, due to the low concentration of

flocculating agents required (<12 μM) and the high density of the cell culture used (6×10^7 cell/ml). However, flocculant demand will probably also be a function of the particular algal species since coagulation properties are dependent upon a complex set of characteristics including cell size and extracellular polysaccharide production (Eldridge et al., 2012). In an interesting recent development, it has been shown up to 99% of the biomass can be effectively recovered using ammonia (Chen et al., 2012), which can be recycled into the culture as a source of nitrogen after neutralization of the pH. It is not known if this procedure can be applied to a wide variety of alga species.

Bio-flocculation is a promising and poorly explored alternative. Some algal strains have a natural ability to auto-flocculate under some specific conditions (Olguín, 2012), while others can be flocculated by the addition of a bacterial culture (Kim et al., 2011). This suggests that novel compounds might be found that could be used as flocculants and that would avoid at least some of the disadvantages of presently used chemical flocculants. One example is the newly described flocculant excreted by cultures of *Solibacillus silvestris* which has been shown to efficiently flocculate cultures of the marine microalgae *Nannochloropsis oceanica* and which can be reused (Wan et al., 2012). Likewise, a bioflocculant has been isolated from an autofloculating *Scenedesmus* (Guo et al., 2013). Of course, it is desirable that any flocculant be of use with a wide variety of species.

5.1.4. Filtration

Filtration can be a very effective method of harvest if the species is large enough or grows in filaments. However, again this implies that the desired species be maintained as a nearly homogenous monoculture. Most microalgae are too small to be effectively harvested this way since their small size and extracellular material quickly clog filters that have been tested.

5.1.5. Sedimentation/flotation

Some microalgal species have the peculiar properties of either sedimenting or floating in the absence of sufficient mixing. While this property could be used to advantage in a least an initial dewatering process, once again the applicability of this method would require a high level of species control during cultivation. Moreover, these properties, while possibly leading to low cost harvesting, may also negatively impact mixing requirements since it may be more difficult to maintain these strains as evenly dispersed cells during cultivation.

5.1.6. Biofilm formation

Species that readily form biofilms have been little studied for biofuels production since it is obviously difficult to maintain them as a homogenous suspension in the cultivation medium. However, several recent studies, with two different systems, have shown that this kind of growth mode can offer the ease of simple mechanical harvesting, leading to slurries with a dry weight content of 9–16%. In one case, algae were grown on a rotating drum in what was otherwise an open pond system, and simple mechanical harvesting was achieved by simply unspooling and scraping the cotton "rope" fiber that was used (Christenson and Sims, 2012). In another approach, the algae were grown on a flat surface which was drip-watered. At the end of the growth period the algae were recovered by simple mechanical scraping (Ozkan et al., 2012). Not only was harvesting greatly simplified in both cases, both protocols achieved high rates of biomass production at respectable light conversion efficiencies.

5.2. Lipid extraction and transesterification

The lipids produced by microalgae are usually between 12 and 22 carbons long and can be saturated or unsaturated (Medina et al., 1998). These can be directed for membrane synthesis (polar lipids) or stored as carbon reserve (neutral lipids). For biodiesel production, saturated fatty acids between 12 and 16 carbons are desirable (Srivastava and Prasad, 2012), with the ideal proportion varying depending upon the local climate (Dunn and Bagby, 1995).

In line with the efforts to find a solution for low cost harvesting of microalgal biomass, a great deal of research on lipid extraction is examining wet extraction methods since the harvest process can be simpler and cheaper if biomass with a very low water content is not required. Direct or wet transesterification, is simply the omission of the extraction step, using the whole biomass as feedstock for the reaction. Surprisingly, the exclusion of the extraction step was found to raise efficiency, increasing the lipid yield per gram of biomass (Griffiths et al., 2010). The major drawbacks of this method are the variation of efficiency when applied to different strains, and the use of volatile solvents which are dangerous pollutants.

A more ecological option would be extraction using switchable solvents These solvents can be either a polar or a non-polar, and can be switched between the two by bubbling with N_2 or CO_2 respectively (Jessop et al., 2005). In a polar configuration, they are highly miscible with water, facilitating entry into the cell and contact with the neutral lipids. Once switched back to a non-polar state, they will extract the lipids out of the cells and out of the aqueous phase. Recovery of the lipids and solvent can be performed by switching to polar and then back to nonpolar, avoiding the distillation process commonly used for volatile solvents, and increasing the recovery rate. This green chemistry has been tested with vegetable oil (Phan et al., 2009), yeast, and microalgal biomass (Young et al., 2010; Boyd et al., 2012). Lipid extraction of unconcentrated algal cultures itself might be feasible (Samorì et al., 2010).

When just a simple extraction is used, the fraction, primarily TAGs, must be transesterified to produce molecules, acyl-esters of the free fatty acids. This involves the substitution of an alcohol for the glycerol found in the TAG, with either methanol or ethanol being used, giving FAMEs (fatty acid methyl esters) or FAEEs (fatty acid ethyl esters). This reaction requires a catalyst, either an acid or a base, to occur at reasonable rates at relatively low temperatures and pressures. In practice, the same compounds, methanol (which is cheaper than ethanol but produced from fossil fuel), and sodium (or potassium) hydroxide or sodium methoxide, the same reagents used in production of biodiesel from oil seeds, are commonly used.

6. Biotechnology of microalgal biofuels

Throughout this review various issues that apply to the biotechnology of microalgal biofuels have been discussed. Here we specifically highlight some specific biotechnology issues that are important in the development of large scale algal biofuels production.

6.1. Water and nutrient supply

At large scale the demands for water, for makeup for evaporative losses (especially relevant to open ponds in arid areas), and for nutrients, are enormous. If indeed production is to be done in a fashion that does not compete with food production, and in terms of cost-effectiveness, cheap sources of the major nutrients require, especially the major ones, nitrogen and phosphorous, must be used. In fact, both conditions can be met by using suitable waste-

water. Of course, different wastewater streams vary widely in their composition and nutrient removal (uptake) appears to be a complex function of a number of factors including nutrient levels and species (Cai et al., 2013). The use of algae for nutrient removal from municipal wastewater has been extensively investigated and in general this nutrient stream provides a good microalgal growth medium. Other wastestreams promise to also provide most of the nutrients for abundant microalgal growth (Cabanelas et al., 2013; Cho et al., 2013). Coupling biofuels production with wastewater treatment makes sense since it results in considerable energy savings, important in improving the NER of an algal production process (Beal et al., 2012).

6.2. Strain selection, cultivation and harvesting

The various cultivation strategies were discussed above for the general case of microalgal biomass production. With existing technology it is obvious that for a low value product such as a biofuel open ponds must be used, photobioreactors are simply too expensive, as discussed above (Section 4). A simple calculation based on possible solar energy inputs and maximum photosynthetic conversion efficiencies shows that the resulting energy value per square meter allows very little capital expenditure for cultivation facilities. Therefore, although specific laboratory strains are attractive since they have been shown to produce high levels of lipids, i.e. *Botryococcos braunii*, they cannot be used in practice in an open system since they will quickly be overrun by indigenous species. One solution is to isolate species native to the particular locale, more likely to be able to compete and prosper under the prevailing climatic conditions if provided in a healthy enough inoculum. Cost-effective harvesting is of course a major unsolved challenge, and several new advances have already been discussed above (Section 5.1.1).

6.3. Oil extraction and transesterification

A variety of different novel extraction and conversion procedures are under active investigation with the goal of obtaining high biodiesel yields in an energy efficient manner that doesn't require extensive use of toxic solvents. As pointed out above, the use of organic solvents, the traditional method for oil extraction from oil seeds, should be avoided both from the perspective of eliminating possible toxic pollutants, but also from an energetic point of view given the energy intensive processes required for solvent recovery. One elegant way around this impasse is the use of switchable solvents (Boyd et al., 2012). Since they can be interconverted between having a polar and a nonpolar character simply by using CO_2, solvent recovery through distillation is not required. Moreover, relatively environmentally benign solvents can be used.

A promising recent development is the demonstration of a wet lipid extraction procedure (Sathish and Sims, 2012). In this procedure the harvested algae do not require complete drying prior to extraction, close to 80% of the lipids susceptible to transesterification could be recovered from wet algal biomass (84% moisture content). Other technologies aim at increasing extraction yields through some form of cell disruption, facilitating solvent access. Pulsed field electroporation seems particularly promising in this regard due to its relatively low energy demand (de Boer et al., 2012).

6.4. Economic analysis

Of course, before moving to very large scale microalgal biofuels production, the production system needs to be subjected to a detailed LCA (life cycle assessment), to determine possible environmental impacts, a determination of NER (net energy ratio), and an economic analysis. However, to do this in a meaningful way requires specific inputs on system components, and since many of the outstanding questions raised here; cultivation method (open ponds versus photobioreactors), harvesting technologies, and even extraction and transesterification reactions, remain to be answered, this cannot really be done in a meaningful way at present.

Moreover, an economic analysis which compares the price at the pump of a biofuel with that of a fossil fuel is in fact wrong. For biofuels a metaeconomic analysis is necessary that takes into account indirect costs associated with fossil fuel production and use. A quick overview suggests that there are in fact many hidden costs to fossil fuel use and that the "real" cost of gasoline or diesel is significantly higher than the price paid by the consumer at the pump. The additional costs of course must be paid either now or later in other ways, typically through a higher tax burden. There are of course direct subsidies to the fossil fuel industry, estimated at about $50 billion (USD) over the next ten years in the US alone (EESI, 2011).

Although this number is significant, the real hidden costs of fossil fuel use are much higher. Damages from external effects, such as impacts on the health system, but not including those related to climate change, ecosystems, infrastructure and security were estimated at $120 billion for the US in 2005 alone (National Research Council, 2010). To this of course must be added the costs of climate change due to fossil fuel use. One way to estimate the damage is to look at the cost of adapting to climate change, although this does not provide the actual full costs incurred since this represents less than full mitigation. An initial international study estimated these costs at $49 to 171 billion (USD) per year (UNFCCC, 2007) and it has been argued that this is in fact an underestimate (Parry et al., 2009). Of course, these estimates are highly dependent on the accumulated of atmospheric CO_2 burden over time as well as a great deal of uncertainty as to actual impacts. Thus, determining what the competitive cost of a biofuel really should be will require detailed economic analysis. In addition, as mentioned above, detailed costing is not possible given the many uncertainties in the design specifics of a practical algal biodiesel plant. Thus, a realistic cost analysis is impossible at present.

7. Conclusion

The production of biofuels using microalgae is promising since of all photosynthetic organisms they have the highest growth rates, and they can be cultivated using non-arable land with wastewater as a source of nutrients. However, much research is still needed before the practical production of biofuels from microalgae can become a reality due to uncertainties as to cultivation strategies, the lack of effective low cost harvesting methodologies, and the need for an oil extraction and biodiesel conversion technology adapted to algal biomass. However, recent advances in some of these areas are encouraging, and the next decade will probably see the successful demonstration of algal cultivation for biodiesel production on the pilot scale or larger.

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Chapter 13
Algae Oil

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Keywords Microalgae • Open pond • Photobioreactor • Dewatering • Harvesting • Autotrophic • Heterotrophic • CO_2 mitigation

13.1 The Hope and the Hype

Over the past 5 years or so, there has been a tremendous interest in developing microalgae as a novel, renewable source of biodiesel. This subject has captured the public interest, with frequent headlines seeming to suggest that cheap renewable "green" oil is just around the corner, as well as attracting significant interest in the investment world, with well over one billion dollars of venture and big oil capital (Mascarelli 2009) being invested in more than one hundred algal biofuels start-ups (Waltz 2009b). In this chapter, we examine some of the promising results that have emerged from research on microalgae (Box 13.1) that have encouraged hope in this approach. Unfortunately, an uncritical listing of the proposed advantages of microalgae for biodiesel production, with encouraging numbers extrapolated from laboratory experiments carried out under specific, idealized conditions, has sometimes led to extravagant claims. In some cases, these have even been extended to the level of hype, with claims that go beyond theoretical limits, making algae oil the "snake oil" of the twenty-first century. Some of the proposed advantages of using microalgae for biofuels production rather than more conventional plants are listed in Table 13.1, and discussed in detail in what follows. However, realizing the (realistically stated) hopes of fuels from microalgae will require that a number of very challenging barriers to overcome. In reality, each potential advantage is counterbalanced by a potential limiting downside, or even downright dismissal (Table 13.1). Thus, before

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Box 13.1 Microalgae, cyanobacteria and biofuel production

Microalgae as discussed in this review are simple microscopic, nonvascular eukaryotic plants. Therefore, their photosynthetic capacities are contained in chloroplasts. In the past, other microorganisms capable of water-splitting photosynthesis, the prokaryotic cyanobacteria, were included by botanists in the algae and called blue-green algae. They have been excluded from the main part of this review mostly because no known example of a hyper lipid accumulating strain exists, but also since, apart from the fact that they also carry-out plant type photosynthesis, they are quite different genetically and physiologically. They could become important autotrophic (CO_2-fixing) producers of liquid biofuels in the future due to the relative ease with which they can be manipulated genetically.

Table 13.1 Advantages and downsides to microalgae for oil production

Putative Advantages	Cautionary notes
– Can be grown on marginal lands, urban areas or industrial parks: no competition with food production	– cultivation much more technologically challenging than traditional crops
– Rapid growth under optimal conditions	– optimal conditions, pH, temp, pCO_2, light intensity difficult to maintain – relatively easily over-run by "weeds" – subjet to plagues of "pests"
– High lipid content	– only a few species and under stringent conditions – High diversity of fuel quality/ characteristics
– Sequester or mitigate CO_2 emissions from fossil fuel power plants	– "Enron style" repo 101 accounting, CO_2 immediately released when fuel combusted
– Production possible throughout the year	– Low productivity during winter months, heating may be necessary

algal biofuel production becomes a practical reality, if indeed it ever does, a large number of algal biofuels start-ups are predicted to fail (NY Times, 2011), just as in nature algae often bloom in the spring, only to die off as the season progresses.

There has been a great deal of debate over food versus fuels in contemplating future large-scale expansion of biofuels production. Microalgae offer the advantage of not competing, either directly or indirectly, with food production since they can be cultivated on marginal, nonarable lands, or perhaps even urban areas and industrial parks. Thus, microalgae would appear to be more suitable than more traditional plants, especially food crops (corn, wheat, and soybean). However, humans have tens of thousands of years of experience in cultivating and harvesting vascular plants

and highly efficient mechanized agricultural systems have been developed. As will be seen in more detail below, cultivation and harvesting of microalgae on a large scale is technologically much more challenging than traditional agriculture. Another potential advantage of microalgae is that they have a much more rapid growth rate than plants, doubling their biomass in as little as 24 h, and can, under the proper conditions, be grown year round. Thus, algae would seem once again preferable to vascular plants. However, as opposed to traditional plants, microalgal cultivation requires the strict maintenance of a number of environmental parameters, pH, temperature, pCO_2, and light intensity, to achieve high productivities. Moreover, at least in open ponds, microalgae are much more susceptible to being overrun by "weeds" (unwanted alien algal species) and to being decimated by plagues of zooplankton, with an exploding population capable of quickly "crashing" a high-rate pond operation. Although microalgae can potentially be grown year round, thus increasing overall productivity growth will be diminished at reduced winter light intensities and cultivation systems may need to be heated, indeed even covered, in cold climates.

Another potential advantage of microalgae over traditional plant sources for biodiesel production is their sometimes very high content of lipids. Typical seeds from plants that are grown for their oil have between 18 (soybean) and 41 (canola) oil % dry weight (Mata et al. 2010), thus these figures do not count the total plant weight (stem, leaves, stalk, and roots). On the other hand, some microalgae can be manipulated to contain as much as 90% oil on a total dry weight basis. However, only select species are capable of reaching such high yields, these are basically under nongrowing conditions and not all algae oil may be suitable for biodiesel due to the quality and/or characteristics of the lipids that are produced.

Finally, as discussed in more detail below, microalgal growth is stimulated at higher than atmospheric levels of CO_2 and thus many scenarios site future microalgal growth facilities near industries emitting high levels of carbon dioxide, either power generating plants burning fossil fuels, or fermentation facilities, etc. Some claim that this is an additional benefit; that the microalgae are thus carrying out CO_2 mitigation. However, as detailed in Sect. 13.7, this is in fact spurious accounting and, although beneficial in the sense that algal growth will not require the import of carbon dioxide compressed elsewhere, this could have no rational place in any carbon trading scheme.

13.2 Microalgae

Microalgae form a wide and heterogenous group with species spread among different phyla. Although there are many exceptions, they are commonly defined as oxygen producing photosynthetic microorganisms containing chlorophyll "a." They are mainly found as solitary cells, showing little or no cellular differentiation. Most species occur in aquatic habitats and can be isolated from fresh, brackish or saline waters, although some species can be found in the soil or rocks, in moist or even relatively dry environments. The simplest example of these organisms would be a

single cell floating in a water column producing and storing its own sugar using sunlight and reproducing itself by simple binary cell division. This example would describe thousands of prokaryotic (cyanobacterial) and eukaryotic species that, being capable of using dissolved carbon dioxide as sole carbon source, have relatively simple nutritional demands.

Of course, the actual metabolic diversity is large, not surprising given the heterogeneity of distribution of these organisms in the tree of life and their long history of evolutionary adaptation. Thus, obligatory heterotrophic species are known, most of which contain a defective plastid (chloroplast) incapable of carrying out photosynthesis and thus making the cells dependent on external carbon sources. In some cases, the obligatory heterotrophs live as parasites. However, many species are metabolically versatile and can either grow autotrophically or heterotrophically, depending upon the environmental conditions.

13.2.1 Distribution and Phylogeny

Microalgae can be isolated from virtually any aquatic environment, from fresh to hypersaline waters. Some species are even found in nonaquatic environments such as rocks or soil. Many microalgae can survive in very dry or cold habitats, entering into a metabolically dormant state until enough moisture becomes available to resume metabolism (Graham et al. 2009). They are, together with the seaweeds and cyanobacteria, the only primary producers in the oceans, supporting directly or indirectly most of the life on 71% of the Earth's surface (Andersen 2005). In addition to the marine environment, they also play a crucial role in fresh or brackish water lakes, rivers, and soil, either directly supporting the food chain with their biomass created by photosynthetically drive carbon fixation or by recycling nutrients.

The term "algae" is an artificial attempt to group organisms with an incredible variety of morphologic and physiologic characteristic. There are over 30 thousand species already described, whereas some authors estimate that this number could easily reach a million (Bell and Hemsley 2004). Detailed analysis using new techniques and more recently the advent of phylogenetic studies using rDNA data (Box 13.2), have shown that many species derive from critical differentiation events occurring prior to the common ancestor of plants, thus they are now wide spread in five Kingdoms among the domains Prokaryota and Eukaryota (Fig. 13.1).

Of course most of the species described are capable of autotrophic growth, using photosynthesis to provide the energy necessary for carbon fixation and the formation of sugars and other cellular components, including lipids. However, the ability to hyperproduce lipids as an energy reserve is not taxon specific and is present "randomly" in species of distant groups (Table 13.2). Three kingdoms group most of the lipid producers known and will be described further: Protozoa, Chromista, and Plantae.

Protozoa: One species of Dinoflagellate is already being used for the industrial production of a nutritional supplement for infant formulas, a PUFA (polyunsaturated fatty acid) containing DHA (docosahexaenoic acid). However, this group is more likely to be associated with the production of high market value products than with biofuels.

Box 13.2 The use of phylogenetics for the taxonomic classification of microalgae

Traditional taxonomy uses the morphological and physiological characteristics of an organism to classify it in an ordered scheme. For example, the presence of a notochord during the embryonic phase characterizes the organism as a member of the Phylum Chordate. These phenotypes are associated with its probable order in natural evolution, allowing the construction of a Tree of Life. The development of molecular techniques allows the use of gene sequences for this type of analysis. However, as noted by Carl Woese, a pioneer in this field, the selection of different genes to make such an evolutionary comparison could radically change the results (Woese et al. 1980), especially for bacteria since microbial evolution is affected by both the vertical inheritance of genes (parental) and the lateral transfer of genetic material. Thus, some phenotypes present in a lineage for several or many generations could in fact have originated from a very distant species. Therefore, in phylogenetic studies it is important to focus on very conserved genes, such as the small subunit of ribosomal RNA (SSU rRNA) and its sequence is widely used. This approach provided a strong argument for changing the Tree of Life from one with five kingdoms as originally proposed by Whittaker (Whittaker 1959) to one with three main divisions. Based on SSU rRNA sequences, many microorganisms have been repositioned to more appropriate clades, and algae have been found to have differentiated prior to the common ancestor of plants (Kingdom Plantae).

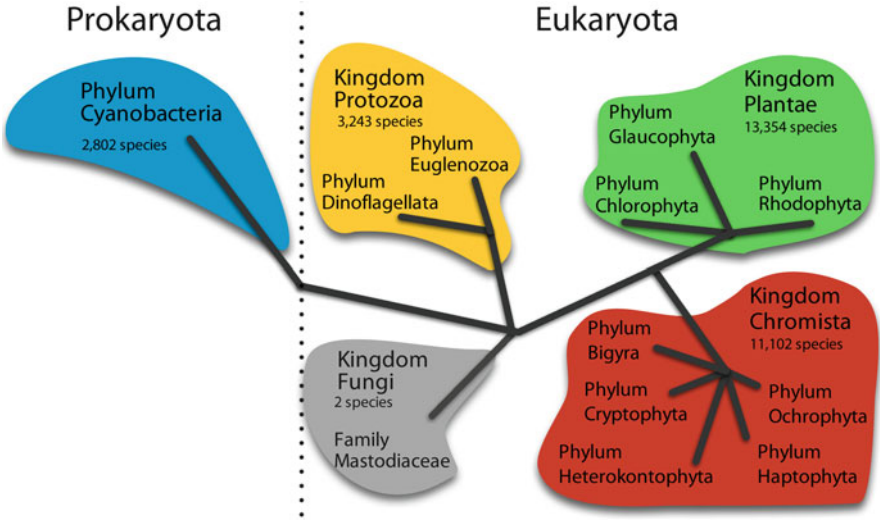

Fig. 13.1 Distribution of algae among groups in the Tree of Life as recognized by the ITIS and Species 2000 (http://www.itis.gov and http://www.catalogoflife.org) in 2011. The deep classification of algae is the subject of great debate and even the higher clades have been discussed and revised recently (Woese et al. 1990; Cavalier-Smith 2009; Cavalier-Smith 2004; Cavalier-Smith and Chao 2006)

Table 13.2 Some groups of microalgae with hyperlipid producing members. This characteristic is not shared with all members of the group, and therefore is not considered a clade factor [Bisby et al. 2011, Guiry and Guiry 2011, Graham et al. 2009, Bell and Hemsley 2004]

Taxon	Rank	Kingdom	Habitat	Reserves	Examples
Bacillariophyceae	Class	Chromista	Marine, fresh water, terrestrial	Fat, chrysolaminarin	Diatoms Navicula sp.
Chlorophyta	Phylum	Plantae	Marine, fresh water, terrestrial	Starch, inulin, fat	Green algae *Chlorella sp.*
Dinophyceae	Class	Protozoa	Marine and fresh water	Starch, fat	Dinoflagelates *Crypthecodinium sp.*
Haptophyta	Phylum	Chromista	Mostly Marine	Fat, chrysolaminarin	Golden brown *Pavlova lutheri*
Chrysophyceae	Class	Chromista	Marine and fresh water	Fat, chrysolaminarin	Golden algae *Chrysocapsa sp.*
Xanthophyceae	Class	Chromista	Mostly fresh water, damp soil	Fat, chrysolaminarin	Yellow-green *Pleurochloris sp.*

Chromista: Among the organisms in this kingdom, the diatoms (Bacillariophyceae) are the most popular in studies of production of biodiesel. Among other characteristics, they have a fast growth rate and are likely to out compete other species in nutrient-rich and relatively cold systems. Some species were shown to accumulate large quantities of lipids. Some haptophytes have also shown to be good prospects for oil production, like Pavlova lutheri, which has a good balance between growth rate and lipid production per dry weight (Griffiths and Harisson 2009).

Plantae: The green algae are the group where most efforts have been focused. Much work has been done with well-defined species, some molecular tools are already available, and some biotechnology companies claim that they were able to enhance the production through metabolic engineering, with, however, no data to this effect being shown yet. Organisms of this group can be found on moist soil and from fresh to saline water environments. Under optimal conditions strains of *Chlorella sorokiniana*, *Ankistrodesmus falcatus*, *Ettlia oleoabundans*, and *Botryococcus braunii* have shown very promising results.

13.2.2 Growth Modes: Autotrophic and Heterotrophic

Many algae are capable of both autotrophic growth, where they obtain the necessary reduced carbon compounds by actively fixing CO_2, and heterotrophic growth, where the necessary carbon compounds are synthesized by assimilating exogenously supplied sugars. Thus, in principle, either growth mode could be used in a scheme using

microalgae for biodiesel production. However, it should be noted that each algal species, even if it is capable of heterotrophic growth, has its own specific capacity for uptake and utilization of organic compounds, and thus this needs to be taken into account when designing and operating a heterotrophic facility (Kröger and Müller-Langer 2011).

While it would perhaps seem counterintuitive to use standard fermenters fed with plant-derived sugars to produce biodiesel with a normally photosynthetic organism, this in fact at least leads to a technologically more simple process since both sugar production and fermentation processes are well understood. Thus, there are no apparent technical barriers to producing biodiesel in this way and, although no detailed cost analyses are available, it should be feasible at moderate cost, probably only somewhat higher than producing ethanol from corn. Yields of conversion of glucose to lipid are in the range of 19–31% with predicted energy efficiencies, glucose to biodiesel, of 29–75% (not taking into account nutrient supply and the energy required for operations) (Kröger and Müller-Langer 2011). In fact, this is the basis for the process being developed by the highly rated biofuels company, Solazyme, which has received over $100 million in funding. It should be pointed out however that producing algal biodiesel heterotrophically does not benefit from many of the proposed advantages of algal biofuels (Table 13.1) since the actual substrate is produced using traditional agricultural methods. In fact, at present, this would make biodiesel produced by microalgae using heterotrophic metabolism, a first-generation biofuel, no better than biodiesel from soy or ethanol from corn.

Nonetheless, most schemes for producing biodiesel from microalgae are based on the ability of these organisms to capture sunlight and carryout photosynthesis with water as the substrate, using the metabolic energy that is generated to fix carbon dioxide. Of course, carbon fixation proceeds by the well-known Calvin–Benson–Bassham cycle using the key enzyme Rubisco. This enzyme has a relatively low turnover rate, as well as a low affinity for CO_2, and consequently, synthesis of large amounts are necessary, making Rubisco the most abundant protein on earth. In fact, the cellular content of this enzyme is so high that it is usually found in an almost crystalline form, often sequestered in special organelles, carboxysomes in prokaryotes such as cyanobacteria, or pyrenoids in some eukaryotic algae. The energy requirement for CO_2 fixation by this pathway, both NADPH and ATP, is supplied by photosynthesis; ATP by photophosphorylation and NADPH though the reduction of $NADP^+$ by reduced ferredoxin. Three carbon intermediates are withdrawn and used to produce hexose sugars, or broken down to form TAGs (Fig. 13.2). The energy requirement for the formation of a six carbon sugar is shown in the following formula which represents six successive turns of this cycle.

$$6CO_2 + 18ATP + 12NADPH + 24H^+ \rightarrow \text{Hexose} + 18ADP + 16P_i + 12NADP^+$$

Thus, this is an energy intensive process. Since each two electron reduced ferredoxin is produced by two electrons which have been extracted from water and boosted in energy through both photosystem II (PSII) and photosystem I (PSI), each of which gained the energy to do this through absorbing a photon for each electron, the generation of the reducing power (12 NADPH) to fix six CO_2 to create one hexose requires

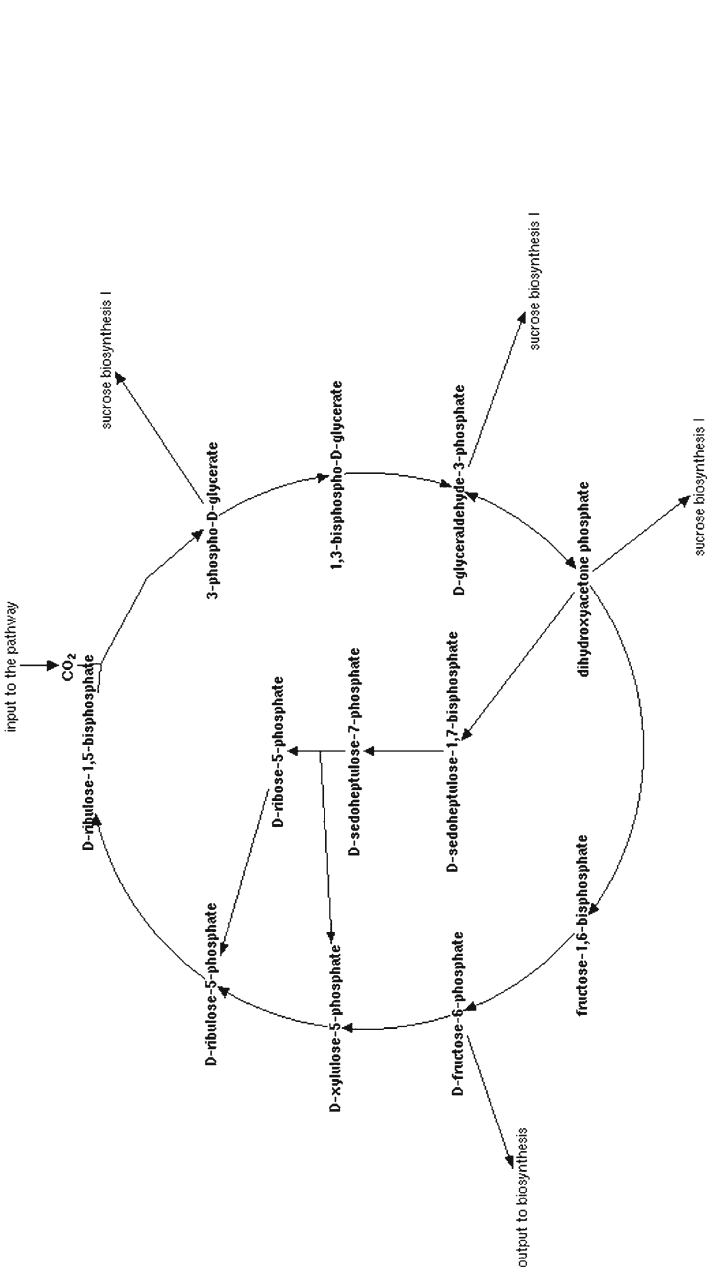

Fig. 13.2 Schematic of the Calvin–Benson–Bassham cycle used by microalgae to fix CO_2. Not shown are the energetic requirements, NADPH and ATP, supplied by photosynthesis

the capture of 48 photons. Each electron passing from PSII to PSI drives the translocation of $3H^+$. Current models of ATP synthase suggest that $12H^+$ are required for the synthesis of three ATP, so in total the passage of the 24 electrons involved in reducing the required amount of $NADP^+$ could generate the necessary ATP ($3 \times (72/12)$). Thus, the fixation of enough carbon to form a six carbon sugar requires the capture of 48 photons or 8 photons per carbon fixed. This is one of the factors that helps to set an absolute limit to the maximum theoretical photosynthetic efficiency attainable (see Sect. 13.3). This quantum requirement is of course higher when biomass synthesis is considered since the biosynthesis of constituents like lipids, proteins, and nucleic acids requires additional energy. Thus, it can be estimated that the light requirement for the fixation of one CO_2 into biomass is more likely 10 or 12 photons.

In a novel variation, a two-stage system has recently been proposed where the microalgae are first grown autotrophically (in the light), expressing high levels of Rubisco, fixing CO_2, and increasing cell biomass. At the end of log phase (120 h), the algal cells are collected by allowing them to settle overnight, and then resuspended in a nitrogen limited medium that supports heterotrophic growth and favors lipid production (45 g/L glucose, 2 g/L glycine) (Xiong et al. 2010). Somewhat surprisingly, these cells had a much (70%) higher lipid yield (0.3 g/g glucose) than cells that had been incubated solely under heterotrophic conditions (0.18 g/g glucose). Among possible reasons for this effect is the suggestion that autotrophically pregrown cells are more efficient since they retain Rubisco and are thus able to refix the CO_2 given off during glucose breakdown (pyruvate decarboxylation). A process based on this concept has recently been patented: US 7,905,930 issued to Genifuel ("A process for production of biofuels from algae, comprising: (a) cultivating an oil-producing algae by promoting sequential photoautotrophic and heterotrophic growth, (b) producing oil by heterotrophic growth of algae wherein the heterotrophic algae growth is achieved by introducing a sugar feed to the oil-producing algae; and (c) extracting an algal oil from the oil-producing algae.")

13.3 Photosynthetic Efficiencies

One of the basic insurmountable constraints on algal production of biodiesel is the maximum theoretical photosynthetic efficiency. Of course, this applies to the production of any biofuel from a resource that is ultimately derived from the solar driven biological fixation of CO_2. This sets an absolute upper limit to the amount of fuel that can be derived per square meter of collector area per year. A series of physical and biological factors combine to reduce total possible energy recovery to only a small fraction of the incident solar radiation (Table 13.3). These issues are covered in great detail elsewhere (Tredici 2010).

First, only slightly less than half (45%) of the solar spectrum can be captured by the photosynthetic pigments of living organisms. An additional amount, estimated as 105, is lost through reflection from the surface of the reactor (or leaf). The reaction center, where the process of charge separation is initiated, leading to conversion of the light energy to chemical energy, is composed of a special chlorophyll *a*, P700,

Table 13.3 Photosynthetic efficiency train

Minimum energy loss		Percent remaining
Radiation outside useable range (non-PAR)	55%	45%
Reflection	10%	41.5%
Transfer to reaction center	21%	32.8%
Conversion to chemical energy	65%	11.5%
Respiration	20%	9.2%
Photosaturation and photoinhibition	40%	5.5%

which absorbs at 700 nm. This creates a downhill gradient for efficient transfer of the excitation energy captured by the antenna pigments which absorb light of shorter wavelengths, but this also means that this fraction of the energy in photons of shorter wavelength is lost (21%). The conversion of the energy which reaches the reaction center to the chemical energy in the fixed carbon compounds that are formed (glucose for example) is only 35% efficient. Some of the chemical energy that is made must be used for respiration to supply the necessary energy to support vital functions of the cell during darkness (20%). Finally, as much as 40% on the average of the light energy that is captured by the photosynthetic apparatus cannot be used by the cells since high light intensities saturate the process; photons are received faster than they can be used and the energy is wasted as heat or fluorescence. Thus, maximum photosynthetic efficiencies cannot be higher in theory than 5.5%, and in practice achieving efficiencies of 1 or 1.5% are exceptional.

13.4 Oil Production

The dependence of photosynthetic organisms on sunlight as their primary energy source necessitates that they have the capacity to synthesize energy-rich reserve compounds to avoid starvation during the nighttime or prolonged periods in the shade. Thus, accumulation of different fixed carbon compounds inside the cell is to be expected, and microalgae are capable of producing many different molecules with high energy content (Table 13.2), including fatty acids (FA) and TAGs which can be converted into biodiesel. The profile of the FAs (and consequently TAGs) produced by microalgae varies considerably between species and even strains and may also vary according to specific culture conditions (Table 13.4) (Abou-Shanab et al. 2011).

The FA composition has a large impact on the potential production of biodiesel since FA length and degree of saturation will greatly influence the resulting fuel properties. For example, the difference between various petroleum derived fuels is basically the length of the hydrocarbon chain, with gasoline being a mixture of saturated chains containing from 6 to 12 carbons, while diesel is basically composed of molecules with chain lengths between 12 and 18 (Srivastava and Prasad 2000). Thus, the length and degree of saturation contained in the FA profile of the microalgae will directly affect the properties of the biodiesel (see Chap. 12 Table 12.4 for details). Although considered as high-value products by the nutraceutical industry, polyunsaturated fatty acids

Table 13.4 Fatty acid composition of some microalgal species (Abou-Shanab et al. 2011)

Specie	Lipid Content (% dry wt)	Lauric acid C12:0	Palmitic acid C16:0	Stearic acid C18:0	Oleic acid C18:1	Linolenic acid C18:3	Others
Scenedesmus obliquus	29%	11%	29%	17%	20%	23%	0%
Chlamydomonas pitschmannii	51%	10%	26%	20%	13%	23%	8%
Chlorella vulgaris	26%	5%	22%	5%	53%	8%	7%
Chlamydomonas mexicana	29%	34%	50%	6%	0%	0%	10%

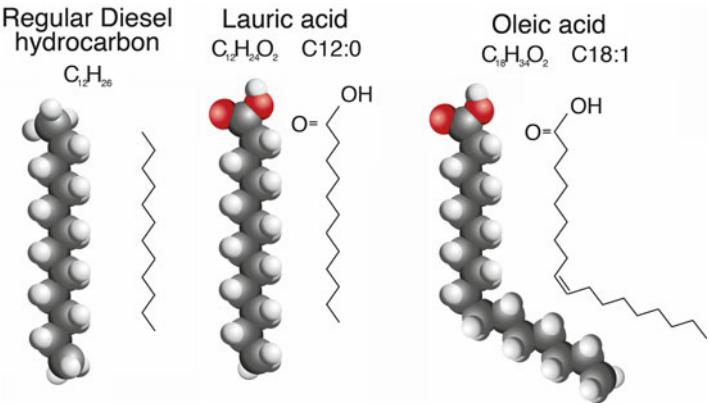

Fig. 13.3 Three different fatty acids differing in chain length and degree of saturation are shown

(PUFAs) are not suitable for the production of biodiesel due to the great structural divergence between these molecules and petrodiesel hydrocarbons (Fig. 13.3).

13.4.1 Biochemistry and Regulation of Lipid Biosynthesis

Of particular interest for the potential production of biodiesel from microalgae are species that are capable of producing high levels of TAGs. TAGs are water insoluble and therefore when they are hyperproduced they are accumulated in lipid bodies, cytoplasmic inclusions apparently surrounded by a membrane containing the normal complement of glycolipids. When lipid bodies were purified from a cell wall-less strain of *Chlamydomonas reinhardtii*, they were found to be about 10% FFAs (free fatty acids) and 90% TAGs (principally C_{16} and C_{18}, 50% saturated and 50% unsaturated) (Wang et al. 2009). Genetic engineering has also been successfully applied to increase oil production in *C. reinhardtii* (Wang et al. 2009). Introduction of a mutation (sta6) that prevents starch accumulation and thus channels more carbon into lipid (TAG) synthesis resulted in a two-fold increase in lipid body content over the wild-type strain.

A major drawback in strategies to improve algal TAG production is the fact that relatively little is known about the details of TAG biosynthesis in microalgae (Hu et al. 2008). Based on sequence homologies among the genes examined, or the enzymatic properties of the few enzymes which have been isolated and characterized, it is believed that FA and TAG synthesis in microalgae follow the same metabolic pathways established for higher plants or for fungi for that matter. Details on TAG synthesis in fungi are given in Chap. 12. The total lipid content of the cell can change drastically under the influence of a number of factors. Understanding the molecular mechanism(s) behind this effect is obviously of great importance in using naturally occurring oleaginous strains or in designing new ones. However, in reality no details are known yet. A number of mechanisms could be at play, either separately or together. Increased enzyme synthesis could lead to higher levels of TAG production. Alternatively, metabolic control processes might exist that would partition carbon flux differently depending upon environmental and physiological factors.

What is known is that, although some species seem to have naturally higher levels of TAGs, most microalgae capable of TAG hyperproduction do not do so during exponential growth, a factor that needs to be taken into consideration when considering the biomass productivity versus oil content question (see below). A number of factors appear to influence TAG content with the best studied being nutrient deprivation. Absence of a required growth factor, most noticeably fixed nitrogen (or silica for diatoms), causes premature growth arrest and diversion of biosynthetic capacities to TAG production (Hu et al. 2008). Phosphate or sulfate limitation has also been shown to increase the lipid content of some microalgal species.

Other environmental factors can affect lipid content and/or composition, but their effects are probably indirect. For example, temperature has a major impact on the cellular fatty acid composition with the degree of saturation increasing with increasing temperature. This is most likely an effect on the polar membrane lipids. Likewise, light levels can affect lipid composition, with low light increasing the synthesis of polar levels. Both these effects are likely due to modulation of the membrane lipids, increasing saturation of the normal complement of membrane lipids with temperature, and stimulation of chloroplast membrane synthesis as the photosynthetic apparatus enlarges to adapt to low light intensity. Obviously, a more thorough understanding of the regulation of lipid biosynthesis on the physiological and molecular level would have a great impact on the ability to control overall oil content and productivity for maximum algal biodiesel production.

13.4.2 Productivity Versus Oil Content

Industrial production systems using microalgae will probably need to be specifically tailored on a case by case basis. Several variables play key roles in microalgal processes and some will likely be project specific, for example, the geographical site and local climate which directly affect annual variations in humidity, temperature and solar radiation may change the optimum for certain variables. Other factors

Table 13.5 Some of the desirable characteristics for an algal strain for large scale culture

Characteristics	Advantages
High growth rate	Higher biomass productivity, reduced area requirement, resistant to invasion
High lipid content	Higher value of biomass, higher productivity
High value by-products	Decrease in production cost
Large cells, colonial or filamentous	Ease of harvest
Planktonic	Less growth on surfaces, easier to harvest and maintain
Tolerance to variations in culture conditions	Lower requirement for control of pH, temperature and others
CO_2 uptake efficiency	Lower cost required to supplement CO_2
Tolerance to contaminants	Potential growth on very eutrophic waters or flue gases
Tolerance of shear force	Allows cheaper pumping and mixing methods to be used
No excretion of autoinhibitors	Higher cell density expected: higher biomass productivity.
Naturally competitive	Harder to be overcome by invading species

Adapted from [Griffiths and Harisson 2009]

to be considered include desired products and/or by-products, outdoor or indoor culture, species to be cultivated, harvesting approach, and others. Strict optimization may not be required for high-value products where the production scale is low. However, biodiesel is a high-volume low-value product with high demand, and under current carbon trading schemes (or the lack of them) the production cost must be low enough so it can compete with petrodiesel.

