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Isolation and Identification of Native Microalgae for Biodiesel Production

Par
Ahmed Elsayed Mohamed Abdelaziz

Département de Microbiologie, Infectiologie et Immunologie
Faculté de Médecine

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Présenté par:

Ahmed Elsayed Mohamed Abdelaziz

a été évalué par un jury composé des personnes suivantes

Dr. Catherine Paradis-Bleau, Ph.D.
Président-rapporteur

Dr. Patrick C. Hallenbeck, Ph.D.
Directeur de recherche

Dr. Marc Drolet, Ph.D.
Membre du jury

Résumé

La demande croissante en carburants, ainsi que les changements climatiques dus au réchauffement planétaire poussent le monde entier à chercher des sources d'énergie capables de produire des combustibles alternatifs aux combustibles fossiles. Durant les dernières années, plusieurs sources potentielles ont été identifiées, les premières à être considérées sont les plantes oléagineuses comme source de biocarburant, cependant l'utilisation de végétaux ou d'huiles végétales ayant un lien avec l'alimentation humaine peut engendrer une hausse des prix des denrées alimentaires, sans oublier les questions éthiques qui s'imposent. De plus, l'usage des huiles non comestibles comme sources de biocarburants, comme l'huile de jatropha, de graines de tabac ou de jojoba, révèle un problème de manque de terre arable ce qui oblige à réduire les terres cultivables de l'industrie agricole et alimentaire au profit des cultures non comestibles.

Dans ce contexte, l'utilisation de microorganismes aquatiques, tels que les microalgues comme substrats pour la production de biocarburant semble être une meilleure solution. Les microalgues sont faciles à cultiver et peuvent croitre avec peu ou pas d'entretien. Elles peuvent ainsi se développer dans des eaux douces, saumâtres ou salées de même que dans les terres non cultivables. Le rendement en lipide peut être largement supérieur aux autres sources de biocarburant potentiel, sans oublier qu'elles ne sont pas comestibles et sans aucun impact sur l'industrie alimentaire. De plus, la culture intensive de microalgues pour la production de biodiesel pourrait également jouer un rôle important dans l'atténuation des émissions de CO_2.

Dans le cache de ce travail, nous avons isolé et identifié morphologiquement des espèces de microalgues natives du Québec, pour ensuite examiner et mesurer leur potentiel de production de lipides (biodiesel). L'échantillonnage fut réalisé dans trois régions différentes du Québec: la région de Montréal, la gaspésie et le nord du Québec, et dans des eaux douces, saumâtres ou salées. Cent souches ont été isolées à partir de la région de Montréal, caractérisées et sélectionnées selon la teneur en lipides et leur élimination des nutriments dans

les eaux usées à des températures différentes (10 ± 2°C et 22 ± 2°C). Les espèces ayant une production potentiellement élevée en lipides ont été sélectionnées. L'utilisation des eaux usées, comme milieu de culture, diminue le coût de production du biocarburant et sert en même temps d'outil pour le traitement des eaux usées. Nous avons comparé la biomasse et le rendement en lipides des souches cultivées dans une eau usée par apport à ceux dans un milieu synthétique, pour finalement identifié un certain nombre d'isolats ayant montré une bonne croissance à 10°C, voir une teneur élevée en lipides (allant de 20% à 45% du poids sec) ou une grande capacité d'élimination de nutriment (>97% d'élimination).

De plus, nous avons caractérisé l'une des souches intéressantes ayant montré une production en lipides et une biomasse élevée, soit la microalgue *Chlorella sp*. PCH90. Isolée au Québec, sa phylogénie moléculaire a été établie et les études sur la production de lipides en fonction de la concentration initiale de nitrate, phosphate et chlorure de sodium ont été réalisées en utilisant de la méthodologie des surfaces de réponse. Dans les conditions appropriées, cette microalgue pourrait produire jusqu'à 36% de lipides et croitre à la fois dans un milieu synthétique et un milieu issu d'un flux secondaire de traitement des eaux usées, et cela à 22°C ou 10°C. Ainsi, on peut conclure que cette souche est prometteuse pour poursuivre le développement en tant que productrice potentielle de biocarburants dans des conditions climatiques locales.

Mots-clés : Les biocarburants; la durabilité; les algues; les eaux usées; le traitement des eaux usées; biodiesel; nutriments; la récolte; extraction de pétrole; espèce indigène; phylogénie; La méthodologie des surfaces de réponse.

Abstract

The continuing increase in fuel demands, the dramatic situation in climate changes and the global warming are bringing the worldwide attention to the identification of alternative energy source for the production of combustibles that can replace fossil fuel. In last years, a lot of potential sources have been identified: the first potential biofuel feedstock that have been evaluated were oleaginous plants, but the utilization of vegetable, or vegetable oils, that may also be used for human feeding, could lead to the increase of food-grade oils costs and also generate ethic questions. Nevertheless, also using as biofuel sources not-edible oils, like oils from jatropha, tobacco seed or jojoba, the common problem for both edible and not-edible crops is the need to subtract arable land from agriculture and food industry.

In this context, the utilization of aquatic microorganisms like microalgae as substrate for the production of biofuel seems to be the better solution. Microalgae are easy to cultivate and can grow with little or no attention, they can grow in fresh, brackish or salt water and in non-arable lands, moreover they are not edible with no consequences on food industry, and the oil productivity, with respect to the other potential biofuel sources, can be much higher. In addition, the intensive cultivation of microalgae for biodiesel production could also play an important role in CO_2 mitigation.

In this study, we isolated and morphologically identified Québec native micro algal species, surveyed and screened their potential for lipid (biodiesel) production. The sampling efforts made in three different regions of Québec: Montreal area, Gaspesie and Northern of Quebec; on fresh, brackish or saline water. One hundred strains were isolated from the Montreal area, characterized and screened for their lipid content and wastewater nutrient removal under different temperatures (10±2 ºC and 22±2 ºC). The high potential lipid producing algal species were selected. The use of wastewater as a substrate media decreases the economic cost realted to the biofuel production from microalgae as well as an interesting tool for wastewater treatment. We compared the biomass and lipid productivity of these strains

on wastewater to a synthetic medium and identified a number of isolates that showed good growth at 10 ºC, gave a high lipid content (ranging from 20% to 45% of dry weight) or a high capacity for nutrient removal (>97% removal).

Furthermore, we characterized one of the interesting strains that revealed high lipid and biomass productivity, the novel microalga *Chlorella sp.* PCH90. Its molecular phylogeny was established and lipid production studies as a function of the initial concentrations of nitrate, phosphate, and sodium chloride were carried out using Response Surface Methodology. Under the appropriate conditions this microalga could produce up to 36% lipid and grew well in both synthetic medium and secondary effluent from a wastewater treatment plant at both 22°C and 10°C. Thus, this strain is promising for further development as a potential biofuels producer under local climatic conditions.

Keywords: Biofuels; sustainability; algae; wastewater; wastewater treatment; biodiesel; nutrients; harvesting; oil extraction; indigenous specie; Phylogeny; Response Surface Methodology.

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Abbreviations

ANOVA	Analysis of variance
APS	Advanced pond system
ATP	Adenosine triphosphate
BBM	Bold Basal Medium
BOD	Biological oxygen demand
BP	Biomass productivity
COD	Chemical oxygen demand
CTAB	Cationic *N*-Cetyl-*N*-*N*-*N* trimethyl ammonium bromide
DAF	Dissolved air flotation
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dwt	Dry weight
ECF	Electro-coagulation–flotation
EOM	Extracellular organic matter
EPS	Extracellular polymeric substance
FAME	Fatty acid methyl ester
FAs	Fatty acids
GHG	Greenhouse gases
GMOs	Genetically modified organisms
HHV	Higher heating value
HRAPs	High rate algal ponds
HRT	Hydraulic retention time
HTL	Hydrothermal liquefaction
IOMMs	Iron oxide magnetic microparticles
LCA	Life cycle assessment
LP	Lipid productivity
ML	Maximum likelihood

MP	Maximum parsimony
N	Nitrogen
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced form
Nd	Not determined
NER	Net energy return
NJ	Neighbor-joining
NR	Nile red
Nr	Not reported
OD	Optical density
P	Phosphorus
PBR	Photobioreactors
PCR	Polymerase chain reaction
PGA	Phosphoglycerate
PUFAs	Polyunsaturated fatty acids
PVA	Polyvinyl alcohol
R&D	Research and development
RSM	Response surface methodology
Rubisco	Ribulose-1, 5-bisphosphate carboxylase oxygenase
SAF	Suspended air flotation
SDS	Sodium dodecylsulfate
SFE	Supercritical fluid extraction
siROS	Specific intracellular reactive oxygen species
TAG	Triacylglycerol
TCL	Thermochemical liquefaction
TFA	Total fatty acid
TKN	Total Kjeldahl nitrogen
TN	Total Nitrogen
TP	Total Phosphorus
Wt	Weight
WW	Wastewater

This thesis is dedicated to the loving memory of my beloved father ***Elsayed M. Abdelaziz,*** *who would have been happy to see me finishing this work, i miss him everday. "Dad, I love you and you will always live on in my heart".*

to my loving mother, who provided unflagging patience, unconditional love and support.

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Footnotes

Notes in the text of the Introduction

During my M sc. study, I authored a number of reviews on algal biofuel production and that have been used in the introduction of this thesis.

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Chapter 1 : Adapted from my contribution in our published review "**Ahmed E.M. Abdelaziz,** Gustavo B. Leite & Patrick C. Hallenbeck (2013), *Addressing the challenges for sustainable production of algal biofuels: I. Algal strains and nutrient supply,* Environmental Technology, 34:13-14, 1783-1805".

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Chapter 1: Addressing the challenges for sustainable production of algal biofuels: I. Algal strains and nutrient supply

Ahmed E. M. Abdelaziz, Gustavo B. Leite, and Patrick C. Hallenbeck[a,b*]

[a] Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP 6128, Centre-ville, Montréal. PQ H3C 3J7 Canada,

[b] Life Sciences Research Center, Department of Biology, United States Air Force Academy, 2355 Faculty Drive, USAF Academy, Colorado 80840

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Chapter 1: Addressing the challenges for sustainable production of algal biofuels: I. Algal strains and nutrient supply

Ahmed E. M. Abdelaziz, Gustavo B. Leite, and Patrick C. Hallenbeck[a,b*]

[a] Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP 6128, Centre-ville, Montréal. PQ H3C 3J7 Canada,

[b] Life Sciences Research Center, Department of Biology, United States Air Force Academy, 2355 Faculty Drive, USAF Academy, Colorado 80840

[*]Corresponding author

"This chapter 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. The chapter focuses on the various sustainability problems around algal cultivation, including land use issues and nutrient supply"

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

Abstract

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 providing 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.