The best microalgal species to be cultivated in a given system strongly depends on those variables, thus selecting the proper strain might be a challenge in itself. Until now, there is no consensus about which group of algae would be the most appropriate for large scale/low cost TAG production. Considering known algal diversity, very few strains are currently under study for biodiesel production. Moreover, although a strain from a culture collection might be well characterized and present characteristics favoring its laboratory study, it is very questionable if these strains could adapt to different local climates or would be able to compete with indigenous strains. Thus, although time consuming and labor intensive, bioprospecting for local microalgal species capable of high levels of lipid production might be advisable. Some of the properties considered desirable in an algal strain for mass culture are given in Table 13.5. A database containing the characteristic of local microalgal species would have extreme utility for different projects for algal biodiesel production, such as their use in tertiary treatment in municipal sewage treatment plants or for treatment and biofuels production from local industrial wastewater (Fig. 13.4).

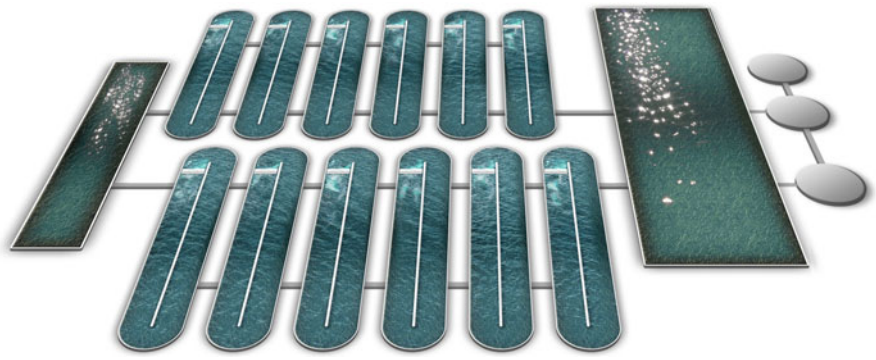

Fig. 13.4 Schematic of an Open Pond System built in conjunction with a wastewater treatment plant

High overall TAG productivity is obviously one of the major keys to the successful production of biodiesel from algae. Overall TAG production is the result of three interacting variables: growth rate, lipid content, and metabolic yield. Obviously, for the strict photosynthetic production of TAGs, cellular metabolism is directly constrained by the availability solar radiation and the efficiency of its conversion. Restriction at this level limits the availability of fixed carbon and the cell must prioritize its use according to current needs (e.g., "house keeping," secondary metabolite production, cell division, carbon reserves). Thus, fast growth (i.e., high cell division rates) does not necessarily translate to high level lipid productivity. In fact, with respect to growth versus lipid content in a specific strain, three basic scenarios are expected:

A. Faster growth, but lower lipid content
B. Medium growth with medium lipid content
C. Slower growth with higher lipid content

Figure 13.5 exemplifies the different behavior of four species when grown in nutrient replete medium (Griffiths and Harisson 2009). *Chlorella sorokiniana* and *Chaetoceros calcitrans* show opposite metabolic strategies, while *C. sorokiniana* invests heavily in growth rate, *C. calcitrans* is "preoccupied" with storing energy. Both, *P. lutheri* and *Chlorella vulgaris* showed average to slightly high growth rates and lipid content. Thus, different species have their own metabolic particularities and often their response may be different depending upon culture conditions. The three scenarios mentioned will have different set points for each strain which therefore be analyzed individually. Of course, lipid productivity is a function of both growth rate and lipid content, and the best strain may not be the one with the highest lipid content. For example, as shown in Fig. 13.4, although having the highest oil content, *C. calcitrans* was shown to have the lowest lipid productivity.

Although it is not possible to overcome the natural limitation on lipid productivity due to the inverse relationship between growth rate and lipid accumulation, several strategies can be used to improve lipid yields. Growth can be carried out in two stages with improved cellular oil content after a first stage of fast growth. The idea here is to use strains with natural fast growth under nutrient-rich conditions until

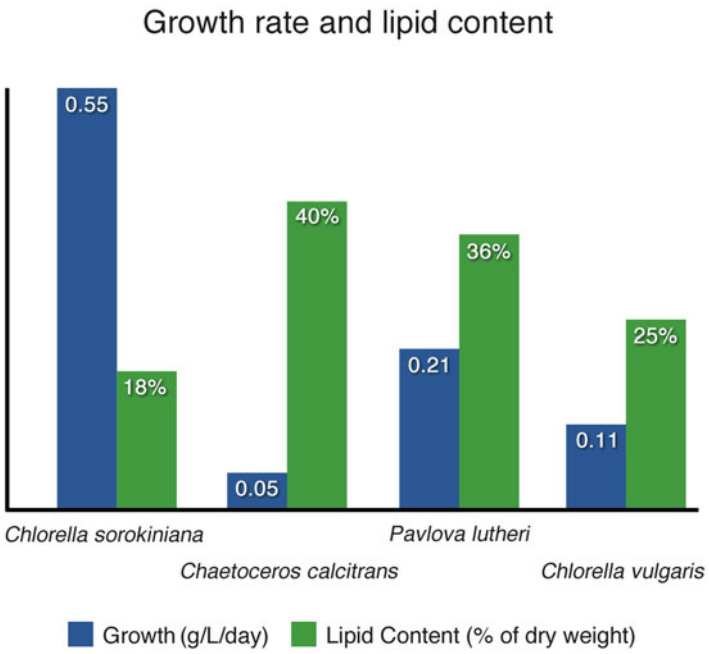

Fig. 13.5 Growth rate and lipid content of four different species under optimal conditions. The best lipid productivity is not always found in the species with higher lipid content

Table 13.6 Enhancement of lipid production in different microalgae

Species	Rich media	Nitrogen deficient	Reference
Chlamydomonas applanata	18%	33%	Shifrin and Chisholm 1981
Chorella emersonii	29%	63%	Illman et al. 2000
Chorella minutissima	31%	57%	Illman et al. 2000
Chorella vulgaris	18%	40%	Illman et al. 2000
Ettlia oleoabundans	36%	42%	Gatenby et al. 2003
Scenedesmus obliquos	12%	27%	Ho et al. 2010
Selenastrum gracile	21%	28%	Shifrin and Chisholm 1981

they reach the appropriate density, whereupon they are induced to accumulate lipids. Lipid induction has been achieved in many species through nitrogen deprivation (Table 13.6), and is thought to lower the costs of harvesting considerably. This is the simplest way so far found to artificially induce the production of fatty acids. In fact, many unnecessary secondary metabolites, at least from the point of view of biodiesel production, are normally made, and nitrogen deprivation shuts down their synthesis, driving metabolism toward the synthesis of fatty acids. New molecular tools for algae are being developed and it is thought that through manipulating cell signals and rerouting carbon flux it should be possible to enhance lipid production.

Massive production of biodiesel will be required to make a significant impact on the use of fossil fuels. In 2010, the USA alone consumed 220 billions of liters of diesel (*http://*www.eia.gov). To reach the level of production necessary to satisfy the current blend limit of biodiesel in petrodiesel, 20%, would require 44 billion liters, while only 1.3 billion are currently produced annually. Of course it is impractical, if not impossible, to supply this quantity using biodiesel derived from oil seeds and waste oil, and attention is turning to oil from microalgae as a possible solution.

One of the problems in this field is the highly exaggerated lipid productivity projections that are sometimes made. These are based on the dubious extrapolation of the best case scenario results obtained under highly controlled, optimized laboratory conditions and projected values as high as 137,000 L/ha/year (137 tons/ha/year) have been proposed (Chisti 2007). However, in reality, practical yields for any kind of large-scale outdoor production will be much lower. A number of relatively large-scale production studies under optimal conditions with raceway ponds indicate that biomass productivities of around 20–30 g/m^2/day are probably achievable (Sheehan et al. 1998). If this could be sustained year round, 73 tons biomass/ha/year would be produced. If the microalgal biomass were 30% lipid, a high value considering that these productivities are obtained under nutrient sufficient conditions, only 20 tons of biodiesel/ha/year would be produced. While this is higher than oil crops, about three times that of palm oil (6 tons/ha/year), it is a far cry from the numbers that originally sparked a "green gold rush."

Confirmation of this more realistic view is given by a recent pilot-scale project, which used outdoor photobioreactors (PBRs) and achieved an extrapolated annual production of 20 tons of oil per hectare (Rodolfi et al. 2009). However, if this extrapolation can be confirmed in any future very large algae farm is quite uncertain. There are many known and unknown risks involved in massive algal cultures and there is in reality no data available about large-scale production at this level. At any rate, it is evident that the reproduction of laboratory results on such a macroscale is just not possible.

Nevertheless, as pointed out above, algae oil productivity per hectare is still very attractive when compared to regular oil crops. However, a largely answerable question is what the actual delivered cost of algal biodiesel would be. In fact, a thorough economic analysis is quite difficult at present given the many unknown variables in ultimate achievable biomass productivity, the scale of production that is feasible, and suitable technologies for harvesting and oil refining that have yet to be developed. Thus, many studies have tried to estimate the putative price of algae oil under different production circumstances, but the disparity between the values found highlight the lack of data from large-scale cultures. In general, realistic projected prices are too high to make biodiesel competitive with petrodiesel under current market conditions. For example, one study predicted bringing in algal biodiesel at a projected price of between $25.00/gallon ($6.60/L) and $2.50/gallon ($0.66/L), with the major factor driving the price differential being the difference between low and high productivities (Pienkos and Darzins 2009). The challenges to be met in this respect can be seen by comparing current (July 2011) pump prices for diesel, $1.03/L[3] with crude oil selling at $95 per barrel (http://www.eia.gov/)

with the market price of palm oil, $1.15/kg = $1.08/L. Thus, despite the promise (sometimes overblown), developing practical systems for biodiesel production from algae faces many formidable challenges.

13.5 Cultivation

Of course, achieving anywhere near realistic photosynthetic conversion efficiencies and productivities depends critically on the geometry and physical properties of the cultivation system used. This is not as straight forward as one might naively think since the important nutrient here, sunlight, is used differently with respect to dilution rate than a nutrient that is dissolved in the liquid phase. Thus, there is a disconnect between growth rate and productivity (Tredici 2010). Maximum specific growth rates (i.e., doubling time of cell biomass) are obtained under conditions of photosaturation, obtained only with very dilute cultures. In practice, mass algal cultures need to be run under conditions of photolimitation to maximize areal productivity. Under these conditions, the increased density of the culture ensures that all the impinging photons are captured, but consequently self-shading is increased with negative effects on growth rate. Two basic types of cultivation systems have been proposed and studied: photobioreactors and open ponds, each with their own advantages and disadvantages (Table 13.7). First, these are briefly reviewed, and then they are compared for used in biofuels production.

Table 13.7 Algae Cultivation with Open Ponds Versus photobioreactors

Advantages	Disadvantages
Open pond	
– Low cost construction	– Easily contaminated • Overun by alien algae • Grazing by zooplankton
– Easily scaled	– Lower productivity
– Low cost maintenance	– Evaporative water loss
– Relatively low energy inputs	– Process control difficult, suboptimal culture conditions
– Easy maintenance	– Large areal requirement – Poor mixing, CO_2 and light utilization
Enclosed photobioreactor	
– Higher level of process control possible	– Expensive
– More resistant to contamination	– Scale up difficult
– Little evaporative water loss	– Wall growth
– Higher yield of biomass	– Cooling may be required
– Outdoor and indoor capability (winter)	– Energy intensive – Sophisticated construction – pH, dissolved CO_2 and CO_2 gradient within the tubes, depending on the model

13.5.1 Photobioreactors

Photobioreactors are enclosed, and not necessarily sterilized, culture vessels that are transparent and usually designed to maximize surface to volume ratio in order to maximize volumetric productivity. Being enclosed allows tighter process control and prevents gas exchange with the environment and contamination by alien microbes. Obviously, they reduce evaporative water losses and, depending upon the design, can be oriented with respect to the incoming solar radiation to maximize light capture while at the same time maximizing productivity (Carvalho et al. 2006; Lehr and Posten 2009; Weissman et al. 1988). A wide variety of designs of differing levels of sophistication and widely variable costs have been developed over the years.

These are the type of systems of choice for laboratory scale studies and small-scale operations where high-value products are being made. However, there are a number of disadvantages with these systems that make their potential applicability to large-scale micoalgal culture for biofuels production doubtful (see also below). In natural sulight, they would be prone to overheating in most situations, necessitating further system control, and addition of some type of cooling system which could add appreciably to the costs and energy requirements of the system. The energy requirements for adequate mixing could well be ten times that for open ponds (Weissman et al. 1988), and, since they are enclosed carbonation becomes easier but at the price of greatly increased risk of photo-oxidation caused by the buildup of oxygen produced by photosynthesis to toxic levels. Wall growth could be problematic, necessitating either shutdown and extensive cleaning, or sophisticated cleaning equipment. They are obviously more expensive to construct and operate than open ponds, and scale-up would be required to ascertain if the increase in productivity, yet to be demonstrated on any even moderate scale, could ever justify the cost.

13.5.2 Open Ponds

Open ponds are relatively easily constructed as long as the land area to be used is relatively level. Liners can be installed to prevent water loss through seepage, or alternatively, some soils contain enough clay to negate this need. Effective mixing can be provided by relatively low energy paddle wheels, and oxygen build up is much less of a problem due to the greater volume and the ease of gas exchange with the environment. Overall, the energy and maintenance requirements are relatively low, and there is already some experience with large-scale outdoor ponds, either operated as part of government funded programs or in some places as part of the wastewater treatment process.

However, some features of open ponds suggest that their use for biofuels production from microalgae could also be problematic. It is thought that they could be relatively easily contaminated and over rum, especially if a noncompetitive

species is being grown (Sheehan et al. 1998). In general, process control is more difficult than with photobioreactors and there would be greater evaporative losses and perhaps more difficulty in efficient carbonation. Their biggest advantage is, however, the relatively low cost with which large-scale systems can be built and managed.

13.5.3 PBRs Versus Open Ponds

There is presently a great deal of discussion as to whether future micoalgal biofuels facilities will consist of open ponds or closed photobioreactors. One practical view on the open system versus photobioreactor debate is provided by a look at how industries currently producing microalgae and cyanobacteria for the nutraceutical market are cultivating their microorganisms. This is a relatively high-value product compared to the value of algae grown for biofuel production; ~$5,000/ton versus ~ $875/ton. However, even at approximately ten times the anticipated value of algae for oil, presently operating plants (Cyanotech, Earthrise Nutritionals, etc.) are all invariably open pond systems.

This is a real indication of the difficulty of making the economics of photobioreactors work for the large-scale production of very low-value products. “Anyone working on closed photobioreactors has got a problem,” says Benemann. “And there are dozens of these companies out there,” he says. “Just like in agriculture, you have to keep it as simple as possible and as cheap as possible. You can’t grow commodities in greenhouses and you can’t grow algae in bioreactors.” (Waltz 2009a). A number of companies are presently producing photobioreactors and touting them for use in making biodiesel. Unfortunately, some of these companies use as their selling points productivity numbers that are so high that they are not even theoretically possible (Tredici 2010). There are serious obstacles to developing photobioreactors for use in biofuels production, problems that will rear their ugly head sooner or later and are ignored at one’s peril, as the following quote shows. “The old algae world has produced some old-timers who are negative. We’re trying not to listen to them, Bob Metcalf, Polaris Venture Partners, investor in Greenfuel Technology.” (Waltz 2009a). Greenfuel Technology, a photobioreactor provider, went bankrupt 5 months later.

13.6 Harvesting and Downstream Processing

In a typical culture of microalgae grown to produce a product, cells are evenly dispersed in the medium and highly dilute with biomass concentrations (dry weight/liter) usually varying between 0.3% and 1.0% (3–10 g/L) when grown autotrophically and 11.6% (116 g/L) when grown heterotrophically (Gouveia and Oliveira 2009; Wu and Shi 2007). Since the lipids or other products are confined inside the

cells, it is usually necessary to concentrate the algae prior to product extraction. Different harvesting techniques are currently applied and although some are already in place as industrial processes, they have a substantial cost which greatly impacts the final value of the product and thus are only justified if this product has a high market value. However, since biodiesel is a low-value product, use of these processes could represent 30–57% of the final price (Grima et al. 2003; Hende et al. 2011). Therefore, in addition to increasing biomass/lipids production, significant advances in harvesting and lipid processing are necessary if algal biodiesel is to become economically viable.

Therefore, just as with other crucial points in a microalgal biodiesel production system, the selection of the appropriate harvesting method is crucial. One or more steps of solid–liquid separation might be necessary to recover the microalgal biomass, and a systematic analysis of the methods already applied in other systems followed by a careful adaptation will be necessary to lower the cost of production (Brennan and Owende 2010). Usually, when separating the biomass from the aqueous solution, a bulk harvesting process, effecting most of the dewatering is made first, forming an algal slurry. If needed, a second step is made to form an algal paste (Uduman et al. 2010). These steps can be made by a combination of flotation, flocculation, filtration, and centrifugal sedimentation methods.

Flotation harvesting consists of collecting the cells at the surface of a tank and is based on the natural tendency of some species to float, especially when they have a high lipid content. Flotation can be enhanced through the use of dispersed micro-air bubbles. The major advantage of this techniques that there is no addition of chemicals; however, it is limited to a few species and might not be technically or economically viable on a large scale (Brennan and Owende 2010).

Flocculation is the formation of aggregates that precipitate. This happens due to the reaction of a flocculating agent with a target compound. Microalgal biomass flocculation can be done by adding different flocculating agents (chemical coagulation), through an electrolytic process, or by auto-flocculation. Auto-flocculation is based on the tendency of old microalgal cultures to precipitate. In this process, the negatively charged cell membranes react with Ca^{2+} ions present in the medium, forming aggregates which precipitate. This reaction is due to the elevation of the pH in the medium caused by photosynthetically driven CO_2 consumption and can be enhanced artificially by elevating the pH of the solution (Sukenik and Shelef 1984). In electrolytic flocculation, a flocculating agent is formed either by oxidation driven by an electrode (electrocoagulation), or simply by attracting the cells toward the electrode, which neutralizes the charge carried on the cell membrane allowing aggregates to form. Chemical flocculation can be achieved through the addition of organic or inorganic salts that acts as flocculating agents. Ferric and aluminum salts are the most commonly used inorganic salts, and successful harvesting of Scenedesmus and Chlorella has been achieved using this method (Grima et al. 2003). However, chemical flocculation has several major drawbacks as it is highly sensitive to pH, its efficiency can vary depending on the species, and large amounts of iron or aluminum salts have to be used, which consequently contaminates the end product and the solution. Multistep flocculation can be performed, combining different types of flocculating agents (Chena et al. 2011).

Gravity sedimentation can be used for certain species, depending of course on the cell density. Filtration, on the other hand, depends on cell size, which with small cells is a crucial limiting factor. It can be energy intensive and sensitive to the algal concentrations, high biomass densities might block the filter (Chena et al. 2011). Centrifugation is the most reliable harvesting method, feasible for most of the species, with high biomass recoveries, but very energy intensive. Differences in equipment and species to be harvested mean that different guidelines may be required, and may result in harvested biomass with varying water content. In addition, cells may be damaged or even disrupted due to the gravitational force which is required. High biomass recovery efficiencies are observed only above $13{,}000 \times g$ (Grima et al. 2003). Different species require different harvesting strategies and therefore a common protocol for different species grown in different systems can probably not be established. Some species characteristics that may be desirable for ease of harvest include cell size, cell density, and the capacity to perform auto-flocculation.

Once the biomass has been recovered in a concentrated form, the lipid content must be extracted relatively soon to avoid spoilage (Grima et al. 2003). Several methods are available for lipid extraction, from the traditional and simple press to the use of high intensity ultrasound assisted by microwave (Cravotto et al. 2008). However, for biodiesel production, it is always important to keep it simple and cost effective, thus solvent extraction is usually the method of choice. Although this requires a relatively dry biomass, and if the harvesting process did not include a centrifugation step, the concentration of the recovered slurry might be too low (around 15% dry solid content is necessary) an additional drying step might be required (Brennan and Owende 2010). Among the methods currently in use in microalgal culture are sun drying, low-pressure shelf drying, spray drying, drum drying, fluidized bed drying, freeze drying, and others (Prakash et al. 1997; Desmorieux and Decaen 2006; Leach et al. 1998; Grima et al. 1994). The least expensive, sun drying has several limitation, such as large area needed, possibility of loss of material and extensive time required, while the most effective, spray drying and freeze drying are quite expensive and therefore not suitable for mass biodiesel production (Brennan and Owende 2010). Wet lipid extraction is under study and could use the biomass just after the centrifugation step, which would represent an interesting option to reduce operational costs (Converti et al. 2009; Widjaja et al. 2009; Levine et al. 2010; Leea et al. 2010). The method to convert algal lipids (TAGs) into biodiesel is the same as that used in the production of first-generation biodiesel from plant oils, using a short chain alcohol, usually methanol, in the presence of a acid or alkali catalyst. The TAG is reacted with the alcohol to form glycerol and three FAMES (fatty acid methyl esters), which can be used as biodiesel (Fig. 13.4).

13.7 CO_2 Sequestration?

When grown autotrophically, the ultimate source of the carbon in algae oil is derived from the CO_2 which the microalgae have fixed using captured solar energy. The Calvin–Benson–Bassham cycle is universally used for carbon fixation by microalgae.

Here the key enzyme is Rubisco, which has a low affinity for CO_2, and consequently, for most species would only be half-saturated at normal atmospheric levels of CO_2. In addition to its CO_2 reactivity (carboxylase activity), Rubisco can also act as an oxygenase, interacting with O_2 and oxidizing a molecule of ribulose 1,5 bisphosphate and therefore removing it from the CO_2 fixation process. Thus, oxygen reduction competes with CO_2 reduction, an effect that becomes significant at low CO_2 concentrations. For these reasons, many microalgae have evolved CCMs (carbon concentrating mechanisms) giving them the capacity to create locally elevated levels of CO_2 in the vicinity of Rubisco (Giordano et al. 2005). In fact, the only microalgae that appear to lack some sort of CCM are almost all freshwater chrysophyte and synurophyte (heterokont) algae.

Given the large degree of diversity in the microalgae, it is perhaps not surprising that there are a number of different CCMs used, depending upon the species. A detailed discussion of this subject is available (Giordano et al. 2005). Regardless of the detailed mechanisms that differ in how substrate is delivered to Rubisco in the interior of the cell, all CCMs depend upon active transport of either HCO^- or CO_2 into the cell. However, even though these organisms have thus adapted to relatively low atmospheric concentrations of CO_2, growth is enhanced by increasing the supply of CO_2, probably due to a number of factors. Rapid inorganic carbon uptake will cause local depletion and hence an increased pCO_2 will help maintain levels above those needed to saturate transporters. As well, at high enough levels, CO_2 diffusion through the membrane could lessen the need to use transporters and expend energy in the process. The energy savings would therefore translate into more energy available for cell growth and other processes, leading to growth enhancement.

Regardless of the exact mechanism, it can be empirically shown that supplementation with CO_2 greatly increases growth and productivity, sometimes by a factor of 5. Thus, CO_2 enrichment is taken as a given when projecting algae oil productivities and when planning growth facilities. This is perhaps one of the most important factors in practical algal culturing as a fivefold increase in productivity directly translates into a fivefold decrease in land footprint, water resource requirements, and operational costs and energy demands. Obviously, the price of CO_2 becomes an issue, and thus most projections of future facilities call for the use of flue gas, an essentially free source of CO_2. Of course, one can question whether there is actually enough flue gas available at the right locations to permit large-scale culture of algae using this source (Pate et al. 2011).

Unfortunately, this requirement has been turned by some into a selling point, claiming that algal cultures thus carryout CO_2 mitigation, or even sequestration (Possible Fix for Global Warming? Environmental Engineers Use Algae to Capture Carbon Dioxide Science Daily April 7, 2007)! A little reflection will show that this is a shady accounting practice, analogous to the deceptive repo101 used by the now defunct and discredited Enron. In that case, liabilities were removed from the books prior to issuing quarterly statements by selling them to dummy companies, and then repurchased once the glowing reports had been issued. In this case, it is true that the algae absorb CO_2 emitted by fossil fuel burning power plants, thus preventing immediate release into the atmosphere. However, if the algae are used to produce fuel, the residence time of the CO_2 in the fixed state will only be a matter of weeks

or at most months before it is released by combustion. Thus, the CO_2 coming from flue gases that is fixed by the algae cannot be taken off the books for enough time to make any difference. This reasoning has recently been recognized by the Advertising Standards Authority of the UK, which issued a judgment against ExxonMobil for an advertisement in which they had a scientist claiming "In using algae to form biofuels, we're not competing with the food supply, and they absorb CO_2, so they help solve the greenhouse problem as well." (March 9, 2011, ASA Adjudication on Exxon Mobil UK Ltd. – Advertising Standards Authority http://www.asa.org.uk/ASA-action/Adjudications/2011/3/).

13.8 Valuable Co-products?

Microalgae, depending upon the species, are capable of producing a large number of high- and medium-value products; including various food supplements, principally polyunsaturated fatty acids (omega-3), also under study as possible pharmaceutical agents, various pigments (chlorophyll), and as livestock feed. When faced with the severe challenges and dismal economics of large-scale production of a high-volume, low-value product such as a biofuel, many propose improving the economics by introducing a co-product generating scheme (Singh and Gu 2010). In this type of scenario, often called a biorefinery, biofuel production is essentially subsidized by profits derived from the sale of the much higher valued product.

However, the problem with this approach is that at the production scale, needed to generate a significant amount of biofuel as a replacement fuel, so much co-product is produced that its price plummets. A now classic example of this is the glycerol produced as a side product of biodiesel manufacture. In the early days of biodiesel production, the glycerol was a value-added product. As biodiesel production has grown significantly, a glut on the glycerol market was created and the bottom has dropped out of the market with the price of glycerol falling over tenfold. Biodiesel manufacturers are now basically forced to burn it as it has changed from a valuable byproduct to a nuisance hazardous waste. Of course, the petrochemical industry survives through the numerous revenue streams generated by its refineries, but in this case hundreds, even thousands, of different medium- and high-value products are generated which help the economics of the production of relatively low-value fuels. Thus, if the biorefinery concept is to work, multiple products must be made, something more difficult given the chemical composition of microalgae than the panoply of compounds available in crude oil.

13.9 Challenges for the Development of Practical Systems

Development of practical biofuels porduction from microalgae faces a number of significant challenges, although there are some possible worarounds (Table 13.8). In One LCA (life cycle analysis) study, based on projecting current laboratory

Table 13.8 Challenges and Workarounds in Cultivation of Microalgae for Biofuels

Challenges	Workarounds
– Large amounts of water needed for growth	– Use wastewater or brackish water
– Small size, difficult to harvest	– Develop novel harvesting technologies • Screens (large species) • Natural sedimentation • Add flocculating agents
– High water content, dewatering challenging	– Develop novel downstream processing • In situ transesterification • wet oil extraction • Engineered strains
– Require high levels of nutrient input	– Use waste water, agricultural run-off
– Expensive production costs	– Production of byproducts – Wastewater treatment credit

observations and current practices in the first-generation biodiesel production industry, open raceway ponds were conceptualized for the cultivation facility, photobioreactors were considered too expensive even considering the possible increased productivity (Lardon et al. 2009). Even using optimistic assumptions, it was concluded that only wet extraction of low-N grown microalgae had a positive energy balance, a reflection of the preponderance of total energy consumption taken up by lipid extraction (90% dry extraction, 70% wet). It was concluded that the development of a sustainable, net energy producing system will require minimizing the energetic demands of the production, harvesting and extraction steps, minimizing nitrogen fertilizer use, and extraction of the energy and recycling of the minerals in the oil cakes through efficient anaerobic digestion.

One of the potentially cost intensive inputs to an algal cultivation system is the supply of macro- and micronutrients. Lowering the cost and increasing the sustainability of such a process requires that cheap, or even "free" sources of fixed nitrogen and phosphate be found. An obvious solution, one which can even at least also partially satisfy the appreciable water requirement, is to use some kind of waste stream, probably domestic wastewater. In essence, if operated as a pond, which in all likelihood it would be, this would be a high-rate algal treatment pond operated for biofuel production (Craggs et al. 2011; Park et al. 2011; Pittman et al. 2011; Rawat et al. 2011). This would appear to be a much more environmentally sound and economically attractive option than a dedicated algal biofuels production unit using large amounts of freshwater with the addition of fertilizers. However, a number of challenges would have to be met; provision of CO_2 for maximum productivity, control of the species which are grown to assure high lipid content and suitability for downstream processing, control of grazers, and suitable harvesting strategies, possibly bioflocculation (Park et al. 2011). Some at least partial solutions to these potential limiting factors are on the table. As noted above, CO_2 could potentially be supplied through the use of flue gas, or alternatively, from the CO_2 remaining after use of the biogas stream coming from anaerobic digestion of the residual algal biomass.

Stable operation of high-rate treatment ponds for biofuels production would require the establishment of a regime capable of maintaining the desired strain (one with naturally high lipid content) as the dominant species over a reasonably long period of time, i.e., several months at least. However, at present, the factors that enable one species to dominate are not well understood and are probably a combination of a wide range of environmental (temperature, light, water quality), operational (pH, HRT), and biological (preadaptation, resistance to predators, etc.) variables. Attempts to grow introduced species invariably fail due to the cultures becoming overrun by native algae or being decimated by zooplankton. This is the major challenge for effective use of high-rate treatment ponds, or indeed any open pond system, for biofuels production. One possible strategy would be to use some form of biomass recycle where a fraction of the desired algae are collected and reintroduced into the system, thus effectively increasing their apparent growth rate over that of other species. Of course for this to work the desired species has to have some easily used specific characteristic, for example, filamentous species could be selectively retained over unicellular forms by screening with nylon mesh and a fraction reintroduced (Weissman and Benemann 1978).

One of the greatest challenges in producing biodiesel from microalgae is the need to develop low cost, effective harvesting. Most of the microalgae so far known showing promise for either biodiesel production or wastewater treatment are small, highly negatively charged (self-repelling) and have a similar buoyant density, making their harvesting problematic. Both centrifugation and chemical flocculation are highly effective but too energy or cost intensive to be used in any large-scale practical process. One promising avenue that requires further research to determine if a practical application is possible is to select strains which, under proper conditions, are capable of auto- or bio-floculation. Cells capable of forming large aggregates could then be harvested by gravity sedimentation and final dewatering could potentially use centrifugation, cost effective if the solids concentration obtained through gravity sedimentation is high enough and therefore only small volumes need to be treated.

Although we are perhaps a long way from large-scale deployment of combined waste treatment and biodiesel production processes, some initial laboratory scale research has given promising results. Cultivation of a freshwater alga, Chlorella ellipsoidea on actual effluent from several different secondary treatment processes has shown that high biomass yields are possible (425 mg/L in the secondary effluent with the highest phosphate concentration) with high levels of lipid accumulation (35–40%) in stationary phase while at the same time removal of nitrogen and phosphorous was above 95% (Yang et al. 2011).

Although the biomass residue after oil extraction could be used as a feedstock for a "biorefinery," as noted above the potential for deriving value by making high cost by-products is limited, and the use of residual material as animal feed is questionable given the need to transport it, a costly option considering its value. Nevertheless, something needs to be done with the residual material as otherwise it becomes an immense waste disposal problem. Probably, the best option is to develop anaerobic digestion methods suitable for converting much of the mass into biogas. The produced

methane could be used to power plant operations, and, at the same time, this would allow recovery of some of the fixed nitrogen, phosphate, and trace elements necessary to continue algal growth operations. Some initial studies have shown the feasibility of this approach, with yields, 0.2–0.3 m^3/kg VS, on the lower end of the range for what is typical of standard anaerobic digestors (Ehimen et al. 2011; Wiley et al. 2011).

13.10 Future Developments in Photosynthetic Biodiesel Production

A number of possible advances could be made in the future through the application of genetic engineering technologies. Use of genetic engineering to improve strains useful in biodiesel production has been severely hampered by the dearth of methods that work with productive microalgae and to a lack of genomic information. The near future should see appreciable genomic sequence information become available, and there is even a dedicated Web site now established as a repository for information pertaining to algae potentially useful in biofuels production, Energy algae DB which has already collected 18 completed sequences (http://www.bioenergychina.org:8989/about.html). Recently, what is already known about the genomes of dinoflagellates and Mamiellophyceae (phylum Chlorophyta) have been published (Lin 2011; Piganeau et al. 2011; Radakovits et al. 2010). Of course, an attractive alternative is to take an organism for which the genetic tools are already well developed and turn it into a biodiesel producer. Thus, a cyanobacterium has recently been engineered for fatty acid biosynthesis (Liu et al. 2011).

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CHAPTER

22

Engineered Cyanobacteria: Research and Application in Bioenergy

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OUTLINE

INTRODUCTION

Paleontological and geochemical data as well as molecular analysis of the plastid genome point to a single prokaryote as the origin of several groups of organisms scattered throughout the tree of life, including the entire kingdom of Plantae (Knoll, 2008; Yoon, 2004). A cyanobacterial ancestor is believed to be the only organism ever to couple together two photosystems, harvesting electrons from water to produce energy-rich molecules such as adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Knoll, 2008) (Figure 22.1). These molecules provide the necessary chemical energy, protons and electrons for cellular reactions and the synthesis of other molecules, most importantly powering CO_2 fixation through the Calvin-Benson-Bassham cycle. This event is thought to have happened between the mid-Archean and early Proterozoic eras (2000–3000 millions of years ago). The atmosphere was poor in oxygen and rich in CO_2, and the oceans were rich in salts and minerals; perfect conditions for the first algal blooms. The invention of oxygenic photosynthesis conferred a great advantage to this ancient cyanobacterium, starting widespread speciation and changing the composition of the atmosphere through the oxidation of water into protons and molecular oxygen (Figure 22.1). This was probably the first universally relevant instance of primary production and established a food chain by transforming inorganic nutrients into organic molecules that could be used by heterotrophic organisms (Knoll, 2008). The role of primary producers, so important in fully establishing life on earth, is still equally important today, when cyanobacteria are thought to be responsible for 25% of all carbon dioxide fixation and together with eukaryotic microalgae sustain most of oceanic life, fixing CO_2 and carrying out important steps in various biogeochemical nutrient cycles (Field et al., 1998).

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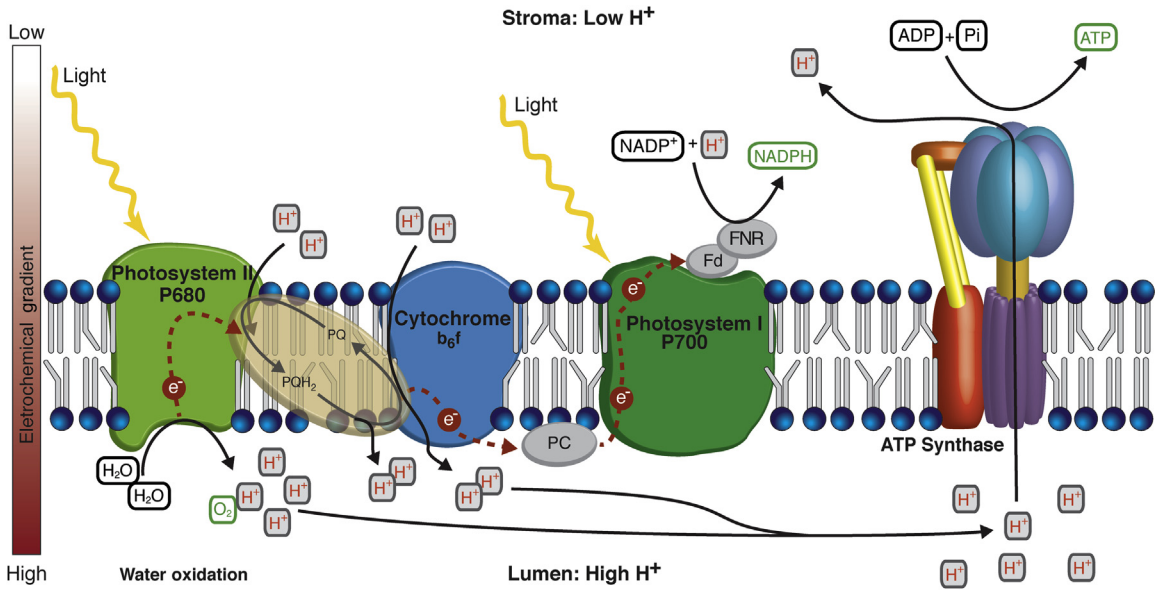

FIGURE 22.1 Scheme of light reactions in oxygenic photosynthesis. Photosystem II oxidizes a water molecule, harvesting the electron that will be used to synthesize NADPH, and producing an electrochemical gradient through the release of protons (H^+) that will be used by ATP synthase to drive phosphorylation of ADP. The ATP and NADPH that are produced are used by the Calvin cycle for CO_2 fixation (dark reactions). (For color version of this figure, the reader is referred to the online version of this book.)

Humanity is totally dependent on photosynthesis for food and fuel. As well as a source of organic carbon, mankind relies on photosynthesis as energy source, through the use of fossil fuels, ancient photosynthetic products stored and cooked under pressure for millions of years, the burning of readily available biomass, or more recently through the use of biofuel crops as a new source of liquid fuels. Sugarcane or corn ethanol and biodiesel have been produced from crops for more than 40 years, with a greatly increased role the last two decades. These first-generation biofuels are presently being produced at large scale, with worldwide production of ethanol and biodiesel of 50 billion and 9 billion liters, respectively, in 2007. Even though these seem like significant quantities, biofuels still represent a miniscule fraction of the world's primary energy use; in 2011, 161 tons per day of renewable liquid biofuels were produced, whereas 12 million tons per day of crude oil were consumed (BP, 2012).

Humans have been constantly perfecting agricultural technology since the dawn of civilization, and with the green revolution, food crop yields have shown considerable increases decade after decade, although this progress is now stagnating in many food producing areas (Ray et al., 2012). At any rate, given the enormous demand for energy and the predicted increase in the world's population to 9 billion by 2050, it is evident that there is not enough arable land to satisfy both nutritional and energy demands through food and fuel crops. Of course, in addition to renewable energy derived through photosynthesis, other sources of sustainable energy exist: solar, wind, geothermal, hydroelectric, etc., but together these energy sources cannot supply the quantity and types of energy demanded worldwide since electricity is not suitable for all applications. Modern society is built around liquid and gaseous fuels, which are very efficient energy carriers suitable for a variety of applications, in particular mobile power. Liquid biofuels are essentially photosynthetically derived compounds, at present sustainably produced through the cultivation of energy crops, but as discussed above, this directly competes with the production of food crops.

A possible and promising alternative for sustainable energy production system is intimately related to crude oil formation over the previous millions of years. Before the appearance of vascular land plants on earth, ancestral cyanobacteria were already occupying a large variety of environments and now, after a long period of evolution, cyanobacteria and the microalgae formed through endosymbiosis of cyanobacteria, can be isolated from virtually any natural water sample, from extremely fresh water to hypersaline lakes, from snow in the Arctic Circle to hot or relatively dry environments. The richness of this speciation over billions of years can be appreciated through the variety of morphological forms that are found. These organisms show themselves to be a promising system for the production of hydrocarbons and other desirable products. Cultivation can be carried out using nonarable land; seawater and wastewater have been shown to support growth, bioremediating effluents while fixing atmospheric carbon dioxide into

possible commercial products. The rather simple nutrition requirements of these organisms highlight the capability of their metabolism to produce all the molecules needed for cellular growth. Their pathways frequently contain metabolites with commercial interest that can be readily used or easily processed into a final product (Figure 22.2).

Although cyanobacteria and eukaryotic algae share these attributes, cyanobacteria have the additional advantage of being relatively easily manipulated genetically. Thus, using cyanobacteria, if a desired product is not naturally produced, genetic engineering techniques allow the insertion of genes or even entire pathways to make novel products, either high-value compounds or

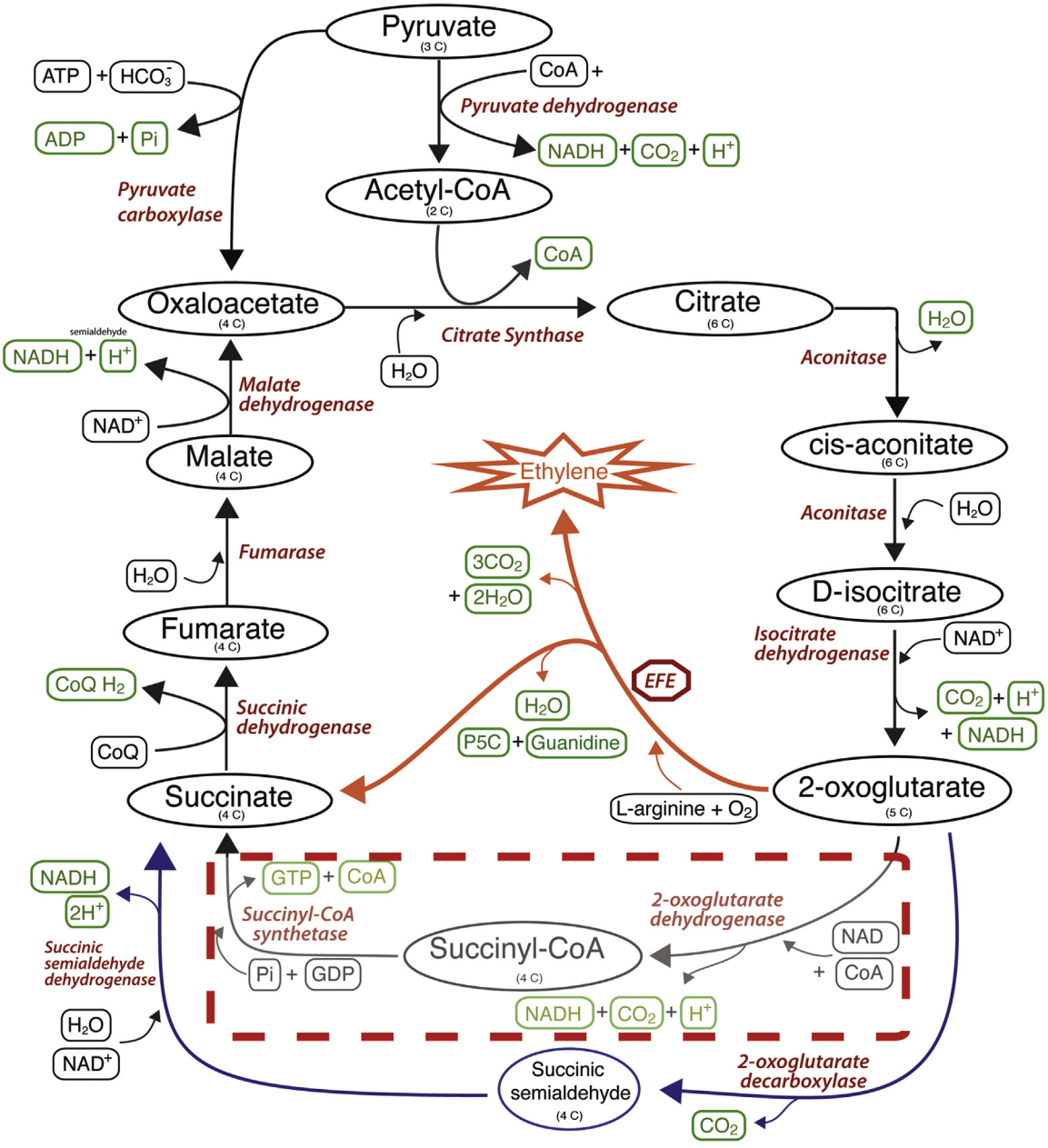

FIGURE 22.2 Scheme of the TCA cycle with the alternatives proposed for cyanobacteria, blue pathways on the bottom (Zhang et al., 2011), and for production of ethylene through the ethylene-forming enzyme isolated from *P. syringae* (orange pathway in the center). The lack of homologous genes for 2-oxoglutarate dehydrogenase in cyanobacteria led to the idea that they have an incomplete TCA cycle, working as two branched chains of reactions (oxidative and reductive) generating succinate from fumarate. However, a new 2-oxoglutarate decarboxylase recently described in *Synechococcus* sp. was the missing piece that closes the TCA cycle in cyanobacteria. Homologs to this gene were found in all cyanobacteria already sequenced with the exception of *Prochlorococcus* and marine *Synechococcus* sp. *Source: Zhang et al., 2011.* (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

commodity chemicals such as biofuels. Of course any molecule that is produced using cyanobacteria could be produced in other microorganisms, especially fermentation workhorses such as *Saccharomyces cerevisiae* or *Escherichia coli*, but these are heterotrophs requiring carbon compounds previously fixed through photosynthesis, i.e. agriculturally produced.