1.0 Introduction

Concerns about climate change driven by fossil fuel combustion and for 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 to 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 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], 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 to 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 sustainability NER concerns are the subject of an accompanying paper.

2.0 Algae

From a biofuels perspective it is important to appreciate the vast heterogeneity of the micro-organisms 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 thousand species of microalgae described taxonomically and, although very debated, they can be defined as eukaryotic microorganisms containing chlorophyl “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 harboring 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 by consuming organic carbon available in the environment, i.e. a 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 bio-prospecting 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 to 16 carbons [27]. These molecules are stored as TAGs (triacylglycerol) 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 reestablish 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 2-fold increase in triacylglycerol (TAG) and 3-5-fold increase in total lipid production compared to 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.

3.0 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 (Bo), 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 the 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. protothecoides* [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. protothecoides* had a lipid content (55 wt %) that was four times higher than 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 byproducts, 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 kilograms of CO_2 per liter 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 to 0.40 kg N and 0.022 to 0.055 kg P is required per liter of algal oil produced [66], or 0.29 kg N and 0.063 kg P per liter 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, nutrient inputs are consequently lower. For example, in the case of continuous ethanol synthesis by cyanobacteria is has been estimated that only 0.002 kg N and 0.0001 kg P per liter 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.

3.1 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 adequate supply of carbon dioxide is critical for 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 alkalinisation of the medium.

3.2 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 range from one percent 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^-), nitric acid (NO_3^-), 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 prokaryotic cyanobacteria can fix atmospheric nitrogen (N_2) into ammonia [77]. The process of assimilation begins with the inorganic forms of nitrogen passing through the algal 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 is no redox reactions involved in its metabolism, so it is the preferred nitrogen source. If both ammonium and 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 (*Chlorella 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 % to 70 % under nitrogen limitation [84, 86]. C*hlorella 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 (triacylglycerols) are produced instead of polar lipids [90]. The pathway the algae will select under nitrogen depletion is species 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*hlorella 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 in Microalgal Biomass Production, a very good option in this regard could be provided by municipal wastewater treatment facilities.

3.3 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 μg 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 *Chlorella vulgaris* biomass was 30 to 40 % lower in phosphorus limited cultures (0.147 mM) then when the nutrient was replete (1.47mM) [99, 100]. Phosphorus starvation effects included 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 affected by pH which affects not only phosphorus uptake, but 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 tons of phosphate are mined annually in the USA, and, already due to its limited supply, fertilizers used for agriculture 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 tons of phosphate would be required annually to completely replace conventional petroleum by algal biofuels, a challenge 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 Program 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.

3.4 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. Sulfur plays a key role in electron transport and is important for protein synthesis, lipid metabolism and algal growth. Thus, sulfur deficient conditions limit algal density and stunt growth [107]. Potassium and most of the other required elements are readily available at the low concentrations at 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 macro nutrients. Thus, the use of wastewaters is a cost effective means to 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.

3.5 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 LCA (life cycle assessment) 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 one 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.

3.6 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 for 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 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 process discharge water is referred to as primary effluent [110] (Fig.1.1).

Secondary treatment uses some type of biological process to consume the large quantities of organic matter present in 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

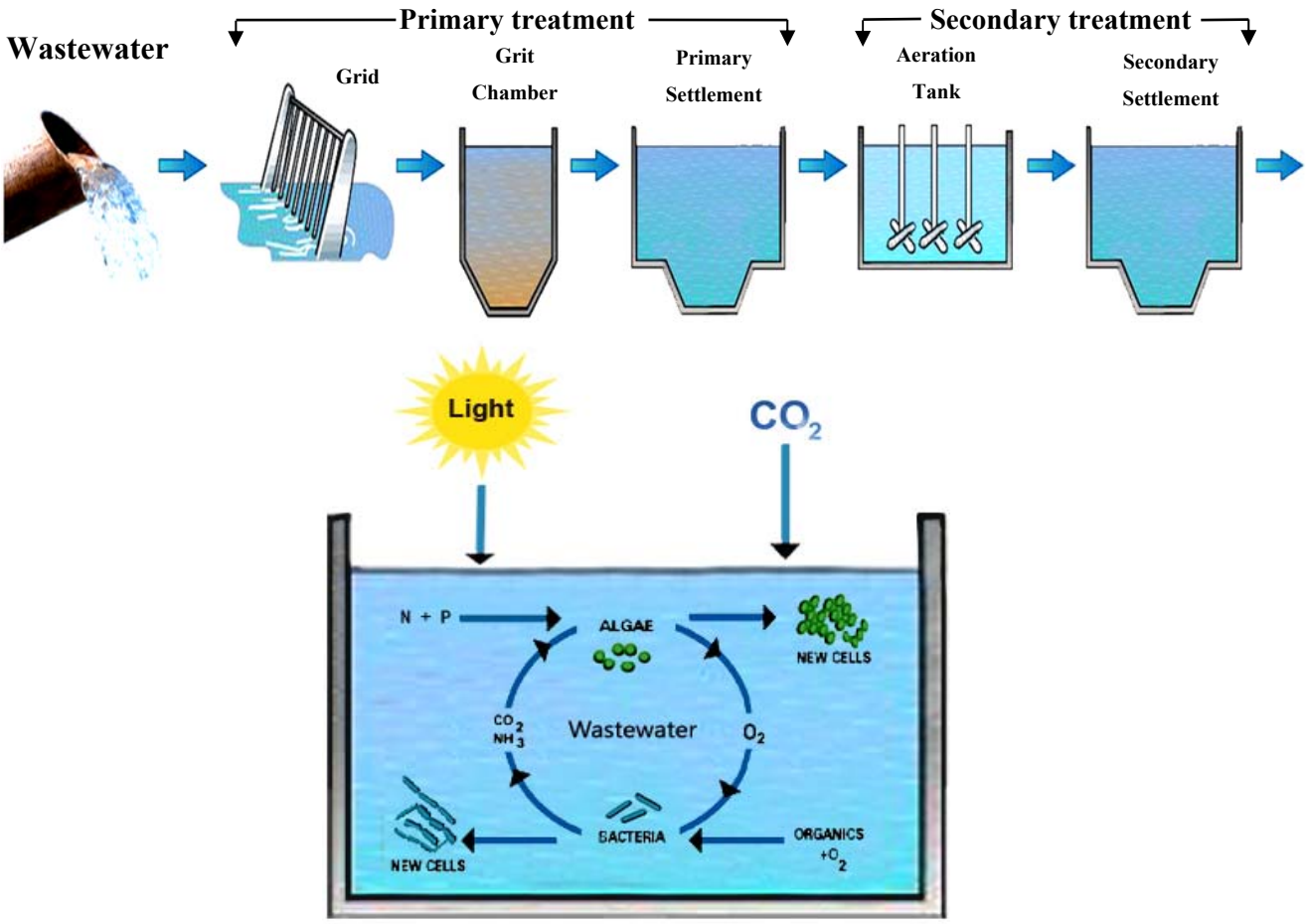

Figure 1.1 Contrast between conventional wastewater treatment and wastewater treatment using algal ponds.

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, a 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).

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, ortho-phosphate, polyphosphate and organic phosphate, the latter two forms are converted to ortho-phosphate during aerobic treatment. Ortho-phosphate 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.

4.0 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 1.1). 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 [114]. 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 in fact 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 contents (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, 123, 179, 183].

Table 1.1 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)*	[115]
	Partially Treated domestic	*(C. vulgaris)*	[116, 117]
	Screened and/or settled domestic sewage	*(Auxenochlorella protothecoides)*	[118]
		(Algae and bacteria,"activated algae")	[119, 120]
		(Algae and other microorganisms)	[121]
		(Algae, duckweed, and macrophytes)	[122]
		(Euglena sp.)	[123]
	Primary (settled) treated ww	*(C. vulgaris)*	[124, 125]
		(C. vulgaris and Bacteria*)*	[126]
	Primary treated sewage / seawater mixture	*(*Marine isolates; *Phaeodactylum tricornutum* and *Oscillatoria sp.)*	[127]
	Secondary treated wastewater (Secondary effluent)	*(Oocystis sp.)*	[128]
		(Chlorella pyrenoidosa and *Scenedesmus sp.)*	[129]
		(Botryococcus braunii)	[130]
		(Scenedesmus obliquus)	[131, 132]
		(Phormidium bohneri)	[133]
		(Scenedesmus sp.)	[134]
		(Multispecies microalgal cultures)	[135]
		(Attached algae, an algal biofilm)	[136]
		(C. vulgaris)	[137, 138]
	Settled & activated secondary treated sewage	*(Chlorella pyrenoidosa)*	[139]
	Pretreated sewage from ponding system	*(*Dominant *Euglena sp., Chlamydomonas sp., Scenedesmus sp.* and *Coelastrum sp.)*	[140]
		*(*Dominant *Chlamydomonas sp., Phacus sp.and* lower no. of *Euglena sp., Chlorella sp., Micractinium sp.and* occasionally *Scenedesmus quadricauda)*	[141]
	High-rate ponds	(Algal biomass)	[142]
	Municipal/ Agricultural ww	(Algal Biomass)	[143]
Agricultural Wastewater	Swine/piggery (Manure) ww	(Gradual succession of Microalgae)	[144]
		(Spirulina maxima and *Phormidium sp.)*	[145]

		(Chlorella sp., Scenedesmus obliquus, and *Phormidium bohneri)*	[146]
		(Spirulina maxima)	[147]
		(C. vulgaris)	[148]
		(Phormidium sp.)	[149]
		(Spirulina platensis)	[150, 151]
		(Chlorella sp.)	[152]
		(Chlorella pyrenoidosa)	[153]
		(Chlorella zofingiensis)	[154]
		(Scenedesaceae sp.)	[155]
		(C. vulgaris, Chlamydomonas mexicana, Nitzschia cf. pusilla, Scenedesmus obliquus, Ourococcus multisporus,and Micractinium reisseri)	[156]
	Raw swine manure effluent	(Freshwater algal consortia)	[157]
	Settled swine ww/ sewage mixture	*(C. vulgaris* and Bacteria*)*	[158]
	Dairy (Manure) wastewater	*(Cyanobacteria)*	[159]
		(Freshwater algal consortia)	[160]
		(Chlorella sp.)	[161]
		(Neochloris oleoabundans)	[162]
		(Six algal genera)	[163]
	Cattle feedlot effluent	*(Scenedesmus quadricauda* and Bacteria*)*	[164]
	Pretreated cattle manure	*(C. vulgaris, Chlorella kessleri and Scenedesmus quadricauda)*	[115]
	Aquaculture	(Photosynthetic algae)	[165]
		(Microbial flocs)	[166]
		(Scenedesmus sp. and *C. vulgaris)*	[167]
	Poultry effluent	*(Spirulina platensis)*	[168]
		(Chlorella minutissima, Chlorella sorokiniana and *Scenedesmus bijuga)*	[169]
		(Algal-bacterial biomass)	[170]
		(C. vulgaris and *Scenedesmus dimorphus)*	[171]
	Agro-industrial wasteater	(Cyanobacteria) Review	[172]
Industrial	Olive oil mill effluent	*(Scenedesmus obliquus)*	[173]
Wastewater		*(Chlorella pyrenoidosa* and *Scenedesmus obliquus)*	[174]
	(Phenols removal)	*(Ankistrodesmus braunii* and *Scenedesmus quadricauda)*	[175]

Textile effluent	*(C. vulgaris)*	[176]
Carpet mill effluent / municipal sewage mix	(Microalgae from different taxa)	[114]
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)	[114]

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 (Table 1.2). Algae offer solutions to those obstacles, as using algae based treatment will reduce sludge formation, be more cost effective [186], exhibit lower energy - quirements, recover nutrients as algae biomass, reduce greenhouse gas emissions, and, in addition, produce useful algal biomass (Table 1.2).