Thus, the cyanobacteria are uniquely positioned to carry out CO_2 fixation driven by solar energy capture while at the same time being amenable of genetic engineering to produce a wide variety of liquid and gaseous biofuels. In this chapter the current achievements on research toward the production of biofuels and crude oil substitutes using cyanobacteria as a model organism are reviewed. As will be seen, although much has already been achieved in terms of engineering toward the production of biofuels, in most cases productivity is the greatest bottleneck, although some steps in downstream processing also present many challenges. Thus, at present, use of a cyanobacterial system for commercial production of biofuels at cost-effective levels still faces significant hurdles.

ENGINEERING CYANOBACTERIA

The major argument for using cyanobacteria or eukaryote microalgae for biofuel production is the possibility to directly couple photosynthesis with product formation. This strategy could have sustainable and economic advantages. The financial appeal is related to the production chain, with CO_2 fixation directly producing the desired fuel in a single organism. Thus, the biofuel is recovered at the production site, avoiding as a consequence the processing of photosynthetically produced sugars in a second-stage microbial fermentation. This process also has great ecological and sustainability appeal since atmospheric CO_2 is being recycled into fuels without using the conventional agriculture system, leaving arable land available for food crops. Nevertheless, the inherently low value and high demand characteristics of fuels present a challenge for the development of biofuel production. The volume of fuel required to fulfill the needs of the transportation sector is massive, in contrast to their low market value, which must be at least as cheap as bottled water. The achievement of this goal requires the solution of major challenges in civil and mechanical engineering, chemistry, and biology.

In the biological arena, the main challenge is strain development. The ideal cyanobacterium for biofuel production would have a high quantum efficiency of photosynthesis and well-defined carbon partitioning, where the CO_2 fixed would be primarily directed to "housekeeping" metabolism and the targeted product. To achieve this goal, two main venues are being followed: high-throughput bioprospecting, which seeks naturally occurring species, enzymes and pathways adaptable for cultivation and economic exploitation, or the use of genetic engineering, where a model organism is genetically modified to introduce and/or to enhance the production of a desired molecule. In this section the available tools are discussed as well as some paths toward the improvement of photosynthetic quantum efficiency.

Strains, Tools and Methods

Originating in an environment without available fixed carbon, cyanobacteria have evolved as versatile organisms, capable of producing a large variety of organic compounds from simple inorganic sources that can be directly used or transformed into a commercial product. When the desired molecule is not naturally produced, genes or entire pathways can be introduced through a variety of methods and product yields can be increased by driving cell metabolism toward the desired product. There are more than 3350 species of cyanobacteria already described, with hundreds available in culture collections (Guiry and Guiry). To date, 87 cyanobacterial genomes have been sequenced and deposited in public databases but only a few strains have been used in genetic manipulation studies (Heidorn et al., 2011). Many molecular tools are currently available and genetic manipulation can be pursued through conjugation, electroporation or natural transformation. These techniques are constantly being revised or optimized for each host species and sample protocols are available elsewhere (Heidorn et al., 2011). So far, no cyanophage able to perform transduction has been described, nevertheless this technique is still the object of great interest (Koksharova and Wolk, 2002).

Natural transformation is an appealing feature found in some cyanobacterial strains, with two standing out as being frequently used in genetic manipulation studies, *Synechocystis* sp. PCC 6803 (Pasteur Culture Collection) and *Synechococcus* sp. PCC 7002 (Grigorieva and Shestakov, 1982). These two strains are of significant interest due to the high yield of mutants achieved through this technique, making it widely used for both pure and applied science, from plant physiology studies to metabolic engineering aiming for the commercial production of biomolecules.

The high frequency of transformants with natural transformation is intimately linked with the nature of the transferred genetic material, with chromosomal DNA reaching up to 100-fold more viable transformants than when replicative plasmids are used as the source of DNA (Golden and Sherman, 1983; Shestako and Khyen, 1970). In fact, this is true specifically for replicative plasmids since most of the transformation efficiency is

recovered when a suicide plasmid is used (Tsinoremas et al., 1994). Thus, it would seem that the final localization of the inserted DNA plays a key role in the transformation efficiency. This is argued to be related to the postreplicative processing of chromosomal DNA together with a putative robust recombination mechanism in these species (Flores et al., 2008). Natural transformation has being reported to be associated with pilus-related genes (Yoshihara et al., 2001; Yura, 1999), a natural machinery putatively adapted to take up exogenous DNA with such high efficiency that different artificial procedures intended to increase the transformation yield fail to improve the frequency of viable mutants (Zang et al., 2007). Unfortunately, natural transformation is not widespread in the cyanobacterial phylum and many species require other techniques for the efficient introduction of exogenous DNA.

Electroporation was first demonstrated in *Anabaena* sp. (Thiel and Poo, 1989) and today has been optimized for many strains. It has been shown to be effective despite the low yield in many cases (Koksharova and Wolk, 2002). Unlike what is observed for green algae (Kilian et al., 2011), the procedures and electric pulse settings are not very different from those used with other bacterial phyla (Heidorn et al., 2011). However, even though it can be an effective method, the ease of natural transformation and the higher yield of conjugation have left electroporation behind as a choice for mutagenesis.

Conjugation is the most commonly used technique for genetic engineering in terms of the diverse species with which it can be used, and, with the filamentous N_2 fixing (heterocyst forming) cyanobacteria, it is the only effective technique thus far described. With the advent of molecular biology, plasmids of cyanobacterial origin were actively sought with the intention of producing shuttle vectors allowing their transfer from *E. coli* to *Synechococcus* (Golden and Sherman, 1983). Since then, *E. coli* has been widely used for conjugation with many filamentous strains, such as *Nostoc* sp. and *Anabaena* sp., and single cell strains, like *Synechococcus* sp. and *Synechocystis* sp. Although incorporation of DNA into the chromosome of many strains has proved to be relatively easily achieved when using linear DNA or suicide plasmids, it has proved challenging to make cyanobacteria harbor replicative plasmids. During conjugation, the plasmid is relaxed and single-stranded DNA is driven to the recipient cell through the type four secretion system by the enzyme relaxase. Once in the recipient cell, the transferred DNA will have its antisense strand resynthesized and this newly reformed plasmid can integrate itself into the genome or autoreplicate. The vectors used in cyanobacteria must contain the replicons for both organisms, donor and recipient, a mobilization site (origin of transfer, e.g. *bom, nic* and *oriT*), a selective marker effective for both organisms, and a codon optimization to avoid the broad range of restriction enzymes harbored by cyanobacteria, which has been found to be an important hurdle to successful conjugation (Elhai et al., 1997; Flores et al., 2008; Wolk et al., 1984). Extra enzymes might be needed to ensure a successful transfer, which could be encoded on secondary (aka helper) plasmids. Among these special enzymes are some endonucleases, intended to cut the cargo plasmid at the bom site and promote transfer, and methylases to protect the transferred DNA against the restriction enzymes in the recipient. Detailed procedures, strategies and strains used are amply reviewed elsewhere (Heidorn et al., 2011).

CYANOBACTERIA AS A PRODUCTION SYSTEM FOR BIOFUELS: CURRENT STATUS

Hydrogen

Frequently cited as the fuel of the future, hydrogen production, storage and utilization are being widely investigated. As a transportation fuel it presents a series of challenges in every link of the chain, from production to storage and distribution. Although having a low volumetric energy density, hydrogen has the highest energy density per mass and the simple fact that its combustion generates almost only water and heat has seduced entire generations. “Yes, my friends, I believe that water will one day be employed as fuel, that hydrogen and oxygen which constitute it, used singly or together, will furnish an inexhaustible source of heat and light, of an intensity of which coal is not capable” (Verne). Cars that could run on water with minimal energy consumption have captured the imagination of many people and, not surprisingly, have inspired frauds like the almost magical conversion of saltwater into fuel using radiofrequency radiation, claimed by John Kanzius and broadcast live countrywide from Philadelphia, or the notorious “StanleyMeyer’s water fuel cell” to be used in an internal combustion engine, where a special device could split water giving an energy output sufficient to generate mechanical energy for the vehicle with enough leftover to power a fuel cell that would provide more hydrogen and oxygen through water splitting. Considering that the combustion of hydrogen and oxygen regenerates water, both systems obviously defy the first and second laws of thermodynamics (Ball, 2007).

Despite the motivation behind these schemes, they touched upon the most limiting step in the development of the hydrogen fuel technology: production. In current industrial practice, hydrogen can be produced by pyrolysis, electrolysis or by steam reforming of hydrocarbons. The last is the dominant method, applied to fossil fuels,

usually natural gas (methane). This makes hydrogen both expensive and unsustainable.

Hydrogen Bioproduction

Molecular hydrogen (H_2) is the lightest gas possible. When released into the atmosphere it diffuses quickly toward the troposphere, thus, at the sea level it can only be found in trace amounts. For this reason, very little naturally occurring H_2 is available and therefore a sustainable production system must be found if this molecule is to be used as a fuel. Efficient biological production of hydrogen could represent a breakthrough in the development of this energy carrier and many different approaches are being followed toward this goal. Undoubtedly, among all the possible fuels that could be produced by cyanobacteria, it is hydrogen that has received the most attention. Here we discuss the biological mechanisms for hydrogen production and advances toward yield improvements in cyanobacteria.

In the light reactions of photosynthesis, light is captured by photosystems I and II, acting together to transform solar energy into chemical energy, splitting water into molecular oxygen and protons (H^+) and the reducing agent NADPH. The transmembrane proton gradient that is formed is used by ATP synthase to combine adenosine diphosphate (ADP) + Pi into ATP (Figure 22.1). This set of reactions is rather interesting because it effectively conserves ubiquitous solar energy in energy-dense molecules using an abundant substrate, water. Ironically, cyanobacteria (and all plants) had been all along for millions of years the very sought after solution for breaking the strong bond between oxygen and hydrogen in the water molecule without using the special radiofrequency of John Kanzius or the mysterious fuel cell of Stanley Meyer.

During the water-splitting process, oxygen is released in its molecular form (O_2), while hydrogen, in the form of protons, is further used to produce two molecules of high-energy content: ATP and NADPH. Together, they feed energy into the Calvin-Benson-Bassham cycle, where CO_2 is fixed into organic molecules, as well as into many other reactions related to cellular homeostasis or secondary metabolism. Alternatively, before it is used to generate NADPH, the high-energy electron generated by photosynthesis can be directly used for the evolution of hydrogen, a process called direct biophotolysis (Benemann and Weare, 1974). Therefore, hydrogen evolution through this route does not require CO_2 fixation, and solar energy and water, together with the required enzymes, are sufficient for H_2 formation (Hallenbeck and Benemann, 2002). The major problem with this process is that hydrogenases, the hydrogen-evolving enzymes, are extremely sensitive to oxygen (O_2) and are irreversibly inactivated by even small concentrations of this gas. Thus, hydrogen evolution is usually a short-lived process, with a burst of hydrogen evolution when transitioning from a dark cycle into light as increasing oxygenic photosynthesis quickly inactivates the hydrogenase. Some species, especially filamentous ones (e.g. *Anabaena* sp. and *Nostoc* sp.), capable of forming specialized cells called heterocysts, can be shown to produce hydrogen over prolonged periods in light, as the heterocysts provide an oxygen-free environment that protects the hydrogenase against inactivation. In indirect biophotolysis, the captured light energy is used to fix CO_2 and the organic molecules that are produced are stored as reserve material. Under normal conditions, part of these carbon reserves will be oxidized over the dark period to maintain cellular homeostasis. However, under proper conditions such a culture can be induced to produce hydrogen, thus separating hydrogen evolution temporally and spatially from the oxygen evolved by oxygenic photosynthesis (Hallenbeck, 2011). Thus, hydrogenase activity is maintained and the simultaneous production of hydrogen and oxygen, an explosive mixture when concentrated in the headspace of a bioreactor, is avoided.

Hydrogen-Evolving Enzymes

Hydrogenases in cyanobacteria have been studied for over 35 years (Benemann and Weare, 1974; Hallenbeck and Benemann, 1978) and many variations of hydrogenases have been described in different bacterial phyla (Vignais and Billoud, 2007). These enzymes are frequently classified into three different groups: nitrogenase, the reversible hydrogenase (Hox), and the uptake hydrogenase (Hup) (Ghirardi et al., 2007).

HUP—HYDROGEN UPTAKE ENZYME

Hup is a [NiFe] hydrogenase that occurs associated with the thylakoid membrane (Seabra et al., 2009). This enzyme shows the least sensitivity to oxygen among the three classes. Its function is in the oxidation of H_2, returning the captured electrons to cellular electron transfer reactions. To date it has been found only in N_2-fixing strains and appears to have an intimate relationship with nitrogenase (Marreiros et al., 2013). Under natural conditions, nitrogenase functions to reduce atmospheric N_2 to NH_3, producing H_2 in an unavoidable side reaction. It is thought that Hup functions to recycle the recently formed H_2, which is oxidized back into protons or reacted with O_2 in a respiratory oxyhydrogen reaction, protecting the nitrogenase from O_2 inactivation, avoiding an excessive build up of H_2 in the cell and recovering part of the ATP used in its formation (Bothe et al., 2010; Tamagnini et al., 2007). In the nitrogen-fixing cyanobacteria, transcription of the Hup-encoding genes *hupSL* is associated with the nitrogen depletion response and

is under the regulation of the NtcA, the global nitrogen regulator (Weyman et al., 2008). Hup inactivation increases the production of H_2 two- to threefold in most cyanobacteria (Ludwig et al., 2006; Tamagnini et al., 2007).

NITROGENASE—A GRATUITOUS HYDROGENASE

In nature this complex enzyme carries out a critical function, breaking the three covalent bonds of molecular nitrogen (N_2) providing ammonia to the cell and closing the nitrogen cycle. This process consumes a large amount of energy in the form of ATP and high-energy electrons (Eqn (22.1)), producing NH_3 with the coproduction of hydrogen in an unavoidable side reaction.

$$N_2 + 10H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP \quad (22.1)$$

The most common nitrogenase is the Mo-Fe nitrogenase, which is characterized by a complex iron-sulfur cluster containing molybdenum. While performing nitrogen fixation, up to one-fourth of the electron flux goes toward the reduction of hydrogen. Variations of this enzyme includes the substitution of the molybdenum by vanadium or iron (V-Fe and Fe—Fe nitrogenases, respectively), which, although a greater proportion of electrons are allocated to hydrogen production, in fact show a lower net flux of electrons to hydrogen since their overall reaction rates are much lower than that of the Mo-Fe enzyme, limiting the application of these variants in bioproduction systems. One option that is an interesting strategy for H_2 production, to increase the electron flux into H_2, is cultivation in the absence of N_2, since nitrogenase turnover continues, but now the electron flux goes totally toward hydrogen evolution. In addition, the growth arrest caused by the nutrient limitation is of interest as this decouples hydrogen evolution from biomass production, therefore potentially leaving more energy available for H_2 production (Benemann and Weare, 1974). Even so, the expression of an oxygen-sensitive enzyme in an O_2 rich milieu is counter productive. To overcome this problem, temporal separation between N_2 fixation and photosynthesis can be used, where during the day the photosynthetic machinery works toward the carbon fixation, which then can be consumed to power nitrogenase and consequently proton reduction. Interestingly, the peak of hydrogen production in indirect biophotolysis occurs when the cell is reilluminated, possibly due to a burst in ATP synthesis before the oxygen formed by PSII (Figure 22.1) reaches a toxic level for the nitrogenase.

Heterocyst forming species on the other hand can perform direct biophotolysis by carrying out nitrogen fixation in the differentiated cell during the day. The heterocyst can maintain an internal anoxic environment since the expression of PSII is repressed. Hydrogen production therefore is supported through the use of carbon compounds delivered by the neighboring vegetative cells.

REVERSIBLE HYDROGENASE (HOX)

In addition to nitrogenase, N_2-fixing cyanobacteria can have a second hydrogen-evolving enzyme, the so-called reversible hydrogenase (Hox). This enzyme is a heteropentameric complex that is formed by a hydrogenase module (HoxHY) and a diaphorase module (HoxEFU), which transfers electrons from NAD(P)H to the hydrogenase module (Bothe et al., 2010). Like Hup, Hox is a [NiFe] hydrogenase, but in this case it shows a high sensitivity to O_2. Its expression is totally independent from that of nitrogenase and varies among species. In some cases it is under the control of the circadian clock, where it is shown to promote hydrogen production in the dark (Hallenbeck and Benemann, 1978; Schmitz et al., 2001). The bidirectional hydrogenase is not taxon specific, being found in many different groups of cyanobacteria, and its location and organization in the chromosome are also heterogeneous. Recent studies regarding Hox transcription factors have elucidated many aspects of its regulatory mechanisms, which are reviewed elsewhere (Oliveira and Lindblad, 2009).

Hydrogen Bioproduction

As discussed above, nonbiological production of hydrogen is energy intensive and often associated with the production of greenhouse gas. Biologically, hydrogen can be produced by a variety of microorganisms possessing one of several different hydrogenases. In the cyanobacteria, enzymes involved in hydrogen metabolism belong to one of the three families discussed above: Hox, Hup or nitrogenase. The uptake hydrogenase (Hup) is not useful for hydrogen evolution since it is poised to work unidirectionally, toward the recycling of H_2 into H^+. When hydrogen is produced by a heterotrophic organism, an organic carbon source (ultimately derived from photosynthesis) is used to provide protons and chemical energy to fuel hydrogen evolution. Ironically, this is also true for cyanobacteria carrying out direct or indirect biophotolysis, at least on the molecular level. As discussed above, a complete photosynthetic apparatus uses water as proton donor, releasing molecular oxygen (Figure 22.1). Thus, the high sensitivity of hydrogenase to this gas dictates that both reactions cannot occur in the same place at the same time. The solution found by Nature was the most obvious one: changing the timing (indirect biophotolysis) or the space (direct biophotolysis).

In indirect biophotolysis the cell uses the chemical energy stored through the capture of sunlight, as

NADPH and ATP (Figure 22.1), to fix CO_2 into organic compounds. These energy reserve molecules are then consumed in the dark to drive cellular metabolism, including nitrogen fixation by nitrogenase. The separation of these reactions occurs naturally in several cyanobacterial species by circadian control and in these strains dark hydrogen production by either nitrogenase or the bidirectional hydrogenase is frequently reported (Prabaharan et al., 2010; Troshina et al., 2002). An interesting characteristic found in many of these strains is a burst of hydrogen production when cells are reilluminated. This phenotype was characterized as a function of the bidirectional hydrogenase and hydrogen production ceases quickly as the O_2 produced by photosystem II (Figure 22.1) accumulates in the cell, inactivating hydrogenase The production of H_2 is thought to serve as an electron sink, helping the cell return to the proper redox state for carrying out the light reactions. In practice, indirect biophotolysis could possibly be done as a large-scale production using a two-stage cultivation system. In a first stage, the cells are cultivated in the light and biomass is formed through photosynthesis. When the desired cell concentration is achieved and the cells have stored enough fixed carbon, a dark anaerobic cultivation could follow, favoring proton reduction to hydrogen by hydrogenase. Thus, the water-splitting reaction is separated from H_2 production in time and space. This system has being already demonstrated, where nitrogen limitation was also used to induce glycogen accumulation and increase hydrogen production yield in the second stage through the nitrogenase enzyme (Huesemann et al., 2009). In a similar approach with *Synechococcus* sp., the carbon accumulated in the first stage was converted into hydrogen in a second stage by a [NiFe] hydrogenase (McNeely et al., 2010).

H_2 PRODUCTION BY HETEROCYSTOUS CYANOBACTERIA

Solar energy capture and hydrogen evolution by some filamentous cyanobacterial strains proceeds naturally in the presence of oxygen by confining the oxygen-sensitive processes to the heterocyst, a cell type that emerged shortly after the oxygenation of the earth's atmosphere in what has been called the Oxygen Catastrophe or Great Oxidation Event 2.6 billion years ago (Kumar et al., 2010; Mariscal and Flores, 2010). In this case the evolved hydrogen is produced by nitrogenase whose expression is restricted to the heterocyst under normal aerobic conditions (Murry et al., 1984). A number of mechanisms are employed to protect nitrogenase from oxygen damage; heterocysts lack photosystem II so do not produce oxygen, gas diffusion into the heterocyst is restricted by a unique cell wall structure, and heterocysts possess a very active membrane-bound respiratory system that consumes trace amounts of entering oxygen. Even so, some continual synthesis of nitrogenase is necessary to replace oxygen-damaged nitrogenase (Murry et al., 1983).

As discussed above, since heterocysts lack a complete photosynthetic apparatus, the necessary reductant is derived from fixed carbon imported from the neighboring vegetative cells through specialized interconnecting pore structures (Mariscal and Flores, 2010). The imported sugar is sucrose (Lopez-Igual et al., 2010) and it is metabolized though the oxidative pentose pathway (Summers et al., 1995) Thus, hydrogen production by heterocysts is essentially indirect biophotolysis on a microscopic scale, and since the energy captured by photosynthesis is first stored as fixed carbon, the maximal possible theoretical conversion efficiencies are reduced.

However, this system has been attractive due to its inherent robustness and has been studied for almost four decades (Benemann and Weare, 1974). Very reasonable conversion efficiencies, sustained for days to weeks, were achieved in early studies using nitrogen-limited cultures. Under laboratory conditions where higher efficiencies can be expected, conversion efficiencies (total incident light energy to free energy of hydrogen produced) were shown to be 0.4% (Weissman and Benemann, 1977). Cultures incubated under natural sunlight (Figure 22.3) were able to attain an average conversion efficiency of 0.1% (Miyamoto et al., 1979a). Remarkably, even though there have been a large number of studies since, very little improvement in yields

FIGURE 22.3 Tubular photobioreactors operating under "airlift"conditions were used to demonstrate prolonged (over 30 days) simultaneous oxygen and hydrogen evolution by nitrogen-limited cultures of the heterocystous cyanobacterium, *Anabaena cylindrica*. *Source: Miyamoto et al 1979d.* (For color version of this figure, the reader is referred to the online version of this book.)

has been obtained. Thus, recent reports of conversion efficiencies found ≈0.7% under laboratory conditions (Berberoglu, 2008; Sakurai and Masukawa, 2007; Yoon et al., 2006) and 0.03−0.1% with natural sunlight (Sakurai and Masukawa, 2007; Tsygankov et al., 2002). Similar low efficiencies have been found with thermophilic strains, which at least have the possible advantage of requiring less cooling (Miyamoto et al., 1979b,c). There should be room for improvement as theoretical efficiencies with this nitrogenase-based system have been calculated to be around 4.6% (Hallenbeck, 2011).

Since observed conversion efficiencies are lower than predicted, different strategies might be employed in order to improve overall performance, which is critically important since light conversion efficiencies directly impact on the photobioreactor footprint (doubling efficiency should halve the required surface area for the same amount of fuel production). For one thing, genetic engineering could be applied to optimizing the size of the photosynthetic antenna, since part of the reduction in efficiency is thought to be due to inefficient use of light energy at high intensities where more photons are captured than can be used and the excess energy is wasted. Another point that could be addressed is the hydrogen producing catalyst. Since half of the photon requirement is needed to provide ATP to nitrogenase action, replacing it with a hydrogenase, which does not require ATP for proton reduction, should in principle have an energy sparing effect. In a recent attempt to verify this, the [FeFe] hydrogenase from *Shewanella oneidensis* was expressed in *Anabaena* sp. under the control of a heterocyst-specific promoter with the required maturation genes (Gartner et al., 2012). Although it could be shown that active hydrogenase was made under the proper conditions, the increase in hydrogen production above the levels due to the coexisting nitrogenase was disappointingly small. Of course, under these conditions the two enzymes compete for the reductant; the true test would be to do this in a strain lacking nitrogenase activity. Finally, it might in principle be a possible way to increase hydrogen production by increasing heterocyst frequency. However, heterocyst frequency might already be close to optimal since even in long-term studies the H_2/O_2 ratio is close to the desired stoichiometry of two, what one would expect for optimal coupling between oxygen-generating photosynthesis in the vegetative cells and hydrogen production by heterocysts.

H_2 PRODUCTION BY NONHETEROCYSTOUS CYANOBACTERIA

Although the heterocyst/nitrogenase-based system has been the most studied, some other known cyanobacterial hydrogen-producing reactions could potentially be used for biological hydrogen production. These include the unicellular and nonheterocystous filamentous cyanobacteria, which possess nitrogenase and are able to fix nitrogen in nature. Two strategies are employed to avoid oxygen inhibition. In some unicellular species, oxygen evolution and nitrogen fixation (or hydrogen production) are separated in time since photosynthesis and nitrogen fixation are under circadian control with photosynthesis taking place during the day and nitrogen fixation being maximal during the night period. The filamentous cyanobacterium *Trichodesmium* uses a strategy of spatial segregation where nitrogen fixation occurs in cells located in the middle of the bundle carrying out the oxygen-sensitive nitrogenase reactions and the others carrying out the normal photosynthetic reactions (Berman-Frank, 2001).

The unicellular cyanobacterium *Cyanothece* has been the subject of a number of recent studies demonstrating prolonged hydrogen production in the light mediated by nitrogenase. In one study, considerable hydrogen production (up to 465 μmol per milligram of chlorophyll per hour) was shown, the growth conditions were very stringent and hydrogen production was only observed when the culture was submitted to nitrogen starvation, sparged with argon to remove any oxygen formed through photosynthesis, supplemented with glycerol and cultivated under low light (Bandyopadhyay et al., 2010; Min and Sherman, 2010). Glycerol, in addition to serving as a possible additional energy source to support nitrogenase activity, appears to release nitrogenase from diurnal control (Aryal et al., 2013). Another recent study found appreciable hydrogen and oxygen production with nitrogen-depleted cultures that were incubated under continuous illumination (Melnicki et al., 2012). Light saturation curves and photosynthesis inhibition studies indicate that the hydrogen is evolved indirectly from the fixed carbon produced through photosynthesis. Here again, the requirements for continuous illumination (it can hardly be energetically positive to produce hydrogen using artificial illumination) and for argon sparging raise serious hurdles to practicality. Thus, although a nice proof of principle, such a system would hardly be economically viable.

Many cyanobacteria also possess Hox, a soluble reduced nicotinamide adenine dinucleotide (NADH)-linked [NiFe] hydrogenase. This reversible hydrogenase is capable of hydrogen evolution, in particular when dark-adapted cells are reilluminated (Schwarz et al., 2010). As discussed above, this forms an electron valve, readjusting the poise of the photosynthetic apparatus, but activity is quickly inhibited with renewed oxygen evolution. A recent survey showed that a diversity of cyanobacteria contains this enzyme and that there is great variability in both the amounts of hydrogen made by this enzyme and the pattern of hydrogen evolution (Kothari et al., 2012). This enzyme is also responsible for evolution during dark fermentation of endogenous

reserves, principally glycogen, and hydrogen production by this pathway can be enhanced through lowering of the hydrogen partial pressure (Ananyev et al., 2012). At least in *Synechocystis*, hydrogen production by Hox can be increased by eliminating the master regulator AbrB2, which normally represses synthesis of Hox (Dutheil et al., 2012; Leplat et al., 2013). In a recent attempt to increase hydrogen production, heterologous expression of the [FeFe] hydrogenase from *Clostridium acetobutylicum* was carried out in the non-nitrogen-fixing cyanobacterium *Synechococcus* (Ducat et al., 2011). Active hydrogenase was formed under proper conditions, but in vivo light-driven hydrogen production from this system was significant only when the cultures were incubated under an inert atmosphere and oxygenic photosynthesis was completely inhibited.

Ethanol

While hydrogen production, or at least direct biophotolysis, can be driven directly by photosynthesis, all other biofuels must use the capacity of cyanobacteria to drive carbon dioxide fixation with photosynthetically derived energy, ATP and reductant. However, once fixed by the Calvin-Benson-Bassham cycle, the newly recycled carbon can be converted to useful biofuels through the introduction of novel (to cyanobacteria) metabolic pathways (Angermayr et al., 2009). The first such cyanobacterial-derived biofuel that was demonstrated was ethanol (Deng and Coleman, 1999; Dexter and Fu, 2009), and its production is the only cyanobacterial-produced biofuel under active investigation and commercial development (Algenol Biofuels: http://www.algenolbiofuels.com/). Algenol Biofuels is presently claiming production at "around $1.00 per gallon using sunlight, carbon dioxide and saltwater at production levels above 9000 gallons of ethanol per acre per year". At an average solar insolation for Florida of 19.8 MJ/day and since ethanol has a higher heating value of 29.7 MJ/kg, this translates to a claim of a very impressive 2.8% conversion efficiency. Now another company, Joule Unlimited (http://www.jouleunlimited.com/), has stepped into the picture, offering to sell SunFlow-E® through its fuel company, Joule Fuels. Their process uses genetically modified thermophilic cyanobacterium containing *Moorella* alcohol dehydrogenase, and their Web site claims are even more spectacular with targets of up to 25,000 gallons per acre (7.8% conversion efficiency) and $0.60 per gallon at full-scale commercial production.

Cyanobacteria can naturally produce relatively minute amounts of ethanol so at the simplest level, creating a cyanobacterium that produces higher levels of ethanol involves boosting flux through the ethanol pathway through the introduction of the key enzymes for conversion of pyruvate, generated by glycolysis of the fixed carbon, to ethanol, pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*). This alone is sufficient to produce low millimolar levels of ethanol in the medium upon prolonged (5–10 days) incubation and growth. Further increases, obviously necessary for practical production, have been achieved through a variety of means, including better transcriptional control and further metabolic engineering. Most of this development work is being done at private enterprise laboratories, but a recent published report (Gao et al., 2012) shows that impressive increases in yields can be achieved by integrating a foreign *pdc* and a native *adh* into the genome of *Synechocystis* and abolishing carbon flux into polyhydroxybutyrate synthesis.

Ethylene

The chemical industry relies on simpler molecules to build complex compounds, which are used in a variety of applications. Among these organic compounds, ethylene is the building block with the highest demand, being used to manufacture "everyday" products such as polyethylene terephthalate (PET bottles), polyester, antifreeze, and others (Ungerer et al., 2012). Ethylene (or ethene) is the second simplest unsaturated hydrocarbon, it consists of two carbons, with a double bond, and four hydrogens ($H_2C{=}CH_2$). It is one of the products of pyrolysis, and has been used as a fuel since the early nineteenth century as one of constituents of the gaseous fuel in gas lamps. Ethylene is the most important compound in the chemical industry in terms of market volume, it has a heat of combustion higher than that of gasoline or diesel and can be used as a transportation fuel or to produce electric energy in stationary plants. Currently, ethylene is a petroleum derivative produced through steam cracking. It reached a production of 100 million metric tones in 2005, accounting for 30% of all petrochemical commodities (McCoy et al., 2006; Saini and Sigman, 2012). The fluctuation in crude oil prices over the last few years (EIADOE, 2012), the imminent threat of peak oil (Nashawi et al., 2010), and the existence of biological pathways for its production coupled with the ease of harvesting a gas like ethylene, make this chemical a good target for the development of a sustainable biological production system.

The most common occurrence of ethylene in nature is as a hormone found in vascular plants, where it is associated with many effects such as defoliation, responses to temperature stress, mechanical injury, and for promoting fruit ripening (Abeles, 1972). In addition to vascular plants, many other plants and algae have been shown to be able to produce ethylene and even if it not found in animals, this gaseous hormone has been shown to induce regulatory responses in invertebrate and mammalian cells (Perovic et al., 2001).

The most common biosynthetic pathway for ethylene production is the Yang cycle that occurs in plants, where it is produced from methionine in a three-step reaction, having *S*-adenosylmethionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC) as precursors. However, the cellular response to this hormone occurs at very low concentrations, a characteristic that, together with the fast and easy diffusion of this gas into plant tissues, makes the conversion of AdoMet to ACC, catalyzed by ACC synthase, and from ACC to ethylene (ACC oxidase) a tightly regulated process. Both enzymes are multigenic with differential regulation through distinct promoters and operators for groups of genes of the same enzyme (Nakatsuka et al., 1998). The methionine used in this pathway is recycled through the Yang cycle (Taiz and Zeiger, 2002; Wang et al., 2002).

Microbial Production of Ethylene

The great influence of this gaseous hormone on different plant organs made it an interesting target for pathogens. Indeed, the mold *Penicillium digitatum* has been known to produce ethylene since the mid-1950s (Wang et al., 1962) and its production was shown for prokaryotic plant pathogens in the early 1960s (Freebairn and Buddenhagen, 1964). Not surprisingly, the pathways found in these microorganisms are not analogous to the one in plants. So far, two distinct routes for ethylene production have been described in microbes: a 2-oxoglutarate-dependent pathway and the 2-keto-4-methyl-thiobutyric acid (KMBA) pathway (Nagahama et al., 1991, 1992). The latter is the most common among microorganisms, composed of a series of chemical and enzymatic reactions, by which only trace amounts of ethylene are usually produced (Ogawa et al., 1990). The former pathway has been found to be more efficient, with 2-oxoglutarate being used as substrate in a single-step reaction by the ethylene-forming enzyme (EFE). This pathway has been found in several different microorganisms, including *P. digitatum*, *Chaetomium globosum, Phycomyces nitens, Fusarium oxysporum*, and in different pathovars of *Pseudomonas syringae,* where a comparison study found the pv. *phaseolicola* to be the most efficient ethylene-producing strain (Weingart et al., 1999). This enzyme catalyzes simultaneously two reactions (Fukuda et al., 1992b):

$$2-\text{oxoglutarate} \leftrightarrow \text{ethylene} + 3CO_2 + H_2O \qquad (22.2)$$

$$2-\text{oxoglutarate} + \text{L}-\text{arginine} + O_2 \leftrightarrow \text{succinate} + CO_2 + \text{guanidine} + (\text{S})-1-\text{pyrroline}-5-\text{carboxylate} + H_2O \qquad (22.3)$$

These reactions are rather interesting as they keep the tricarboxylic acid (TCA) cycle closed through a shortcut, converting 2-oxoglutarate directly into succinate with the formation of ethylene "as a by-product" (Figure 22.2), and therefore substituting for the steps catalyzed by 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase (Figure 22.2). The original two-step reaction between 2-oxoglutarate and succinate generates one NADH and one guanosine triphosphate, which are not produced by EFE. Thus, competition of the two pathways for substrate 2-oxoglutarate would lower the formation of NADH. Since NADH also has a role as an inhibitor for four enzymes associated with the TCA cycle, pyruvate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and citrate synthase (Figure 22.2), this could potentially upregulate the reactions performed by these enzymes, from the decarboxylation of pyruvate to 2-oxoglutarate. The last is a direct and indirect substrate to the EFE, directly to generate ethylene (Eqn (22.2)) and indirectly, as it is also a substrate for the synthesis of arginine, required for the simultaneous reaction of this enzyme (Eqn (22.3) and Figure 22.2).

Bioproduction of Ethylene Using efe

When producing any molecule of commercial interest with a microorganism, prospecting for the key gene is as important as the choice of the host to be used. The evolutionary convergence of ethylene production is highlighted by the three pathways delineated above: Yang cycle, KMBA and 2-oxoglutarate, with the last having been found to be the most efficient when overproduction is desired. The evolutionary radiation of the mobile plasmid encoding the *efe* gene among the different species and strains might have produced a naturally optimized gene that could be used in commercial production. A comparison between 20 *P. syringae* strains revealed a high amino acid sequence similarity between five pathovars, with *P. syringae* pv. *phaseolicola* PK2 being the most efficient, giving a twofold higher production of ethylene (Weingart et al., 1999). However, these variations are likely to be due to differences in regulation as the sequence of amino acids of *efe* of these five strains differs by only one codon.

Using the *efe* gene encoded by an indigenous plasmid from *P. syringae* pv. *phaseolicola* PK2, ethylene production was reported in *E. coli* with a tenfold increase when compared to the original strain, *P. syringae* (Fukuda et al., 1992a; Ishihara et al., 1995), showing that the *efe* gene alone was sufficient for ethylene production. When a high-copy-number plasmid containing *efe* was transconjugated into *Pseudomonas putida* and *P. syringae*, ethylene production was increased, but surprisingly, production was 27- and 8-fold higher, respectively, than the wild type, whereas the amount of protein produced in the cloned *P. syringae* was 20-fold higher

(Ishihara et al., 1996), suggesting the presence of a post-transcription regulatory system. Using cellulose as substrate, Tao et al. showed the production of ethylene in *Trichoderma viride* through the heterologous expression of *efe* from *P. syringae* pv. *glycinea.* Thus, the use of agriculture wastes as substrate for ethylene production was proved to be feasible, but the recombinant filamentous fungus produced only very small amounts of ethylene (Tao et al., 2008).

So far, cyanobacteria have been shown to be the best model for the bioproduction of ethylene. Of course, many barriers still have to be crossed and commercial production is far from reality at present, but the last few years have seen encouraging reports where the productivity was increased several fold without compromising cell fitness, suggesting that the true production limit might be much higher. The *efe* (EFE) from *P. syringae* was originally cloned into *Synechococcus elongatus* PCC 7942 (Fukuda et al., 1994; Sakai et al., 1997; Takahama et al., 2003). The first problem area, the production of only trace amounts of ethylene by the transformants (Fukuda et al., 1994), was later shown to be due to the nature of the promoter used. A systematic evaluation of different promoters showed the *psbA1* promoter is more efficient for *efe* expression than those (lac and *efe*) previously used in other reports, achieving production rates up to 240 nl/ml h or 451 nl/ml h OD730 (Takahama et al., 2003). However, these recombinants showed high genetic instability. Sequencing of the heterologous gene from mutants that had ceased to produce ethylene showed punctual mutations at a defined sequence of five nucleotides, suggested to be a possible hot-spot site for spontaneous mutagenesis (Takahama et al., 2003). Nevertheless, active ethylene-producing strains showed signs of metabolic stress, evidenced by their yellow-green color. When these strains had ceased ethylene production due to spontaneous mutation of *efe* (genetic instability), they recovered the normal blue-green phenotype.

In another strategy (Ungerer et al., 2012), *Synechocystis* sp. PCC 6803 was used as model organism. Toxicity to ethylene was tested, *efe* was codon optimized and artificially synthesized, eliminating the bases at the putative mutational hot spot by conservative substitution. As well, *efe* was placed under the control of the *psbA1* promoter. A semicontinuous culture using a clone containing two copies of *efe* was sustained over a three-week period, reaching a constant production of 3100 nl/ml h, compared to the previous result of 240 nl/ml h (Takahama et al., 2003). The peak of the specific productivities was 380 nl/ml h OD730 for one *efe* copy and 580 nl/mL h OD730 for two copies, respectively, and when in semicontinuous culture, the average rate was 200 nl/ml h OD730. The additional copy of the *efe* gene presented some production improvement when compared with the previous work from Takahama et al., (451 nl/ml h OD730 compared to 580 nl/ml h OD730) but the real advance for the field can be seen from the healthy state of the culture. The growth rate, the color of the culture and the growth curve were the same for wild type and the mutants containing one or two copies of *efe*. This shows that there is no toxicity either by the product or by the metabolic route used to produce ethylene. In addition to the zero toxicity, the release of five carbons per ethylene formed does not seem to present a burden to the cell, as shown by the growth pattern of the single and double mutant when compared with the wild type. Nevertheless, the metabolic consequences to the cell of a higher rate of ethylene production are unknown and a physiological approach would help to understand how far ethylene production can be pushed and what to target to improve the final yield.

Isoprene

As with ethylene, isoprene is a medium-value biochemical that is produced through steam cracking of oil. It is actually an important by-product of ethylene production and is almost entirely used for production of a synthetic substitute for natural rubber. It is also naturally produced by many plants as a heat stress response, where it was shown to increase the stability of photosynthetic membranes at high temperatures (Sharkey et al., 2001). It can represent as much as 2% of all carbon fixed by oak leaves at a temperature of 30 °C (Sharkey, 1996), showing the physiological importance of this compound. The enzyme isoprene synthase (ispS) was shown to produce isoprene in plants, converting one of the products of the methylerythritol phosphate (MEP) pathway, dimethylallyl-diphosphate (DMADP), into isoprene (Silver and Fall, 1991; Silver and Fall, 1995). Prokaryotes were suggested to be able to produce isoprene after reports of the detection of this compound in the headspace of culture broth on many species (Kuzma et al., 1995), with emphasis on *Bacillus subtilis*. Not surprisingly, sequence analysis of bacterial genome could not identify any gene homologous to the ispS. found in plants (Withers et al., 2007). So far, functional genomics has also failed to identify the pathway for isoprene production in prokaryotes. Sequence-independent methods showed that 19,000 *E. coli* clones transformed with DNA fragments from *B. subtilis* in an environment where DMADP and IPP (isopentenyl pyrophosphate) levels were selectively toxic, showed that no single enzyme was sufficient to convert DMADP to isoprene, where the few clones that managed to survive, preferably converted it to a prenyl alcohol (Withers et al., 2007). As all isoprenoids are thought to be solely produced from DMADP and IPP (Xue and Ahring, 2011), the conversion of the metabolites involved in MEP or mevalonate pathway

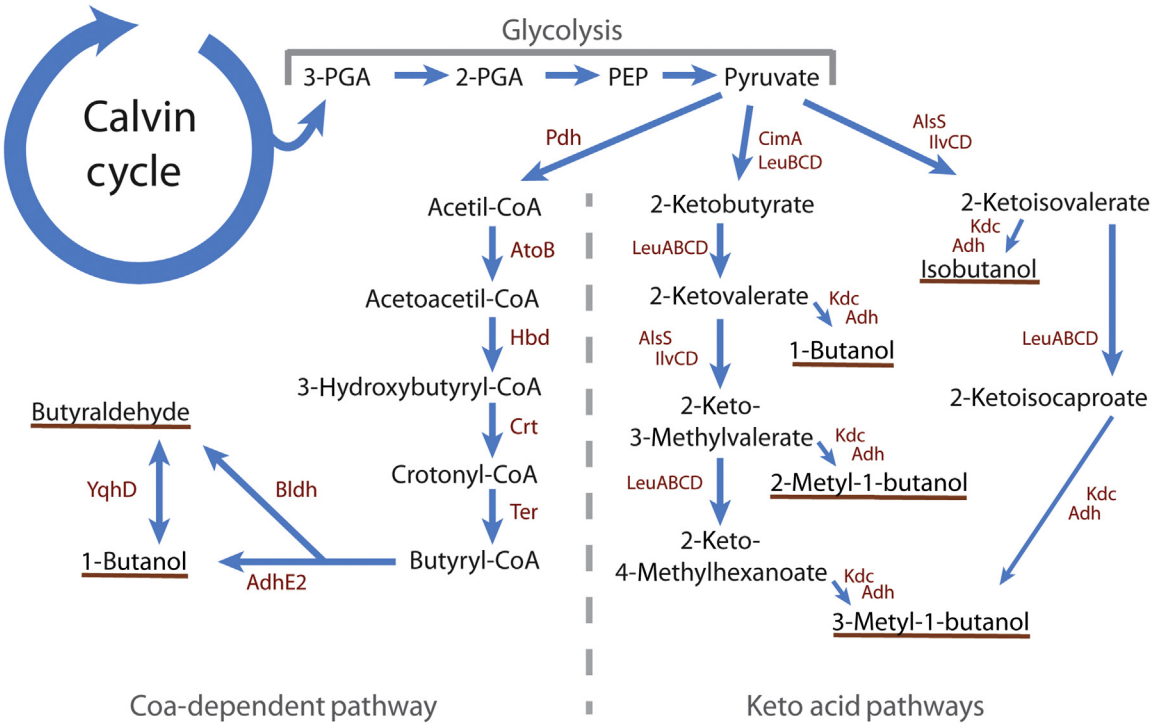

FIGURE 22.4 Pathway alternatives for n-butanol bioproduction. The alcohol n-butanol is naturally produced in different microorganisms in small quantities, where it can be synthesized either through the CoA-dependent pathway or the keto acids pathway. (For color version of this figure, the reader is referred to the online version of this book.)

to isoprene in bacteria could be a phenotype derived from convergent evolution using a multistep reaction diverged from those pathways (Izumikawa et al., 2010; Withers et al., 2007; Xue and Ahring, 2011).

Bioproduction of isoprene is feasible and has already been demonstrated in *E. coli* expressing heterologous *ispS* (Miller et al., 2001; Zhao et al., 2011). Of course productivity is an issue and different strategies were tried to increase isoprene production. Simultaneous expression of heterologous enzymes involved in MEP or mevalonate pathways was shown to be effective in both cases (Yang et al., 2012; Zhao et al., 2011). Julsing et al. also showed that the individual expression of the genes encoding enzymes involved in the MEP pathway did not affect isoprene production with the exception of the *dxs* gene, encoding the enzyme that catalyzes the first reaction of the MEP pathway, which significantly improved isoprene production (Julsing et al., 2007). Cyanobacteria produce DMAPP through the MEP pathway for secondary metabolites and, albeit with no natural production of isoprene being reported yet, transformation and expression of heterologous *ispS* were shown to be sufficient for production of isoprene. Lindberg et al. reported isoprene production using *Synechocystis* sp. PCC 6803 as a model organism harboring *ispS* from *Pueraria montana* (Kudzu) (Lindberg et al., 2010). The transgene was inserted at the *psbA2* locus and mutants did not show any disturbance in growth when compared to the wild type. This was a well-achieved proof of concept, and the low productivity reported, 50 μg per gram of dry cell weight per day, can be much improved through metabolic engineering. However, the use of cyanobacteria to produce isoprene has issues different from metabolic yield: to develop a production system of a molecule with a half-life of only a couple hours in the presence of light is particularly challenging in a photosynthetic organism. To overcome this issue, the development of special photobioreactors is made in parallel to the molecular research, where the properties of isoprene as a volatile hydrophobic compound, easily separated from a culture broth and concentrating in the headspace, are exploited (Lindblad et al., 2012). The production of a gas in microorganisms is an interesting strategy because one does not need to harvest the cells, the product is concentrated in the gaseous phase of the culture. However, the cultivation techniques and the purification of this gas from a complex mixture represents an important step in the production chain and, as shown in this case, should develop together.

Butyraldehyde and Butanol

Butanol has many desirable properties as a fuel and thus is a suitable target for modification of

cyanobacteria. In fact, as a fuel it is superior to ethanol, being less corrosive and less volatile. Thus, it can easily be mixed with hydrocarbon-based fuels and used in the same infrastructure. A number of recent studies have shown that engineered cyanobacteria can in fact make surprisingly high levels of this compound, at rates that in fact surpass published rates for ethanol production by engineered cyanobacteria. Since cyanobacteria produce this fuel directly through photosynthetically driven CO_2 fixation, it is appropriate to compare the productivity per area of this process, as presently described, with that required for other biofuels, be it growing corn to produce the necessary sugars, or growing algae to produce biodiesel. Such a comparison shows that butanol production by cyanobacteria could be much better than making fuels from corn and very comparable to making biodiesel from microalgae (Sheehan, 2009).

Many cyanobacteria are capable of producing volatile compounds, including higher alcohols, but the natural production levels are miniscule (Hasegawa et al., 2012). In order to make fuel molecules at significant quantities, new pathways must be introduced as well as changes made to the native metabolic pathways. Studies on creating heterotrophic bacterial strains capable of producing butanol demonstrated that two possible routes were useful: the 2-ketoacid pathway, normally involved in amino acid biosynthesis, and the acetyl-CoA pathway, found in organisms such as *Clostridium* that naturally produce butanol during fermentation (Figure 22.4).

The first successful attempt in this direction was to engineer *S. elongates* to produce isobutyraldehyde through the 2-ketoacid pathway (Atsumi et al., 2009). Isobutyraldehyde is a precursor for isobutanol and other chemicals of interest and has the advantage of being highly volatile, easing its recovery from the culture broth thus removing product inhibition. The strategy applied consisted of boosting carbon flux through the pathway from pyruvate to 2-ketoisovalerate by integration into the genome of three foreign genes, *alsS*, *ilvC* and *ilvD*, catalyzing these steps, as well as *kivd* from *Lactococcus lactis*, the gene encoding the ketoacid decarboxylase enzyme that converts 2-ketoisovalerate to isobutyraldehyde. Overall carbon flux was then increased by integrating an additional copy of Rubisco (*rbcLS*) and the resulting strain produced 6230 μg isobutyraldehyde per liter per hour , a production rate that is higher than any other fuel molecule made by cyanobacteria to date. Additionally, it was demonstrated that isobutanol could be formed if a foreign alcohol dehydrogenase (YqhD from *E. coli*) was introduced, but titers were lower, presumably due to product inhibition.

However, the isomer that is made by the 2-ketoisovalerate pathway is isobutanol, a fuel additive, but not nearly as desirable in itself as a fuel as n-butanol, the product of the acetyl-CoA pathway or the 2-ketobutyrate pathway (Figure 22.4). Metabolic engineering was used to create an n-butanol-producing strain of *S. elongatus* by introducing the *hbd*, *crt*, and *adhE2* genes from *C. acetobutylicum*, the *ter* gene from *Treponema denticola*, and the *atoB* gene (instead of *thl*) from *E. coli* (Lan and Liao, 2011). However, n-butanol was only produced by this strain under anaerobic conditions, either in the light when photosystem II was inhibited by DCMU, or in the dark, which gave the highest production, a meager 20.8 μg/L/h. It was suggested that anaerobic conditions were necessary since some of the enzymes introduced are oxygen sensitive, severely limiting its usefulness. On the other hand, metabolic fluxes are obviously different during dark metabolism than during photosynthesis and the difference could be in the supply of a key metabolite. In line with this, in a more recent attempt to create an n-butanol-producing strain, flux through acetyl-CoA was increased by substituting an irreversible ATP hydrolysis step leading to the formation of acetoacetyl CoA (Lan and Liao, 2011). Other improvements consisted of substituting NADPH-requiring enzymes for NADH enzymes. With these changes, it was possible to demonstrate light-dependent n-butanol production, but at 62.5 μg/L/h this is well below (by a factor of 100) the initial promising results with butyraldehyde. This system would need very significant improvement before it could be considered for practical biofuel production.

Photosynthetic Production of Aliphatic Alcohols and Alkanes

For many fuel purposes, alkanes are more desirable than the other biofuels already discussed. For example, jet fuel standards (Jet-A or JP-8) demand a fuel with high energy density, low viscosity, low freezing point and good physical-chemical compatibility. These criteria cannot be met with fuels such as ethanol or fatty acid methyl esters, biodiesel. Being able to directly make alkanes would have a great payoff as these biofuels are "drop-in" fuels, able to directly substitute for presently used petroleum-based fuels as they could be used with existing infrastructure and would require no engine modification, etc.

Cyanobacteria, like some other bacteria, have long been recognized as being able to synthesize at least very small quantities of alkanes, which in fact can serve as a biogeochemical marker for their presence in the past (Han et al., 1968; Winters et al., 1969). This was taken advantage of in a recent demonstration of the heterotrophic production of alkanes using a modified *E. coli* that expressed the alkane biosynthetic pathway from a

cyanobacterium, consisting of an acyl-carrier protein reductase, which produces a fatty aldehyde, and an aldehyde decarbonylase (Schirmer et al., 2010). This allowed the production and secretion of a variety of C13–C17 alkanes and alkenes. Of course it would be desirable to actually do this in a cyanobacterium, and one study examined this through the heterologous expression of fatty acyl-CoA reductase in *Synechocystis* (Tan et al., 2011), which allowed the production of small quantities of aliphatic alcohols. The *acc* genes, encoding acetyl-CoA carboxylase (ACCase), which catalyses what is believed to be the rate-limiting step of fatty acid biosynthesis, were introduced into the genome in hopes of boosting alkane production, but only insignificant quantities were made. Further work is required to demonstrate significant alkane synthesis by a cyanobacterium. However, it may prove difficult to greatly boost alkane synthesis in this oxygen-evolving organism as the critical enzyme, aldehyde decarbonylase, has recently been shown to be a di-iron enzyme with an unusual mechanism that requires anaerobic conditions for full activity (Das et al., 2011).

CONCLUSION AND OUTLOOK

As discussed in this chapter, recent studies have shown the great promise for biofuels production by cyanobacteria. Unique among possible biofuel producers, cyanobacteria combine the attributes of being able to carry out photosynthesis-driven carbon dioxide fixation and to be easily manipulated genetically. The next few years should see advances in increasing the production rates and titers of the different demonstrated biofuels as well as perhaps the widening of the spectrum of possible biofuels. Nevertheless, for cyanobacterial systems to live up to their potential, a number of serious hurdles must be overcome. These include the development of reliable methods of stable cyanobacterial mass culture at high levels of productivity and the demonstration of cost-effective harvesting strategies. Harvesting presents a real dilemma no matter what the biofuel. If the biofuel is contained within the cell, then the biomass has to be removed from the culture medium, of which it is less than 1% by weight. If the biofuel is an excreted liquid, then this will necessarily be quite dilute and require substantial concentration. If the biofuel is a gaseous product, the culture will have to be enclosed in air-tight transparent material at a substantial cost given the large surface areas that would be required. Of course, the payoff to solving these problems would be enormous and this is likely to inspire future research and development in this area.

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Annexe II: Articles not Discussed in this Thesis

Through the four years of PhD training, I had the opportunity to collaborate with other students. The fruit of some of these collaborations were published as original and review articles. These articles are annexed here in it whole form and also available online at the address below.

Abdelaziz, A. E. M.; **Leite, G. B**.; Belhaj, M. A.; Hallenbeck, P. C. (2014) Screening microalgae native to Quebec for wastewater treatment and biodiesel production. Bioresource Technology. 157, pp. 140-148. http://dx.doi.org/10.1016/j.biortech.2014.01.114

Abdelaziz A. E. M.; **Leite G. B**.; Hallenbeck P. C. (2013) Addressing the challenges for sustainable production of algal biofuels: I. Algal strains and nutrient supply. Environmental Technology, 34(13-14), pp.1783–1805. http://dx.doi.org/10.1080/09593330.2013.827748

Abdelaziz A. E. M.; **Leite G. B**.; Hallenbeck P. C. (2013) Addressing the challenges for sustainable production of algal biofuels: II. Harvesting and conversion to biofuels. Environmental Technology, 34(13-14), pp.1807–1836. http://dx.doi.org/10.1080/09593330.2013.831487

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Screening microalgae native to Quebec for wastewater treatment and biodiesel production

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HIGHLIGHTS

- A novel microalgal screening method using 12 well microplates was used.
- 100 strains from local (Quebec) freshwater lakes and rivers were characterized.
- A number that showed good growth at 10 °C or high (20–45%) lipid content.
- Some showed a high capacity for nutrient removal.

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ABSTRACT

Biodiesel production from microalgae lipids is being considered as a potential source of renewable energy. However, practical production processes will probably require the use of local strains adapted to prevailing climatic conditions. This report describes the isolation of 100 microalgal strains from freshwater lakes and rivers located in the vicinity of Montreal, Quebec, Canada. Strains were identified and surveyed for their growth on secondary effluent from a municipal wastewater treatment plant (La Prairie, QC, Canada) using a simple and high throughput microalgal screening method employing 12 well plates. The biomass and lipid productivity of these strains on wastewater were compared to a synthetic medium under different temperatures (10 ± 2 °C and 22 ± 2 °C) and a number identified that showed good growth at 10 °C, gave a high lipid content (ranging from 20% to 45% of dry weight) or a high capacity for nutrient removal.

1. Introduction

The need for sustainable sources of energy is rapidly increasing due to the increase in the world's population, industrialization and greater demand for transportation. Conventional sources of energy, such as oil, natural gas, and coal, are nonrenewable and their use has caused extensive damage to the environment by increasing the atmospheric load of carbon dioxide and other greenhouse gases (GHGs) that are causing disastrous global climatic changes (Abdelaziz et al., 2013a,b). The highly productive terrestrial bioenergy crops, such as soybean oil and palm, are challenging feedstocks due to their effect on the world food supply. The use of non-edible crops as feedstock, seen by some as desirable, often diverts land from the production of food crops, and neither type can match the potentially high productivity of microalgae (Leite et al., 2013). Biodiesel derived from microalgal lipids has received much attention as it holds the promise to provide low carbon, renewable feedstocks without adversely affecting the food supply or the environment. Although microalgae have many desirable characteristics; faster growth rates, higher photosynthetic efficiencies, greater biomass and lipid productivities, there are however some significant challenges that need to be overcome. Large scale biofuel production will probably require the use of strains that are adapted to and competitive in local environmental conditions, Thus there is a need for the effective and rapid isolation of microalgal strains with potentially high intrinsic lipid content and rapid growth and biomass productivities (Demirbas, 2011; Elliott et al., 2012).

One of the major hurdles in the development of microalgal based biodiesel is that at present the overall cost for microalgal biodiesel production is much higher than that from other bioenergy crops. Thus, selection of an energy and cost effective production strategy will play a very important role in achieving competitive biodiesel prices. Selection of high lipid-producing microalgae, cheap nutrient sources, suitable cultivation locations, rapid cultivation and harvesting methods and efficient oil extraction techniques are criteria that should be considered (Duong et al., 2012). Here, we focus on screening around 100 freshwater strains of

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native microalgae to select the most suitable high lipid-accumulating microalgal strains and the use of wastewater as a production medium, thus potentially greatly reducing microalgal cultivation costs.

Microalgae, in addition to serving as a biofuel feedstock, are potential candidates for wastewater treatment. The discharge of incompletely treated wastewater can lead to eutrophication of surrounding waters and ecosystem damage due to the high amounts of nitrogen and phosphorus (Rawat et al., 2011). The high energy requirements and costs associated with wastewater treatment and nutrient removal with existing chemical and physical based technologies remains a challenge for municipalities, governments and industries (Christenson and Sims, 2011). Using microalgae based wastewater treatment potentially has a number of benefits; wastewater treatment can be coupled to biomass production for biofuel production, offsetting the utilization of unsustainable amounts of freshwater and commercial fertilizers otherwise required for microalgal cultivation. This option promises to reduce microalgal cultivation costs and the energy required for wastewater treatment as well as permitting resource recovery and recycling (Abdelaziz et al., 2013a; Cho et al., 2011; Pittman et al., 2011). Suitable wastewaters, rich in nutrients, in particular nitrogen and phosphorus, are available from slaughterhouse wastes, agricultural/industrial wastes, dairy effluents, compost plant and municipal waste. Growing algae on these waters is an attractive means to decontamination of heavily polluted wastewaters while at the same time providing high yields of biomass for the production of biofuels, organic chemicals, and other commercial products.

Municipal wastewater is one of the main sources of pollution to surface water in Canada, especially since many treatment plants, including those of major cities like Montreal, only carry out rudimentary treatment due to the lack of suitable regulations (Environmental Canada, 2010). An ideal sewage treatment process would consist of three stages; primary treatment to remove heavy solids, secondary treatment, often using microorganisms, to remove BOD (biological oxygen demand), and tertiary treatment to remove the remaining fixed nitrogen and phosphate. Algae can be used either in the secondary treatment process, where they generate the required oxygen through photosynthesis (Oswald et al., 1953), or in tertiary treatment, where they remove the excess nutrients (nitrate and phosphate) (Gutzeit et al., 2005; Munoz and Guieysse, 2006).

Temperature is an important environmental parameter affecting algal growth. Temperatures ranging between 15 and 25 °C are usually considered optimal for algal growth with lower temperatures resulting in decreased growth rates. However, these temperature specific effects most likely vary from one species to another (Goldman and Carpenter, 1974). Although, nutrient uptake and photosynthesis might be expected in general to be lower at lower temperatures, algal strains that are native to cold climates might be capable of achieving treatment goals with high growth rates and good lipid production (Powell et al., 2008). The recent isolation of a novel yellow–green cold tolerant species from snowfields in Colorado, USA, with a lipid content of 55% demonstrates the potential for cold climate algae as strong candidates for biofuel production (Nelson et al., 2013).

Algal samples were collected from five different locations in the vicinity of Montreal, Quebec, Canada. A native culture collection of more than 100 unialgal strains has been established and characterized. As far as we are aware this is the first description of isolation and characterization for biofuels production of any microalgal strains in Quebec. Thus, this work establishes for the first time knowledge about useful properties of microalgae native to Quebec. Here we report on the use of a high throughput 12 well microplate process to survey 100 strains from this collection for growth on municipal wastewater (WW) and synthetic Bold Basal Medium (BBM) at 10 ± 2 °C and 22 ± 2 °C. Additionally, the strains were screened for their capacity for nutrient removal and biofuel production. The results show that the collection microalgae is highly diverse, with genera of various algal classes showing a variety of growth rates under different conditions, different levels of lipid production and differing abilities to carryout nutrient removal.

2. Methods

2.1. Sampling and isolation

Water samples were collected from five different locations; three fresh water lakes (Lac Croche (45°59′24.37″N 74° 0′21.01″W) and Lac Pilon (46°0′14.02″N 74°1′7.09″W), University of Montreal biological station (45°59′17.11″N 74°0′20.55″W)), situated in the Laurentian region north of Montreal, Canada; and two on each side of the Saint Lawrence river, situated approximately 10 km downstream from the confluence with the Ottawa river, where the water of both rivers are not yet totally mixed (45°25′39.12″N 73°49′15.78″W and 45°21′23.36″N 73°48′49.96″W). Sampling at each site was conducted during the spring, summer and fall. Coarse material, potentially including zooplankton, was removed on site by filtration through a 50 μm mesh net and then samples were stored in cool boxes for transportation to the laboratory. Once in the laboratory, the water samples were filtered through a series of membranes of decreasing mesh size (33, 20 and 0.45 μm). The retention products of each membrane was taken using a sterile swab, and directly plated on BBM agar plates (Andersen, 2005) and incubated in a light chamber at 20 ± 2 °C. In all the experiments reported in this study, no special provisions were made for CO_2 supply. Thus, all cultivations were with atmospheric CO_2. Light was provided by warm white fluorescent bulbs at 25 W/m^2 operated on a light/dark cycle of 12/12 h. After growth, different colonies were inoculated in 125 ml Erlenmeyer flasks containing 70 ml of BBM medium and incubated in a light mounted shaker at 20 ± 2 °C, with shaking at 120 RPM and a light intensity of 21.2 W/m^2 using a photoperiod of 12 h light: dark. Isolates were then kept in falcon tubes with the same medium for the further analysis.

2.2. Strain identification

Samples of the different algal cultures were examined morphologically in a light microscope for preliminary identification and confirmation that the cultures were unialgal using a NIKON Eclipse E600 microscope with an attached NIKON digital camera DXM 1200F. Preliminary identification of the algal cultures was made using a field guide (Prescott, 1978).

2.3. Screening for growth

One hundred isolates were assessed for the ability to grow at 10 ± 2 °C or 22 ± 2 °C on the secondary effluent from a municipal wastewater treatment plant (La Praire, QC, Canada) and BBM medium (Andersen, 2005). The nitrate and phosphate content of the wastewater was determined as described below giving an estimated N:P ratio of 37:1 with a phosphate concentration of 3 mg l^{-1} (Table 1). Strains were inoculated (1% v/v of OD_{600} value 1.0) in 12 well flat bottom plates (Falcon tissue culture plates, USA) containing either 4 ml sterile municipal wastewater or BBM medium (both media were sterilized using filtration apparatus using Millipore membrane filter with a 0.45 μm pore size) and incubated for 14 days in a photoincubator at 10 ± 2 °C or 22 ± 2 °C at a light intensity of 40 W m^{-2} and a 12:12 h light/dark cycle. Growth was quantified daily by measuring the optical density (OD_{600})

Table 1
Initial nitrate and phosphate concentrations.

	BBM medium [20]	Municipal wastewater
pH	6.8	7.4
TP (Phosphate) (mg l^{-1})	163	3.00
TN (Nitrate) (mg l^{-1})	182.5	110.3

using a microplate reader (Biotek EL800) after agitating the plates for 30 min on a mini-orbital shaker. This type of screening method presents some variability due a number of different reasons. An analysis of data obtained in this way indicates that variation between biological replicates done at different times is ±25%. Correlation of dry weights and OD_{600} gave the following relationships, allowing for interconversions and comparison with other studies; BBM medium, OD_{600}/gm dry wt = 1.055 ± 0.12; WW medium, OD_{600}/gm dry wt = 0.87 ± 0.16. The complete experimental screening procedure is shown in Fig. 1.

2.4. Measurement of lipid productivity

The cellular content of neutral lipids was assessed at day 14 by measuring the fluorescence intensity of Nile red (NR) stained cultures (Alonzo and Mayzaud, 1999; Chen et al., 2009). Algal cells (80 μL) were placed in micro-centrifuge tubes, treated using a microwave oven at the high power setting (1200 Watt) for 40–60 s, and then mixed with 20 μL of 25% (v/v) DMSO (dimethylsulfoxide). The tubes were then subjected to a second microwave treatment using the previous conditions. 1 μL of Nile red solution (250 μg/ml acetone) were added and the tubes were incubated in the dark for 10 min at room temperature and then the samples were pipetted into 96 well micro-plates. The fluorescence intensity at excitation and emission wavelengths of 535 and 580 nm respectively, was measured using a Perkin Elmer/Packard Fusion Alpha-FP Microplate Fluorescence Analyzer. The untreated microalgal suspension and medium containing Nile red alone, considered as auto-fluorescence, were also measured and subtracted from that measured as Nile red fluorescence. Nile red fluorescence values were converted to dry weight of lipid using a standard curve produced using Triolein (Fischer Scientific, USA) as a lipid standard. It should be noted that under these conditions Nile Red will primarily detect neutral lipids, which in this case are predominately TAGs (triacylglycerols), and hence will directly indicate the potential for biodiesel production (Greenspan and Fowler, 1985; Kimura et al. 2004). If the proper extraction procedure were used, any inhibitors, such as polar lipids and free fat acid, would not be extracted along with the TAGs.

2.5. Analytical methods

Microalgal growth and biomass concentrations were determined via measurement of absorbance and dry weight of the selected strains. Total phosphate and nitrate were determined at the beginning and end of the experimental incubation. Total

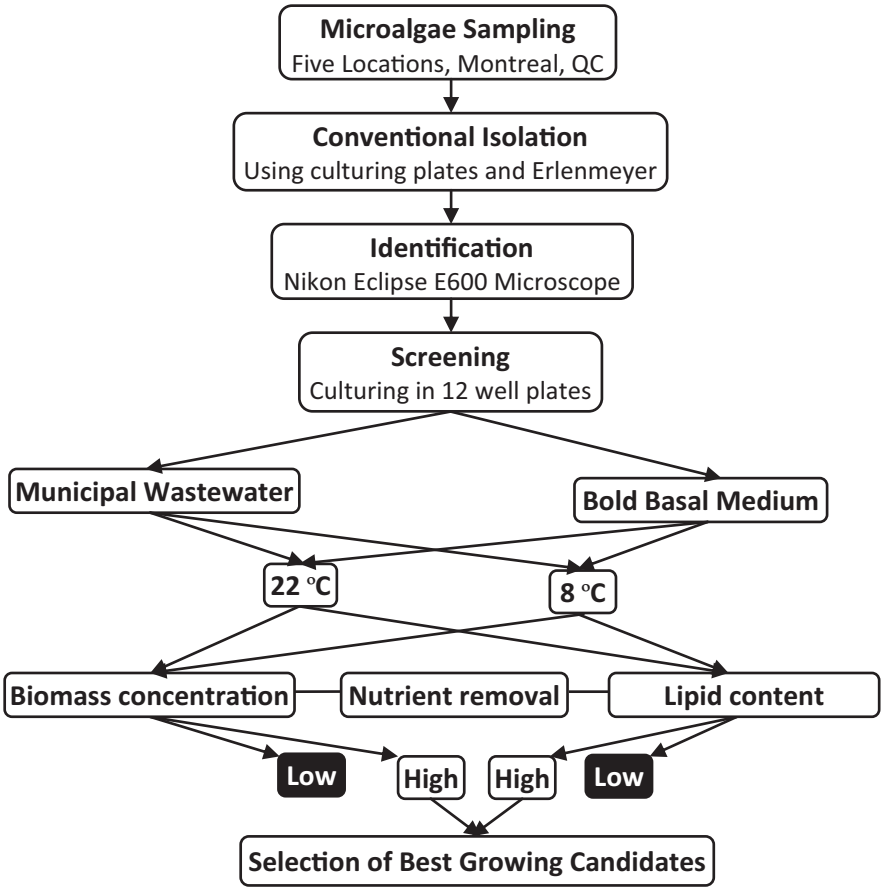

Fig. 1. Screening was carried out using the above procedure: in order; sampling, isolation, identification and screening. Water samples were obtained at five different locations near Montreal, and subjected to conventional isolation and microscopic identification using a Nikon Eclipse E600 Microscope. The screening process was based on culturing the 100 strains in 12 well microplates containing synthetic BBM medium or municipal wastewater (WW) at 10 °C and 22 °C. Growth was monitored daily by measuring the optical density of the wells. Nile red fluorescence intensity of the stained algal cells, a measure of the cellular neutral lipid content, was detected via a Fusion microplate reader. Strains showing comparatively higher biomass productivity and/or lipid content were chosen for discussion.

phosphate (Total-P) was determined colorimetrically at 610 nm (Hitachi UV-2101PC, Japan) using ammonium heptamolybdate and malachite green (Cogan et al., 1999). Nitrate was determined colorimetrically using diphenylamine (Bartzatt and Donigan, 2004). The 96 well microplates were read at 630 nm (BioTek Microplate Reader EL800). Biomass productivity was calculated from the following equation: BP ($g\,l^{-1}\,d^{-1}$) = $(B_1 - B_0)/t$, where B_1 (g/l) was the biomass concentration at the end of the cultivation, B_0 (g/l) was that at the beginning, and t was the duration of cultivation (14 days). The cellular content of lipid was determined by Nile red (Greenspan and Fowler, 1985; Kimura et al. 2004) and was calculated using the equations: C_1 (g/g) = W_L/W_B, and % Lipid/Dry weight biomass = $(W_L/W_B * 100)$, where W_L (g) is the weight of lipid and W_B (g) the algal biomass dry weight. Lipid productivity was calculated from the equation: LP ($g\,l^{-1}\,d^{-1}$) = $(C_1B_1 - C_0B_0)/t$, where C_0 (g/g) is microalgae lipid content at the beginning and C_1 (g/g) is that at the end of cultivation, B_0 and B_1 (g/L) are the biomass concentrations at the beginning and end, and t the duration of the experiment (14 days). A Varian Vista MPX ICP-OES spectrophotometer was used to measure the partial elemental composition of the wastewater.

3. Results and discussion

3.1. Culture collection of native microalgae

The sampling protocol and isolation procedure used here was successful in establishing a culture collection of over one hundred local microalgae (Fig. 1). Microscopic examination demonstrated that a high degree of diversity had been obtained with a variety of microalgae from different algal groups including cyanobacteria, green algae, and diatoms, with the majority apparently being green algae (Chlorophyta). Some representative photomicrographs are shown in Fig. S1. Following isolation, 100 strains were screened using a high throughput 12 well microplate method for growth potential and lipid production in wastewater, and compared to what was observed when they were grown on synthetic medium (BBM) (Fig. 1). Most of the isolates grew robustly in synthetic BBM medium in comparison to WW. However, as discussed in what follows, some strains grew better on WW, and others grew rapidly and constantly under all experimental conditions.

3.2. Growth on synthetic medium (BBM)

As to be expected, in general growth on synthetic medium gave the highest cell density as ascertained by measuring optical density. (For representative samples see Figs. 2 and 3). At 22 °C, about 50 isolates showed an optical density (OD) between 0.8 and 1.5, among them, two isolates PCH22 and MA1B1 were fast growing and distinguished themselves by reaching stationary phase in only 7 days (OD = 1.0), while most of the other strains generally took between 9 and 12 days to reach the same cell density and with some growth had not yet plateaued at the end of the 14 days of incubation. At 10 °C, algal growth was in general slower as might be expected for a lower temperature. Twenty-five strains had achieved an OD between 0.8 and 1.3 and were still growing at

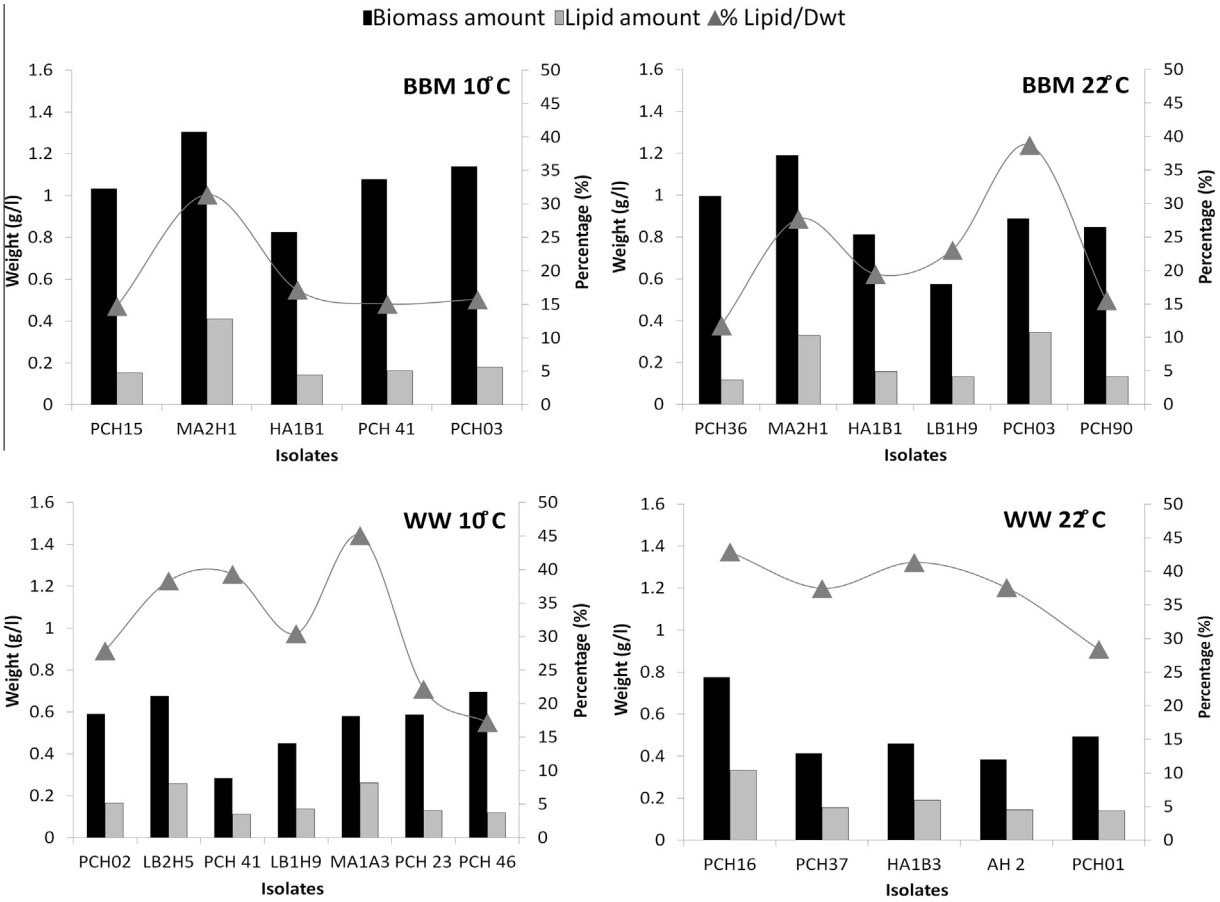

Fig. 2. Biomass and lipid content of selected strains. The lipid and biomass content as well as the percentage of lipid per dry weight of biomass for each of the selected strains at the end of the cultivation period (14 days) are shown. Biomass and lipid were determined as described in Section 2. The five best strains (out of one hundred) were chosen for each experimental condition. The highest biomass and lipid content were shown by strain MA2H1 (~1300 mg l^{-1} & 31.4% respectively) in BBM at 10 °C and strain PCH03 with a biomass content of 889 mg l^{-1} and a lipid content of 38.7% in BBM at 22 °C, while in wastewater at 10 °C, strain MA1A3 showed a biomass and lipid content of ~580 mg l^{-1} & 45% respectively) and at 22 °C, strain PCH16 gave 775 mg l^{-1} and 43%.

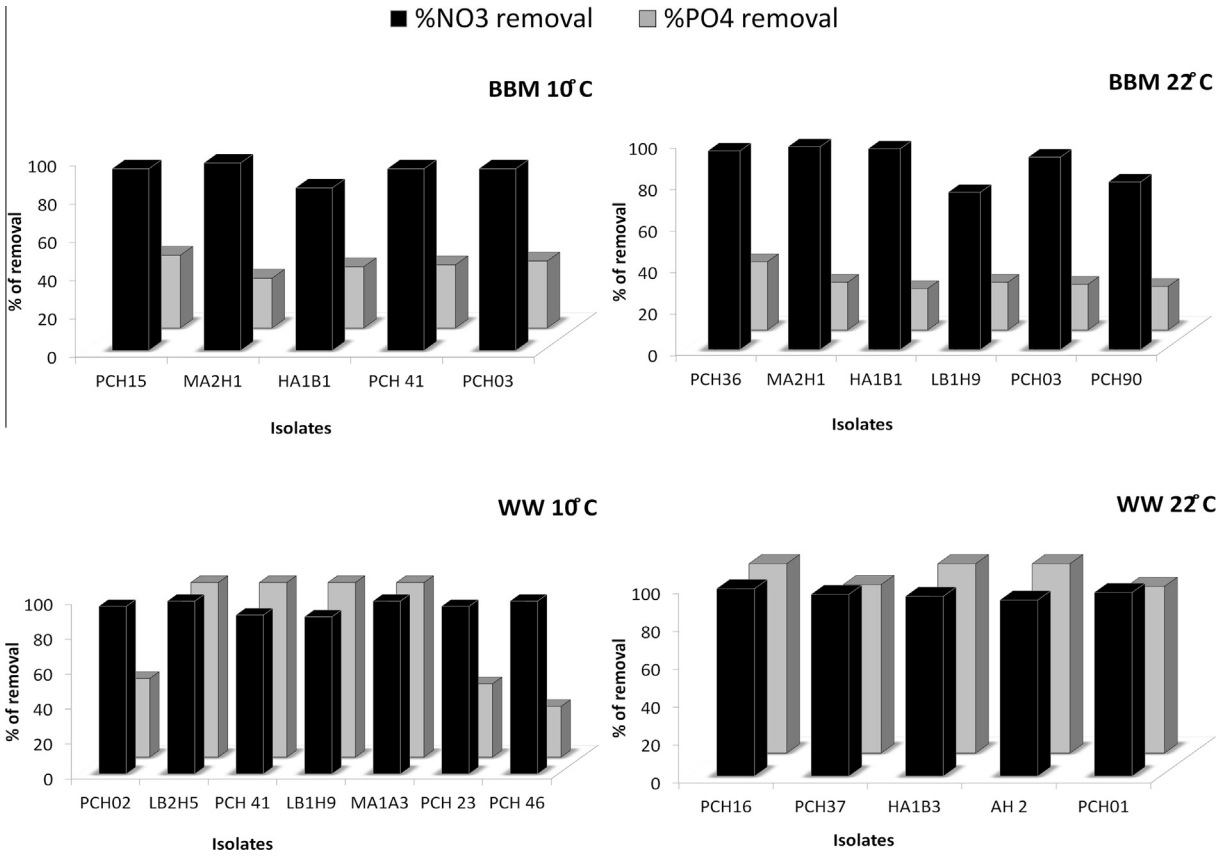

Fig. 3. Nile red fluorescence and Optical density measurements of the selected microalgae. Growth (as optical density) and lipid content (as Nile red fluorescent) for each of the selected isolates under the different experimental conditions are shown.

the end of the 14 days of incubation, while most of the other strains showed an extended growth phase of 9–12 days before reaching stationary phase. There was a wide variation in growth rates of course, but roughly six out of the hundred had specific growth rates at 22 °C of between 1 and 1.5 day^{-1}. This is quite good considering that growth conditions may not have been optimal and is higher than reported in at least some other strain collection studies (Abou-Shanab et al., 2011a,b). In this later study higher rates are quoted in the text, but an examination of the data as shown in the figures shows that the highest growth rates found were in fact close to 0.4 day^{-1}. An equal number of strains gave growth rates at 10 °C of between 1 and 1.5 day^{-1}. As far as we are aware no comparable studies have been done at low temperatures like this.

After 14 days of growth, the highest amount of biomass was achieved at 22 °C by isolate PCH22, which only had a low neutral lipid content (about 4.5%) under these conditions (Fig. 3). The biomass concentrations from the highest lipid producing strain MA2H1 at 10 °C and 22 °C, were 1.31 g l^{-1} and 1.19 g l^{-1}. The corresponding biomass productivities were 93 mg l^{-1} d^{-1} and 85 mg l^{-1} d^{-1}, respectively (Fig. 2, Table 2).

Amongst all the strains, five strains (PCH15, MA2H1, HA1B1, PCH41 & PCH03) from the cultures grown in BBM at 10 °C and six strains (PCH36, MA2H1, HA1B1, LB1H9, PCH03 & PCH90) grown in BBM at 22 °C were selected based on their high lipid production regardless of their biomass productivity (Figs. 2 and 3). Strain MA2H1 was the highest lipid producing isolate, producing ~0.41 g lipid l^{-1} (a lipid content of 31.4% of biomass dry weight) at 10 °C after 14 days. Surprisingly, this strain gave higher lipid production as well as biomass at low temperature (10 °C) (about 0.41 g l^{-1} lipid and 1.305 g l^{-1} biomass dry weight) compared to the amounts produced at 22 °C (about 0.33 and 1.191 g l^{-1}, respectively) (Fig. 2). This suggests that this organism might have an optimum range of growth between these two temperature points

Table 2
Biomass/lipid content and productivity for the selected microalgae strains.