4.1 Municipal wastewater

Municipal wastewater is mainly generated from domestic sewage and small enterprises in addition to some environmental runoff 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 volume available and to its content in nitrogen and phosphorus. 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 1.1). A typical composition of municipal wastewater is given in Table 1.3.

Table 1.2 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	[119, 136, 186-188]
Energy Requirements	High Energy Requirements : 45 to 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, color and odor 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	[130, 134, 146-150]
Additional Disadvantages	Large amounts of sludge requiring disposal	Large land requirement, expensive harvesting to meet suspended solids limits, algal biomass production limited by the environmental conditions	[96, 109, 187, 195, 196]

4.2 Agricultural wastewater

Agricultural wastewater, often derived from manure (animal farms), agricultural operations and live-stock 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 [156, 171]. 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 [198]. Table 1.3 gives the typical composition of some agricultural wastewaters.

4.3 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 [199]. 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 [199]. Although somewhat dependent upon the source, most industrial wastewaters contain less nitrogen, phosphorus and more heavy metals in comparison to both municipal and agricultural wastewaters [200].

Some studies that have investigated the use of microalgae for nutrient removal (N & P) and biofuel production are summarized in Table 1.4

Table 1.3 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	[201-
wastewater	Medium Domestic	40-50	8-12	220	500	203]
	Strong Domestic	85-90	15-20	400	1000	
	Raw Sewage Sludge	190	86.4	Nr	43844	[204]
Agricultural	Dairy	185	30	Nr	Nr	[205]
wastewater		167	36	Nr	Nr	[206]
	Dairy Lagoon Water	244-1081	Nr	Nr	Nr	[207]
	Dairy Anaerobic Lagoon Sludge	556-4420	141-3263	Nr	Nr	[208]
	Dairy Anaerobic digestion effluent	3456	249.7	Nr	Nr	[161,
	Dairy Manure As Excreted (AE)	2370	240	Nr	32700	209]
		5294	824	Nr	129400	[210]
	Swine	1290-2430	264-324	Nr	Nr	[205]
	Raw flushed swine manure	1501	566	3046	16,758	[211]
	Swine after solid–liquid separation	895	168	923	3122	
	Poultry	96-802	30-50	Nr	Nr	[205]
	Poultry Manure	1381-1825	382-446	420-5900	1753-12052	[212]
	Poultry anaerobic digestion effluent	1580	370	370	1800	[213]
	Beef feedlot	63	14	Nr	Nr	[205]
	Beef fresh Manure	8.2-19 (lb/ton)	2.7-12 (lb/t)P_2O_5	25004	127095	[214]
Industrial	Dairy Industry	58-115	9.7-28	1034-3203	2148-5134	[215]
wastewater	Textile industry	42.7–161	9.4–27.9	400–490	773–1290	[216]
	Winery industry	0-425	3-188	8858	15553	[217]
		0.0–142.8(TN)	3.3–188.3	125–130000	738–296119	[218]
		67-71	7.0-8.5	1740-1970	3112-3997	[219]
	Olive mill industry	532 (TN)	182	30600	97000	[220]
	Paper mill industry	13 (TN)	4	230	420	[220]
	Tannery industry	273	21 PO4	1860	6200	[221]
		90 – 630	Nr	210 - 4300	180 - 27000	[222]
	Tomato Cannery industry	0.1-5.6 (Nitrate)	0.3-7.4	29-1100	Nr	[185]
	Pharmaceutical industry	5166 (TN)	Nr	15250	28540	[223]
	carpet industry/sewage mix	32.6–45.9	26-49	331–487	1412	[114]

All the values are in mgL^{-1} otherwise specified.

Table 1.4 Some studies of uses microalgae in wastewater treatment (nutrient removal) and biofuel production

WW	Wastewater type	Wastewater Composition (mgL^{-1})			Species Used	BP ($mg\ L^{-1}\ d^{-1}$)	Lipid Content (%DW)	LP ($mgL^{-1}\ d^{-1}$)	TN Removal (%)	TP Removal (%)	COD Removal (%)	Ref.
		N	P	COD								
Municipal wastewater	Urban wastewater effluent (secondary treated)	28.1 (NH_4)	8.7 – 11.8 (PO_4)	Nd	*Scenedesmus 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	[143]
	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)	[117]
	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)	[161]
	Dairy manure supported by polystyrene foam led	517 (TN) 309 (NH_3)	770 (TP)	Nd	*Chlorella sp.*	2.57 g m^{-2} d^{-1}	9.01 (TFA)	0.23 g m^{-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	*Chlorella 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)	[153]
	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	[156]
					Scenedesmus 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	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)	99.6 – 99.8 (In 5 days)	Nd	[231]

	treated swine								69.4 – 88.5 (NO_3) (In 5 days)			
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, 114]
					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	[114]
	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]

All the values are in mgL^{-1} except otherwise specified. Lipid content, Nitrogen, phosphors and COD removal are expressed by Percentage (%).

5.0 Microalgal Biomass Production

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

5.1 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 2 to 4 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 Fig. 1.1). 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 BOD (Biochemical Oxygen Demand), 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 in the pond effluent.

5.1.1 High Rate Algal Ponds (HRAPs) for Wastewater Treatment

In practice, high rate algal ponds (HRAPs), also known as raceways 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 proposed as a method for combined wastewater treatment and biofuel production on a large scale more than fifty 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 to 150 kg BOD_5 ha^{-1} day^{-1}, depths varing between 0.25–0.6 m, and hydraulic retention times, depending upon the season, from 3–4 days in the summer and 7–9 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 absorbtion [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 compared to 10–15 t ha^{-1} $year^{-1}$ for wastewater treatment HRAPs in moderate latitudes and Mediterranean climates) [238, 239]. One method to stabilize the species in the 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 favor 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 to 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 neighboring 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 throughout 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 arranged in series; facultative ponds, high rate algal ponds (HRAP), 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 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.

5.1.2 Enclosed photobioreactors

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 photobioreactors, 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 photobioreactors or Algenol-type reactors [9].

Closed systems, in particular tubular photobioreactors have several advantages compared to 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 to 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 bio-fouling [242, 271].

5.1.3 Hybrid Systems

As discussed above, open ponds are relatively cheap and very efficient methods for algal cultivation but can be easily contaminated with undesirable microbial species. On the other hand, photobioreactors (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 favor biofuel production [272] .

5.1.4 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 of using immobilized or attached algal processes that help reducing this challenge. Immobilized

cultures have the benefits of increased culture densities, easy to harvest, as well as, reducing water and land requirements could be achieved via future design innovations [273], however, the economics associated with this methods were prohibitive.

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 favor the formation of algal biofilms, naturally immobilized systems. The presence various organic molecules on submerged surfaces can create favorable locations for microbial growth and biofilm formation [278]. Compared to 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 to 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 to 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].

6.0 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, 132, 283-286]. Effective operation of HRAP ponds for wastewater treatment and biofuel production over a fifteen month time period has already been demonstrated at the hectare scale [284]. Obviously, sustainability concerns favor 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 [132]. Even though domestic wastewater in the US 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 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].

7.0 Conclusions

Many technical barriers remain to 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 in regards 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 for 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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Chapter 2: Addressing the challenges for sustainable production of algal biofuels: II. Harvesting and conversion to biofuels

Ahmed E. M. Abdelaziz, Gustavo B. Leite, and Patrick C. Hallenbeck[a,b*]

[a] Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP 6128, Centre-ville, Montréal. PQ H3C 3J7 Canada,

[b] Life Sciences Research Center, Department of Biology, United States Air Force Academy, 2355 Faculty Drive, USAF Academy, Colorado 80840

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Chapter 2: Addressing the challenges for sustainable production of algal biofuels: II. Harvesting and conversion to biofuels

Ahmed E. M. Abdelaziz, Gustavo B. Leite, and Patrick C. Hallenbeck[a,b*]

[a] Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP 6128, Centre-ville, Montréal. PQ H3C 3J7 Canada,

[b] Life Sciences Research Center, Department of Biology, United States Air Force Academy, 2355 Faculty Drive, USAF Academy, Colorado 80840

[*]Corresponding author

"This chapter is considered as part two for chapter one, it also examines the different sustainability issues potentially involved in large scale algal biofuel production and to suggest ways in which these challenges might be met, but in this chapter we examine the different algal harvesting techniques, the conversion of the algal biomass to fuels, and energy investments and discuss how all these factors can impact on the biofuel sustainability"

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

Abstract

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 NER (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.

1.0 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 severe problems with pretreatment, deconstruction and conversion to fermentable sugars (Table 2.1). 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 wastwater as source of water and nutrients. Algae exhibit faster growth rates than seed oil plants, with some species reaching more than 50 % lipid per dry weight. Algal oil (lipids) can be converted to biodiesel, or 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 byproducts; nutraceuticals, animal feed, etc. Thus algal biofuels show promise for biofuel production that is more sustainable and with a higher net 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 [1] and in the private sector, an investment of $600m 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 [2]. There are a variety of on-going collaborative R&D partnership efforts between algal tech companies and major industrials for biofuel and bioproducts production, for example, Algenol-Dow, BP-Martek, Shell-HR and Chevron-Solazyme [3]. Additionally, more than $300m 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 [3]. In July 2013 the US Department of Energy (DOE) announced an

investment of $13m to accelerate the development of next generation biofuels with the goal of producing drop-in biofuels at $3 per gallon by 2017 [4]. In Japan, a project of biofuel production and sewage treatment from algae has been recently started with a total budget of around $9m [5].