Strain ID	Biomass concentration (g l^{-1})	Lipid content (% of Dwt)	BP (mg l^{-1} d^{-1})	LP (mg l^{-1} d^{-1})
Synthetic media (BBM) 10 °C				
PCH15	1.0335	14.739	73.8	10.9
MA2H1	1.3055	31.375	93.3	29.3
HA1B1	0.8262	17.188	59	10.1
PCH41	1.0784	15.093	77	11.6
PCH03	1.1402	15.770	81.4	12.8
Synthetic media (BBM) 22 °C				
MA2H1	1.1912	27.670	85.1	23.5
HA1B1	0.8119	19.471	58	11.3
PCH90	0.8463	15.544	60.5	09.4
LB1H9	0.5750	23.069	41.1	09.5
PCH03	0.8893	38.746	63.5	24.6
Municipal wastewater (WW) 10 °C				
PCH02	0.5914	27.935	42.2	11.8
PCH23	0.5885	22.162	42	09.3
PCH46	0.6958	17.204	49.7	08.6
LB2H5	0.6760	38.320	48.3	18.5
LB1H9	0.4511	30.465	32.2	9.82
MA1A3	0.5805	45.066	41.5	18.7
Municipal wastewater (WW) 22 °C				
PCH16	0.7748	42.872	55.3	23.7
PCH37	0.4129	37.468	29.5	11.1
HA1B3	0.4596	41.342	32.8	13.6
AH2	0.3840	37.575	27.4	10.3
PCH01	0.4930	28.423	35.2	10.0

with a preference for low temperatures. Despite the high productivities of this strain in synthetic medium, it showed only low growth and low lipid content in wastewater with slightly better results at 10 °C where it produced 0.24 g biomass l^{-1} and 0.07 g lipid l^{-1} as compared to 0.18 and 0.028 g l^{-1} at 22 °C. Again, this suggests this microalga prefers low temperature conditions. In the same manner, strain PCH03 gave a higher biomass content in BBM at 10 °C (about 1.14 g l^{-1}) compared to (0.89 g l^{-1}) in BBM at 22 °C. However, this strain produced higher lipid amounts in BBM at 22 °C compared to cultures at 10 °C, about 38.7% and 15.8% of dry weight, respectively (Fig. 2). This might indicate that, although preferring low temperatures for growth, in contrast to MA2H1, which also produced greater amounts of lipid at low temperature, PCH03 produced more lipids at the higher temperature.

The growth of strain LB1H9 was very similar under all experimental growth conditions with only slight differences in neutral lipid content, showing that temperature or media composition had little or no effect on this strain in terms of biomass and lipid productivities (Fig. 3). Similarly, several strains, such as PCH36, PCH38, PCH43, MA1A2 LA1H13 and HA1B3, although showing slight differences in growth contained almost the same amount of neutral lipid under all growth conditions (Figs. 2 and 3). Most of these strains demonstrated high nitrate removal capability (>76%), with strains such as MA2H1, HA1B1 being able to carry out 95–97% removal at 10 °C and 22 °C. On the other hand, phosphorus removal was low, ranging from 20% to 38%, with this medium which had a relatively high phosphorus concentration (Fig. 4).

3.3. Growth on municipal wastewater

Not surprisingly, given the relatively lower content of nitrate and phosphate (Table 1), growth on wastewater gave lower cell densities (between 0.2 and 1.1), about half of that on BBM, and with a shorter exponential phase, compared to their growth in synthetic medium. Apparently the trace element content of this wastewater; As, 0.034; Be, 0.00059; Ca, 32; Cd, 0.00081; Co, 0.0034; Cr, 0.0048; Cu, 0.085; Fe, 0.0086; Li, 0.055; Mg, 17; Mn, 0.0027; Mo, 0.011; Ni, 0.015; Pb, 0.019; Se, 0.080; V, 0.010; Zn, 0.051 (in ppm), was sufficient to satisfy nutritional requirements. The majority of the strains took about 4–10 days to reach stationary phase. As stated above, the low cell density and different growth kinetics are presumably due to the depletion of nutrients in a shorter period of time. There was a wide variation in growth rates of course, but roughly six out of the hundred had specific growth rates on WW (wastewater) at 22 °C of between 1 and 1.5 day^{-1}. This is quite good considering that growth conditions may not have been optimal and is higher than reported in at least one other strain collection studies where the highest growth rates found on wastewater were between 0.455 and 0.472 day^{-1} (Zhou et al., 2011). Three strains gave growth rates on WW at 10 °C of >1 day^{-1}. As far as we are aware no comparable studies have been done at low temperatures like this.

Seven strains (PCH02, PCH23, PCH41, PCH46, MA1A3, LB2H5 & LB1H9) were selected as high lipid producers at 10 °C and five strains (PCH01, PCH16, PCH37, AH2 & HA1B3) were selected as

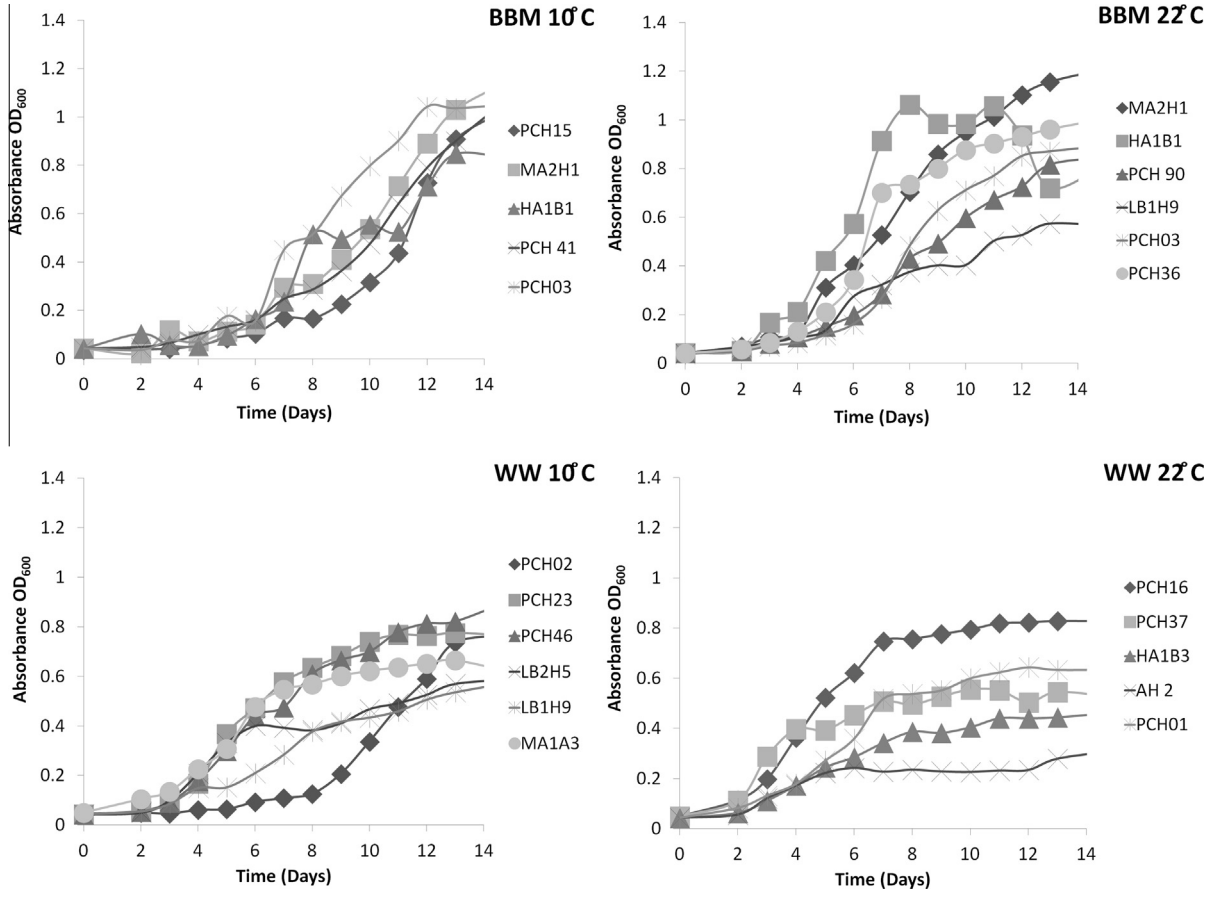

Fig. 4. Nutrient (PO_4 & NO_3) removal the selected strains under the different experimental conditions. Nitrate and phosphate concentrations were determined before and after 14 days of growth for each of the strains as described in Materials and Methods. This allowed the calculation of percent removal. Strains LB2H5, MA1A3 (grown in WW @10 °C) and PCH16 (grown in WW @22 °C) revealed complete phosphate removal and >99% nitrate removal at the end of the culturing period (14 days) while in the synthetic BBM medium, the strains showed around 80–98% and 20–40% removal of NO_3 & PO_4, respectively.

high lipid producers at 22 °C (Fig. 2, Table 2). The greatest lipid producer was isolate PCH16 which had a lipid content of 43% w/w when grown in WW at 22 °C (Fig. 2). This strain also showed complete (>99%) nitrate and phosphate removal (Fig. 4). Although synthetic medium supported better growth, this strain showed only a very low amount of lipid in synthetic medium but showed a maximum lipid content in cultures grown in WW at 22 °C (Fig. 3). One likely explanation is that this high lipid production was due to nutrient stress brought about by its depletion of both nitrate and phosphate under this growth condition. Nutrient depletion is well known as a trigger that can redirect algal pathways towards higher lipid productivity and oil accumulation.

On the other hand, strain LB1H13 showed a different growth pattern with very similar growth kinetics under all experimental conditions; in both wastewater and synthetic medium, and at 10 and 22 °C (Figs. 5 and 6). Surprisingly, strains PCH23 and PCH46, which had only a relatively low lipid content, showed fast growth which was better in wastewater than in synthetic medium (Fig. 6). In the same way, strain LB1H3 showed better growth performance in wastewater at 22 °C than under other conditions (Fig. 6).

In summary, after 14 days of cultivation in either municipal wastewater or BBM synthetic medium, several strains demonstrated high lipid content and biomass concentrations (Fig. 2, Table 2). In wastewater at the lower temperature of 10 °C, strain LB2H5 showed an oil content of around 38% of lipid per dry weight of biomass, and the biomass dry weight was around 676 mg l^{-1} (containing 259 mg lipid l^{-1}) (Fig. 2). The highest amount of lipid, 410 mg l^{-1} (lipid productivity of 29 mg l^{-1} d^{-1} (Table 2)) was produced by strain MA2H1 when grown in BBM at 10 °C (compared to 330 mg l^{-1} lipid at 22 °C). This strain showed only weak growth on wastewater (Fig. 6). The highest amount of biomass was achieved at 22 °C by isolate PCH22 which had a low lipid content (about 4.5%) (Fig. 3).

In terms of neutral lipid production, taken as Nile red fluorescence, several strains accumulated nearly equal amounts of lipid under all four experimental conditions, PCH36, PCH38, PCH43, MA1A2, HA1B1, LA1H13, PCH90, AH31, PCH41, LB1H9 and HA1B3 (Fig. 3 shows the fluorescence and optical density of each of the selected strains). Strains such as PCH22, PCH37, LB1H7 and PCH98 produced similar amounts of neutral lipids in both synthetic and wastewater media at 22 °C but only very low lipid content at 10 °C. On the contrary, strains PCH06 and PCH15 showed high lipid production in both media at 10 °C compared to very little at 22 °C. Strains such as PCH16, PCH23, PCH34 and AH2 gave lipid production only in wastewater at both temperatures, with greater amounts at 22 °C but only very low amounts in synthetic medium. For example, PCH16 had 8200 Nile red fluorescence units in WW at 22 °C compared to 2994 in WW at 10 °C but only very low amounts in BBM at 10 °C or BBM at 22 °C, 188 and 230, respectively. Under all cultivation conditions this strain achieved almost the same final optical density but with highest growth rates at 22 °C in the synthetic medium.

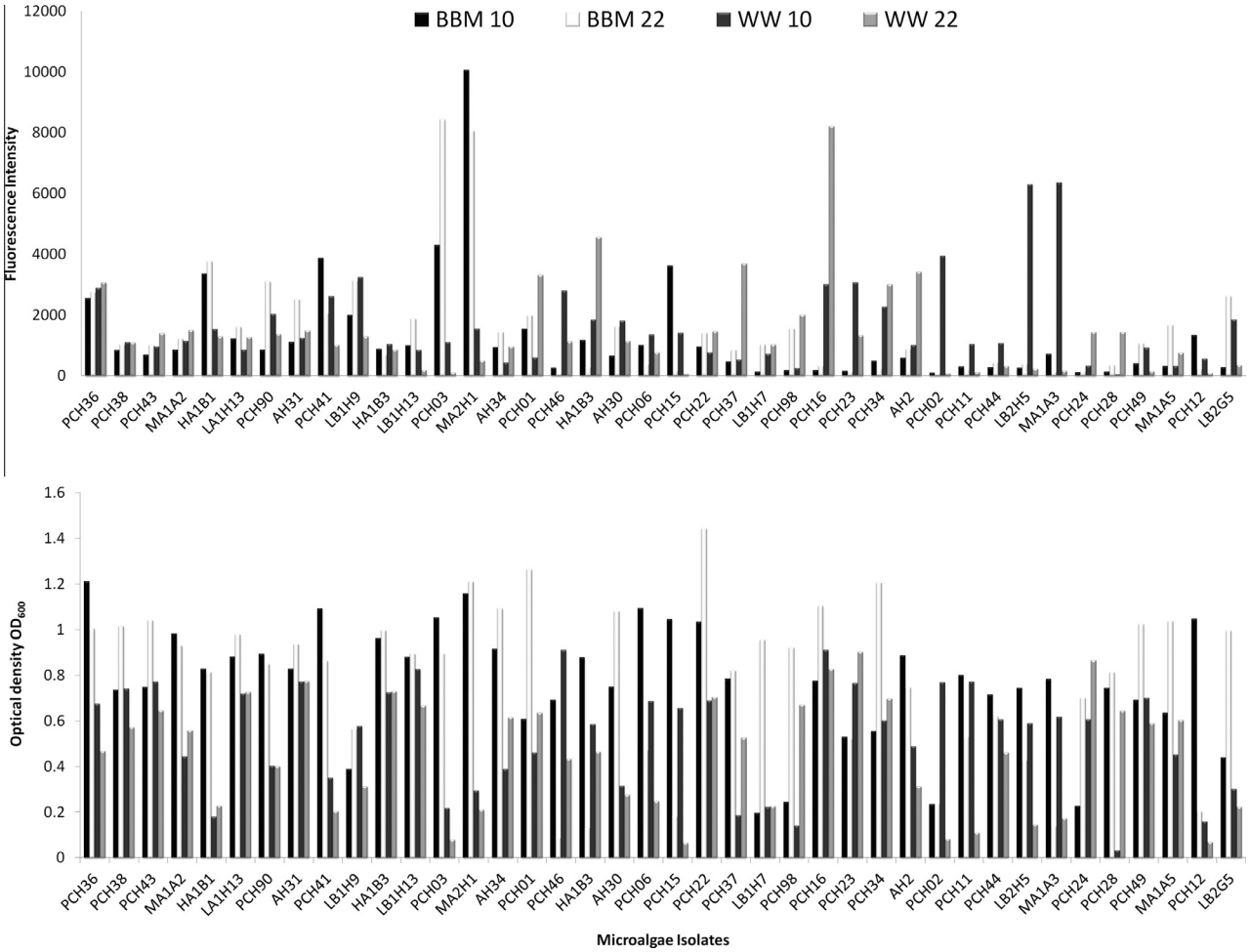

Fig. 5. Growth curves for selected strains on BBM Strains were inoculated (1% v/v of OD_{600} value 1.0) in 12 well flat bottom plates (Falcon tissue culture plates, USA) containing 4 ml BBM medium and incubated for 14 days in a photoincubator at 10 ± 2 °C or 22 ± 2 °C at a light intensity of 40 W/m^{-2} and a 12:12 h light/dark cycle. Growth was quantified daily by measuring the optical density (OD600) using a microplate reader (Biotek EL800) after agitating the plates for 30 min on a mini-orbital shaker. An analysis of data obtained in this way indicates that variation between biological replicates done at different times is +25%.

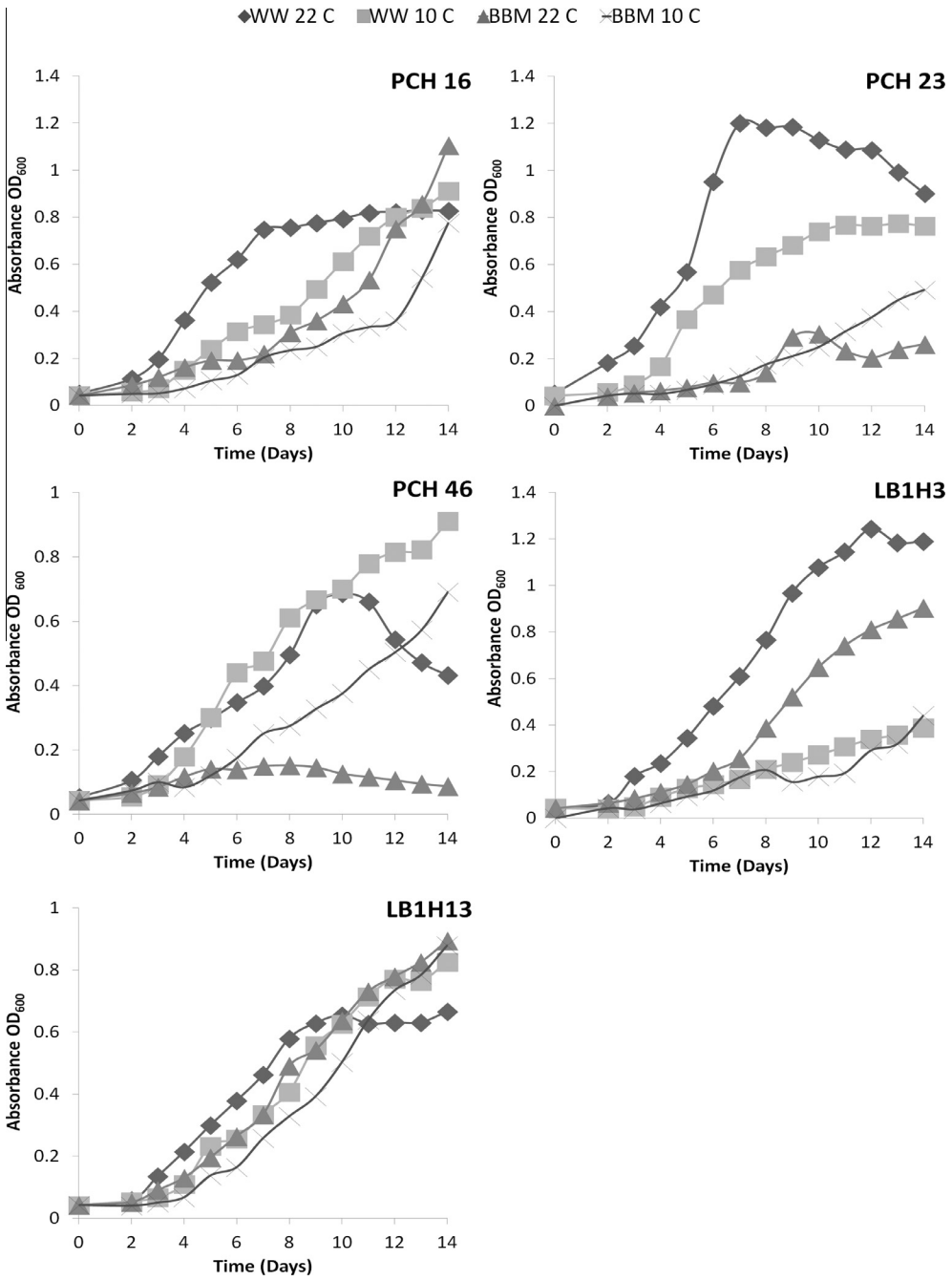

Fig. 6. Growth curves for selected strains on WW Strains were inoculated (1% v/v of OD600 value 1.0) in 12 well flat bottom plates (Falcon tissue culture plates, USA) containing 4 ml municipal wastewater and incubated for 14 days in a photoincubator at 10 ± 2 °C or 22 ± 2 °C at a light intensity of 40 W m^{-2} and a 12:12 h light/dark cycle. Growth was quantified daily by measuring the optical density (OD600) using a microplate reader (Biotek EL800) after agitating the plates for 30 min on a mini-orbital shaker. An analysis of data obtained in this way indicates that variation between biological replicates done at different times is +25%.

3.4. Nutrient removal

To be successful, algal production facilities will probably need to source their nutrients at least in part from wastewater, which could also create the benefit of generating wastewater treatment credits. More than half of the strains examined here in detail removed >70% of the wastewater nitrogen and phosphate under both temperature conditions. Strains MA1A3, LB2H5, HA1B3, and PCH16 also showed a high neutral lipid content while removing >94% and 100% of the wastewater nitrate and phosphate. Of course, given the higher nutrient content, nutrient removal efficiency with the synthetic BBM medium was in general lower. With BBM medium, most of the strains removed about 65% of the initial nitrate concentration but only around 20–30% of the phosphate. Among the high lipid producing strains, strains MA2H1 (at 10 °C) and PCH03 (at 22 °C) with final lipid contents of 31% and 39%, showed nitrogen removal efficiencies of 97%, 93% and phosphate removal efficiencies of 26%, 22% (Fig. 2).

4. Conclusions

This study demonstrates the 12 well microplate high throughput screening of native microalgae suitable for a wastewater

treatment process that combines nutrient removal and algal neutral (TAG) lipid production for potential use as a biofuel feedstock. Highly efficient strains carried out the nearly complete removal of nitrate and phosphorus from municipal wastewaters and selected wastewater-grown strains had a neutral lipid content and productivity as high as 45% and 29 mg l^{-1} d^{-1}. A number of strains showed good growth at low temperature (10 °C) and might be useful in a waste-to-biofuel process that would provide wastewater treatment and lipid production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.01.114.

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Addressing the challenges for sustainable production of algal biofuels: I. Algal strains and nutrient supply

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Microalgae hold promise for the production of sustainable replacement of fossil fuels due to their high growth rates, ability to grow on non-arable land and their high content, under the proper conditions, of high energy compounds that can be relatively easily chemically converted to fuels using existing technology. However, projected large-scale algal production raises a number of sustainability concerns concerning land use, net energy return, water use and nutrient supply. The state-of-the-art of algal production of biofuels is presented with emphasis on some possible avenues to provide answers to the sustainability questions that have been raised. Here, issues concerning algal strains and supply of nutrients for large-scale production are discussed. Since sustainability concerns necessitate the use of wastewaters for supply of bulk nutrients, emphasis is placed on the composition and suitability of different wastewater streams. At the same time, algal cultivation has proven useful in waste treatment processes, and thus this aspect is also treated in some detail.

Keywords: biofuels; sustainability; algae; wastewater; wastewater treatment; biodiesel; nutrients; harvesting; oil extraction

Introduction

Concerns about climate change driven by fossil fuel combustion and future energy security are driving intense R&D activity in replacement biofuels production. While a number of different scenarios look promising, the enormous scale at which biofuels would need to be produced introduces a number of sustainability issues for any biofuel. Already the production of first-generation biofuels, bioethanol and biodiesel, most of which are derived from edible plants grown on arable land, has increased to such a magnitude that they are seen as possibly competing with food production and are thus unsustainable. Second-generation biofuels, derived from lignocellulosic feedstocks, might seem better in this regard, but even if the deconstruction problem, the breakdown of the complex substrate into its components, were solved, would require enormous tracts of land and incur significant energy debts due to feedstock transportation, decreasing the net energy return (NER).

Microalgae are being pursued as a possible source of third-generation biofuels, principally biodiesel produced by the transesterification of algal-derived lipids. This route appears attractive since the high growth rate of microalgae compared with traditional crops means that smaller surface areas are required and their cultivation does not require the use of arable land. In addition, at least under some conditions, their lipid content can be much higher than that of the oil seeds typically used at present in biodiesel production. However, there are still a number of technical challenges to be solved before commercial production of biofuels from algae becomes a reality.[1–12] As well, large-scale production of microalgae would also pose a number of significant sustainability issues.[13] The aim of this review, and the accompanying paper, is to examine in some detail the different sustainability issues potentially involved in large-scale algal biofuel production and to suggest ways in which these challenges might be met.

Although the land use issue is diminished compared with other biofuels due to the smaller footprint, there is still a problem in this area due to the requirement for siting production facilities close to sources of CO_2, as CO_2 enrichment is required for achieving the desired high productivities. However, even more significant are the potential water and nutrient requirements. Here, we examine how the choice of algae and culture conditions, including in particular nutrient supply, are potentially constrained by sustainability concerns. Finally, as with any other fuel, an important consideration is the amount of energy investment required in order to produce a biofuel with a reasonable NER. This sustainability constraint means that attention has

to be paid to the methods used for harvesting and the conversion to fuel process that is adopted. In what follows, we examine some of these issues, first introducing algae and some of their important properties, including those that could be improved, followed by a discussion of nutrient requirements and how these might be met by various wastewater streams. Harvesting issues and methods available at present for conversion to fuel and how these are constrained by sustainable NER concerns are the subject of an accompanying paper.

Algae

From a biofuels perspective, it is important to appreciate the vast heterogeneity of the microorganisms that are collectively called algae. With hundreds of thousands species already described, algae are an artificial aggregation of a very heterogeneous array of species taxonomically grouped into five different kingdoms on the tree of life.[14] Several macroalgal species have been systematically cultivated and harvested for over a thousand years,[15] whereas microalgal exploitation is relatively new. There are more than 35,000 species of microalgae described taxonomically and, although very debated, they can be defined as eukaryotic microorganisms containing chlorophyll 'a' and a plastid. This definition excludes the prokaryote group of cyanobacteria but most of the literature concerning microalgae includes a section about cyanobacteria due to their obvious relatedness to the group in terms of the formation of chloroplasts through endosymbiosis.[14,16,17]

The variety of this group is rather easy to understand when examining its evolution. The phylogeny of the plastid genome points towards a single microorganism as the origin of the association of the two photosystems, using light to drive water oxidation and storing energy in molecules such as NAD(P)H and ATP.[18] This organism, the common ancestor of the cyanobacterial clade, is also the origin of eukaryotic algae. An endosymbiotic event is thought to be responsible for both red and green algae, where the later differentiated into Chlorophyta and Charophyta, the closest living ancestor of land plants.[19–21] Red and green algae also underwent additional endosymbiotic events, as suggested by chloroplasts harbouring three and four membranes and corroborated by plastid genome analysis of algal groups in the Kingdom Protozoa (green algae endosymbiosis) and Chromista (red algae endosymbiosis). As an ancient group of organisms, algae started differentiating early in evolution, producing their notable radiation in the tree of life. Of course such speciation was followed by a large metabolic diversity, allowing these organisms to inhabit almost any aquatic niche, from cold arctic salt water to fresh thermal springs or even relatively dry environments. The majority of species are autotrophic, but species containing a defective plastid and living exclusively on organic carbon sources are not rare. Some individuals show additional metabolic versatility as they are able to thrive by both fixing carbon dioxide through oxygenic photosynthesis and consuming organic carbon available in the environment, i.e. mixotrophic growth.[1] The great diversity evident from their evolutionary history suggests that there are many strains with properties of interest in biofuels production remaining to be discovered.

Commercial exploitation of microalgae is a relatively new activity, becoming more significant over the last few decades. Rather than individual compounds, this market has mostly focused on biomass for the nutraceutical industry with a high market value.[22–24] The cultivation of microalgae directed towards the extraction of lipids for biofuel production faces several challenges as the end product has a low market value, demanding an efficient and optimized production chain. The choice of strain for this application is an important part of this optimization as the genetic variability found in algal strains may decide the fate of the business plan. More than the cultivation system to be used, environmental variables play key roles in 'crop' success. Two obvious paths towards choosing the right strains are the selection of well-studied strains deposited in culture collections or bioprospecting in the natural environment. While the characteristics of a strain deposited in a collection can be somehow controlled, native strains can demonstrate impressive robustness against predators and competition.[25,26]

Of course, being able to flourish is not sufficient and a successful commercial strain must produce the desired molecule, preferably in large quantities. In the case of biodiesel production, the best lipids will have a saturated chain of 12–16 carbons.[27] These molecules are stored as triacylglycerols (TAGs) and represent a carbon reserve for when the cell does not have enough light for photosynthesis. However, lipids are not the only strategy found for carbon storage and algal strains may produce other molecules at different rates. Here, a high yield of the metabolic flux into the lipid production is a key characteristic. Also, by definition, a cell cannot grow fast and fat, one must be sacrificed for the benefit of the other since, as an autotroph, the amount of carbon fixed depends on photosynthetic efficiency versus time exposed to light.[25] Different species have different photosynthetic efficiencies as some are adapted to shade and others for direct sunlight, but it is important to understand that there is a natural limitation due to photosynthetic efficiency.[28] A strategy commonly used to overcome this issue is the cultivation of a fast growing strain until a certain density is reached at which point it undergoes growth arrest due to, for example, nitrogen deprivation. Different kinds of stress will prevent the cell from growing and could trigger lipid production.[29,30] It is a rather obvious adaptation not to waste an important nutrient and, when growth is prohibited by limitation in protein synthesis (for the example of nitrogen deprivation), light energy capture will continue, providing the necessary NADPH and ATP for the Calvin–Benson–Bassham cycle of carbon fixation, but the 3-phosphoglycerate (3PGA) molecule synthesized

in this case will be preferentially driven to the production of carbon reserves, allowing the cell to re-establish fast growth when the limiting nutrient is once again available, even if that occurs during the dark. Finally, a key characteristic for an algal biodiesel production strain would be a high quantum yield into lipids, which can be measured in different points of the growth curve depending on the strategy used.

The use of genetically modified organisms (GMO) is also a tempting solution, where unnecessary routes (at least for us) for 3PGA usage can be avoided, thus artificially producing a higher yield of lipids. In fact, there are a variety of targets for strain improvement that are potentially amenable to genetic engineering.[31,32] In a recent study, a transgenic strain of the diatom *Thalassiosira pseudonana* in which lipase had been decreased gave, under silicon-limited conditions,more than a two-fold increase in TAG and three- to five-fold increase in total lipid production compared with the wild-type.[33] These results suggest that metabolic engineering of lipid catabolism could be a feasible method for increasing lipid yields in microalgae without decreasing growth.[33]

Besides all the technical issues involved in the large-scale cultivation of a GMO, metabolically engineered strains are unfortunately often characterized by a loss in inherit robustness, limiting even more their applicability. This issue was suggested as one of the main reasons that drove a giant oil company to curtail investments in algal biofuels projects.[34] As well, Exxon, which initially announced a $600 million investment in the Venter Institute/Synthetic Genomics and Exxon Research for algal biofuels has recently announced that it will be refocusing its efforts after an initial spend of $100 million failed to produce the results it had anticipated (http://www.bloomberg.com/news/2013-05-21/exxon-refocusing-algae-biofuels-program-after-100-million-spend.html). Regardless, a number of genetic engineering projects aimed at increasing biofuels production are underway in both academia and private enterprise, and it is not too soon to begin discussions on appropriate methods of risk assessment and various containment strategies given the likelihood of inadvertent release when algal production ramps up to scale, especially in open ponding systems.[35]

Although a great many different strains of algae have been studied, with different ones showing interesting lipid accumulation properties, for several reasons it is not possible to choose a single strain, or even several, to use in large-scale production of biofuels. Very few studies have reported the successful long-term growth of a single strain, and even if one were shown to be capable of being maintained and producing good quantities of biofuels in one location it is not evident that this strain would be successful under different conditions elsewhere. More success is to be expected from developing biofuel production strategies based on local variants that are adapted to existing climatic conditions and that are competitive against other native strains.

Nutrient requirements

All organisms require basic nutrients for growth and multiplication, and most microalgae can meet all their cellular needs for their growth with a few key compounds; macronutrients, micronutrients (trace elements) and vitamins.[16] Two macronutrients, nitrogen (N) and phosphorus (P), are the most important for microalgal growth and are required in relatively large amounts. In addition, silica (Si) is required for cell wall production by diatoms, and some chrysophytes and silicoflagellates. Although required in lesser amounts, sodium (Na), potassium (K), sulphur (S) and magnesium (Mg) are also considered macronutrients. Micronutrients (trace elements) are only required in intermediate or small quantities and these include iron (Fe), copper (Cu), calcium (Ca), chloride (Cl), manganese (Mn), zinc (Zn), boron (B), cobalt (Co) and molybdenum (Mo). Some microalgae also require vitamins such as B1 (thiamine), B12 (cyanocobalamin) and H (Biotin) for growth. These are the basic compounds that microalgae must source from any medium used for cultivation.

Although most microalgae typically grow autotrophically when supplied with light, CO_2 and the macro- and micronutrients mentioned above, some can also grow mixotrophically [36–42] or even heterotrophically [38,43–47] using organic substrates such as sugars; glucose, [37–41,45] fructose,[40,44,45] maltose [40] or sucrose,[40,46] acetate,[37,39] glycerol [41,42,47] or amino acids.[48] *Spirulina sp.* [37–39,49], *Chlorella vulgaris*,[41,50–53] *C. prototheco*ides,[54] *Scendesmus acutus*,[52] *Haematococcus pluvialis*,[55,56] *Anabaena variabilis*[40,44] and *Micractinium pusillum* [57] have been shown to grow heterotrophically in the dark using glucose, acetate or other organic substrates or mixotrophically in the light. It has been suggested that heterotrophic growth is economically superior to phototrophic growth for biomass production with microalgae [58] but this is obviously true only for high value products where the cost of added substrate can be justified. Moreover, since this reduces an algal-derived biofuel to a first, or at best second-generation biofuel, the sustainability of such a production process if carried out on a large scale is dubious. Nevertheless, there are some positive aspects to heterotrophic growth, including a possible increase in nutrient removal.[59] Heterotrophic growth may also enhance lipid production. For example, heterotrophically grown *C. prototheco*ides had a lipid content (55 wt%) that was four times higher than that when grown under autotrophic conditions,[60] a condition shared by mixotrophic growth.[30] Many heterotrophic and mixotrophic algae may also have great utility as biological agents for treatment of wastewater from municipal, industrial or agricultural activities.[61] Indeed, various industrial by-products, such as glycerol, acetate and ethanol, have been shown to support the mixotrophic growth of microalgae.[62–64] Therefore, in some cases there may be a great opportunity to couple microalgal cultivation with the use of industrial

waste streams, coupling heterotrophic growth with efficient treatment of otherwise polluting effluents.[59]

The stoichiometry of usage of the major nutrients, carbon, nitrogen and phosphorus may vary somewhat with the algal strain, but in general the stoichiometry of carbon to nitrogen to phosphorus is of the order C:N:P = 106:16:1, an almost universal constant known as the Redfield ratio.[65] The total algal population of natural bodies of water adhere to this ratio, which may represent an average of species-specific C:N:P ratios. Carbon dioxide requirements for algal cultivation and biofuel production have been estimated at between 3.69 and 9.23 kg of CO_2 per litre of biodiesel.[66,67] Similarly, estimates have been made for the other major nutrients that are required. Based on algal cultivation in open pond systems without nutrient recycling, it is thought that 0.16–0.40 kg N and 0.022–0.055 kg P is required per litre of algal oil produced [66] or 0.29 kg N and 0.063 kg P per litre of biodiesel.[68] This demand reflects the fact that the biomass must be harvested to recover the biofuel, which represents only a fraction of the total biomass, and hence nutrient input. Therefore, if the biofuel can be recovered separately from the biomass, then nutrient inputs are consequently lower. For example, in the case of continuous ethanol synthesis by cyanobacteria it has been estimated that only 0.002 kg N and 0.0001 kg P per litre of gasoline equivalent are required.[69] Obviously, there are challenges to providing sufficient and sustainable supplies of nitrogen and phosphorus (and silicon for the cultivation of silicon-requiring taxa, such as diatoms) for large-scale algal production. In what follows next, we examine some of the individual nutrients and how they might be supplied in a sustainable manner.

Challenges in supplying carbon

Carbon is an essential element required for the cellular synthesis of organic molecules within the cell such as carbohydrates and lipids, which can be converted to biofuels. Obviously under autotrophic conditions, the carbon for algal biomass production comes from carbon dioxide fixation, and thus an adequate supply of carbon dioxide is critical for maximal algal production.[70] CO_2 levels affect the activity of the primary carbon fixation enzyme, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This enzyme is less than half saturated at CO_2 levels in equilibrium with the atmosphere, thus limiting the rate of photosynthetic carbon fixation.[71,72] Although CO_2 is naturally available from the atmosphere, its diffusion across the air–water interface limits its availability.[73] Thus, CO_2 supplementation, either in gaseous form or as bicarbonate, is required for efficient algal production and maximum biomass yield, with biomass often doubling when CO_2 concentrations are increased over ambient.[74] However, this can be a problematic solution due to the expense of CO_2 capture and transport. The only possibly cost-effective strategy for large-scale microalgal production would be to build the algal culturing facilities close to industries that emit CO_2, where the algae can at least temporarily recycle the CO_2, providing algal biomass that can be processed to biofuel and valuable products. In this scenario, the microalgae capture fossil CO_2 that would otherwise be immediately emitted to the atmosphere. However, there is no net carbon-sparing effect if the algae are used for biofuel or any other short-term purpose. The only way such a scheme could be used for sequestration would be to bury the biomass for essentially long geological times. Even then, the process would be unsustainable if unfractionated algae were used, since an unacceptable amount of nutrients would be put out of circulation.[75] A consequence of carbon dioxide injection into a culture is the acidification of the medium. If a buffer cannot be used due to cost factors, an alternative is to supply CO_2 as needed to regulate the culture pH, since over time algal growth causes an alkalinization of the medium.

Challenges in supplying nitrogen

Nitrogen is the most critical nutrient for algal biomass after carbon. Although the nitrogen content of algal biomass varies according to the algal group (e.g. lower in diatoms), it has been estimated to generally lie in the range from 1% to somewhat more than 10%.[76] Microalgae, depending upon the species, can assimilate nitrogen in different inorganic forms such as; nitrate (NO_3^-), nitrite (NO_2^-), or ammonium (NH_4^+ and NH_3), converting it into various organic compounds required for growth; peptides, amino acids, proteins, enzymes, nucleic acids, etc. In addition, some cyanobacteria can fix atmospheric nitrogen (N_2) into ammonia.[77] The process of assimilation begins with the inorganic forms of nitrogen passing through the cytoplasmic membrane and undergoing several enzymatically driven reductions to form ammonium, which is then incorporated into amino acids and other aminated compounds in the cytoplasm.

Ammonium assimilation requires less energy and there are no redox reactions involved in its metabolism, so it is the preferred nitrogen source. If both ammonium, nitrate or nitrite are present in medium, algae will utilize ammonium until depletion and then use the other forms, nitrite followed by nitrate.[70] However, even though ammonium is the preferred form, its use in algal culturing has several drawbacks: (1) excess ammonium can negatively affect algal growth, since, depending upon the species, algae can only tolerate from 25 to 1000 μM, and (2) significant quantities of ammonium can be removed by volatilization at moderate temperatures and high pHs in a process known as ammonium stripping.[70,78–81] Thus nitrate is the nitrogen form most commonly used for algal cultivation, since it is chemically stable in oxidized aquatic environments.[82]

Nitrogen limitation can cause a variety of responses, including fixed nitrogen mobilization through phycobilisome degradation,[83] or induction of carbon storage where cells either produce lipids (*C. vulgaris*) [84] or carbohydrates (many *Dunaliella* strains).[85] At high levels

of nitrogen, algae can produce up to 20% (poly unsaturated fatty acids) with cellular lipid content increasing up to 45–70% under nitrogen limitation.[84,86] *C. vulgaris* lipid content varies from 14% to 63% of dry weight under different nitrogen concentrations.[84] In addition, nitrogen limitation can cause changes in pigment content, a decrease in chlorophyll (chlorosis),[83] an increase in carotenoids, for example β-carotene production in *Dunaliella*,[87] or an accumulation of astaxanthin, its acylesters and production of oleic acid rich triacylglcerols (as in case of *Haematococcus pluvialis*).[88,89] In general, if the response is augmented carbon storage through the production of lipids, neutral lipids (TAGs) are produced instead of polar lipids.[90] The pathway the algae will select under nitrogen depletion is very specific, for example, some strains of *Chlorella* are found to accumulate large amounts of starch whereas others accumulate neutral lipids instead.[91]

Several strategies can be applied to maximally harness this ability including the use of a two-stage process in which cells are grown under nutrient-sufficient conditions until a significant amount of biomass has been produced followed by a nutrient deprivation stage for enhanced lipid accumulation.[92] However, it has been suggested that the most effective strategy for high lipid production in *C. vulgaris* is to let cells deplete nitrogen normally rather than transferring cells to nitrogen-lacking medium.[93]

Given the enormous quantities of fixed nitrogen that would be required for large-scale cultivation of microalgae, sustainability concerns require at a minimum either strict nutrient recycling and/or use of wastestreams with sufficient quantities of this nutrient. As discussed below under Microalgal biomass production, a very good option in this regard could be provided by municipal wastewater treatment facilities.

Phosphorus challenges

Phosphorus represents less than 1% of algal biomass and is required at a level of about 0.03–0.06% in the culture medium.[94,95] Inorganic forms of phosphorus (PO_4^{-3}, HPO_4^{-2}, $H_2PO_4^-$ and H_3PO_4) are readily used for algal metabolism,[96] but some species are also capable of using phosphorus found as organic esters. Most algae have a tolerance for phosphorus in the range of 1 μM to 20 mM (50 $\mu g\,L^{-1}$ to 20 $mg\,L^{-1}$).[97] Under phosphorus excess conditions, algae are able to store phosphorus mainly as polyphosphates and metaphosphate granules, which are mobilized under conditions of phosphorus deficiency.[97,98]

As already discussed for nitrogen, phosphorus limitation can affect biomass production. The amount of *C. vulgaris* biomass was 30–40% lower in phosphorus limited cultures (0.147 mM) then when the nutrient was replete (1.47 mM).[99,100] Phosphorus starvation effects include an increase in lipid or carbohydrate content, a decrease in chlorophyll [98] and the accumulation of carotenoids (astaxanthin). Phosphorus is not only removed from wastewater by algal cell uptake but it is also affected by abiotic processes triggered by external conditions such as elevated pH and high levels of dissolved oxygen. Although algal cells can use both inorganic and organic forms of phosphorus, orthophosphate is the form commonly used in large-scale cultivation. The availability of added phosphate is strongly influenced by pH, which affects not only phosphorus uptake but also its ability to be assimilated. At alkaline pH, it can react with Mg^{2+}, Ca^{2+}, CO_3^{-2} or Fe^{2+} and precipitate, thus becoming unavailable for algal uptake. This needs to be taken into consideration in practical algal production systems.