Thus, there has been a great deal of interest recently in developing algal biofuel production systems as a means to 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 fuels as this is the driving force behind considerations of sustainability and NER (net energy return) [6, 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 liters, 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 tons per day of renewable liquid biofuels were produced, whereas 12 million tons per day of crude oil were consumed [34]. The aim of this chapter, and the previous chapter, 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 positive 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.

Table 2.1 Comparison between the different generation biofuels

Biofuel Type	Biofuel	Advantages and Disadvantages	Socio- economical impacts	Production	Ref.
1st 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 fertilisers 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.	Yield (gal/ac/yr) • Corn 18 • Soybeans 48 • Sunflower 102 • Safflower 83 • Rapeseed 127 • Palm oil 635	[6] [7] [8] [9] [10] [11] [12]
2nd Generation Biofuel	• Thermal production from biomass giving biochar, bio-oil and syngas • Bioproduction 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 (GHG) emissions are reduced compared to 1st generation biofuels. • Avoids soil degradation and land use change. • NER (switchgrass & *Jatropha)* biodiesel maybe as high as 5.4:1 & 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) overcome	[6] [11] [13] [14] [15] [16] [17]
3rd Genetation 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 unfavorable 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., Botryococcus braunii* can contain up to 75 % lipid	[6] [18] [19] [20] [21] [22]

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 [21, 35] . Likewise, given the nascent stage of development, a LCA (life cycle assessment) analysis is a dubious exercise for a number of reasons [35]. First, it is difficult to define the necessary pertinent boundary conditions. Secondly, LCA was conceived to deal with already existing supply chains for which retrospective historical data is available, not the case here where in fact future developments and scale-up could have enormous, but 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.

2.0 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 (TAGs) 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% to 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 30μm, the energy input for this step can represent a major proportion of the total NER [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 [21]. 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 ~80 to 90%, 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 [21].

2.1 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 [65]. . 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 to 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 [21, 66]. 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 g cm^{-3} and 1.025 g cm^{-3} at 20°C respectively) [65, 67]. 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 rates for this species, 3.575 m day^{-1} [68], 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 m day^{-1} and 0.2 m day^{-1} for green algae and diatoms [69].

Table 2.2 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, 43-45]
Centrifugation	Rapid, easy, effective, high capture efficiency (>90%) & preferred method for lab & small scale.	Requires high investment, operation costs & 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, 45, 46]
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 hardly 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, 45, 47]
Bioflocculation	High efficiency, cell structure preserved, successfully used in use harvesting the microalgae cultures in wastewater treatment ponds.	High bioflocculant costs, cultivation of producing species required.	[47, 48]
Physical flocculation	No chemical or biological contamination, efficient at lab scale.	Difficult to apply at large scale, costly (ultrasound more expensive than centrifugation).	[48]
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.	[48, 49]
Electroflocculation	Very efficient, easy operation, 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	[43, 45, 50, 51]
Electrocoagulation	Low electricity (marine algae)	Recovered biomass contaminated with metals,	[45,

flocculation		energy consumption of (1.5 kWh m^{-3}).	48]
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, 45, 52]
Cross flow membrane	Pathogen removal, water recycle, low filter cake formation.	Membrane associated problems (fouling). High material cost.	[53]
Submerged membrane microfiltration	Economically feasible, low shear stress, pathogen removal.	Membrane fouling, problems with scale up.	[54]
Microstrainers	Simple structure, operation and function, low cost, high capability, requires little maintenance required.	Cell size and concentration dependent, not suitable for small cells, energy-intensive, Incomplete solids removal, buildup of bacterial and algal slime, periodic cleaning required.	[40, 43, 55]
Sand Filtration	Simple and inexpensive construction and operation.	Slow and impractical, back-wash water issues, removal of some algal species marginal.	[56, 57]
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 floatation, low recovery yield.	[58, 59]
Ozone fractionation	Efficient, small footprint, causes cell lyses, pure disinfected product, no toxic chemicals, complete separation.	High cost (ozone).	[60, 61]
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, 43]
Suspended air flotation (SAF)	Quick, low energy requirements, economical.	Oversized bubbles break up the floc.	[62, 63]
Magnetic separation	Quick, low running cost, energy saving, simple operation.	Complex and expensive fabrication.	[64]

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 the 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 [70]. 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.

2.2 Centrifugation

Centrifugation is a highly efficient and reliable method, where most microalgae particles can be recovered from the liquid cultures, with about 95–100% and 80–90% efficiency using centrifugation at 13,000g and at 500–1,000g, respectively [39, 71]. 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 that $115m^2$ and 0.3m deep in one hour. However this is very energy intensive (1-8 kWh m^{-3}) and can easily bring the NER (net energy 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 one hour, the centrifuge would be able to harvest 35,000 liters, yielding 7kg of algal biomass containing 2.1kg of lipids. An oil extraction/transesterification with 90% efficiency would give 1.89kg of fatty acid methyl esters (FAMES) containing 19.8kWh. Thus, the centrifugation operation alone would consume 49kWh to produce only 19.849 kWh. Certainly, this type of operation is far from being sustainable [66].

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 a NER greater than one. It is important to remember that the cultivation process is by itself an energy intensive process 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 positive net energy balance. In addition, cell structure may be damaged due to the high centrifugal and shear forces [42, 46, 72]. 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 [73].

On 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 [74]. 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 meter 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 1.73kWh per liter of algal oil produced (containing 9.72kWh). Using this scenario, estimated harvest costs were \$1.868 l^{-1} compared to the current cost of \$4.52 per liter of oil estimated by the US Department of Energy for centrifugal harvesting [74]. These numbers are still far from an economically viable scenario, but open an avenue for optimization.

2.3 Flocculation and autoflocculation

Flocculation is a process where the dispersed particles in suspension are aggregated together by the addition of chemicals (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 surpass this characteristic is an efficient and common solution widely used in similar applications (e.g. wastewater 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 [46]. 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, sensitivity to pH [75], the fact that some coagulants work for some microalgae species but not 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 [76]. 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 aluminum-based coagulants, will disrupt the stability of the system, neutralize or reduce the cells surface charge leading to successful settling and harvesting [39, 77]. Low pH has been found to assist the efficiency of inorganic flocculants [40]. A screen of twelve different salts for the harvest of *Chlorella minutissima* showed that chloride, ferric and sulfate salts of aluminum were the most efficient coagulants [78], 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 [77].

Organic flocculants using high molecular weight bridging (polyelectrolytes) polymers [65], aluminum sulfate followed by certain polyelectrolytes [75], biodegradable natural chitosan [79], or cationic flocculants [80], have been found to be very effective in microalgal harvesting. Anionic and nonionic polyelectrolytes fail to flocculate microalgae due to charge repulsion or insufficient bridging distance [81], 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 10-10.6 [46].

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 microalgae flocculant [82]. 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 [79, 80]. As expected, pH plays an important role in the efficiency of this method through effects on the protonation of chitosan amino groups [83]. 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 [84]. A modified form of chitosan, nano-chitosan has been developed and tested for harvesting *Nannochloropsis sp.*[85].

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*. [86]; organocays doped with Al^{3+} and Mg^{2+} backbone which have been shown to havest oleaginous *Chlorella sp*. with 100% efficiency [87]; 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 [88]. Other organic flocculants tested include poly (γ-glutamic acid) [89], and *Moringa oleifera* [90].

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 the flocculation by cationic polymers [80].

Combined flocculation is a multistep 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) [61] and ozone oxidation followed by the addition of a flocculant or a cationic starch [91, 92]. For example, a recent report analyzed 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, chitosan), flocculant dosage and sedimentation time on flocculation efficiency in the harvest of *Scenedesmus sp.* [93].

Triggering self-flocculation (autoflocculation) could be a cost effective, nontoxic 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 [72]. Cultivation under elevated pH and limited CO_2 supply assists autoflocculation [49]. In addition, in some cases microalgae-associated bacteria may play an important role in algal flocculation and sedimentation by increasing the floc size [94]. The use of flocculants for bulk harvest could represent an important step towards a positive NER. Nevertheless, 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 [95].

2.4 Filtration and Screening

Filtration is a very effective solution at the laboratory scale, but in 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 aspect of this technique [42]. However, it might be cost effective for harvesting filamentous species such as *Spirulina sp.* or large colonial (ca. >70 μm) microalgae such as *Coelastrum sp.* and *Micractinium sp.* [21, 96, 97]. For small cells, techniques like microfiltration [54], ultra-filtration [98], or membrane-filtration can be used, however not for large scale production as the membranes are prone to plugging [21, 99, 100]. 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 [99], 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, 101]. A cross-flow membrane filtration system equipped with an anti-fouling membrane (surface-coating with

hydrophilic polyvinyl alcohol (PVA) polymer) to reduce fouling formation has been used for *Chlorella sp.* harvesting [102].

Micro-strainer 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,[103]. 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 fiber types of 66-93%, 54-90%, 43-71% and 27-75% respectively [104]. 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 ≤ £0.15 per m^2 kg^{-1} of algae m^{-3} using a stretch cotton filter [104]. Sand filtration [57], or sand filtration combined with solar drying [105] or ozonation [106] has also been studied as potential methods for harvesting micro-algal biomass.

2.5 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 [107]. Addition of polyelectrolyte salts (such as aluminium and iron salts or formulations of charged organic polymers) to the liquid could be useful step prior to flotation since they 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 [108]. The flotation process can be divided into dissolved air flotation (DAF), or dispersed flotation based on the bubble size.

Dissolved Air Flotation (DAF) 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 μm by collision and subsequent adhesion between the bubble and the particle [109]. This process is capable of working with large volumes [110] 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 pretreatment step of flocculation was applied [65]. One possible drawback is the rather intense energy demands 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.

Dispersed Air Flotation (DAF) is a method that mainly works by generating 700-1500 μm bubbles with a high-speed mechanical agitator and an air injection system [111]. 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 (CTAB) being relatively efficient (90 %) while the anionic sodium dodecylsulfate (SDS) and the nonionic Triton X-100 were only 10% [108]. 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 (consumes only 0.015 kWh m^{-3}) and can efficiently compete with the other commonly used harvesting technologies [58].

2.6 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 [112]. 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 [113, 114], where with *Nannochloropsis sp.,* > 97% of biomass was recovered with no significant changes in biomass quality [115]. The use of electro-coagulation prior to centrifugation could drastically decrease the energy demand for harvesting, from 60 to 90% [115].

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) [116] with a total cost estimated to be \$0.19 kg^{-1} of ash free dry mass [50]. The effect of initial cell density, ionic strength, coagulant dosage, and medium pH on inorganic electrolyte flocculation harvesting have been examined using *Nannochloris oculata* [117].

Electro-coagulation–flotation (ECF) technology has been shown to be an effective approach, technically and economically, for algae removal, [118] where under optimal conditions (Al electrode, 1 mA cm^{-2} pH = 4–7, 18–36 °C, algal density of 0.55×10^9–1.55×10^9 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 aluminum anode to be more efficient than an iron anode and concluded that the ECF method is more efficient than centrifugation under optimum conditions [119]. Thus, a limited number of studies have suggested that electrochemical methods might be safe, cost effective, environmentally friendly and energy efficient [112].

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 [120].

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 [121]. 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 algal from freshwater [122] and the recovery of *Botryococcus braunii*, *Chlorella ellipsoidea* and *Nannochloropsis maritima* from a culture broth using Fe_3O_4 nanoparticles [64]. Up to 99% separation efficiency of *Chlorella vulgaris* from a highly diluted suspension has been claimed using novel microwave synthesized iron oxide magnetic microparticles (IOMMs) [123].

2.7 Biologically based methods

Biologically based methods include; bioflocculation, caused by secreted biopolymers (such as extracellular polymeric substance (EPS) or extracellular organic matter (EOM)) [124], or microbial flocculation of algae caused by adding flocculating microbes to an algal culture [96, 125]. For example, a flocculating microalga can be used to concentrate and recover a non-flocculating microalga of interest [126] or a bioflocculant from a bacterium can be used, as was the case where *Chlorella vulgaris* was harvested using a bioflocculant from *Paenibacillus* [125]. Novel alternative techniques have been described such as the co-cultivation of microalgae with fungi [127] where for example the pellet-forming filamentous fungae *Aspergillus oryzae* is grown with *Chlorella vulgaris* [128]. Finally, in an ecosystem approach, an algae eating fish such as tilapia can be used and the algae harvested from the sedimented droppings by a conveyor belt [129, 130].

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 [131]. Different genetic modifications can induce cellular flocculation as it was the case of the cell wall-deficient mutant of *Chlamydomonas sp.* [132].

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 [133], 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 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, 134]. 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 2.2.

3.0 Biofuel 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 (Fig. 2.1, 2.2 and 2.3). First we examine the more traditional routes of biofuels production from algae, biodiesel, bioethanol and biogas production. Then we examine newer proposed novel routes, some of which have given promising results, as summarized in the Table 2.3.

Table 2.3 Conversion using Pyrolysis, Liquification or Gasification

Species	Scale	Conversion process and Conditions	Yields (% dry wt.)					Ref.
			Solid	Liquid Bio-Oil			Gaseous	
			charcoal	Content	HHV (MJ kg $^{-1}$)	Properties/ elemental composition		
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},a vapor residence time 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]
Chlorella 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}, a vapor residence time 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]
Chlorella 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]
Microcystis 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]
Chlorella vulgaris	lab-scale fixed-bed	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	Nr	Syngas H_2 emission rate 50.75 ppmv/s at	[142]

					at 900 ˚C was 1.3391 (ppmv kJ)/ L kW h		900 ˚C, CO 102 ppmv/s at 800 ˚C	
Scenedesmus sp.	Bench scale isothermal spouted bed /dynamic	Fast pyrolysis, 480 ˚C and 100 kPa with a 2 s vapor residence time and 2 hours 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 ˚C – 450 ˚C	48 & 55 at 300 ˚C	55 & 40.5 at 450 ˚C	27 & 33 at 400 ˚C & 450 ˚C	Low Oxygen content (33.2) Lower acidity, higher aromatics.	23 & 34 at 400 ˚C	[144]
Chlorella pyrenoidosa	Lab scale a stainless-steel batch	Non-catalytic hydropyrolysis, temperature 310 °C, time 60 min and H_2 pressure 3MPa	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]
Spirulina 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	29.30	O (11.3)[a], C (67.5), H (9.82), N (10.7)	19.2	[146,
		Thermochemical liquefaction (TCL), at 350 ˚C, 60 min, heating rate 3.5˚C min^{-1}, 2Mpa	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	147]
Chlor ella 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]
Chlor ella sp.	pilot-scale	Microwave- assisted pyrolysis, catalyst, power of 500, 750, 1000 and	~ 25.0 at 750W	28.6 at 750W	30.7 at 750W	O (16.5)[a], C (65.4), H (7.84), N (10.3) a density of 0.98 kg/L (at 30 ˚C),	27.0 at 750W	[149]

		1250 W, (462–627 ˚C), 20 min,				a viscosity of 61.2 cSt at 750W		
Raw Scenedesmus biomass	Lab scale	Slow pyrolysis	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		at 450 ˚C, reaction time of 2 h	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	Hydrothermal liquefaction (HTL)	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	triplicate	at 300 ˚C, pressure ranging from 10	06.0	36.0		O (8.2), C (72.6), H (8.9), N (10.0)	41.0	
Spirulina biomass	batch	to 12 MPa	11.0	31.0		O (9.2), C (71.2), H (9.0), N (9.2)	35.0	
Chlorogloeopsis fritschii	lab-scale	Hydrothermal liquefaction, 300 °C	~ 10.0	38.6	32.0	O (19.0)[a], C (66.5), H (07.2), N (06.8)	~ 13.0	[151]
Spirulina platensis	batch	and 350 °C (accompanied with	~ 02.0	35.5	36.1	O (11.5)[a], C (72.7), H (08.8), N (06.3)	~ 05.0	
Chlorella vulgaris	reactor, high	Nutrient recycling of aqueous phase)	~ 08.0	46.6	37.5	O (09.3)[a], C (75.9), H (09.0), N (05.3)	~ 12.0	
Scenedesmus dimorphous	pressure		~ 18.0	27.1	33.6	O (12.6)[a], C (73.0), H (08.2), N (05.7)	~ 08.0	
	reactors							
Chlorella vulgaris,	Lab scale	Hydrothermal liquefaction, 350 °C, ~	~ 03.0	~ 39.0	337.1	O (14.8)[a], C (73.6), H (10.7), N (5.9)	~ 28.0	[152]
Nannochloropsis occulta	batch	200 bar in either[b] pure distilled water,	~ 07.0	~ 37.0	39.0	O (18.9)[a], C (74.7), H (10.6), N (4.3)	~ 48.0	
Porphyridium creuntum	reactor	or 1 M base Na_2CO_3 or 1 M of the	~ 10.0	~ 27.0	36.3	O (13.3)[a], C (72.8), H (09.1), N (05.7)	~ 15.0	
Spirulina sp.		organic acid HCOOH	~ 07.0	~ 27.0	36.8	O (10.9)[a], C (75.4), H (10.8), N (07.0)	~ 32.0	
Botryococcus braunii	Lab scale	Thermochemical liquefaction, 300˚C,	Nr	64.0	45.9	Nr	Nr	[153]
		3 Mpa						
Dunaliella tertiolecta	Lab scale	Thermochemical liquefaction, 340 ˚C,	Nr	33.6 –	36.0	Viscosity 150 - 330 mPas	Nr	[154]
		10 Mpa, 250–340 ˚C, 5–60 min		40.4				
Spirulina sp.	Lab scale	Gasification, 1000 ˚C, 0.101 Mpa	Nr	Nr	Nr	Nr	64.0	[155]
Nannochloropsis gaditana	Lab scale	Gasification, 850 ˚C, a particle size	Nr	Nr	Nr	Nr	~ 52.0 H_2,	[156]
	flux bed	from 100 to 250 μm and a heating					~35.0 CO,	
	reactor	rate of 40 ˚C min^{-1}, 7.3% in Argon					~14.5 CO_2	

[(a)] Oxygen content was determined by difference and [(b)] Result as the maximum values.

3.1 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 [73, 160]. Microalgal biodiesel is produced by transesterification of the extracted lipid, resulting in fatty acid methyl esters (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 *Chlorella vulgaris, Chlorella protothecoides, Nannochloropsis sp., Nitzchia sp., Chlamydomonas reinhardtii, Schizochytrium sp., Scenedesmus obliques, Neochloris oleabundans* [6, 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 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 (triacylglycerols (TAGs)) by growing microalgae under unfavorable 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 stresses which have been studied as tools to improve lipid productivity, with nutrient starvation being the most widely used option extensively studied. Some of those studies are summarized in Table 2.4.

The microalgal lipid content, as well as the quality of the fatty acid 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 fatty acid composition and vice versa [208, 209] . Low light intensity favors the synthesis of polyunsaturated fatty acids (PUFAs) and induces polar lipid formation whereas high light intensity favors saturated and mono unsaturated fatty acids, 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 [21]. 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.

Table 2.4 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]
Chlorella 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]
Scenedesmus 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]
Neochloris oleoabundans	Nitrogen deficiency	Increase in TAGs accumulation from 1.5% to 12.4% w/w	[171]
Chlorella 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]
Dunaliella 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., 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 Fatty acids	[180]
Chlorella sp.	Phosphate, potassium, iron	Lipid productivity of 49.16 mg L^{-1} d	[175]
Chlamydomonas reinhardtii	Sulphur limitation	2-fold increase of the phosphatidylglycerol	[181]
Chlamydomonas reinhardtii	Sulphur limitation	Increase in TAG	[182]
Cyclotella cryptica	Silicon deficiency	Total lipids increase from 27.6% to 54.1%	[183]
Scenedesmus sp., 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% & 5.7% to 35.9%, 30.2%, 28.4% & 14.7%, respectively	[185]
	Temperature stress		
Rhodomonas sp.	Temp. range of 27 °C to 30 °C	Increase in lipid production by 15.5%	[186]
Cryptomonas sp.	Temp. range of 27 °C to 30 °C	Increase in lipid production by 12.7%	[186]

Isochrysis sp.	Temp. range of 27 °C to 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]
Chlorella 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	Greatly increase in saturated FAs C15:0 and C17:0	[190]
Dunaliella 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} /18h light: 6h 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}/ 12:12h, 100 µmol m^{-2} s^{-1}/24:0h, and 50 µmol m^{-2} s^{-1} /24:0h 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}/12:12h regime.	[197]
T. pseudonana	100 µmol m^{-2} s^{-1}/ 12:12h, 100 µmol m^{-2} s^{-1}/24:0h, and 50 µmol m^{-2} s^{-1} /24:0h 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)	[189]
Chaetoceros muelleri	UV-A radiation	Increase in monounsaturated FAs	[198]
Nannochloropsis sp.	UV-A radiation	Increase in ratio of saturated FAs to PUFAs	[199]
Chaetoceros simplex	High UV-B radiation	Increases in saturated fatty acids	[200]
Tetraselmis sp.	UV-B radiation	Increase in saturated and monounsaturated FAs and decrease in PUFAs	[201]

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]
Chlamydomonas reinhardtii	Defective in Isoamylase gene	Increase in lipid and starch production	[202]
Chlamydomonas reinhardtii	Defective in ADP-glucose pyrophosphorylase	Increase in TAGs accumulations	[203]
Chlamydomonas 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, 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]
Thalassiosira pseudonana	Targeted knock down of Thaps3_264297 gene (Defective in Lipase) + Silicon limitation	2-folds increase in triacylglycerol (TAG) and 3-5-folds in total lipid	[207]

The Solvent extraction method is where oil is extracted from the algae in the liquid medium 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 the two-step method, using methanol to extract the lipid, followed by hexane to purify them [212] .This method has several drawbacks 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 to 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.