One major constraint that needs to be taken into consideration is the fact that only very limited quantities of phosphate are available for use in large-scale algal production systems. Less than 40 million tonnes of phosphate are mined annually in the USA, and, due to its limited supply, fertilizers used for agriculture already contain less than optimal concentrations of phosphates.[101,102] Obviously, phosphate supplied in this form is not renewable, and even with efficient phosphorus recycling from algal ponds, substantial 'make-up' phosphate would be needed. It has been estimated that an additional 53 million tonnes of phosphate would be required annually to completely replace conventional petroleum by algal biofuels, a challenge that is difficult to meet through increasing mining outputs, which would only provide a temporary solution at best given looming 'peak phosphate'.[95]

Since nitrogen and phosphorus are the two most limiting nutrients for algal growth, optimal growth requires that they be found in the medium in a molar ratio matching the stoichiometric ratio of the algal biomass, the Redfield ratio of 16:1.[103] Thus, when using wastewater as a medium, addition of nitrogen and/or phosphorus may be required to achieve the proper ratio. Of course, this ratio is optimal for growth and not necessarily for lipid production. Stress conditions, primarily nutrient starvation, have intensively applied to algal cultivation for biodiesel production where they are intentionally used to increase lipid production. Much of the US Department of Energy's Aquatic Species Programme was focused on this process.[90] Unfortunately, even though lipid content is increased by nutrient limitation, the cell's slower growth rate means that in general no overall increase in lipid productivity is seen.

Other nutrient challenges

Most of the other nutrients required for microalgal growth are needed only in relatively very small quantities and are readily available in water with the exception of silicon. Silicon is important for several different groups of algae, especially diatoms. It is an essential nutrient for their growth and is a major component of their cell wall.[104] It is one of the most abundant elements and usually present

in solutions as orthosilicic acid. Silicon deficiency prevents diatom division and therefore protein, DNA and chlorophyll synthesis halts. In addition, energy producing processes such as photosynthesis and glycolysis are diminished.[105] Iron is an important nutrient since it plays an important role in many cellular metabolic pathways, including photosynthesis, respiration, electron transport, DNA synthesis and nitrogen fixation.[76] Chlorophyll a and phycocyanin may be degraded under iron-limiting conditions.[106] The oxidized form of iron is not optimal for algal uptake and iron may become limiting when it is present at small concentrations under highly oxidizing conditions. Sulphur plays a key role in electron transport and is important for protein synthesis, lipid metabolism and algal growth. Thus, sulphur-deficient conditions limit algal density and stunt growth.[107] Potassium and most of the other required elements are readily available at the low concentrations in which they are required, and, if needed, they could be supplemented by adding nutrient-rich wastewater to algal growth facilities. Of the various waste streams that might be available, algal biofuel production facilities might be run in conjunction with anaerobic digestion to recover additional energy from the algal biomass, giving a nutrient-rich effluent, and thereby effectively recycling some of the micro- and macronutrients. Thus, the use of wastewaters is a cost-effective means for meeting challenges in nutrient availability and supply.[108] While in some particular cases, other perhaps more productive uses for wastewater might be found (use in irrigation, etc.), in the majority of the cases the wastewater effluents represent a nuisance that must be treated in some fashion before discharge.

Nutrient supply and sustainability

Thus, nutrient supply for algal biofuels production raises major sustainability concerns. Any biofuels process needs to be subjected to a rigorous life cycle assessment (LCA) before going to scale to ensure the practicality of the system. In brief, LCA is an analysis of the environmental impact of a product from cradle to grave. Among other things, an inventory of energy and materials inputs and releases is made. In terms of water and nutrients, one study estimated that without recycling, generating 1 kg of biodiesel could require 3726 kg water, 0.33 kg nitrogen and 0.71 kg phosphate.[68] As might be expected, in this analysis, recycling water after harvest would reduce water and nutrients usage by 84% and 55%, respectively. However, the most sustainable solution would be to use wastewater, thereby decreasing the water requirement 90% and virtually eliminating the need for nutrients as discussed next.

Wastewater as a sustainable source of nutrients

Algal cultivation on wastewater has received a great deal of attention over the past few decades. Many different wastewaters support algal growth, which carries out tertiary treatment through the removal of nitrogen, phosphorus and other elements. In addition, this process has the potential to produce large quantities of biomass, which could be used as a source of biodiesel and valuable products.[54–56] In fact, different analyses of potential scenarios for algal biofuel production have concluded that large-scale algal biodiesel production is unlikely to be viable without the use of wastewater.[61,109] A convenient and abundant wastewater source is municipal wastewater which must, in any case, be subjected to some sort of treatment process.

In general, conventional municipal wastewater treatment involves a series of processes. During primary treatment, large material is removed by screening and heavy or suspended solids are allowed to settle in sedimentation tanks before they are incinerated or put into landfill. The processed discharge water is referred to as primary effluent.[110] (Figure 1).

Secondary treatment uses some type of biological process to consume the large quantities of organic matter present in the primary effluent. Traditional treatment processes use microorganisms in aeration tanks to carryout oxidation of the organic matter. Primary effluent is mixed with air in the presence of bacterial sludge and left for several hours; bacteria breakdown the organic matter into relatively harmless smaller and simpler molecules (CO_2, PO_4, NH_3, etc.). Another sedimentation tank is used to remove excess bacteria and sludge.[110] However, together primary and secondary treatments are not sufficient to completely remove inorganic nutrients from wastewater and the resulting secondary effluent can cause eutrophication of rivers and lakes due to a high content of nitrogen and phosphate.[111] Tertiary treatment is designed to remove these nutrients and minimize ecological impacts on the environment. Physico-chemical methods, such as air stripping of ammonia, ion exchange and breakpoint chlorination, or biological methods can be used to remove nitrogen.[112] The most common removal process is denitrification where nitrate is reduced first to nitrite and finally to nitrogen gas, easily released into the atmosphere.[96] On the other hand, phosphorus is often removed by chemical precipitation using metal salts. While three forms of phosphorus are usually present in the initial wastewater, orthophosphate, polyphosphate and organic phosphate, the latter two forms are converted to orthophosphate during aerobic treatment. Orthophosphate is efficiently precipitated using chemical reactions through addition of metals salts like ferric sulphates, ferric chlorides, etc.[112]

However, the pioneering work of Oswald showed that these nutrients, nitrogen and phosphate, can be assimilated by algae leading to biomass, which can be removed and used for the production of biodiesel and other commercial products.[113] In fact, a number of different wastewater systems have been devised which use the metabolic properties of microalgae.

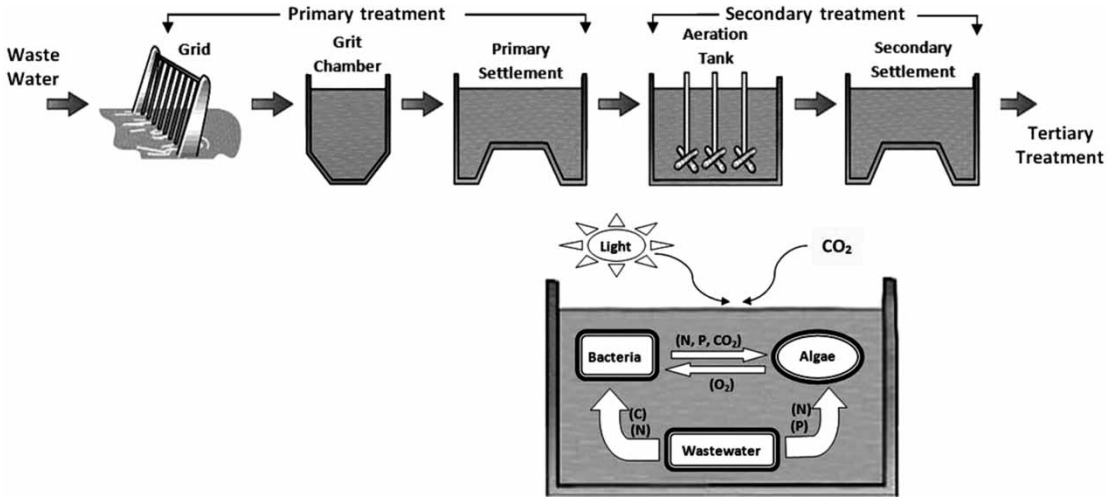

Figure 1. Contrast between conventional wastewater treatment and wastewater treatment using algal ponds.
Note: At the top is shown a schematic for a conventional wastewater treatment process. The majority of the BOD (biological oxygen demand) is removed during secondary treatment through the mechanical addition of oxygen, which drives BOD breakdown through microbial respiration. O_2 addition can represent one of the major energetic, and hence cost, inputs into the process. At the bottom is shown, in schematic form, an algal pond (HRAP-see text) treating the same waste. The algae supply the necessary oxygen through their photosynthetic process and grow using the nutrients in the wastewater. In reality, a series of ponds may be required after a first primary treatment stage. (See the text for details).

Algae-based wastewater treatment

Different processes are presently used for wastewater tertiary treatment Algal-based methods offer the possibility of coupling bioremediation with biofuel production. Many species of microalgae are potentially able to grow in wastewater from different sources (Table 1). Various contaminants are present in wastewater depending upon its source but are typically organic and inorganic nitrogen, phosphorus, pathogens, pharmaceuticals and inorganic particles. Extensive studies have been conducted to investigate the use of algae for nutrient removal, in particular nitrogen and phosphorus, from wastewater. In general, the efficiency of nutrient removal is variable, from rather poor to several studies announcing almost complete removal.[176] Although, it is widely thought that environmental factors such as temperature and the amount of sunlight present challenges that restrict algal-based wastewater treatment to tropical countries where the temperature and sunlight are optimum, this is an effective process that is in fact used in the Canadian Artic.

The major wastewater classes to be treated are municipal, agricultural (e.g. confined animal facilities including dairy, swine and poultry), industrial (e.g. food processing including olive oil mill, textile, paper, etc.) and other eutrophic waters with high nutrient content (e.g. agricultural drainage).[185]

Municipal wastewater is one of the main sources of surface water pollution. As discussed above, ideal treatment includes three stages. Secondary treatment using microorganisms requires a constant supply of oxygen, which is expensive and requires intense operations, energy input, manpower and expertise. Growing microalgae in the ponds and tanks where the treatment is carried out is a good alternative solution to this problem, since algal growth and photosynthesis will release substantial amounts of oxygen. At the same time, the microalgae will remove nutrients (nitrogen and phosphorus), incorporating them into biomass, and thus carrying out tertiary treatment of the wastewater before it is released into the environment.[108,122,179,183]

Traditional wastewater treatment practices have a number of major disadvantages, including the costs associated with the handling and disposal of the huge amounts of sludge generated, the substantial energy input, operation and maintenance requirements and emission of greenhouse gases (Table 2). Algae offer solutions to those obstacles, as using algae-based treatment will reduce sludge formation, be more cost effective,[186] exhibit lower energy requirements, recover nutrients as algal biomass, reduce greenhouse gas emissions, and, in addition, produce useful algal biomass (Table 2).

Municipal wastewater

Municipal wastewater is mainly generated from domestic sewage and small enterprises in addition to some environmental run-off as snowmelt or storm water.[197] Although its composition can vary significantly in terms of place and time due to differences in water consumption and seasonal variations, its characteristics worldwide show roughly the same pattern due to shared similarities in human lifestyles.[197] Municipal wastewater is a preferred source of wastewater for algal cultivation due to the large available volume and to its nitrogen and phosphorus

Table 1. Studies of using wastewater as a medium for microalgae cultivation.

WWs	Wastewater source	Species used	Ref.
Municipal wastewater	Raw sewage	(*C. vulgaris, Chlorella kessleri* and *Scenedesmus quadricauda*)	[114]
	Partially treated domestic	(*C. vulgaris*)	[115,116]
	Screened and/or settled domestic sewage	(*Auxenochlorella protothecoides*)	[117]
		(Algae and bacteria,'activated algae')	[118,119]
		(Algae and other microorganisms)	[120]
		(Algae, duckweed, and macrophytes)	[121]
		(*Euglena sp.*)	[122]
	Primary (settled) treated wastewater	(*C. vulgaris*)	[123,124]
		(*C. vulgaris and* Bacteria)	[125]
	Primary treated sewage/seawater mixture	(Marine isolates; *Phaeodactylum tricornutum* and *Oscillatoria sp.*)	[126]
	Secondary treated wastewater	(*Oocystis sp.*)	[127]
	(Secondary effluent)	(*Chlorella pyrenoidosa* and *Scenedesmus sp.*)	[128]
		(*Botryococcus braunii*)	[129]
		(*Scenedesmus obliquus*)	[130,131]
		(*Phormidium bohneri*)	[132]
		(*Scenedesmus sp.*)	[133]
		(Multispecies microalgal cultures)	[134]
		(Attached algae, an algal biofilm)	[135]
		(*C. vulgaris*)	[136,137]
	Settled and activated secondary treated sewage	(*Chlorella pyrenoidosa*)	[138]
	Pretreated sewage from ponding system	(Dominant *Euglena sp., Chlamydomonas sp., Scenedesmus sp.* and *Coelastrum sp.*)	[139]
		(Dominant *Chlamydomonas sp., Phacus sp.* and lower no. of *Euglena sp., Chlorella sp., Micractinium sp.* and occasionally *S. quadricauda*)	[140]
	High-rate ponds	(Algal biomass)	[141]
Municipal and agricultural wastewater		(Algal Biomass)	[142]
Agricultural wastewater	Swine/piggery (Manure) wastewater	(Gradual succession of Microalgae)	[143]
		(*Spirulina maxima* and *Phormidium sp.*)	[144]
		(*Chlorella sp., S. obliquus*, and *Phormidium bohneri*)	[145]
		(*Spirulina maxima*)	[146]
		(*C. vulgaris*)	[147]
		(*Phormidium sp.*)	[148]
		(*Spirulina platensis*)	[149,150]
		(*Chlorella sp.*)	[151]
		(*Chlorella pyrenoidosa*)	[152]
		(*Chlorella zofingiensis*)	[153]
		(*Scenedesaceae sp.*)	[154]
		(*C. vulgaris, Chlamydomonas mexicana, Nitzschia cf. pusilla, S. obliquus, Ourococcus multisporus*, and *Micractinium reisseri*)	[155]

	Raw swine manure effluent	(Freshwater algal consortia)	[156]
	Settled swine wastewater/sewage mixture	(*C. vulgaris* and Bacteria)	[157]
	Dairy (Manure) wastewater	(*Cyanobacteria*)	[158]
		(Freshwater algal consortia)	[159]
		(*Chlorella sp.*)	[160]
		(*Neochloris oleoabundans*)	[161]
		(Six algal genera)	[162]
	Cattle feedlot effluent	(*S. quadricauda* and Bacteria)	[163]
	Pretreated cattle manure	(*C. vulgaris, C. kessleri and S. quadricauda*)	[114]
	Aquaculture	(Photosynthetic algae)	[164]
		(Microbial flocs)	[165]
		(*Scenedesmus sp.* and *C. vulgaris*)	[166]
	Poultry effluent	(*Spirulina platensis*)	[167]
		(*Chlorella minutissima, Chlorella sorokiniana* and *Scenedesmus bijuga*)	[168]
Agro-industrial wastewater		(Algal–bacterial biomass)	[169]
		(*C. vulgaris* and *Scenedesmus dimorphus*)	[170]
		(Cyanobacteria) Review	[171]
Industrial wastewater	Olive oil mill effluent	(*S. obliquus*)	[172]
		(*C. pyrenoidosa* and *S. obliquus*)	[173]
	(Phenols removal)	(*Ankistrodesmus braunii* and *S. quadricauda*)	[174]
	Textile effluent	(*C. vulgaris*)	[175]
	Carpet mill effluent/municipal sewage mix	(Microalgae from different taxa)	[176]
	Parboiled rice effluent	(*Aphanothece microscopica N¨ageli*)	[177]
	Paper industry effluent	(*Chlorella and diatom species were the dominant*)	[178]
	Tannery effluent	(*Oscillatoria formosa, Navicula lanceolata and Nitzschia scalaris*)	[179]
		(*Spirulina sp.*)	[180]
	Steel making facility effluent	(*C. vulgaris*)	[181]
	Hazardous wastes	(Algae–bacteria) (microalgae)	[182,183]
	Oil refinery	(*Nannochloropsis sp.*)	[184]
Industrial-municipal wastewater		(Microalgae from different taxa)	[176]

Table 2. Mechanical versus algal-based wastewater treatment.

Category	Mechanically aerated systems	Algal ponding systems	Ref.
Cost	High costs-requires expensive energy inputs and processes	Low costs more cost effective reduction in biochemical oxygen demand (BOD), pathogens and nutrients	[118,135,186–188]
Energy requirements	High energy requirements 45–75% from the total energy costs of wastewater treatment plant required for supply of oxygen to the aerobic bacteria	Low energy requirements-algae provide the needed oxygen through photosynthesis and consume nutrients with much smaller energy input	[96,187,189,190]
Sludge formation	Hazardous-potential use of hazardous chemicals for sludge elimination, colour and odour removal	Safe-fewer chemicals used, potentially less sludge is produced, more possible uses for sludge	[187,191–193]
Greenhouse gases emission	High potential emission of large quantities of CO_2	Low-algae consume CO_2 during growth	[187,194,195]
Additional advantages	Successfully used world-wide for many years	Potential heavy metal removal, algal biomass can be used to produce biofuels, potential treatment of agricultural drainage	[129,133,145–149]
Additional disadvantages	Large amounts of sludge requiring disposal	Large land requirement, expensive harvesting to meet suspended solids limits, algal biomass production i limited by the environmental conditions	[96,109,187,195,196]

content. However, municipal wastewater also contains considerable amounts of heavy metals such as zinc, lead and copper, which might interfere with algal cultivation.[77] Microalgae cultivation in municipal wastewaters has been most extensively studied (Table 1). A typical composition of municipal wastewater is given in Table 3.

Agricultural wastewater

Agricultural wastewater, often derived from manure (animal farms), agricultural operations and livestock production (such as beef cattle, dairy cattle, swine and poultry), is another major source of wastewater which can be very high in nitrogen and phosphorus, rendering it a suitable substrate for microalgal cultivation and sustainable algal biodiesel production.[155,170] The Redfield ratio should be considered with respect to the nitrogen and phosphorus ratios, further supplementation with some nutrient might be necessary to sustain algal growth. Expansion of livestock operations in the past few decades has resulted in excessive nutrient concentrations within livestock production areas.[221] Table 3 gives the typical composition of some agricultural wastewaters.

Industrial wastewater

Industrial wastewater is one of the most serious pollution sources affecting aquatic environments. Significant amounts of highly polluted water have been discharged into rivers, lakes and coastal areas during the past decades, resulting in serious problems for both ecosystems and human health.[222] The composition of these wastewaters varies significantly depending upon the industry. Each sector has its own particular combination of pollutants which may require specific treatment processes.[222] Although somewhat dependent upon the source, most industrial wastewaters contain less nitrogen and phosphorus but more heavy metals in comparison with both municipal and agricultural wastewaters.[223]

Some studies that have investigated the use of microalgae for nutrient removal (N and P) and biofuel production are summarized in Table 4.

Microalgal biomass production

Algal culturing methods are usually divided into either suspension cultures; open ponds, closed reactors and hybrid systems or immobilized cultures; matrix-immobilized systems and biofilms. The most widely used systems for wastewater treatment and biofuel production are based on suspension cultures. Algal culturing with suspension cultures using open pond systems, either natural water such as lagoons, lakes and ponds, or artificial ponding systems such as raceway ponds, has received extensive interest.[234,235] The method of choice for commercial microalgae production has been high-rate algal ponds (HRAPs), but a variety of systems including; facultative ponds, maturation ponds

Table 3. Compositions of some typical wastewater from different streams.

WW	Type	TKN	TP	BOD_5	COD	Ref
Municipal	Weak domestic	15–20	4–5	110	250	[198–200]
	Medium domestic	40–50	8–12	220	500	
	Strong domestic	85–90	15–20	400	1000	
	Raw sewage sludge	190	86.4	Nr	43,844	[201]
	Dairy	185	30	Nr	Nr	[202]
		167	36	Nr	Nr	[203]
	Dairy lagoon water	244–1081	Nr	Nr	Nr	[204]
	Dairy anaerobic lagoon sludge	556–4420	141–3263	Nr	Nr	[205]
	Dairy anaerobic digestion effluent	3456	249.7	Nr	Nr	[160]
		2370	240	Nr	32,700	
	Dairy manure	5294	824	Nr	129,400	[206,207]
Agricultural Wastewater	Swine	1290–2430	264–324	Nr	Nr	[202]
	Raw flushed swine manure	1501	566	3046	16,758	[208]
	Swine after solid–liquid separation	895	168	923	3122	
	Poultry	96–802	30–50	Nr	Nr	[202]
	Poultry manure	1381–1825	382–446	420–5900	1753–12,052	[209]
	Poultry anaerobic digestion effluent	1580	370	370	1800	[210]
	Beef feedlot	63	14	Nr	Nr	[202]
	Beef fresh manure	8.2–19 (lb/ton)	2.7–12 (lb/t) P_2O_5	25,004	127,095	[211]
Industrial Wastewater	Dairy industry	58–115	9.7–28	1034–3203	2148–5134	[212]
	Textile industry	42.7–161	9.4–27.9	400–490	773–1290	[213]
	Winery industry	0–425	3–188	8858	15,553	[214]
		0.0–142.8 (TN)	3.3–188.3	125–130,000	738–296,119	[215]
		67–71	7.0–8.5	1740–1970	3112–3997	[216]
	Olive mill industry	532 (TN)	182	30,600	97,000	[217]
	Paper mill industry	13 (TN)	4	230	420	[217]
	Tannery industry	273	21 PO_4	1860	6200	[218]
		90–630	Nr	210–4300	180–27,000	[219]
	Tomato cannery industry	0.1–5.6 (Nitrate)	0.3–7.4	29–1100	Nr	[185]
	Pharmaceutical industry	5166 (TN)	Nr	15,250	28,540	[220]
	Carpet industry/sewage mix	32.6–45.9	26–49	331–487	1412	[176]

Notes: TN, total nitrogen; TKN, total Kjeldahl nitrogen; TP, total phosphorus; BOD, biochemical oxygen demand; COD, chemical oxygen demand; Nr, not reported. All the values are in mgL^{-1} otherwise specified.

and high-rate algal ponds, have been widely used either separately or in combination in wastewater treatment.

Algal ponding systems

Facultative ponds, in use worldwide for the biological treatment of municipal, agricultural and industrial wastewater, are ponds where a combination of aerobic, anaerobic and facultative microbes carryout secondary treatment. In these ponds, often operated as a series of two to four ponds, microalgae grow using sunlight, CO_2 and nutrients (N, P) obtained from the wastewater and produce oxygen through photosynthesis. The O_2 they produce promotes bacterial degradation of the organic matter in the wastewater, releasing more CO_2 and nutrients which in turn are assimilated by the microalgae, producing additional algal biomass and O_2.[113,236] (see Figure 1). Facultative ponds are typically operated at an organic loading rate of 50–100 kg BOD_5 ha^{-1} day^{-1}, a depth of 1–1.5 m, and with a 30–60 day hydraulic retention time. While facultative ponds are fairly efficient at reducing biochemical oxygen demand (BOD), removal of nutrients (N, P) and pathogens is often highly variable and relatively poor. More importantly for biofuels production, the annual algal biomass productivity of facultative ponds is quite low, 10–15 tonnes (dry wt) ha^{-1} $year^{-1}$.[237,238] As well, during normal wastewater pond operation, a major issue associated with the use of facultative ponds is the high cost of large-scale harvesting technologies needed to prevent the discharge of the algal biomass contained in the pond effluent.

HRAPs for wastewater treatment

In practice, HRAPs, also known as raceway ponds, are the most commonly used large-scale production systems. HRAP is a technology developed by Oswald and colleagues for wastewater treatment, where it demonstrates a capability for a high-rate removal of nutrients (N, P) and wastewater organic compounds, as well as a significant reduction in pathogens.[113,236,239,240] Originally

Table 4. Some studies of uses of microalgae in wastewater treatment (nutrient removal) and biofuel production.

WW	Wastewater type	Wastewater composition ($mg\,L^{-1}$) N	P	COD	Species used	Biomass productivity ($mgL^{-1}\,d^{-1}$)	Lipid content (%DW)	Lipid productivity ($mgL^{-1}\,d^{-1}$)	Total nitrogen removal (%)	Total phosphorus removal (%)	COD removal (%)	Ref.
Municipal wastewater	Urban wastewater effluent (secondary treated)	28.1 (NH_4)	8.7–11.8 (PO_4)	Nd	*S. obliquus*	26	31.4	8	100 (in 7.8–7.8 days)	98 (in 3.93 days)	Nd	[224]
	Secondarily treated sewage	15.3 (NO_3) $mg\,dm^{-3}$ 15.0 (NH_4) $mg\,dm^{-3}$	11.5 $mg\,dm^{-3}$ (PO_4)	49.71 mg O_2 dm^{-3}	*Botryococcus braunii*	345.6	17.85	62	Nd	Nd	Nd	[225]
	Municipal (centrate) wastewater	128.6 (TKN) 67 (NH_3)	120.6 (TP)	Nd	*Chlamydomonas reinhardtii*	2000	25.25	505	83 (in 10 days)	14.45–15.4 (in 10 days)	Nd	[226]
	Primary clarifier effluent supplemented with CO_2	39 (NH_4) 51 (TN) or (TKN)	2.1 (PO_4)	Nd	*Polyculture* (*Mix. of Chlorella sp., Micractinium sp., Actinastrum sp.*)	270.67	9	24.4	96–>99 (in 3 days)	96–>99 (in 3 days)	Nd	[142]
	Highly concentrated wastewater	82.5 (NH_3) 116.1 (TN)	212.0 (PO_4)	2304	*Chlorella sp.*	920	11.04 (FAME)	120	93.9 (NH_4) 89.1 (TN) (in 14 days)	80.9 (in 14 days)	90.8 (in 14 days)	[116]
	Anaerobically digested municipal wastewater effluent (diluted (3%))	80 (TN) 68 (NH_4)	11.43 (TP)	80	*Nannochloropsis salina*	82	35	30	99 (in 10 days)	99 (in 10 days)	Nd	[227]
Agricultural wastewater	Anaerobic digested dairy manure (diluted (20%))	691.2 (TKN) 446.4 (NH_3)	49.94	4752	*Chlorella sp.*	81.4	13.7 (TFA)	11 (TFA)	100% (NH_3), 78.3 (TKN) (in 21 days)	71.6 (in 21 days)	34.3 (in 21 days)	[160]
	Dairy manure supported by polystyrene foam led	517 (TN) 309 (NH_3)	770 (TP)	Nd	*Chlorella sp.*	2.57 $gm^{-2}\,d^{-1}$	9.01 (TFA)	0.23 $gm^{-2}\,d^{-1}$ (TFA)	61–79 (TN) 94–99.96 (NH_3) (in 15 days)	62–93 (TP) (in 15 days)	Nd	[228]
	Fermented swine urine	86.4 (TN)	20.2 (TP)	Nd	*Scenedesmus sp.*	6	0.9 (TFA)	0.54 (TFA)	Majority	Majority	Nd	[229]
	Pretreated piggery wastewater (undiluted up to 80% diluted)	510–85 (TN) 264–60 (NH_4)	54.3–13.3 (TP)	833–197	*C. vulgaris*	6.67–33.33	28	2.3–9.5	41–68 (NH_4) (in 30 days)	11–41 (in 30 days)	21–42 (in 30 days)	[230]

	Diluted primary piggery wastewater (secondary treated)	25–100 (TN) 35–140 (NH_3)	4–16	250–1000	*Chlorella pyrenoidosa*	12–38	13–23	5–6.3	91.2–95.1 (NH_4) 54.7–74.6 (TN) (in 10 days)	31.0–77.7 (in 10 days)	36.5–57.6 (in 10 days)	[152]
	Anaerobic/oxic-treated piggery wastewater effluent	53 TN 4.5 (NH_4) 16.8 (NO_3)	7.1 TP 11.4 (PO_4)	Nd	*Chlamydomonas mexicana*	60	33	15.5	60.4–63 (in 20 days)	28.2–62 (in 20 days)	Nd	[155]
					S. obliquus	50	31	12	58.5–60 (in 20 days)	23.9–60 (in 20 days)		
					Chlorella vulgaris	47.5	29	10.5	49–51 (in 20 days)	18.4–57 (in 20 days)		
	Combination of anaerobic/aerobically treated swine	418.8 (NH_4) 11.3 (NO_3)	5.4 (PO_4)	199.8	*Nannocholropsis oculata*	420–590	30	35–177	74.7–99.9 (NH_4) 69.4–88.5 (NO_3) (in 5 days)	99.6–99.8 (in 5 days)	Nd	[231]
Industrial wastewater	Carpet industry effluents mixed with 10–15% municipal sewage (untreated)	32.6–45.9 (TKN) 17.6–25.9 (NH_4) 0.21–28.13 (NO_3)	5.47–13.83 (P) 20.3–35.1(PO_4)	1412	*Chlorella saccharophila*	23	18.1	4.2	Nd	Nd	Nd	[61,176]
					Dunaliella tertiolecta	28	15.2	4.3				
					Pleurochrysis carterae	33	12	4				
					Botryococcus braunii	34	13.2	4.5				
	Carpet industry effluents mixed with 10–15% municipal sewage (treated)	3.97–5.53 (TKN) 0.57–3.61 (NH_4) 1.39–3.91 (NO_3)	3.47–7.89 (P) 17.6–21.9 (PO_4)	106 –183	*Consortium of 15 algal isolates*	41	12.2	11.12 L ha^{-1} d^{-1}	99.7–99.8 (in 3 days)	96.1–99.8 (in 3 days)	Nd	[176]
	Untreated industrial wastewater aerated with 5% CO_2	38.4 (NH_4) 3.1 (NO_3) 16.2 organic N	44.7 (PO_4)	42.2	*Chlamydomonas sp.*	150	18.4	28	100 (NH_4), 100 (NO_3) (in 2 days) 0 organic N (in 10 days)	33 (in 10 days)	Nd	[232]
	Artificial wastewater (50% dilution)	16.86 (NO_3) 25.17 (NH_4), 42.03 (N)	2.14 (PO_4)	Nd	*Scenedesmus sp.*	126.5	12.8	16.2	66 (daily removal)	>50 (daily)	Nd	[61,233]

Notes: WW, wastewater; COD, chemical oxygen demand; FAME; fatty acid methyl ester; TFA, total fatty acid content rather than total lipids; TN, total nitrogen; TP, total phosphorus; Nd, not determined. All the values are in mgL^{-1} except otherwise specified. Lipid content, as well as, nitrogen, phosphors and COD removal are expressed by percentage (%).

proposed as a method for combined wastewater treatment and biofuel production on a large scale more than 50 years ago,[241] there has been a marked resurgence in interest in this field in the past decade. Structurally, HRAPs are open, relatively shallow ponds, gently mixed using paddle wheels. Thus, they are relatively cheap to construct and easy to operate. HRAPs are typically run at organic loading rates of 100–150 kg BOD_5 ha^{-1} day^{-1}, depths varying between 0.25 and 0.6 m, and hydraulic retention times, depending upon the season, from three to four days in the summer and seven to nine days in the winter. However, they can also suffer from several limitations that can affect any pond system, including low productivity due to microbial and predator contamination, high evaporation rates, inefficient light distribution (dark zones), relatively poor mixing, large areal footprint and inefficient CO_2 absorption.[9,242]

Thus, HRAP production levels are theoretically high, and in fact, levels of 50–60 g m^{-2} day^{-1} (180–200 t ha^{-1} $year^{-1}$) have been achieved on exceptional days,[26] in practice, 10–20 g m^{-2} day^{-1} (36–40 t ha^{-1} $year^{-1}$) are hard to achieve on a consistent basis.[243] Of course, algal production in wastewater treatment HRAPs varies with the climate, but even so gives productivities that are about two-fold higher than those achieved with facultative ponds (30 t ha^{-1} $year^{-1}$ for wastewater treatment HRAPs in moderate latitudes and Mediterranean climates compared with 10–15 t ha^{-1} $year^{-1}$).[238,239] One method to stabilize the species in HRAP and to increase harvest efficiency is to carry out species control using partial algal biomass recycle.[244,245]

A variety of factors limit HRAP productivity. Rapid assimilation of dissolved CO_2 can cause significant pH increases, depressing algal growth rates and productivity.[226,240,246] Likewise, high pH will favour the formation of free ammonia which can reach inhibitory levels.[247,248] The intense daytime photosynthetic activity of HRAPs can lead to oxygen supersaturation (typically to 200–300% normal saturation), inhibiting algal productivity, especially at high pH and carbon limitation.[249] Selection of algal strains that can thrive under the sometimes harsh environmental conditions of an HRAP; high light intensities, supersaturated dissolved O_2, diurnal temperature fluctuations and unstable pHs, may be a necessary approach for achieving increased algal productivity in HRAPs.[249]

Certainly, one factor that often limits productivity in HRAPs is carbon limitation brought about by the low C:N ratio of wastewaters and the high level of photosynthetic uptake of CO_2 and bicarbonate.[236,250,251] Municipal wastewater typically has a C:N ratio of 3:1 to 4:1, relatively low compared to that of algal biomass, which ranges from 10:1 to 5:1 (typically 6:1).[252] Thus, domestic wastewater contains insufficient carbon to drive the removal of all the nitrogen and phosphorus present through direct assimilation into algal biomass. Therefore, increasing carbon availability by addition of CO_2 to wastewater treatment HRAPs maintains the pH in the optimum range (pH 7.5–8.5) for growth and, at the same time, promotes nutrient removal through assimilation into algal biomass, potentially doubling biomass productivity.[195,239,244,247,250,251,253] Thus, addition of CO_2 enhances at the same time wastewater treatment and algal production by a HRAP and the harvested biomass can subsequently be converted into a biofuel.[239, 254–256] Although CO_2 may be provided from flue gases from neighbouring power plants or other industrial sources, CO_2 supply and distribution would be problematic for very large-scale algal production.

Achieving high HRAP algal productivity also requires strict control of herbivorous zooplankton, such as cladocerans and rotifers, which can rapidly reduce algal biomass concentrations to very low levels within a few days causing pond crashes.[239,257–260] Although such measures are probably impractical on a truly large scale, zooplankton growth can be inhibited by using certain chemicals or invertebrate hormone mimics or by increasing pH levels to 11.[261–263] On the other hand, no practical control methods have yet been developed that are effective for fungal parasitism and bacterial or viral infection, which can also inhibit and deplete the algal population within a few days.[264–266]

Maturation ponds are yet another type of ponding system sometimes used in the final stages of wastewater treatment where they act primarily for tertiary treatment, the removal of pathogens and nutrients. These are essentially shallow (usually 0.9–1 m depth), allowing light penetration to the bottom, and consequently creating aerobic conditions in the whole depth of the pond. These are only effective if the majority (>80%) of the BOD has been previously removed.

Thus, an advanced pond system (APS), typically composed of a series of four types of ponds; facultative ponds, HRAPs, algal settling ponds which harvest the algal biomass by gravity sedimentation and maturation ponds that mainly provide additional disinfection via exposure to sunlight UV radiation, can be a very efficient method for wastewater treatment.[240,267–269] However, despite the benefits of this technology, which include, highly efficient wastewater treatment, biogas recovery and algal harvesting, APS has only been relatively sparingly used. Some of the reasons behind the lack of use of this technology are: the lack of the professional skill set required for operation and maintenance, the requirement for large land areas and the relatively expensive current harvesting technologies that must be used since the gravity settling method has not been found to be either reliable or efficient. In addition, nitrogen removal efficiency is a complex function of the algal biomass concentration making management of these systems difficult.

Enclosed photobioreactors (PBRs)

A variety of designs and different configurations of closed systems for algal production have been tested at either the

laboratory or pilot scale, including vertical, horizontal, helical, flat plate, plastic bags operated in batch mode and various forms of tubular PBRs, which are either mechanically pumped or mixed by air-lift. It has been suggested that helical reactors are the easiest to scale-up.[270] However, so far the only type used for large-scale productions have been tubular PBRs or Algenol-type reactors.[9]

Closed systems, in particular tubular PBRs have several advantages compared with open ponds, including attaining higher cell densities, providing better protection against culture contamination, less evaporative losses, better mixing and better operational (pH, light and temperature) control.[242] Thus, these systems in general give higher productivities. For example, a productivity level of 20–40 $g\,m^{-2}\,day^{-1}$ was reported in one study.[243] In spite of these benefits, tubular bioreactors suffer from some serious faults that probably render them unusable for large-scale use. These problems include accumulation of oxygen to toxic levels since oxygen removal is very difficult at scaled up proportions,[270] adverse pH and CO_2 gradients, large material and maintenance costs, high energy requirements, overheating and biofouling.[242,271]

Hybrid systems

As discussed above, open ponds are a relatively cheap and very efficient method for algal cultivation but can be easily contaminated with undesirable microbial species. On the other hand, PBR, while too expensive for mass algal culture, are an excellent method for maintaining an uncontaminated culture. Thus one option is to use a hybrid system, essentially a two-stage cultivation method where PBRs provide a very efficient and cost-effective method for inoculum preparation for the larger scale open pond system. In one scenario, inoculation of an algal strain that was grown under suitable conditions in a PBR into a low-nutrient open pond could favour biofuel production.[272]

Immobilized cultures

Apart from the pros and cons of using suspended algae culturing methods (either open, closed or hybrid ponds), biomass recovery (harvesting) is considered a significant challenge associated with the use of these methods, therefore, there is a growing interest in using immobilized or attached algal processes that help meet this challenge. Immobilized cultures have the theoretical benefits of increased culture densities, ease of harvest, as well as the reduction of water and land requirements.[273] However, the economics associated with these methods are prohibitive and their use would depend upon future design innovations.

In the past, immobilization of microalgal cells has been shown to lead to more efficient nutrient removal in wastewater applications [274] and to enhanced lipid and pigment content.[275,276] However, the use of this method is usually prohibitively expensive due to the high cost of immobilization.[277] One solution is to favour the formation of algal biofilms, naturally immobilized systems. The presence various organic molecules on submerged surfaces can create favourable locations for microbial growth and biofilm formation.[278] Compared with suspension cultures, algal biofilm systems can better integrate production as well as harvesting and dewatering operations, thus reducing downstream processing costs. Coupling a trickling filter with a raceway pond has been shown to help with algae harvesting [277] and attached cultures have shown greater yields compared with suspension cultures grown under the same conditions.[228] Several studies have examined a design consisting of a plastic mesh used for filamentous algae attachment, called an Algal Turf Scrubber, which showed efficient nutrient uptake and biomass productivity (15–27 $g\,m^{-2}\,day^{-1}$ [279] and 5–20 $g\,m^{-2}\,day^{-1}$ [280,281]). The estimated costs of producing algae using attached growth systems vary widely, with one basic economic analysis study suggesting that an attached system may be a good option for low-cost algal production and wastewater treatment.[282]

Sustainability and the way forward

A number of recent studies have indeed suggested that the operation of HRAPs is a feasible way to produce biofuels if they are coupled to wastewater treatment driven by a need for tertiary treatment (removal of nitrogen and phosphorous).[2,61,131,283–286] Effective operation of HRAP ponds for wastewater treatment and biofuel production over a 15-month time period has already been demonstrated at the hectare scale.[284] Obviously, sustainability concerns favour the use of wastewater for the supply of macronutrients such as fixed nitrogen and phosphorous for microalgal culture and by the same token, algae are uniquely suited for recovering these nutrients from relatively dilute solutions such as wastewaters. Typically, 50% or more removal of N and P, is routinely obtained.[131] Even though domestic wastewater in the USA does not of course contain sufficient amounts of N and P to grow enough algae to completely meet biofuel needs, it can nevertheless be estimated to be sufficient to produce 77.6 million kg of algae per day!.[2] Of course, these calculations are based on the Redfield ratio for nutrient usage and not all algae under all cultivation conditions may follow this rule as some may assimilate and store excess phosphate, for example.[287] The recovered biomass, after use for biofuel production, can either be first subjected to anaerobic digestion and the nutrients be recovered as effluent, or the biomass can be directly used as a source of nutrients for either further algal culture or for some types of agriculture. Of course, this is contingent upon the development of an effective algal harvesting technology.[2,61,283,285]

Conclusions

Many technical barriers remain for making algal biofuels a practical reality. One overarching concern should be the sustainability of this, or any other biofuels production process. Here, we have examined the role that algal species and nutrient supply play with regard to sustainability issues. Finding or creating an optimal algal species is important in this regard, as maximum productivity means fewer demands on land use and water requirements. At scale, algal biofuels production would require enormous amounts of nutrients, principally nitrogen and phosphorus, and this alone would threaten fertilizer supply and cost of food production. Thus, wastewaters are a very attractive nutrient source, and a number of wastewater streams have the necessary composition to support abundant algal growth. Other sustainability issues and technical challenges revolve around harvesting technologies and processes for conversion of algal biomass to fuel. These are dealt with in the second article in this series.

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Addressing the challenges for sustainable production of algal biofuels: II. Harvesting and conversion to biofuels

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In order to ensure the sustainability of algal biofuel production, a number of issues need to be addressed. Previously, we reviewed some of the questions in this area involving algal species and the important challenges of nutrient supply and how these might be met. Here, we take up issues involving harvesting and the conversion of biomass to biofuels. Advances in both these areas are required if these third-generation fuels are to have a sufficiently high net energy ratio and a sustainable footprint. A variety of harvesting technologies are under investigation and recent studies in this area are presented and discussed. A number of different energy uses are available for algal biomass, each with their own advantages as well as challenges in terms of efficiencies and yields. Recent advances in these areas are presented and some of the especially promising conversion processes are highlighted.