Supercritical fluid extraction (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 fatty acid yields compared to the solvent extraction when the microalgae *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 expositing 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 fatty acids 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 it up [221].

3.2 From algal starch to bioethanol

Bioethanol is usually produced by fermentation of starch, sugars and lignocellulosic feedstocks [222]. The extracted starch can be hydrolyzed to produce glucose, metabolized by yeasts (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 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 2.5) 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 % [82]. Extraction of the carbohydrates from the microalgal biomass can be carried out via different methods such as ultrasonic process, explosive disintegration [224] or enzymatic hydrolysis conversion of the biomass into simple fermentable feedstock [225]. *Chlamydomonas reinhardtii* [226], *Chlorococum littorale* [227] and *Chlamydomonas perigranulata* [228] were found to produce bioethanol by dark fermentation in a energy efficient process. However, the yield of bioethanol was too low to be used for commercial scale, about 1-2.07 % (w/w). On the other hand, several species of microalgae produce large quantities of carbohydrates (Table 2.5), which can be potentially processed for bioethanol production [73, 229, 230], 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) [231, 232]. As well, marine microalgal strains have been screened for their potential to store carbohydrates [233] and more than 70 strains were found to contain a carbohydrate content of 40 to 53 %. *Chlorella vulgaris* (37% starch content) yielded a 65% ethanol-conversion rate compared to the theoretical rate by fermentation [226]. Temperature, biomass concentrations, cell wall disruption through a pre-treatment stage, for example sulfuric 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*. [232]. Iron supplementation [234] and nutrient starvation (P, N or S) [230] have been shown to increase the starch content in *C. vulgaris* [235]. Dilute acid pretreatment has been used with *Chlorococcum humicola* to obtain an ethanol yield of 520 mg ethanol g^{-1} dry wt biomass [236], but this pretreatment may also result in converting the glucose and xylose into hydroxymethylfurfural and furfural [237], which inhibit ethanol fermentation. Thus, pretreatment 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. In 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.

Table 2.5 The starch or carbohydrate content of some microalgae suitable for bioethanol production

Microalgal source	Starch or Carbohydrate Content (% of dry wt)	Ref.
Chlamydomonas reinhardtii	43.6 – 55.0 (Starch)	[225, 226, 238]
Scenedesmus sp.	13.0 – 20.0 (Starch)	[239]
Chlorella vulgaris	37.0 – 60.0 (Starch)	[226, 235, 240, 241]
Nannochlorum sp.	25.0 (Starch)	[228]
Nostoc muscorum	33.5 (Starch)	[239]
Phormidium angustissimum	28.5 (Starch)	[239]
Chlorococcum sp.	17.0 – 26.0 (Starch)	[239]
Scenedesmus obliquus	23.7 (Starch)	[239]
Oscillatoria sp.	19.3 (Starch)	[239]
Tetraselmis subcordiformis	62.1 (Starch)	[242]
Nostoc sp.	30.7 – 32.9 (Starch)	[239]
Oscillatoria sp.	19.3 (Starch)	[239]
Anabaena variabilis	09.2 (Carbohydrate)	[243]
Porphyridium cruentum	40.0 – 57.0 (Carbohydrate)	[244]
Chlamydomonas reinhardtii UTEX90	60.0 (Carbohydrate)	[226]
C. vulgaris CCAP 211/11B	55.0 (Carbohydrate)	[245]
S. obliquus CNW-N	46.7 (Carbohydrate)	[246]
Pavlova pinguis	41.0 (Carbohydrate)	[247]
Tetraselmis suecica F&M-M33	50.0 (Carbohydrate)	[248]
Anabaena cylindrica	25.0 – 30.0 (Carbohydrate)	[249]
Dunaliella salina	32.0 (Carbohydrate)	[249]
Spirulina platensis	31.2 (Carbohydrate)	[147]
Spirulina maxima	13.0 –16.0 (Carbohydrate)	[215, 249]
Nannochloropsis sp.	12.0 (Carbohydrate)	[250]
Porphyridium purpureum	40.0 – 57.0 (Carbohydrate)	[231]
Phaeodactylum tricornutum	26.0 (Carbohydrate)	[251]
Dunaliella tertiolecta	20.0 (Carbohydrate)	[252]
Neochloris oleoabundans	08.0 (Carbohydrate)	[251]
Nannochloropsis gaditana	36.0 (Carbohydrate)	[251]

3.3 Biomethane (Biogas) by anaerobic digestion

Microalgal biomass is a source of a wide range of organic biopolymers, carbohydrates, lipids and proteins, that 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 are 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 sulfide [253] should be removed before using the methane for use in electricity generation. Additionally, carbon dioxide (around 30-40%)fuel gas [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. As well, sodium ions have been found to be toxic to some anaerobic microorganisms as well. 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 to the anaerobic digestion of the algal biomass alone [255].

Anaerobic digestion is appropriate for feedstocks with a high moisture content (80–90% moisture) [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 to 0.65 m^3 kg^1 of dry biomass [9]. 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 (Fig. 2.1). Surplus energy could be sold to the grid, thus improving overall process economics. [9]. 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 [245, 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.

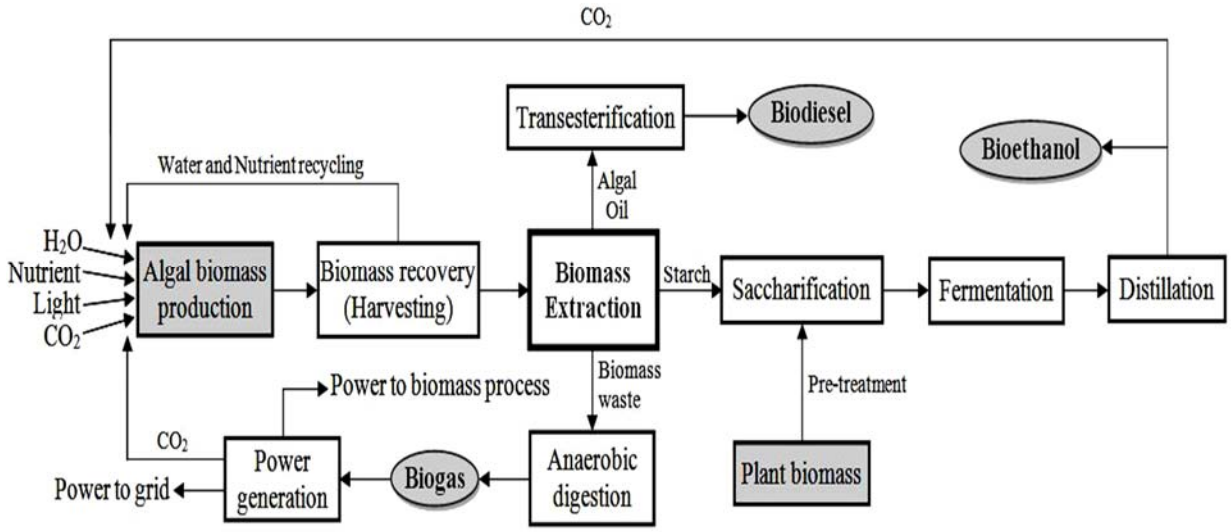


Figure 2.1 Integrated system for biodiesel, bioethanol, biogas and power generation production.

3.4 Liquefaction

Thermochemical liquefaction is a method used to convert the wet algal biomass into liquid fuel [160] by heating the biomass at high temperatures (200 to 500 ◦C) and pressures (greater than 20 bar) in the presence of a catalyst to yield bio-oil [160, 260] . Although the high ability of this method to convert the biomass to energy, 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 a bio-oil yields of 37% to 54%. The choice of the 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 a high methane yields, whereas, iron sulfide 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].

3.5 Bio-Oil by Pyrolysis

Pyrolysis is the thermal decomposition process of materials (algal biomass), in the complete absence 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 vapor residence times (5 - 30 min), thus favoring production of tars and char compared to fast pyrolysis with much higher heating rates (e.g. 1000 ˚C min-1) and shorter vapor residence times (seconds), favoring direct production of liquid fuels [13]. The advantage of this technology is that the entire microalgal biomass can be processed. However, the high energy costs that are required are a hindrance to its practical development. Only a few studies have examined the pyrolytic characteristics of microalgae (Table 2.3).

This process leads to products in three phases: vapor; 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°C to 500°C for less than 2 seconds), and fast pyrolysis (heated to between 350°C -500°C for 10-20 seconds) is associated with liquid fuels of 75 and 50 % respectively, and if carried out at higher temperatures will result in more gas production [265] . In one 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 for power generation through internal combustion (diesel or gas turbine engines or by co-firing with diesel or natural gas [265, 267, 268]. Algal biomass is distinguished by its inherent small size with no fibrous tissue, making it preferred when comparing to 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 *Chlorella 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].

3.6 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°C to 700°C) in a gasifier, resulting in hydrogen [270] with yields ranging from 5 to 56%, carbon monoxide (9 to 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 (~ 4–6 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 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 -1000°C) showed that at 1000°C a high yield of methanol (0.64 g methanol/g of algae biomass) could be obtained [155].

3.7 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 catalyzed 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 biohydrogen 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].

3.8 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 [228, 285]. The produced ethanol can be collected from the headspace of the culturing reactor in a low energy intensive process. 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.

3.9 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 pretreatment processes such as drying or grinding which will add cost [222, 260].

3.10 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 "Heteroboost" to induce the production of additional biofuel and bio-products which are then extracted from the algae (Fig. 2.2).

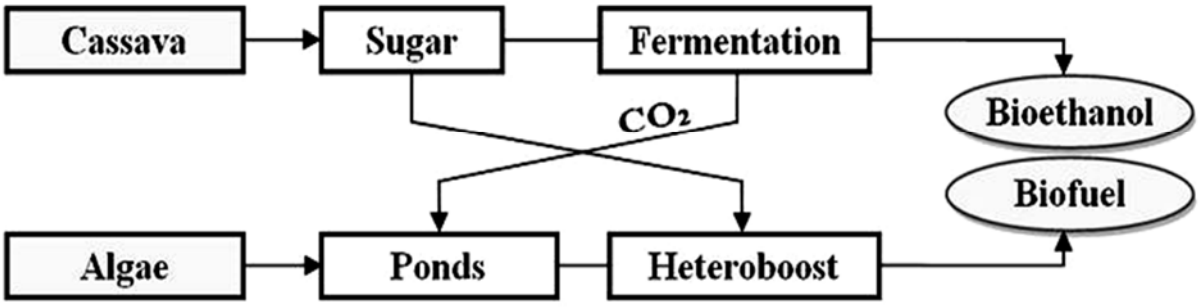


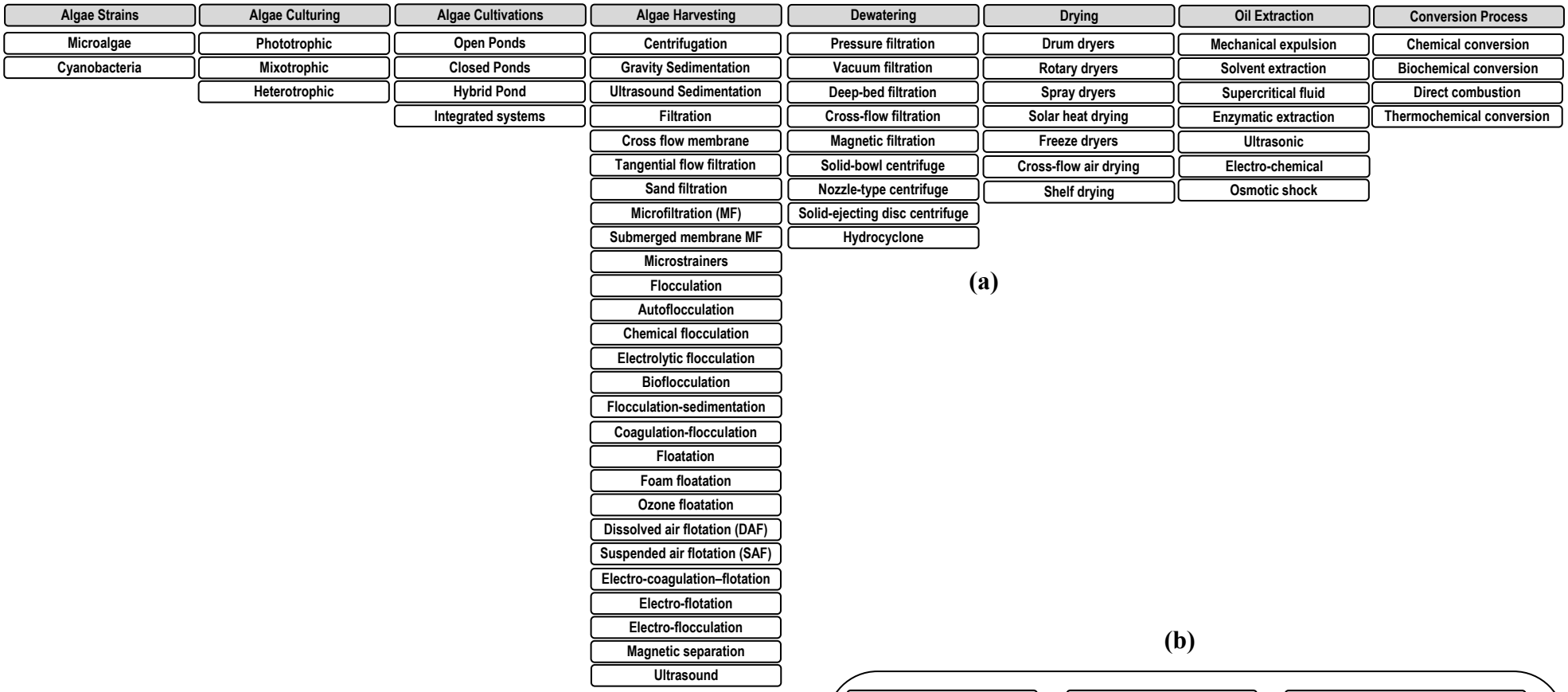
Figure 2.2 Phycal integrated advanced technology.

Some studies that have sued combined processes or that have used algal biomass as additive feedstock in biofuels process are given in Table 2.6

Table 2.6 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 (Bio-diesel)	Lipid productivity 0.223 $gL^{-1}d^{-1}$	[287]
Spirulina platensis, Rhodotorula glutinis	Microalgae/yeast mixed cultivation using monosodium glutamate wastewater		Lipid content 12.71%	[288]
Chlorella sorokiniana	Culturing of oleaginous yeast and algae in food waste and municipal wastewater for lipid production		Lipid content 18.7% - 28.6%	[289]
Arthrospira (Spirulina) platensis	Microalgae used the CO_2 from ethanol fermentation by the yeast *Saccharomyces cerevisiae*		Lipid content 8.39 %	[290]
Two *Botryococcus braunii , Chlorella vulgaris, Chlorella pyrenoidosa*	Production of Biodiesel catalyzed by immobilized, *Penicillium expansum* lipase and *Candida antarctica* lipase B		Lipid content 40.7%	[291]
Chlorella 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]
Chlamydomonas reinhardtii UTEX 90 , *C. vulgaris* IAM C-534	Enzymatic or hydrothermal acid treated algal biomass fermented to ethanol by *Saccharomyces cerevisiae*	Bio-ethanol	29.2% 235 mg ethanol /1.0 g biomass	[225, 226, 293]
Chlamydomonas reinhardtii, C. vulgaris, Undaria pinnatifida	Acid hydrolyzed-enzymatic treated algal biomass was fermented by four different strains of *E. coli*		0.4 g ethanol/1.0 g biomass	[294]
Chlamydomonas reinhardtii	Ethanol-hexane-sulfuric acid treated algal biomass fermented by *S. cerevisiae*		0.44 g ethanol/1.0 g glucose	[295]

Chlorococum sp.	Supercritical lipid-extracted microalgae biomass was fermented by the yeast *S. bayanus*		Maximum ethanol conc. 3.83 g L^{-1}	[232]
Synechococcus leopoliensis	HCl-acid treated saccharified algal biomass (growth supported with CO_2) was fermented by yeast *Saccharomyces* sake		0.42 g ethanol /1.0 g glucose	[296]
Microcystis aeruginosa , Anabaena variabilis	Super Critical fluid pre treated algal biomass hydrolyzed and fermentation by *S. cerevisiae*		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 &yield 30.03 ml/g VS	[298]
Chlorella sp.	Algal biomass was simultaneously hydrolyzed and fermented using sewage sludge consortia via one-step process		Nd	[299]
- C. reinhardtii, Dunaliella tertiolecta	- Algal biomass is fermented by *Lactobacillus amylovorous* and *Rhodobacter sphaeroides*		- H_2 yield of 61% & 52%, respectively	[300]
- C. reinhardtii, Dunaliella tertiolecta, Cholrealla pyrenoidosa	- Algal biomass was liquefied/fermented using a starch-hydrolyzing lactic acid *L. amylovorus* then fermented by *Rhodobacter sphaeroides* RV		- The conversion yield was 5 mol H_2/mol of starch glucose)	[301]
Spirulina maxima, Chlorella sp. , Scenedesmus sp., Phaeodactylum tricornutum	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]
	Biomass anaerobically digested by potato anaerobic treated sludge in a lab-scale anaerobic membrane bioreactor		75.3% of methane	[304]
Chlorococcum sp.	Distillery waste with algal biomass is anaerobically treated by acidogenic/ methanogenic culture in two stage setup		Biogas was 6 L day^{-1}	[305]



(a)

(b)

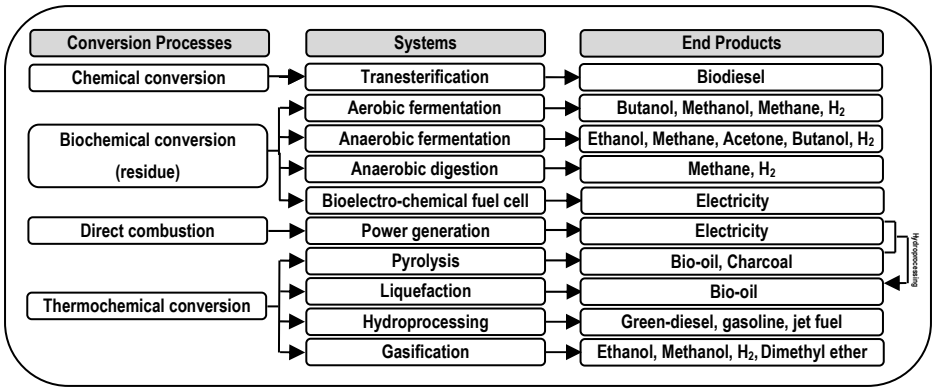


Figure 2.3 From cultivation to algal biofuel production steps

(a) Summary for the steps toward production of algal biofuel.

(b) Biofuel production via microalgal biomass conversion processes

4.0 Conclusions

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.

5.0 Acknowledgements

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Objectives of the present study

The goal of the research presented in this thesis is to screen native Quebec algae strains, well adapted to local conditions, for potential use in simultaneous biodiesel production and wastewater nutrient removal of second effluents from municipal wastewater at high efficiency. Moreover, I wished to examine the operational conditions (e.g. variation in the medium nutrient concentrations) that can facilitate maximum biomass and/or biodiesel production. The specific objectives of this research are to:

I. To screen the various isolated native Quebec microalgal strains to identify these with high potential to grow in and treat wastewater and produce biodiesel under different temperature conditions (10±2 ºC and 22±2 ºC). One hundred strains were isolated and surveyed for their growth and neutral lipid accumulation in synthetic medium as well as in municipal wastewater at two different temperatures. The growth, lipid content, and wastewater nutrient removal were determined using a high-throughput fast and reliable screening method by using 12 and 96 well plates. This method is distinguished by the ease of its use, as well as minimizing the effort, lab space and time required to study a large number of strains at once. This research will greatly improve knowledge about algae native to Quebec.