Keywords: biofuels; sustainability; algae; wastewater treatment; biodiesel; nutrients; harvesting; oil extraction

Introduction

First-generation biofuels, bioethanol and biodiesel suffer from severe sustainability problems and second-generation biofuels, to be produced mainly from lignocellulosic materials, have yet to be realized due to significant problems with pre-treatment, deconstruction and conversion to fermentable sugars (Table 1). Microalgae have been proposed as a third-generation biofuel. Unlike corn and sugarcane, feedstocks for first-generation biofuels, and other energy crops, many forms of algae can be grown on non-arable land under harsh conditions and in different environments, using wastewater as a source of water and nutrients. Algae exhibit faster growth rates than oil seed plants, with some species reaching more than 50% lipid per dry weight. Algal oil (lipids) can be converted to biodiesel, and algae, or their biomass, can be used to produce a wide variety of other biofuels, including jet fuel, biogas, ethanol, etc. with the potential to produce useful by-products; nutraceuticals, animal feed, etc. Thus, algal biofuels show promise for biofuel production that is more sustainable and with a higher net energy return (NER) than other biofuels.

Interest in algal-based biofuels has been shown in a variety of countries by both governmental agencies and private enterprise. For example, in the US funding has been awarded for algal and biomass fuels [18] and in the private sector, an investment of $600 m was reported for a collaborative strategic R&D partnership between ExxonMobil and Synthetic Genomics Inc. (SGI) for algal-based biofuels. Recently (May 2013), SGI announced a new co-funded research program with ExxonMobil that will focus on using synthetic genomic science to improve production.[19] There are a variety of on-going collaborative R&D partnership efforts between algal tech companies and major industrials for biofuel and bio-products production, for example, Algenol–Dow, BP–Martek, Shell–HR and Chevron–Solazyme.[20] Additionally, more than $300 m towards algal biofuels commercialization has been invested by a consortium of governmental, commercial and philanthropic organizations, including the US Department of Energy (DOE), Chevron, BP, Carbon Trust and the Gates as well as Rockefeller Foundations.[20] In July 2013, the US DOE announced an investment of $13 m to accelerate the development of next-generation biofuels with the goal of producing drop-in biofuels at $3 per gallon by 2017.[21] In Japan, a project of biofuel production and sewage treatment from algae has been recently started with a total budget of around $9 m.[22]

Thus, there has been a great deal of interest recently in developing algal biofuel production systems as a means of meeting the challenges of climate change and diminishing fossil fuel reserves. A significant amount of R&D is ongoing in this area, both at the academic-fundamental level and the industrial-applied level. Although there are a number of technical challenges to be met before algal biofuels could be deployed at even a modest scale, it is worthwhile considering the scope of the need for replacement

Table 1. Comparison between the different generation biofuels.

Biofuel	Advantages and disadvantages	Socio-economic impacts	Production Yield (gal/ac/yr)	Ref.
First-generation biofuel				
Bioethanol by fermentation of starch (from corn, wheat, barley) or sugars (sugarcane, sugar beet). Biodiesel using oil seed plants (sunflower, palm, coconut, rapeseed etc.) and transesterification	Large-scale production possible with current technology Production uses existing, well-established technology Low sustainability (NER (ethanol from corn) = 0.8–1.5:1) Feedstock competes with edible crops Requires large land areas and intense water use Significant CO_2 and NO emissions Large quantities of fertilizers required. Soil erosion and degradation, crop residue removal	Increases rural economies May increase food prices as growers switch to supplying feedstock for biofuels Attracts foreign investors. Engine modifications might be required in case of high biofuel blends	Corn 18 Soybeans 48 Sunflower 102 Safflower 83 Rapeseed 127 Palm oil 635	[1–7]
Second-generations biofuel				
Thermal production from biomass giving bio-char, bio-oil and syngas Bio-production of ethanol using lignocellulosic materials (e.g. straw, wood, and grass) Bioethanol and biodiesel production from non-food energy crops (*Cassava, Jatropha, or Miscanthus)*	More sustainable Feedstock is inedible biomass and crop residues, mainly cellulosic and lignocellulosic material or wastes; food wastes, used cooking oil, animal fats, etc. Carbon and other greenhouse gases emissions are reduced compared with first-generation biofuels. Avoids soil degradation and land-use change NER (switchgrass and *Jatropha)* biodiesel maybe as high as 5.4:1 and 1.85 Still requires land that might be used in some cases for edible crop production, some crops (Cassava) used for food in some societies	Increases rural economies. Less effect on food prices Wastes highly available and cheap Land-use change issues (forests cleared) Biosecurity issues related to invasive species Costs related to feedstock transportation Varied use of natural resources	*Jatropha* 202 (gal/ac/yr) Represents less than 0.1% of world biofuel supply (pilot plants) Large potential if technical limitations (lignocellulose deconstruction) are overcome	[1,6,8–12]
Third-generation biofuel				
Produced from algal biomass Biodiesel Bioethanol Hydrogen	Potentially highly sustainable. (NERs between 0.2 and 3.0) Commercial production at present only for high-value products (nutraceuticals) Higher growth rates – superior yields-reduced surface required Large volumes of water and nutrients required at large-scale Geographical challenges associated with unfavourable climatic conditions Variety of technical challenges in cultivation, harvesting and conversion Large-scale production needs more R&D of organisms and processing to be economic	No competition with food since non-arable land can be used Can be used for wastewater treatment, minimizing water and nutrient requirements. Potential for introduction of invasive species Provides employment opportunities. Potential high infrastructure costs	Algae oil yield 1000–6500 (gal/ac/yr) *Schisochytrium* sp., *B. braunii* can contain up to 75% lipid	[1,13–17]

fuels as this is the driving force behind considerations of sustainability and NER.[1,23–33] In this optic, scale factors alone remove first-generation biofuels, bioethanol from corn, wheat, and sugar cane and biodiesel from plant oils, from serious consideration. This is because even though with worldwide production of ethanol and biodiesel of 50 billion and 9 billion litres, respectively, in 2007, in reality insignificant quantities of these biofuels are being made, since these amounts represent only a minute fraction of the world's primary energy use; in 2011, 161 tonnes per day of renewable liquid biofuels were produced, whereas 12 million tonnes per day of crude oil were consumed.[34] The aim of this review, and the accompanying paper (Part 1), is to examine in some detail the different sustainability issues potentially involved in large-scale algal biofuel production and to suggest ways in which these challenges might be met. In Part I, we examined the various sustainability problems around algal cultivation, including land-use issues and nutrient supply. Here, we examine different algal harvesting techniques and the conversion of the algal biomass to fuels. One important consideration that has emerged, especially as concerns harvesting, is the amount of energy investment required in order to produce a biofuel with a reasonably high NER. Of course, reducing energy input improves sustainability. In addition, conversion processes can also be energy intensive and, in addition, require the use of toxic organic solvents, both of which will negatively impact sustainability.

In what follows we examine these issues and attempt, where enough is known to make this feasible, to compare the NERs and other sustainability indices of the different prospects under study. Of course, many would like to be presented with an economic assessment of algal biofuels production, but it has been concluded that in reality this would be a futile exercise given the very premature state of the technology in this area.[16,35] Likewise, given the nascent stage of development, a life cycle assessment (LCA) analysis is a dubious exercise for a number of reasons.[35] First, it is difficult to define the necessary pertinent boundary conditions. Second, LCA was conceived to deal with already existing supply chains for which retrospective historical data are available, not the case here where in fact future developments and scale-up could have enormous, yet difficult to predict, impacts on material flows necessary for this type of analysis. Because of these uncertainties, there are large variations in NERs that have been reported for the overall process, from 0.28 to over 3. Therefore, realistic assessments of this type will require the construction and operation of large-scale demonstration plants from which real time data can be obtained.[36] Finally, many have proposed the generation of co-products as a way to improve the overall economics. This subject will not be treated here since this is only viable at small to medium scale. At the scale necessary to make a significant impact in terms of replacing fossil fuels, the value of any single co-product would fall precipitously. This follows from basic precepts of economics, the law of supply and demand, once market saturation is achieved high-value products become low-value ones. Perhaps the only co-products with appreciable demand at large scale would be animal feeds or fertilizer, in themselves high-volume low-value commodities.

Harvesting

Nearly all microalgal biomass cultivation methods produce a dilute solution, ranging between 0.02% and 0.05% solids.[37] When the molecule of interest is restrained inside the cell, as is the case for the triacylglycerol (TAG) used for biodiesel production and most other biofuels, it is necessary to separate the biomass from this green broth. Effective harvesting is one of the major challenging factors in algal biofuels development due to its potentially intense energy usage which may represent 20–30% of total production costs.[38,39] Indeed, the nature of microalgal cultures demands continuous harvest operation and, as the cell size is at most 30 μm, the energy input for this step can represent a major proportion of the total energy input.[39] Centrifugation of algal biomass may require up to 8 MJ kg^{-1}. The challenge is to concentrate cells from a dilute solution through either one or more physical, chemical or biological steps. Common harvesting methods include sedimentation, centrifugation, filtration, flotation and electrophoresis.[40] However, there is no single universal harvesting method suitable for every case. Selection of the harvesting technique is mainly dependent on microalgal properties such as size, density and the final market and value of the desired product.[16] Harvesting efficiency can be strongly affected by cell concentration, pH and ionic strength. In addition to the dilute nature of the algal culture, cells often carry negative charges and have a density equivalent to the medium, keeping the cells in a dispersed state which increases the difficulty of harvesting, and consequently the costs.[41]

Microalgal harvesting is usually a two-step process.[42] The first step, a bulk harvesting, separates the microalgal biomass from the suspension, using flocculation followed by flotation or gravity sedimentation. This concentrates the cells into a green slurry with a solids content of ~2% to 7%, usually too dilute for downstream processing. The second step is called thickening with the main purpose of further concentrating the slurry (dewatering) through filtration, centrifugation or thermal processes, further concentrating the biomass to a solids content of up to 95–99% depending on the requirements for downstream processing. Thickening usually requires more energy than bulk harvesting.[16]

Sedimentation

Gravity sedimentation is a simple method commonly applied in water and wastewater treatment to separate solids. The rationale of the process is to use gravity to separate liquids and/or solids from another liquid with different densities. Although it is an easy and simple

method, it has several drawbacks since it is time and space consuming and the separation of low-density microalgal particles is often unsuccessful.[43] It is influenced by the density and radius of the particle, or in this case microalgal cells, as well as their sedimentation velocity (0.1–2.6 cm h^{-1}). Of course, when the density of the components being separated is similar, the process can be very slow. The settling speed can be theoretically calculated through Stokes' Law using the density and radius of the particles, as well as the density of liquid in which they are suspended.[16,44] The limitation of using gravity settling alone is demonstrated by the case of *Chlorella* sp., whose density (1.070 g cm^{-3}) is very close to the density of fresh or salt water (around 0.998 and 1.025 g cm^{-3} at 20°C, respectively).[43,45] The theoretical settling speed calculated for *Chlorella* sp. in fresh water has been calculated to be only 0.1 m day^{-1}. Nevertheless, a recent report showed a faster settling rate for this species, 3.575 m day^{-1},[46] but of course at the cost of efficiency, since only 60% of the biomass was recovered. However, the average settling time for microalgae is usually much slower, ranging between 0.1 and 0.2 m day^{-1} for green algae and diatoms.[47] Moreover, the actual sinking rate depends on many variables, including cell shape and settling tank geometry, which might leave some room for improvement in this method.

In a recent report, the design and the operation of a sedimentation tank were explored in an attempt to increase the settling efficiency. Using a novel design characterized by the shallow angle of the tank and non-continuous sludge recovery, they claimed to have achieved a bulk sludge of 59 g L^{-1} (5.9% w/w dwt) from a dilute culture of *Chlorela* sp. (0.7 g L^{-1} (0.07% w/w dwt)), an interesting concentration to start with for a subsequent thickening step.[48] Previously used standard industrial settling tanks have an angle of operation of $\theta = 55°$ and employ continuous recovery of the settled particles from the bottom of the tank. The new proposed design operates at $\theta = 8°$ and works through the accumulation of biomass by recovering it through the resuspension of particles after a several fold concentration. Among the particularities of the system is the inability to use it with flocculants or any pre-concentration method since the in-flow must be a dilute suspension of small particles.

Gravimetric sedimentation has an intrinsically low-energy input (0.1 kWh m^{-3}), produces no chemical contamination and efficiently recovers the water used in cultivation so that it can be recycled back into the cultivation system or discharged. However, its applicability is limited since it is land intensive and time consuming, and is somewhat species dependent. Thus, further development and pilot-scale demonstration is needed before it can be considered a practical process that is economically viable.

Centrifugation

Centrifugation is a highly efficient and reliable method, where most microalgae particles can be recovered from liquid cultures, with about 95–100% and 80–90% efficiency using centrifugation at 13,000 *g* and at 500–1000 *g*, respectively.[39,49] This is an effective method when dealing with the relatively low biomass concentrations obtained from microalgal cultivation systems with just a slight difference in density with respect to the liquid phase and the small size of microalgal cells. This method is essentially capable of concentrating any particle as it is relatively indifferent to variations in microalgal size and arrangement. Thus, in terms of solids capture efficiency centrifugation is the preferred method. One centrifuge can harvest an algal pond of 115 m^2 and 0.3 m deep in one hour. However, this is very energy intensive (8 kWh m^{-3}) and can easily bring the NER ratio below one. An example of this would be to harvest an algal broth of 0.02% w/w dry weight with an average of lipid content of 30% until an algal paste of 20% w/w is formed. In an hour, the centrifuge would be able to harvest 35,000 litres, yielding 7 kg of algal biomass containing 2.1 kg of lipids. An oil extraction/transesterification with 90% efficiency would give 1.89 kg of fatty acid methyl esters (FAMEs) containing 19.8 kWh. Thus, the centrifugation operation alone would consume 49 kWh to produce only 19.849 kWh. Certainly, this type of operation is far from being sustainable.[44]

A possible solution would be to use a less energy demanding process to generate an algal slurry (e.g. sedimentation). Concentrating this slurry would demand far less energy for centrifugation and could help achieve an NER greater than one. It is important to remember that the cultivation is by itself energy-intensive and, as the algal-to-biodiesel process is a complex multi-step chain, each link has to be efficiently optimized to produce a fuel with a NER >1. In addition, cell structure may be damaged due to the high centrifugal and shear forces.[42,50,51] While this might not matter for some biofuel production scenarios, it would of course shorten the 'shelf-life' of the algal biomass. Thus in most cases, centrifugation is probably more useful as a secondary harvesting method used in a combination with oil extraction.[52]

On the other hand, a recent study suggested that centrifugation can potentially be useful as a primary harvesting technique for microalgae and can be cost effective if the appropriate conditions are employed.[53] This approach is based on relaxing the efficiency of algal recovery through an increase in the flow-through rate. Effective centrifugation of microalgal cells is a fine balance between flow rate and recovery efficiency. The higher the flow rate, the lower the recovery efficiency. At the same time, at higher flow rates, less energy is required per cubic metre processed. This relationship is far from linear, and therefore energy/cost efficiency is not directly proportional to the recovery efficiency. Empirical tests showed that adjusting the flow rate for a recovery of only 28% of the biomass (in contrast with 95% mentioned above) gave the highest NER, using only 1.73 kWh per litre of algal oil produced (containing 9.72 kWh). Using this scenario, estimated harvest

costs were \$1.868 l^{-1} compared with the current cost of \$4.52 per litre of oil estimated by the US DOE for centrifugal harvesting.[53] These numbers are still far from an economically viable scenario but open an avenue for optimization.

Flocculation and autoflocculation

Flocculation is a process where the dispersed particles in suspension are aggregated together by the addition of bacterial or chemical flocculants to form larger particles that can easily settle. The negative charges on the algal cell surface naturally prevent them from self-aggregation and promote dispersion, an important ecological adaptation to improve light and nutrient accessibility.[40] From one point of view, this evolutionary trait is interesting since it might make mixing during growth more efficient. However, for harvesting purposes the dispersion induced by the electric charge increases the difficulty of separating the biomass from the water. The use of flocculants to supress this characteristic is an efficient and common solution widely used in similar applications (e.g. wastewater treatment treatment plants) and has been shown to be efficient with microalgae as well. The rationale is to use positively charged ions or polymers (e.g. $Al_2(SO_4)_3$, $FeCl_3$, $Fe_2(SO_4)_3$ or natural starch derivatives and tannins), which aid coagulation and improve algal biomass settling. The chemical flocculants used (either inorganic or organic) vary in effectiveness depending on their ionic charge and algal strain, but a report claimed to achieve up to 80% harvesting efficiency using pH-induced Fe^{3+} flocculation for various algal species.[50] Nevertheless, this is not a method of choice for cheap and sustainable production because it suffers from several drawbacks, such as the large amounts of flocculant required, sensitivity to pH,[54] the fact that some coagulants work for some microalgae species but not for others, and the contamination of the harvested algal biomass with large amounts of the flocculating agent, reducing the usability of some by-products.

Chemical flocculation is a common pre-treatment step in various solid–liquid separation strategies.[55] There are two main flocculants used: organic flocculants/polyelectrolytes or inorganic compounds. Combined flocculation by using more than one type of flocculant has also been used.

Inorganic coagulants like polyvalent metal salts, iron-based or aluminium-based coagulants, will disrupt the stability of the system, neutralize or reduce the cells surface charge leading to successful settling and harvesting.[39,56] Low pH has been found to assist the efficiency of inorganic flocculants.[40] A screen of 12 different salts for the harvest of *Chlorella minutissima* showed that chloride, ferric and sulphate salts of aluminium were the most efficient coagulants,[57] a result supported by a study of the flocculation of *C. zofingiensis* which found more than 90% recovery at a pH $>$ 4.0 and 100–200 mg L^{-1} ferric chloride.[56]

Organic flocculants using high molecular weight bridging (polyelectrolytes) polymers,[43] aluminium sulphate followed by certain polyelectrolytes,[54] biodegradable natural chitosan [58] or cationic flocculants,[59] have been found to be very effective in microalgal harvesting. Anionic and non-ionic polyelectrolytes fail to flocculate microalgae due to charge repulsion or insufficient bridging distance,[60] an effect that can be bypassed through the adjustment of the pH, as another study has shown $>$80% recovery of marine microalgae using a non-ionic polymer when the pH was between 10 and 10.6.[50]

An important flocculant currently being tested is chitosan. It is a by-product of shrimp and crab industries, produced from the chitin of these animals. It is already widely used in different industries, such as chemical, food, pharmaceutical, and in agriculture. It is a non-toxic and biodegradable polycationic polymer, which has shown promising results as a microalgal flocculant.[61] At low concentrations of chitosan (15 mg l^{-1}) and pH 7.0, *Chlorella* sp., *Spirulina* sp. and *Oscillatoria* sp. were recovered with an efficiency of 90%, later raised to 99% after optimization.[58,59] As expected, pH plays an important role in the efficiency of this method through effects on the protonation of chitosan amino groups.[62] Chitosan has the advantage of being a natural product (from chitin), and therefore potentially allows the harvest of an algal biomass free from toxic or undesirable contaminants, conferring an important advantage if a fraction of the biomass is intended to be used as fertilizer or in human or animal nutrition.[63] A modified form of chitosan, nano-chitosan has been developed and tested for harvesting *Nannochloropsis* sp.[64]

Concerns about the sustainability of harvesting processes are leading to the development of different natural, organic biodegradable flocculating agents. Among these are cationic guar gum, which has been used to flocculate two different green algae, *Chlorella* sp. and *Chlamydomonas* sp. [65]; organoclays doped with Al^{3+} and Mg^{2+} which have been shown to harvest oleaginous *Chlorella* sp. with 100% efficiency [66]; and aminoclay-based microalgae harvesting systems that have been shown to be promising and potentially cost-effective tools for downstream processing in microalgae-based biofuel production.[67] Other organic flocculants tested include poly (γ-glutamic acid),[68] and *Moringa oleifera*.[69]

Flocculation is most effective at high biomass concentrations and low mixing speeds, which avoid excessive shear forces that could disrupt flocs. Other factors that can affect flocculation efficiency include; ionic strength, pH, polymer molecular weight and the charge density of the flocculant.[39] As well, the high salinity of the marine environment can inhibit flocculation by cationic polymers.[59]

Combined flocculation is a multi-step process using more than one type of flocculant. The idea is that with combining different agents it might be possible to decrease the economic and/or environmental impact while retaining

efficiency. Among the combinations tried for algal biomass are: polyelectrolytes with inorganic flocculants (such as ferric chloride or alum) [70] and ozone oxidation followed by the addition of a flocculant or a cationic starch.[71,72] For example, a recent report analysed a combined flocculation method and the effects of the medium pH, flocculant type (alum, $Ca(OH)_2$, $FeCl_3$, $Al_2(SO_4)_3$, polyacrylamide, and chitosan), flocculant dosage and sedimentation time on flocculation efficiency in the harvest of *Scenedesmus* sp.[73]

Triggering self-flocculation (autoflocculation) could be a cost-effective, non-toxic process for algal harvesting. Some species naturally flocculate and others can flocculate in response to environmental stimuli; pH changes, carbon limitation, nitrogen stress, excretion of macromolecules or the level of dissolved oxygen.[51] Cultivation under elevated pH and limited CO_2 supply assists autoflocculation.[74] In addition, in some cases microalgae-associated bacteria may play an important role in algal flocculation and sedimentation by increasing the floc size.[75] The use of flocculants for bulk harvest could represent an important step towards increasing the NER. Nevertheless, it is important to note that changes made to the cultivation medium, such as the addition of salts or changes in the pH to promote flocculation, can interfere with the final product, not only potentially contaminating the biomass, but even decreasing the yield of the main product.[76]

Filtration and screening

Filtration is a very effective solution at the laboratory scale, but at large scale it presents several issues making it an option with limited application. The high maintenance costs (membrane replacement and pumping), energy consumption (0.3–2 $kWh\,m^{-3}$), formation of compressed filter cakes and membrane clogging are the main negative aspects of this technique.[42] However, it might be cost effective for harvesting filamentous species such as *Spirulina* sp. or large colonial (ca. $>70\,\mu m$) microalgae such as *Coelastrum* sp. and *Micractinium* sp.[16,77,78] For small cells, techniques like microfiltration,[79] ultrafiltration [80] or membrane-filtration can be used, however not for large-scale production as the membranes are prone to plugging.[16,81,82] Plugging can be reduced by using tangential flow filtration (also called cross-flow filtration) in which the majority of the liquid flow is across the membrane surface, continuously removing larger particles that might cause blocking. In one study, about 70–89% of freshwater algae were recovered using tangential flow filtration,[81] which has the advantage of maintaining the structure and properties of the collected microalgae, but this method has yet to be successfully scaled-up.[42,83] A cross-flow membrane-filtration system equipped with an anti-fouling membrane (surface coating with hydrophilic polyvinyl alcohol polymer) to reduce fouling formation has been used for *Chlorella* sp. harvesting.[84]

Microstrainer and vibrating screen filters are two attractive primary screening methods for use in microalgae harvesting since they are mechanically simple in function and construction, available in large unit sizes, easily operated, have low energy consumption, require little capital investment and have high filtration ratios. However, inefficient capture or blocking of the screen can occur when applied to organisms approaching bacterial dimensions or high microalgal concentrations.[85] In this case, a flocculation pre-step might be required prior to micro-straining.[39]

Fabric filters such as stretch-cotton, polyester-linen, satin-polyester, in addition to silk were found to be variably efficient in harvesting microalgae using the physical filtration method, with efficiencies of different fibre types of 66–93%, 54–90%, 43–71% and 27–75%, respectively.[86] It was suggested that for 1500 $m^3\,day^{-1}$ wastewater and an algae concentration of 200 $mg\,l^{-1}$, microalgae harvesting cost would be $\leq$ £0.15 per m^2 per kg for algae per m^3 using a stretch-cotton filter.[86] Sand filtration [87] or sand filtration combined with solar drying [88] or ozonation [89] has also been studied as potential methods for harvesting micro-algal biomass.

Flotation

Flotation is a gravity separation process in which air or gas bubbles are introduced into a solid–liquid mixture, which, attaching to the solid particles, brings them to the surface. Auto-flotation of algae by the dissolved oxygen produced by photosynthesis was shown to be a rapid and effective technique for harvesting algae from high-rate pond effluents.[90] Addition of polyelectrolyte salts (such as aluminium and iron salts or formulations of charged organic polymers) to the liquid could be a useful step prior to flotation since it might help to overcome the natural repulsion between the air bubbles and the negatively charged algal particles. As with any flocculation-dependent process, factors such as pH and ionic strength should be optimized before using this technique. It has been noted that flotation is more beneficial and effective than sedimentation in harvesting microalgae.[91] The flotation process can be divided into dissolved air flotation (DAF) or induced flotation based on the bubble size.

Dissolved Air Flotation is a method involves the generation of fine bubbles (10–100 μm) that will adhere to the flocs, rendering them very buoyant.[40] The fine bubbles produced by the decompression of a pressurized fluid can capture particles with diameter of $<500\,\mu m$ by collision and subsequent adhesion between the bubble and the particle.[92] This process is capable of working with large volumes [93] and works well in fresh water. The pressures of the tank, hydraulic retention time, recycle rate and particle floating rate are the main factors affecting DAF harvesting of microalgae, and the contamination of the materials with the floc agent (which may significantly decrease their value) is the main disadvantage of this approach.[39] Chemical

flocculation with DAF has been used to harvest microalgae [40] and it has been found that DAF is more efficient and effective than settling, although a pre-treatment step of flocculation was applied.[43] One possible drawback is the rather intense energy demand of this process. Common operating saturation pressures range from 3 to 6 atmospheres bringing the energy required to pressurize the air saturated water for the dissolved air flotation process to 0.04–0.08 MJ m^{-3}.

Induced Air Flotation (IAF) is a method that mainly works by generating 700–1500 μm bubbles with a high-speed mechanical agitator and an air injection system.[94] A dispersed air flotation process has been evaluated to remove *Scenedesmus quadricauda* from water using three different agents with the cationic *N*-Cetyl-*N*-*N*-*N* trimethylammonium bromide being relatively efficient (90%) while the anionic sodium dodecylsulphate and the non-ionic Triton X-100 were only 10% efficient.[91] It has been proposed that combining dispersed air flotation with foam fractionation to harvest, concentrate and physically separate particles in suspension can be cost effective (consuming only 0.015 kWh m^{-3}) and can efficiently compete with the other commonly used harvesting technologies.[95]

Electrolytic separation

Electrolytic separation is another potential approach for harvesting algae without chemical addition. In one method, hydrogen generated by water electrolysis adheres to the microalgal flocs, driving them to the surface.[96] Electro-coagulation mechanisms involve coagulant formation through electrolytic oxidation of the sacrificial electrode followed by destabilization of the particulate suspension, breaking of any emulsions and aggregation to form flocs.[42] This method has been used to remove microalgae from industrial wastewater, achieving >98% algae recovery when run times and voltage are optimized,[97,98] where with *Nannochloropsis* sp., >97% of biomass was recovered with no significant changes in biomass quality.[99] The use of electro-coagulation prior to centrifugation could drastically decrease the energy demand for harvesting.[99]

Electrolytic flocculation is a method where microalgae move towards an anode to neutralize the carried charge, forming aggregates. This method appears to be efficient (80–95% removal) [100] with a total cost estimated to be \$0.19 kg^{-1} of ash free dry mass.[101] The effect of initial cell density, ionic strength, coagulant dosage and medium pH on inorganic electrolyte flocculation harvesting have been examined using *Nannochloris oculata*.[102]

Electro-coagulation–flotation (ECF) technology has been shown to be an effective approach, technically and economically, for algae removal,[103] where under optimal conditions (Al electrode, 1 mA cm^{-2} pH $= 4 - 7, 18 -$ 36°C, algal density of $0.55 \times 10^9 - 1.55 \times 10^9$ cells L^{-1}), 100% algal removal could be achieved with low energy consumption (as low as 1.4 MJ m^{-3}). Another study found an aluminium anode to be more efficient than an iron anode and concluded that the ECF method is more efficient than centrifugation under optimum conditions.[104] Thus, a limited number of studies have suggested that electrochemical methods might be safe, cost effective, environmentally friendly and energy efficient.[96]

This harvesting method could potentially be linked with downstream processing, such as oil extraction, leading to one-step process; a combination which has been named high-speed algal harvesting.[105]

Magnetic separation is a simple, quick, low energy and potentially low running cost method for capturing of cells and bio-molecules from a solution using functional magnetic particles and an externally applied magnetic field.[106] However, its complexity and the cost of fabrication have hindered adoption of this method. This method has been used proof-of-principle demonstrations in the removal of harmful algae from freshwater [107] and the recovery of *Botryococcus braunii*, *Chlorella ellipsoidea* and *Nannochloropsis maritima* from a culture broth using Fe_3O_4 nanoparticles.[108] Up to 99% separation efficiency of *Chlorella vulgaris* from a highly diluted suspension has been claimed using novel microwave synthesized iron oxide magnetic microparticles.[109]

Biologically based methods

Biologically based methods include; bio-flocculation, caused by secreted biopolymers (such as extracellular polymeric substance or extracellular organic matter),[110] or microbial flocculation of algae caused by adding flocculating microbes to an algal culture.[77,111] For example, a flocculating microalga can be used to concentrate and recover a non-flocculating microalga of interest [112] or a bio-flocculant from a bacterium can be used, as was the case where *C. vulgaris* was harvested using a bio-flocculant from *Paenibacillus*.[111] Novel alternative techniques have been described such as the co-cultivation of microalgae with fungi [113] where, for example, the pellet-forming filamentous fungae *Aspergillus oryzae* is grown with *C. vulgaris*.[114] Finally, in an ecosystem approach, an algae eating fish such as tilapia can be used and the algae can be harvested from the sedimented droppings by a conveyor belt.[115,116]

Genetic modification, although usually done for the purposes of increasing biomass productivity or lipid content, may be a promising approach for improving algal harvesting.[24,25] This is suggested by a study involving strains of yeast genetically modified to contain flocculin in their cell walls, a protein which causes cells to aggregate.[117] Different genetic modifications can induce cellular flocculation as it was the case of the cell wall-deficient mutant of *Chlamydomonas* sp.[118]

The choice of biomass harvesting method is mainly driven by economics and strictly depends on the value of the product. For example, gravity sedimentation (possibly

enhanced by flocculation) may be used for low-value products and sewage-based processes,[119] while high-value products, such as those for food, feed and nutraceuticals, may permit the use of cost-intensive continuous centrifuges. Indeed, there is no universal best method for microalgal harvesting and it mainly depends on algae species, size, density, production costs, growth medium and the end product. Low-cost filtration procedures are usually applicable for large-sized microalgae, while flocculation aids in harvesting of small microalgae. Flotation technologies can be considered for low cell density separation, while sedimentation is good for high cell density harvesting. Moreover, oxygen generated from algal photosynthesis will create super saturation conditions in the medium that will support the use of flotation methods.

After the recovery of an algal slurry by harvesting, dewatering methods, such as belt filter presses, thermal drying and centrifugation, are usually employed to increase the solids content before downstream processing such as oil extraction.[39,120] Heat, methane drum, air, solar, wind or other types of dryers can be used for algae dewatering, however, as usual, cost, space and time need to be carefully considered.

A summary of the advantages and disadvantages of the different harvesting methods is given in Table 2.

Biofuels from microalgae

Microalgae are rapidly growing microorganisms that are able, depending upon the species, to grow in a variety of climates. They are a highly diverse group of organisms with some capable of the synthesis of a variety of valuable products, and they are potentially able to mitigate some environmental pollution problems by taking up CO_2 and removing nitrogen and phosphorus from waste streams. As well, they possess unique properties that make them potentially suited as a sustainable renewable source for biofuel. Their cultivation does not necessarily compete with the world's food supply, in contrast to the traditional biofuel-producing crops. They have the potential to be a source of a diverse spectrum of valuable products such as food, energy carriers (e.g. biodiesel, jet fuel, gasoline, aviation gas, ethanol, etc.), nutritional products, organic fertilizers, biodegradable plastics, medicines and animal feed.[135,136]

Algal biomass can be processed in different ways to yield biofuels or biofuel-related products either by using the whole algal biomass, algal biomass extracts or the wastes after extraction. Algae have been touted as a source for next-generation biofuels, however large-scale industrial production has been thwarted by challenges in cost-effective harvesting, drying and extraction.[42] Microalgae can be converted into biofuel through a variety of processes, including biochemical, thermochemical and other routes (Figures 1, 2 and 3). First, we examine the more traditional routes of biofuels production from algae, biodiesel, bioethanol and biogas production. Then, we examine newly proposed novel routes, some of which have given promising results, as summarized in Table 3. Table 3 is expressing some studies of Pyrolysis, Liquefactions and gasification.

From algal lipids to biodiesel

Biodiesel is one of the most important biofuels, as all industrial vehicles, much motorized transport, and farming machinery are diesel dependent. Biodiesel is the monoalkyl esters of long chain fatty acids (FAME) derived from renewable feedstocks (such as oleaginous crops) [157,158] by transesterification of their oil with alcohol, mainly methanol. It has the advantages of being non-toxic and biodegradable.[159] The use of crop-based biodiesel has arguably created pressure on the arable land used for food production with potential impacts on the food supply, including possible future food shortages and increased food prices. Microalgae have the potential to be a clean environmentally sustainable future feedstock for biofuel that does not compete with the food supply.[52,160] Microalgal biodiesel is produced by transesterification of the extracted lipid, resulting in FAMEs and glycerol as a side product. In general, microalgae are estimated to produce more oil than oleaginous plants and have been shown to contain as much as 73% lipids by weight, with the major component being TAG.[161] Several microalgae strains were screened for choosing the best superior candidates for lipid production, in terms of the biomass productivity and lipid content in addition to the quality of the fatty acid composition, such as *C. vulgaris*, *Chlorella protothecoides*, *Nannochloropsis* sp., *Nitzchia* sp., *Chlamydomonas reinhardtii*, *Schizochytrium* sp., *Scenedesmus obliques* and *Neochloris oleabundans*.[1,162–166]

Algae species vary greatly in terms of growth rates, lipid accumulation and productivity, nutrient requirements, adaptability, etc. For any particular production system, it is important to find the right algal strain. Microalgae are being considered as a sustainable feedstock for lipid-based biodiesel production. However, optimal lipid productivity is a compromise between high growth rate and high lipid accumulation. In order to increase the lipid content, several biochemical, physical, metabolic engineering and genetic approaches have been applied to naturally occurring high lipid producing microalgal species. Stimulation of lipid biosynthesis (TAGs) by growing microalgae under unfavourable environmental or stress conditions imposed by chemical or physical environmental stimuli has been extensively studied.[32,162,167,168] Genetic engineering tools have also been applied but this approach is still in its infancy due to the high diversity of algal metabolic machinery and a lack of understanding at the molecular level of control of microalgal growth, biofuel production and molecular regulation.[169] Nutrient stress (e.g. nitrogen and/or phosphorus starvation), temperature, pH, light irradiation, salinity, heavy metals and other compounds are among the

Table 2. Summary of the advantages and disadvantages of different algal harvesting methods.

Algal harvesting method	Advantages	Disadvantages	Ref.
Gravity sedimentation	Simple, easy, inexpensive (0.1 kWh m^{-3}), water recycling	Slow, species dependent, depends on the particle size and cell density, high moisturize content (need for further drying for downstream processing)	[40,121–123]
Centrifugation	Rapid, easy, effective, high capture efficiency (>90%) and preferred method for lab and small scale	Requires high investment, operation costs and high energy consumption (1–8 kWh m^{-3}), can damage cell structure due to the high speed and shear stress, time consuming and too expensive for large scales	[40,50,123]
Chemical flocculation	Large volumes, effective with a wide range of species, low cell damage, rapid (small harvesting units)	May not be sufficient alone, highly pH dependent, introduction of toxic contaminants, costly and hard to separate from the recovered biomass, large quantity of sludge higher dehydration costs, costly for commercial use (14.8 kWh m^{-3} polymer flocculation), efficiency and costs are dependent on chemical agents used	[39,40,123,124]
Bio-flocculation	High efficiency, cell structure preserved, successfully used in use harvesting the microalgae cultures in wastewater treatment ponds	High bio-flocculant costs, cultivation of producing species required	[124,125]
Physical flocculation	No chemical or biological contamination, efficient at lab scale	Difficult to apply at large scale, costly (ultrasound more expensive than centrifugation)	[125]
Autoflocculation	Spontaneous, very low costs	Elevated pH, recovered biomass contains high amount of minerals, medium ions may precipitate together with the algal biomass, light-dependent	[74,125]
Electroflocculation	Very efficient, easy operation and construction, pH adjustment unnecessary, avoidance of chemical usage, cost effective (0.11 US$ for separation of 1 m^3 of the algal suspension) with low energy consumption (0.33 kWh m^{-3})	Cathode fouling, further research required	[101,121,123,126]
Electro-coagulation flocculation	Low electricity (marine algae)	Recovered biomass contaminated with metals, energy consumption of (1.5 kWh m^{-3})	[123,125]
Filtration	Low cost, easy, energy consumption of (0.4 and 0.88 kWh m^{-3}) in case of natural and pressure filter	Slow, requires pressure or vacuum, not suitable for large quantities and inadequate for small species, membrane fouling and clogging, high energy consumption (vacuum filtration (5.9 kWh m^{-3}))	[39,40,123,127]
Cross-flow membrane	Pathogen removal, water recycle, low filter cake formation	Membrane associated problems (fouling). High material cost	[128]
Submerged membrane microfiltration	Economically feasible, low shear stress, pathogen removal	Membrane fouling, problems with scale-up	[79]
Microstrainers	Simple structure, operation and function, low cost, high capability, requires little maintenance	Cell size and concentration dependent, not suitable for small cells, energy intensive, Incomplete solids removal, build-up of bacterial and algal slime, periodic cleaning required	[40,121,129]
Sand filtration	Simple and inexpensive construction and operation	Slow and impractical, back-wash water issues, removal of some algal species marginal	[87,130]

(*Continued*)

Table 2. Continued.

Algal harvesting method	Advantages	Disadvantages	Ref.
Tangential flow filtration	High filtration rate, cells structure and properties preserved	High energy requirement (2.06 kWh m^{-3}), membrane fouling, unsuitable for large scale	[40]
Foam (flotation) fractionation	Cost effective, no chemicals used, small footprint	Inefficient flotation, low recovery yield	[95,131]
Ozone fractionation	Efficient, small footprint, causes cell lyses, pure disinfected product, no toxic chemicals, complete separation	High cost (ozone)	[70,132]
Dissolved air flotation (DAF)	Easy, low cost, can be applied to large scale	Flocculants/pre-treatment by flocculation required, product extraction may be negatively affected	[40,121]
Suspended air flotation	Quick, low energy requirements, economical	Oversized bubbles break up the floc,	[133,134]
Magnetic separation	Quick, low running cost, energy saving, simple operation	Complex and expensive fabrication	[108]

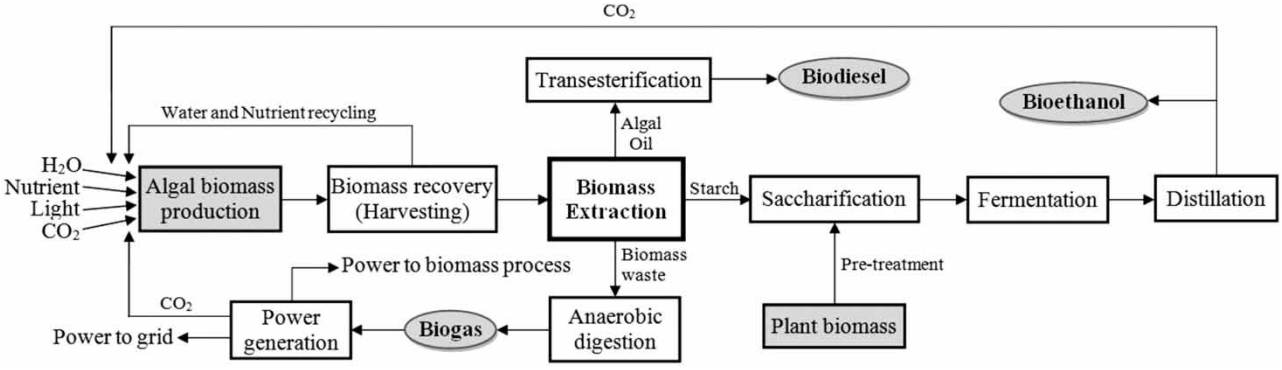

Figure 1. Integrated system for biodiesel, bioethanol, biogas, and power generation.

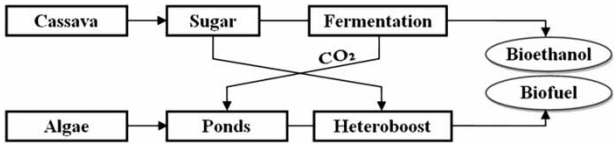

Figure 2. Phycal-integrated advanced technology.

stresses which have been studied as tools to improve lipid productivity, with nutrient starvation being the most widely used option is extensively studied. Some of those studies are summarized in Table 4.

The micro-algal lipid content, as well as the quality of the FA composition, can increase considerably when the cells are subjected to stress conditions, either chemical: nutrient starvation, salinity and pH; or physical, temperature and light intensity, environmental stimuli.[32,164] A decrease in temperature leads to an increase in the unsaturation of the FA composition and vice versa.[208,209] Low light intensity favours the synthesis of polyunsaturated fatty acids (PUFAs) and induces polar lipid formation, whereas high light intensity favours saturated and mono unsaturated FAs, principle components of neutral lipids, decreases the total polar lipid content and increases the amount of neutral storage lipids (mainly TAGs).[197,210]

Algal biofuel has already been tested as a jet fuel by the aviation industry (Continental Airlines in 2009) without engine modification (http://www.nbcnews.com/id/28547191/#.Uc8rivm1Gdc). This algal-derived fuel has the advantages of low flash point, sufficiently low freezing point, high energy densities and reduced CO_2 emissions, up to 78% compared with the currently used petroleum derivate.[16]

Several methods for the extraction of lipids from microalgae have been described, with the most common methods being solvent extraction, expeller/oil press, supercritical fluid extraction (SFE) and ultrasound techniques.

The solvent extraction method is where oil is extracted from the algal cell by adding organic solvents (such as hexane, acetone, chloroform benzene, etc.) The solvent destroys the algal cell wall and extracts the oil, which can then be separated from the solvent by distillation and further processed for biodiesel. Based on the cost and extraction capability, hexane has been found to be the most efficient solvent in lipid extraction.[211] In one case about 80% of the total lipids were extracted by a two-step method, using methanol to extract the lipid, followed by hexane to purify them.[212] This method has several drawbacks,

Table 3. Conversion using pyrolysis, liquification or gasification.

Species	Scale	Conversion process and Condition	Yields (% dry wt.) Solid residues charcoal	Liquid bio-oil Content	HHV ($MJ\,kg^{-1}$)	Bio-oil properties and elemental composition	Gaseous	Ref.
Chlorella prothothecoides	Lab scale	Fast pyrolysis, heterotrophic, at 500°C, 0.101 MPa heating rate of $600°C\,s^{-1}$, a sweep gas (N_2) flow rate of $0.4\,m^3\,h^{-1}$, and a vapour residence time of 2–3 s	11.2	57.2	41.0	Contain an average of low oxygen content O (11.2), C (76.2), H (11.6), A density of $0.92\,kg\,l^{-1}$, viscosity of 0.02 Pa s (at 40°C)	32.0	[137]
C. prothothecoides	Lab scale	Fast pyrolysis, phototrophic, at 500°C, 0.101 MPa heating rate of $600°C\,s^{-1}$, a sweep gas (N_2) flow rate of $0.4\,m^3\,h^{-1}$, and a vapour residence time of 2–3 s	53.8	16.6	30.0	O (19.4), C (62.1), H (08.8) A density of $1.06\,kg\,l^{-1}$, viscosity of 0.10 Pa s (at 40°C)	32.0	[137,138]
C. prothothecoides	Lab scale	Fast pyrolysis, at 775°C, 0.101 Mpa a heating rate of 10 K/s	08.4	55.3	39.7	Nr	36.3	[139]
M. aeruginosa	Lab scale in fluid bed reactor	Fast pyrolysis, phototrophic, 500°C, 0.101 MPa, a heating rate of $600°C\,min^{-1}$ at residence time of 2–3 s	~21.0	24.0	29.0	O (21), C (62.1), H (08.2), A density of $1.06\,kg\,l^{-1}$, viscosity of 0.10 Pa s	~54.0	[138]
Blue-green algae blooms	Lab-scale fixed-bed reactor	Pyrolysis, at 500°C, particle size below 0.25 mm and sweep gas flow rate of $100\,mL\,min^{-1}$	25.0	55.0	31.9	O (14.5)[a], C (67.6), H (8.95), N (7.75), High level of long chain alkanes	20.0	[140]
C. vulgaris	Lab-scale fixed-bed reactor	Catalytic pyrolysis using H^+ZSM-5 catalyst, at 500°C	25.7	52.7	18.6	O (24.8)[a], C (51.4), H (10.4), N (12.4), high hydrocarbons (~ 25%)	21.6	[141]
	Lab-scale quartz tube reactor	Fast pyrolysis, at 500–900°C, heated by using a SK2-4-13 tube furnace,	30 at 500°C	91.09 Biofuel yield at 900°C	Syngas heating based on energy consumption at 900°C was 1.3391 (ppmv kJ)/(L kW h)	Nr	Syngas H_2 emission rate 50.75 ppmv/s at 900°C, CO 102 ppmv/s at 800°C	[142]

(*Continued*)

Table 3. Continued.

Species	Scale	Conversion process and Condition	Yields (% dry wt.)					Ref.
			Solid residues charcoal	Liquid bio-oil			Gaseous	
				Content	HHV ($MJ\,kg^{-1}$)	Bio-oil properties and elemental composition		
Scenedesmus sp.	Bench scale isothermal spouted bed reactor/dynamic	Fast pyrolysis, 480°C and 100 kPa with a 2 s vapour residence time and 2 h total run time	Oil/char = 3.76 by wt	55.0	18.4	Contain an average of O (27.6), C (51.9), H (9.0), N (8.6)	Nr	[143]
Chlorella sp.	Lab-scale fixed-bed reactor	Fast pyrolysis, non-catalytic and catalytic using Na_2CO_3 catalyst, at 300–450°C	48 and 55 at 300°C	55 and 40.5 at 450°C	27 and 33 at 400°C and 450°C	Low oxygen content (33.2) Lower acidity, higher aromatics	23 and 34 at 400°C	[144]
Chlorella pyrenoidosa	Lab-scale a stainless-steel batch reactor	Non-catalytic hydropyrolysis, temperature 310°C, time 60 min and H_2 pressure 3 MPa	12.3	53.2	37.3	Low oxygen content, O (7.6)[a], C (72.9), H (9.8), N (9.7)	18.5	[145]
S. platensis	Lab-scale batch reactor	Pyrolysis at 500°C, 60 min, heating rate 7°C min^{-1}	25.6	29.0	33.62	O (6.81)[a], C (74.7), H (10.8), N (7.13)	28.0	[146]
		Pyrolysis at 350°C, 60 min, heating rate 3.5°C min^{-1}	39.7	23.0	29.3	O (11.3)[a], C (67.5), H (9.82), N (10.7)	19.2	
		TCL, at 350°C, 60 min, heating rate 3.5°C min^{-1}, 2 MPa	05.7–07.0	39.9–41.0	34.21	O (10.1)[a], C (73.7), H (8.90), N (6.30) TCL bio-oil better in quality and stability compared to pyrolsis oil	22.0–23.2	[146,147]

Chlorella vulgaris	Lab scale	Microwave- assisted pyrolysis, power of 750, 1500 and 2250 W	~90 Solid residues	35.8 at 1000 W	Nr	Nr	52.4 at 2250 W	[148]
Chlorella sp.	Pilot-scale	Microwave- assisted pyrolysis, catalyst, power of 500, 750, 1000 and 1250 W, (462–627°C), 20 min,	~25.0 at 750 W	28.6 at 750 W	30.7 at 750 W	O (16.5)[a], C (65.4), H (7.84), N (10.3) A density of 0.98 kg/L (at 30°C), a viscosity of 61.2 cSt at 750 W	27.0 at 750 W	[149]
Raw Scenedesmus biomass	Lab scale	Slow pyrolysis at 450°C, reaction time of 2 h	30.0	31.0	35.0–37.0	O (10.5), C (72.6), H (9.0), N (6.5)	12.0	[150]
Defatted Scenedesmus			33.0	24.0		O (10.5), C (72.2), H (8.9), N (7.8)	21.0	
Spirulina biomass			30.0	24.0		O (9.2), C (72.2), H (9.1), N (8.1)	15.0	
Raw Scenedesmus biomass	Lab scale Triplicate batch reactions	Hydrothermal liquefaction (HTL) at 300°C, pressure ranging from 10 to 12 MPa	07.0	45.0	33.0–40.0	O (8.1), C (73.9), H (9.3), N (7.9)	30.0	[150]
Defatted Scenedesmus			06.0	36.0		O (8.2), C (72.6), H (8.9), N (10.0)	41.0	
Spirulina biomass			11.0	31.0		O (9.2), C (71.2), H (9.0), N (9.2)	35.0	
Chlorogloeopsis fritschii	Lab-scale batch reactor, high pressure reactors	HTL, 300°C and 350°C (accompanied with Nutrient recycling of aqueous phase)	~10.0	38.6	32.0	O (19.0)[a], C (66.5), H (07.2), N (06.8)	~13.0	[151]
S. platensis			~02.0	35.5	36.1	O (11.5)[a], C (72.7), H (08.8), N (06.3)	~05.0	

(*Continued*)

Table 3. Continued.

Species	Scale	Conversion process and Condition	Yields (% dry wt.) Solid residues charcoal	Liquid bio-oil Content	HHV ($MJ\,kg^{-1}$)	Bio-oil properties and elemental composition	Gaseous	Ref.
C. vulgaris			~08.0	46.6	37.5	O (09.3)[a], C (75.9), H (09.0), N (05.3)	~12.0	
Scenedesmus dimorphous			~18.0	27.1	33.6	O (12.6)[a], C (73.0), H (08.2), N (05.7)	~08.0	
C. vulgaris	Lab-scale batch reactor	HTL, 350°C, ~200 bar in either[b] pure distilled water, or	~03.0	~39.0	337.1	O (14.8)[a], C (73.6), H (10.7), N (5.9)	~28.0	[152]
Nannochloropsis occulta		1 M base Na_2CO_3 or	~07.0	~37.0	39.0	O (18.9)[a], C (74.7), H (10.6), N (4.3)	~48.0	
Porphyridium creuntum		1 M of the organic acid HCOOH	~10.0	~27.0	36.3	O (13.3)[a], C (72.8), H (09.1), N (05.7)	~15.0	
Spirulina sp.			~07.0	~27.0	36.8	O (10.9)[a], C (75.4), H (10.8), N (07.0)	~32.0	
B. braunii	Lab scale	TCL, 300°C, 3 Mpa	Nr	64.0	45.9	Nr	Nr	[153]
D. tertiolecta	Lab scale	TCL, 340°C, 10 Mpa, 250–340°C, 5–60 min	Nr	33.6–40.4	36.0	Viscosity 150–330 mPas	Nr	[154]
Spirulina sp.	Lab scale	Gasification, 1000°C, 0.101 Mpa	Nr	Nr	Nr	Nr	64.0	[155]
Nannochloropsis gaditana	Lab-scale flux bed reactor	Gasification, 850°C, a particle size from 100 to 250 μm and a heating rate of $40°C\,min^{-1}$, 7.3% in argon	Nr	Nr	Nr	Nr	~52.0 H_2, ~35.0 CO, ~14.5 CO_2	[156]

Note: HHV, Higher heating value; Nr, Not reported.
[a]Oxygen content was determined by difference.
[b]Result as the maximum values.

Table 4. The impacts of some different stress conditions on microalgal lipid production.

Species	Stress condition	Impacts on the lipid content	Ref.
Nutrient stress			
Neochloris oleoabundans	Nitrogen deficiency	Lipid productivity of 131 mg L^{-1} d	[170]
C. vulgaris	Nitrogen deficiency	Lipid productivity of 146 mg L^{-1} d	[170]
Chlorococcum oleofaciens	Nitrogen deficiency	Lipid productivity of 127 mg L^{-1} d	[170]
S. dimorphus	Nitrogen deficiency	Lipid productivity of 111 mg L^{-1} d	[170]
Chlorella sorokiniana	Nitrogen deficiency	Lipid productivity of 85 mg L^{-1} d	[170]
Scenedesmus naegleii	Nitrogen deficiency	Lipid productivity of 83 mg L^{-1} d	[170]
N. oleoabundans	Nitrogen deficiency	Increase in TAGs accumulation from 1.5% to 12.4% w/w	[171]
C. vulgaris esp-31	Nitrogen deficiency	Lipid productivity of 78 mg L^{-1} d	[172]
Parachlorella kessleri	Nitrogen deficiency	Increase in storage lipid from almost 0–29%	[173]
Chlorococcum infusionum	Nitrogen deficiency	Increase in lipid content from 15–40%	[174]
Chlorella sp.	Nitrogen deficiency	Lipid productivity of 53.96 mg L^{-1} d	[175]
D. tertiolecta	Nitrogen deficiency	Fivefold increase in lipid fluorescence	[176]
Scenedesmus sp.	Nitrogen and phosphorus starvation	Lipids content increased 30% and 53%, respectively	[177]
Phaeodactylum tricornutum, *Chaetoceros* sp. and *Isochrysis galbana*	Phosphorus limitation	Increase in total lipids	[178]
Monodus subterraneus	Phosphorus limitation	Increase in TAGs accumulation	[179]
Chlorella kessleri	Phosphorus limitation	Increase in unsaturated FA	[180]
Chlorella sp.	Phosphate, potassium, iron	Lipid productivity of 49.16 mg L^{-1} d	[175]
C. reinhardtii	Sulphur limitation	2-fold increase in the phosphatidylglycerol	[181]
C. reinhardtii	Sulphur limitation	Increase in TAG	[182]
Cyclotella cryptica	Silicon deficiency	Total lipids increase from 27.6% to 54.1%	[183]
Scenedesmus sp. and *Coelastrella* sp.	pH and N-limitation	Increase in TAG accumulation	[184]
Four green microalgae *Botryococcus* spp. (TRG, KB, SK and PSU)	Nitrogen deficiency, high level of iron and high light intensity	Increase in lipid content from 25.8%, 17.8%, 15.8% and 5.7–35.9%, 30.2%, 28.4% and 14.7%, respectively	[185]
Temperature stress			
Rhodomonas sp.	Temp. range of 27°C–30°C	Increase in lipid production by 15.5%	[186]
Cryptomonas sp.	Temp. range of 27°C–30°C	Increase in lipid production by 12.7%	[186]
Isochrysis sp.	Temp. range of 27°C–30°C	Increase in lipid production by 21.7%	[186]
Nannochloropsis oculata	Increase temp. from 20°C to 25°C	Increase in lipid production by 14.92%	[187]
C. ellipsoidea	Decreasing temperature (Chilling sensitivity)	Increase in unsaturated FAs	[188]
Selenastrum capricornutum	Temp. from 25°C to 10°C	Increase in oleate FAs (18:1)	[189]
Salinity stress			
Schizochytrium limacinum	Salinity of 9–36 g L^{-1} at Temp. range 16–30°C	Large increase in saturated FAs C15:0 and C17:0	[190]
D. salina	Culture transferred from 29.2 g L^{-1} to 204.5 g L^{-1} NaCl (from 0.5 to 3.5 M NaCl)	Increase in the concentration of C18 FAs	[191]
Hindakia sp. PKUAC 169	8.8 g L^{-1} NaCL (0.15 M NaCl)	Three-fold higher lipid productivity compared to N starvation	[192]
Nannochloropsis salina	Increase salinity from 10–22–34–46–58 g L^{-1}	Increase in the lipid content, was highest at 34 g L^{-1}	[193]
Nitzschia laevis	Increase of NaCl from 10 g L^{-1} to 20 g L^{-1} (from 0.17 to 0.34 M NaCl)	Increase in unsaturated FAs of both neutral and polar	[194]
Light irradiation stress			
Nannochloropsis sp.	Light intensity of 100 μmol m^{-2} s^{-1}/18 h light: 6 h dark cycle	Increase in total lipid content as much as 31.3%	[195]
Pavlova lutheri	High light intensities stress	Increase in total lipid content	[196]
Thalassiosira pseudonana	100 μmol m^{-2} s^{-1}/12:12 h, 100 μmol m^{-2} s^{-1}/24:0 h, and 50 μmol m^{-2} s^{-1}/24:0 h light:dark, harvested at the logarithmic phase	Increase in polar lipids (79 to 89% of total lipid) and increase in PUFA in the case of 100 μmol m^{-2} s^{-1}/12:12 h regime.	[197]

(*Continued*)

Table 4. Continued.

Species	Stress condition	Impacts on the lipid content	Ref.
T. pseudonana	100 μmol m^{-2} s^{-1}/12:12 h, 100 μmol m^{-2} s^{-1}/24:0 h, and 50 μmol m^{-2} s^{-1}/24:0 h light:dark, harvested at the stationary phase	Increase in TAGs (22 to 45% of total lipid).	[197]
S. capricornutum	Dark treatment stress	Increase in linoleate FAs (18:2) and sterol	[189]
Chaetoceros muelleri	UV-A radiation	Increase in monounsaturated FAs and increase in ratio of saturated FAs to PUFAs	[198,199]
Nannochloropsis sp.			
Chaetoceros simplex	UV-B radiation	Increases in saturated FAs and monounsaturated FAs and decrease in PUFAs	[200,201]
Tetraselmis sp.			
Metabolic engineering			
Haematococcus pluvialis	Cloning the main key genes for FA biosynthesis	ACP, KAS and FATA may play an important role in FA synthesis	[169]
C. reinhardtii	Defective in Isoamylase gene	Increase in lipid and starch production	[202]
C. reinhardtii	Defective in ADP-glucose pyrophosphorylase	Increase in TAGs accumulations	[203]
C. reinhardtii	Defective in the small subunit of ADP-glucose pyrophosphorylase	8-fold and 3.5-fold increase in neutral and total lipid content (32.6% and 46.4%), respectively	[204]
Parietochloris incisa	Mutagenesis in Δ-5 desaturase	Increase in saturated FAs	[205]
Phaeodoactylum tricornutum	Overexpression of acyl-ACP thioesterase	Increase in saturated FAs	[206]
T. pseudonana	Targeted knock down of Thaps3_264297 gene (defective in lipase) + silicon limitation	2-fold increase in TAG and 3–5-fold in total lipid	[207]

Notes: TAG, Triacylglyceride; FAs, Fatty acids; PUFAs, Polyunsaturated fatty acid.

including the large volumes of solvent that are required and the fact that most organic solvents are toxic and highly flammable.[213]

The oil press or expeller method is very commonly used for extraction of oil from seeds and nuts and likewise it can extract oil from microalgae.[214] Although easy to use and with an efficiency of about 75%, it requires a relatively long extraction time compared with other methods as well as requiring large amounts of sample.[214,215] In this approach, algae are first dried, followed by compression to extract the oil.

SFE is a method which uses high pressures and temperatures to breakdown the microalgal cells.[216] It has the advantage of being extremely time efficient and at least one study found that the temperature and pressure of SFE did not have any effect on the yields of extracted compounds.[217] In a study using *Nannochloropsis* sp., no difference between the extraction yield with SFE and the solvent extraction method using hexane was found.[218] However, another study found that the SFE method gave higher FA yields compared with the solvent extraction when the cyanobacterium *Spirulina platensis* was used.[219] Some of these apprarent contradictions could be due to differences in the actual process used or in the algae which are being extracted. To firmly decide whether this method is more efficient in general will require further study.

Ultrasound is a potentially useful method to extract lipids from algae. It works by exposing the algal cells to a high-intensity ultrasonic wave which produces tiny cavitation bubbles that collapse and emit shockwaves around the cells, shattering and disrupting the cell wall and releasing the oil. More than 90% extraction of FAs and pigments can be achieved from the microalgae *Scenedesmus obliquus* using this methodology.[220] Although there is a high rate of lipid extraction, it will be difficult to apply on a large scale due to the costs, high power consumption and the difficulty in scale up.[221]

From algal starch to bioethanol

Bioethanol is usually produced by fermentation of starch, sugars and lignocellulosic feedstocks.[222] The extracted starch can be hydrolysed to produce glucose, metabolized by yeast (such as *Saccharomyces cerevisiae or Zymomonas mobilis*) to produce ethanol (+CO_2), which is then purified from the mixture by distillation and dehydration. Bioethanol is produced from microalgae mainly by either dark fermentation or yeast fermentation; in addition, it can be also produced thermo-chemically by gasification. In dark fermentation, the microalgae itself consumes its intracellular starch anaerobically and produces bioethanol, while in yeast fermentation the yeast ferment either the

Table 5. The starch or carbohydrate content of some microalgae suitable for bioethanol production.

Microalgal source	Starch or Carbohydrate (% of dry wt)	Ref.
C. reinhardtii	43.6–55.0 (starch)	[224–226]
Scenedesmus sp.	13.0–20.0 (starch)	[227]
C. vulgaris	37.0–60.0 (starch)	[225,228–230]
Nannochlorum sp.	25.0 (starch)	[231]
Nostoc muscorum	33.5 (starch)	[227]
Phormidium angustissimum	28.5 (starch)	[227]
Chlorococcum sp.	17.0–26.0 (starch)	[227]
S. obliquus	23.7 (starch)	[227]
Oscillatoria sp.	19.3 (starch)	[227]
Tetraselmis subcordiformis	62.1 (starch)	[232]
Nostoc sp.	30.7–32.9 (starch)	[227]
Oscillatoria sp.	19.3 (starch)	[227]
Anabaena variabilis	09.2 (carbohydrate)	[233]
P. cruentum	40.0–57.0 (carbohydrate)	[234]
C. reinhardtii UTEX90	60.0 (carbohydrate)	[225]
C. vulgaris CCAP 211/11B	55.0 (carbohydrate)	[235]
S. obliquus CNW-N	46.7 (carbohydrate)	[236]
Pavlova pinguis	41.0 (carbohydrate)	[237]
Tetraselmis suecica F&M-M33	50.0 (carbohydrate)	[238]
Anabaena cylindrica	25.0–30.0 (carbohydrate)	[239]
D. salina	32.0 (carbohydrate)	[239]
S. platensis	31.2 (carbohydrate)	[147]
Spirulina maxima	13.0–16.0 (carbohydrate)	[215,239]
Nannochloropsis sp.	12.0 (carbohydrate)	[240]
Porphyridium purpureum	40.0–57.0 (carbohydrate)	[241]
P. tricornutum	26.0 (carbohydrate)	[242]
D. tertiolecta	20.0 (carbohydrate)	[243]
N. oleoabundans	08.0 (carbohydrate)	[242]
N. gaditana	36.0 (carbohydrate)	[242]

microalgal biomass or the extracted starch.[223] Although some microalgae accumulate excess fixed carbon as lipids and are therefore being studied for the production of biodiesel, others accumulate starch instead (see Table 5 for a partial listing of the starch content of some microalgae). There is little phylogenetic relationship between the species and these metabolic capacities. It has been reported for the strains that do accumulate starch that the microalgal carbohydrate content can reach as much as nearly 70%.[61] Extraction of the carbohydrates from the microalgal biomass can be carried out via different methods such as ultrasonic processing, explosive disintegration [244] or enzymatic hydrolysis conversion of the biomass into simple fermentable feedstock.[224] *C. reinhardtii*,[225] *Chlorococum littorale* [245] and *Chlamydomonas perigranulata* [231] were found to produce bioethanol by dark fermentation in an energy efficient process. However, the yield of bioethanol was too low to be used for commercial scale production, about 1–2.07% (w/w). On the other hand, several species of microalgae produce large quantities of carbohydrates (Table 5), which can be potentially processed for bioethanol production,[52,246,247] for example, *Porphyridium cruentum* (40–57%/dry weight of biomass), *Spirogyra* sp. (33–64%/dwt), *Dunaliella salina* (32%/dwt), *Scenedesmus dimorphus* (21–52%/dwt) and *Prymnesium parvum* (25–33%/dwt).[241,248] As well, marine microalgal strains have been screened for their potential to store carbohydrates [249] and more than 70 strains were found to contain a carbohydrate content of 40 –53%. *C. vulgaris* (37% starch content) yielded a 65% ethanol-conversion rate compared with the theoretical rate by fermentation.[225] Temperature, biomass concentrations, cell wall disruption through a pre-treatment stage, for example, sulphuric acid to release and convert the entrapped complex carbohydrates inside the cell into simple sugars, were all found to improve the bioethanol yield using the microalgae *Chlorococum* sp.[248] Iron supplementation [250] and nutrient starvation (P, N or S) [247] have been shown to increase the starch content in *C. vulgaris*.[228] Dilute acid pre-treatment has been used with *Chlorococcum humicola* to obtain an ethanol yield of 520 mg ethanol g^{-1} dry wt biomass,[251] but this pre-treatment may also result in converting the glucose and xylose into hydroxymethylfurfural and furfural,[252] which inhibit ethanol fermentation. Thus, pre-treatment should be monitored in order to eliminate the formation of these compounds. One synergistic application would be to carryout ethanol fermentation of the residual biomass from oil extraction. This eliminates the need to dry the algal biomass, thus saving energy. This technology therefore would permit the production of both biodiesel and bioethanol from the same biomass. In general, the fermentation method has several advantages such as a low energy requirement; simple process and operation conditions, and the emitted CO_2 can be recycled by the microalgae.

This methodology is rather interesting because instead of extracting the oil and/or starch from the algal biomass, it is possible to process the whole algae into biofuel. Even if some degree of dewatering is required, it would still save the costs associated with the conventional extraction process. On the other hand, if the traditional method is chosen, the residual biomass after extraction for oil, starch and/or high-value products can be used for other purposes. In general, the entire processes should be evaluated for the best choice economically. Several conversion technologies exist for further use of the whole algal biomass including anaerobic digestion, supercritical processing, pyrolysis and gasification.

Bio-methane (biogas) by anaerobic digestion

Microalgal biomass is a source of a wide range of organic biopolymers, carbohydrates, lipids and proteins, which can

be anaerobically digested to produce biogas, a technology which has the benefit of not requiring the costly steps of drying, extraction and fuel conversion. A natural consortium of microorganisms is able to breakdown the organic matter of the algal biomass into simple monomers which can then be converted into a methane-rich gas (about 60–70% methane). Some interfering gaseous impurities like hydrogen sulphide [253] should be removed before using the methane for use in electricity generation. Additionally, carbon dioxide (around 30–40%) might need to be removed if the gas is to be used as a fuel.[254] The residual biomass from the anaerobic digestion can be reprocessed and used as fertilizer. The high protein content in algal biomass may result in low C/N ratios and increased ammonium production, which can affect the performance of the anaerobic digestion. One solution to the problem of low C/N ratios can be the co-digestion of the algal biomass (50:50) with waste paper, which has a high C/N, effectively doubling the methane production rate compared with the anaerobic digestion of the algal biomass alone.[255]

Anaerobic digestion is appropriate for feedstocks with a high moisture content (80–90%) [222] and so is suitable for wet algal biomass. The typical energy content of biogas produced via anaerobic digestion depends upon the nature of the biomass feedstock and ranges from 16,200 MJ m^{-3} to 30,600 MJ m^{-3}. Typical biogas yields vary between 0.15 and 0.65 $m^3\,kg^{-1}$ of dry biomass.[4] Integration of biodiesel production with biogas can add to the carbon neutrality of the production facility since the produced biogas could be used to provide the power required for algal production and processing (Figure 1). Surplus energy could be sold to the grid, thus improving overall process economics.[4] Using a two-stage anaerobic digestion process with different strains of algae, a biogas production of 180.4 mL g^{-1} day^{-1} (with 65% methane concentration) has been determined.[256] The quantity of biogas obtained is strongly dependent on the species.[257] Interestingly, when the methane energetic content of either whole biomass or algal residues after lipid extraction was compared, it was found that together the energetic content of biodiesel and methane obtained from the processed algal residues was higher, with, however higher costs due to the drying and extraction processes.[258] For this type of combined process, it would be desirable to grow the algae under nitrogen starvation conditions allowing for the accumulation of greater quantities of carbon (in forms of starch or lipid) and a significant increase in the caloric value of the biomass.[235,259] For the greatest cost effectiveness, the algal production facility and the biogas fermentation plant should be coupled together in the same place to avoid costs related to the transportation.[257] This technology could be very efficient and cost effective in the case of growing microalgae for wastewater treatment and using the resultant biomass for biogas production.

Liquefaction

Thermochemical liquefaction is a method used to convert the wet algal biomass into liquid fuel by heating the biomass at high temperatures (200–500°C) and pressures (greater than 20 bar) in the presence of a catalyst to yield bio-oil.[160,260] Although the ability of this method to convert the biomass to energy is great, the reactors associated with this method are complex and expensive.[222] *Dunaliella tertiolecta*,[154] *Spirulina* sp.[261] and *Microcystis viridis* [262] have been shown to produce bio-oil yields of 37–54%. The choice of catalyst is important, as it has a large effect on the gaseous products and the quality of the produced bio-oil, for example, Ru and Ni catalysts were able to achieve high methane yields, whereas iron sulphide proved to be feasible for the production of high oil yields, as in case of *Spirulina* (up to 66.9%) [261] with an optimal quantity of catalyst (5–7%).[263] An optimal liquefaction reaction temperature of 340°C, with a 30 min residence time and a 5% catalyst dosage has been suggested.[262]

Bio-oil by pyrolysis

Pyrolysis is the thermal decomposition process of materials (algal biomass), in the complete absence of oxygen or in the presence of less oxygen than that required for complete combustion.[264] Pyrolysis can be divided into slow and fast pyrolysis. Slow pyrolysis uses slower rates (5–80°C min^{-1}) and longer vapour residence times (5–30 min), thus favouring production of tars and char compared with fast pyrolysis with much higher heating rates (e.g. 1000°C min^{-1}) and shorter vapour residence times (seconds), favouring direct production of liquid fuels.[8] The advantage of this technology is that the entire microalgal biomass can be processed. However, the high energy costs are a hindrance to its practical development. Only a few studies have examined the pyrolytic characteristics of microalgae (Table 3).

This process leads to products in three phases: vapour; liquid, a complex mixture (bio-oil) and with its composition depending upon the feedstock and the processing conditions; and solid phase. Slow pyrolysis (400°C) mainly results in high charcoal content (35% char, 35% gas and 30% liquid) but flash pyrolysis (temperatures between 300 and 500°C for less than 2 s) and fast pyrolysis (heated to between 350°C and 500°C for 10–20 s) is associated with liquid fuel yields of 75% and 50%, respectively, and if carried out at higher temperatures will result in more gas production.[265] In a study, algal biomass was submitted to fast pyrolysis with the production of 60–75% liquid bio-oil, 12–25% solid char and 10–20% non-condensable gases, depending on the feedstock used.[266]

The resulting bio-oil may be used to generate power (electricity) through internal combustion (diesel or gas turbine) engines or by co-firing with diesel or natural gas).[265,

Table 6. Integrated studies for biofuel production.

Microalgae species	Conditions	Biofuel product	Biofuel yield	Ref.
Chlorella sp. KKU-S2	Microalgae used the CO_2 emitted from the yeast *Torulaspora maleeae* Y30 fermentation	Algal lipid (biodiesel)	Lipid productivity 0.223 gL^{-1} d^{-1}	[287]
S. platensis, Rhodotorula glutinis	Microalgae/yeast-mixed cultivation using monosodium glutamate wastewater	Biodiesel	Lipid content 12.71%	[288]
C. sorokiniana	Culturing of oleaginous yeast and algae in food waste and municipal wastewater for lipid production	Biodiesel	Lipid content 18.7%–28.6%	[289]
Arthrospira (Spirulina) platensis	Microalgae used the CO_2 from ethanol fermentation by the yeast *S. cerevisiae*	Biodiesel	Lipid content 8.39%	[290]
Two *B. braunii, C. vulgaris* and *C. pyrenoidosa*	Production of biodiesel catalysed by immobilized, *Penicillium expansum* lipase and *Candida antarctica* lipase B	Biodiesel	Lipid content 40.7%	[291]
C. vulgaris	Photobioreactors culturing (cathodic) coupled with yeast fermentors at a bioethanol plant (anodic) to create microbial fuel cells to generate power. The microalgae sequester CO_2 emitted by the yeast fermentors and produce oil, biodiesel	Bioethanol-biodiesel-microbial fuel cell	Nd	[292]
C. reinhardtii UTEX 90 and *C. vulgaris* IAM C-534	Enzymatic or hydrothermal acid-treated algal biomass fermented to ethanol by *S. cerevisiae*	Bioethanol	29.2% 235 mg ethanol/1.0 g biomass	[224,225,293]
C. reinhardtii, C. vulgaris and *Undaria pinnatifida*	Acid hydrolysed-enzymatic treated algal biomass was fermented by four different strains of *Escherichia coli*	Bioethanol	0.4 g ethanol/1.0 g biomass	[294]
C. reinhardtii	Ethanol-hexane-sulphuric acid-treated algal biomass fermented by *S. cerevisiae*	Bioethanol	0.44 g ethanol/1.0 g glucose	[295]
Chlorococum sp.	Supercritical lipid-extracted microalgae biomass was fermented by the yeast *Saccharomyces bayanus*	Bioethanol	Maximum ethanol conc. 3.83 $g\,L^{-1}$	[248]
Synechococcus leopoliensis	HCl-acid-treated saccharified algal biomass (growth supported with CO_2) was fermented by yeast *Saccharomyces* sake	Bioethanol	0.42 g ethanol/1.0 g glucose	[296]
M. aeruginosa and *A. variabilis*	Super Critical fluid pre-treated algal biomass hydrolysed and fermentation by *S. cerevisiae*	Bioethanol	2.66 g/L, 2.28 g/L	[297]
Scenedesmus sp.	Lipid-extracted microalgal biomass residues fermented by anaerobic digested sludge	Bio-hydrogen	H_2 rate 2.82 ml/h and yield 30.03 ml/g VS	[298]
Chlorella sp.	Algal biomass was simultaneously hydrolysed and fermented using sewage sludge consortia via one-step process	Bio-hydrogen	Nd	[299]
C. reinhardtii and *D. tertiolecta*	Algal biomass is fermented by *Lactobacillus amylovorous* and *Rhodobacter sphaeroides*	Bio-hydrogen	H_2 yield of 61% and 52%, respectively	[300]
C. reinhardtii, D. tertiolecta and *Cholrealla pyrenoidosa*	Algal biomass was liquefied/fermented using a starch-hydrolysing lactic acid *Lactobacillus amylovorus* then fermented by *Rhodobacter sphaeroides* RV	Bio-hydrogen	The conversion yield was 5 mol H_2/mol of starch glucose)	[301]

(*Continued*)

Table 6. Continued.

Microalgae species	Conditions	Biofuel product	Biofuel yield	Ref.
S. maxima, *Chlorella* sp. and *Scenedesmus* sp.	Algal biomass was anaerobically digested of by sewage sludge culture	Bio-methane	Methane yield and productivity were 0.26 m^3 kg^{-1} VS added day	[257,302,303]
P. tricornutum	Biomass anaerobically digested by potato anaerobic treated sludge in a lab-scale anaerobic membrane bioreactor	Bio-methane	75.3% of methane	[304]
Chlorococcum sp.	Distillery waste with algal biomass is anaerobically treated by acidogenic/methanogenic culture in two-stage set-up	Bio-methane	Biogas was 6 L day^{-1}	[305]

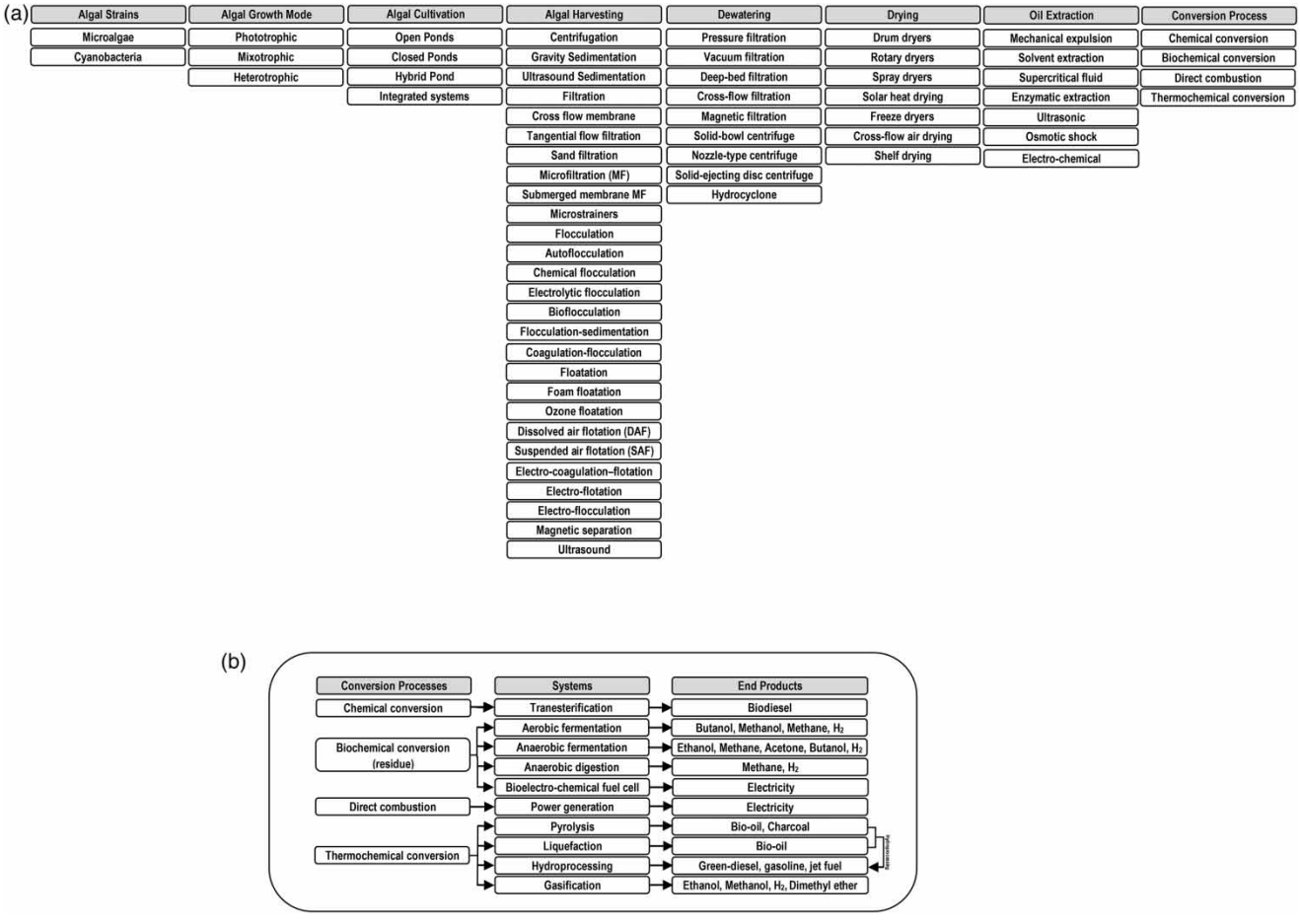

Figure 3. From cultivation to algal biofuel production steps: (a) summary for the steps towards production of algal biofuel and (b) biofuel production via microalgal biomass conversion processes.

267,268] Algal biomass is distinguished by its inherent small size with no fibrous tissue, making it the preferred one when compared with other biomass sources for bio-oil production. Pyrolysis is an extremely fast conversion method with reaction times of seconds to minutes. Several studies have been carried out for microalgal biomass conversation to bio-oil.[137,139,269] Fast pyrolysis of *C. protothecoides* and *Microcystis aeruginosa* and slow pyrolysis of dried and finely ground *Tetraselmis* and *Chlorella* biomass gave 18%, 24%, and 43% bio-oil.[137,138,269]

Fuel gas or syngas by gasification

Gasification is a thermochemical process of reacting the carbonaceous compounds of the biomass with air, steam or oxygen at high temperature (200–700°C) in a gasifier,

resulting in hydrogen [270] with yields ranging from 5% to 56%, carbon monoxide (9–52%) and small amounts of methane.[271,272] Gasification of algal biomass at high temperature though partial oxidation with air, O_2 and/or steam results in a low calorific value combustible gas mixture (~4 to 6 MJ/m^3) [267] which can be burnt directly and used for different energy purposes such as heating, electricity generation and as a fuel for internal combustion engines and gas turbines; or cleaned and upgraded to usable liquid fuels by water–gas shift and carbon monoxide hydrogenation.[273] A study of the production of methanol from the gasification of *Spirulina* biomass with temperatures ranging between 850°C and 1000°C showed that at 1000°C a high yield of methanol (0.64 g methanol/g of algae biomass) could be obtained.[155]

Bio-hydrogen

Hydrogen can be obtained from algae in a number of ways. It can be produced directly by cyanobacteria in a light-dependent reaction which is catalysed by nitrogenase or in the dark under anaerobic conditions by a hydrogenase.[274, 275] Hydrogen is also produced directly by some species of green algae [276–279] or by converting the algal biomass, either whole or after extracting oil and/or starch, into bio-hydrogen by dark fermentation using various strains of anaerobic bacteria that have the capability of producing hydrogen using different carbon sources.[253,280,281] Hydrogen production by both processes has been recently reviewed.[282–284]

Alcohols and alkanes

Algae such as *C. vulgaris* and *C. perigranulata* can produce ethanol and other alcohols via fermentation of intracellular starch or sugars which have been introduced into the medium.[231,285] The produced ethanol can be collected from the headspace of the culturing reactor in a low energy-intensive process. As well, some cyanobacteria have been shown to produce ethanol directly from photosynthesis. In addition to alcohols, alkanes can be directly produced by algae using heterotrophic metabolic pathways. Some strains produce a mixture of hydrocarbons which are similar to light crude petroleum. However, these are only naturally produced in minute amounts and this process has been little studied for biofuels production.

Direct combustion for electricity

Direct burning or incineration of the algal biomass can be used to provide energy as well. Burning algal biomass in the presence of air at high temperature (above 800°C) converts the stored chemical energy in the biomass into hot gases [260] and heat that can be used immediately for power, ranging from very small scale to large industrial scale of 100–300 MW.[222] Combustion is mainly for biomass which contains <50% moisture and usually requires pre-treatment processes such as drying or grinding, which will add cost.[222,260]

Integrated systems

Some have proposed an integrated approach, capable of the co-production of fuels, as a more sustainable fuel and chemical production system with improved economics. This process starts with cultivation of microalgae, followed by harvesting and subsequent lipid extraction to produce biodiesel via transesterification. After oil extraction, starch degrading enzymes are added for formation of fermentable sugars. These are fermented and distilled using a conventional bioethanol production technology.[286] Phycal is developing a hybrid-integrated process where bioethanol is produced from Cassava and oil from algae by combining technologies in what they hope will be a system capable of bringing biofuels to market at competitive prices. Sugars produced from Cassava are used not only for conventional bioethanol fermentation, with the CO_2 that is produced fed to algae growing autotrophically in open ponds, but are also fed to the algae in a process that has been termed as 'Heteroboost' to induce the production of additional biofuel and bio-products which are then extracted from the algae (Figure 2). Some studies that have used combined processes or that have used algal biomass as additive feedstock in biofuels process are given in Table 6.

Conclusion

Practical algal biofuel production is currently limited by the lack of cost-effective, low energy means of recovering the algal biomass from the dilute medium in which it grows. In addition, although a variety of energy uses for algal biomass exist, there are challenges in obtaining efficient, low-cost conversion processes that require minimal energy inputs. A number of innovative harvesting and conversion technologies are in the process of being developed and promise to move this area forward significantly in the near term. Together with advances in algal species and nutrient supply, the future sustainable production of algal biofuels may become a reality.

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