II. To improve biodiesel production by assessing and testing the effect of operational and environmental conditions on accumulation and metabolism of neutral lipids. Nitrogen, phosphorous depletion, and salt sufficiency were found to maximize lipid accumulation in one model strain, *Chlorella sp.* PCH90

Chapter 3: Screening microalgae native to Quebec for wastewater treatment and biodiesel production

Ahmed E.M. Abdelaziz[a], Gustavo B. Leite[a], Mohamed A. Belhaj & Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP 6128, Centre-ville, Montréal. PQ H3C 3J7 Canada

Author contribution: Isolation of strains was carried out by G.L. and P.C.H. Experimental design was assisted by G.L. The wet laboratory experimental work was carried out by A.E.M.A and M.A.B. Dataset analysis and manuscript preparation were performed by A.E.M.A. The article was guided and revised by our supervisor Prof. Patrick C. Hallenbeck.

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Chapter 3: Screening microalgae native to Quebec for wastewater treatment and biodiesel production

Ahmed E.M. Abdelaziz[a], Gustavo B. Leite[a], Mohamed A. Belhaj & Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP 6128, Centre-ville, Montréal. PQ H3C 3J7 Canada

[*]Corresponding author

"This chapter discusses the use of a rapid and high throughput method to screen a hundred strains isolated from five different regions located in the vicinity of Montréal, QC for their potential to produce biofuel and wastewater treatment at two different temperature conditions (10˚C and 22˚C). Several strains revealed high lipid content (>35) and wastewater nutrient removal potential (>95%)"

Keywords: Biofuels; algae; wastewater; wastewater treatment; biodiesel; indigenous specie

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. Here we report on 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 microalgae screening method employing 12 well plates. We compared the biomass and lipid productivity of these strains on wastewater to a synthetic medium under different temperatures (10±2 ºC and 22±2 ºC) and identified a number that showed good growth at 10 ºC, gave a high lipid content (ranging from 20% to 45% of dry weight) or a high capacity for nutrient removal.

1.0 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., 2013b). 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 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 & 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 & Guieysse, 2006).

Temperature is an important environmental parameter affecting algal growth. Temperatures ranging between 15℃ –25℃ 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 & 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 ºC and 22±2 ºC. 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.

2.0 Materials and Methods

2.1 Sampling and isolation

Water samples were collected from five different locations; three fresh water lakes Lac Triton (45° 59' 17.11" N 74° 0' 20.55" W), 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), 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 50µm 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 (33µm, 20µm and 0.45µm). 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 ºC as well as 10 ± 2 ºC. 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 hours.

After growth, different colonies were inoculated in 125ml Erlenmeyer flasks containing 70ml of BBM medium and incubated in a light mounted shaker at 10 ± 2 ºC and 20 ± 2 ºC, with shaking at 120 RPM and a light intensity of 21.2 W/m^2 using a photoperiod of 12 hours 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 of 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 $mg \cdot l^{-1}$ (Table 3.1). 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 $W \cdot m^{-2}$ and a 12:12 h light/dark cycle. Growth was quantified daily by measuring the optical density (OD_{600}) 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 Figure 3.1.

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 & 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 seconds, 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 minutes at room temperature and then the samples were pipetted into 96 well micro-plates. The fluorescence intensity at excitation and emission wavelengths of 535 nm 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 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 & 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 (Table 3.2).

3.0 Results and Discussions

3.1 Native microalgal culture collection

The sampling protocol and isolation procedure used here was successful in establishing a culture collection of over one hundred local microalgae. 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 Figure S 3.1. 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). 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. At 22 ºC, 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 ºC, 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 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 ºC 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.,

2011 a,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 ºC 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 biomass amount was achieved at 22˚C by isolate PCH22 which only had a low neutral lipid content (about 4.5%) (Fig. 3.5). The biomass concentrations from the highest lipid producing strain MA2H1 at 10 °C and 22 °C, were 1.305 g $\cdot l^{-1}$ and 1.191 g $\cdot l^{-1}$. The corresponding biomass productivities were 93 mg l^{-1} d^{-1} and 85 mg l^{-1} d^{-1}, respectively (Fig. 3.2, Table 3.3).

Among 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 (Fig. 3.2). 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 g l^{-1} and 1.191 g l^{-1}, respectively) (Fig. 3.2). This suggests that this organism might have an optimum range of growth between these two temperature points of culturing 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 0.243 g biomass l^{-1} and 0.07 g lipid l^{-1} as compared to 0.181 g l^{-1} and 0.028 g l^{-1} at 22 °C. Again, this suggests this microalga prefers low temperature conditions (Fig. 3.5). 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. 3.2). 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 (as measured by optical density) 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. 3.5). 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 (Fig. 3.5). 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 20-38%, with this medium which had a relatively high phosphorus concentration (Fig. 3.3).

3.3 Growth on municipal wastewater

Not surprisingly, given the relatively lower content of nitrate and phosphate (Table 3.1), growth on wastewater gave lower cell densities (between 0.2 – 1.1), about a half of that on BBM, and with a shorter exponential phase, compared to their growth in synthetic medium. The majority of the strains took about 4 to 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 ºC 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 ºC 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 high lipid producers at 22 °C (Fig. 3.2, Table 3.3). The greatest lipid producer was isolate PCH16 which had a lipid content of 43 % w/w when grown in WW at 22 °C (Fig. 3.2). This strain also showed complete (>99 %) nitrate and phosphate removal (Fig. 3.3). 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. 3.5). 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 (Yeh and Chang, 2011; Praveenkumar et al., 2012).

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 °C and 22 °C (Fig. 3.6). 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. 3.6). In the same way, strain LB1H3 showed better growth performance in wastewater at 22 °C than under other conditions (Fig. 3.6).

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. 3.2, Table 3.3). 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. 3.2). The highest amount of lipid, 410 mg l^{-1} (lipid productivity of 29 mg l^{-1} d^{-1} (Table 3.3)) 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. 3.5).

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 (Figure 3.5 shows the fluorescence and optical density of each of the selected strains). Strains such as PCH22, PCH37, LB1H7 and PCH98 produced similar amounts of lipids in both synthetic and wastewater media at 22 °C but only very low lipid contents 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.

3.4 Nutrient removal

As stated before, to be successful, algal production facilities will probably need to source their nutrients at least in part from wastewater. In doing so, wastewater treatment credits can be obtained if the offending nutrients, principally nitrate and phosphate, are largely removed. Hence, the ability of the various strains to deplete the different media of nitrate and phosphate was assessed as detailed in Materials and Methods. More than half of the strains removed >70% of the wastewater nitrogen and phosphate under both temperature conditions. Strains MA1A3, LB2H5, HA1B3, and PCH16 also showed high neutral lipid content and removed >94 % and 100 % of the wastewater nitrate and phosphate at a temperature of 10 °C and 22 °C, respectively (Fig. 3.3). 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. 3.3).

4.0 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 that would provide wastewater treatment and lipid production.

5.0 Acknowledgements

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Table 3.1 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

Table 3.2 Elemental analysis of wastewater	
Element	**ppm**
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

Table 3.3 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 ºC				
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 ºC				
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 ºC				
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 ºC				
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

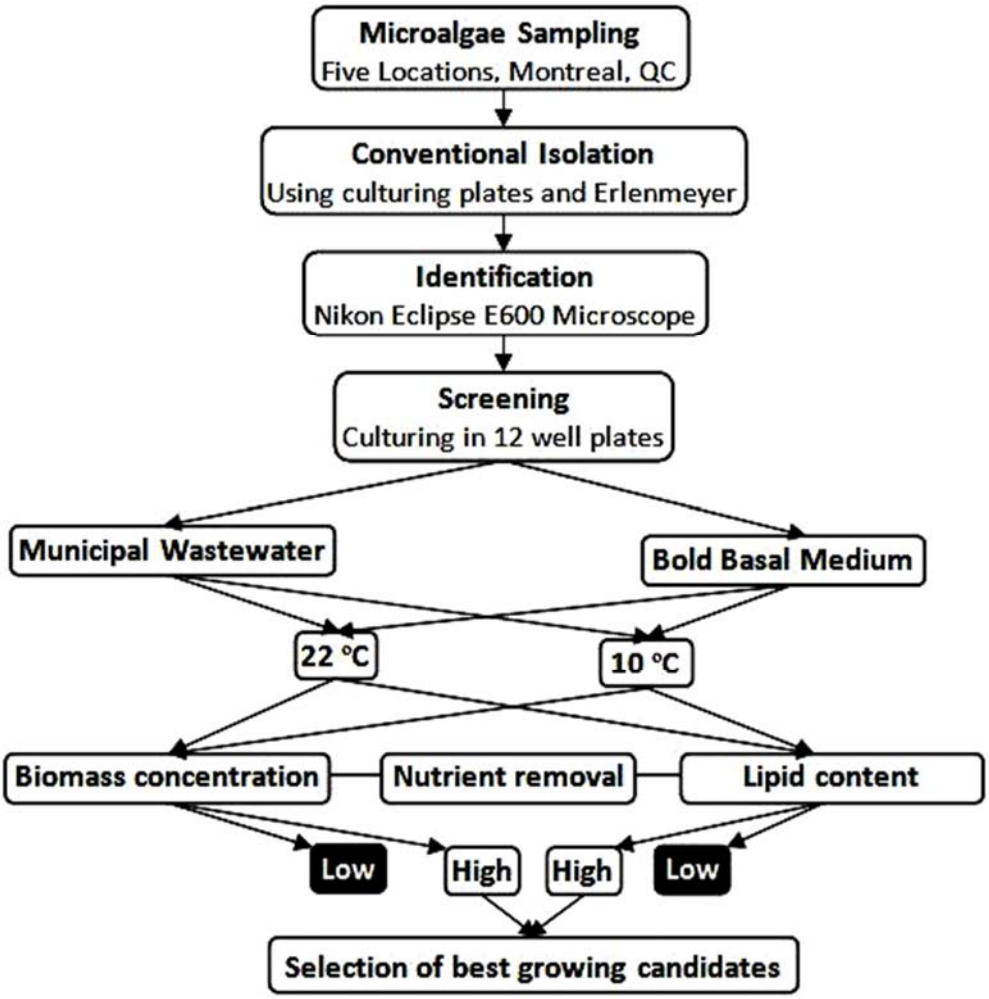

Figure 3.1 Microalgae strain screening procedure.

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℃ and 22℃. 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

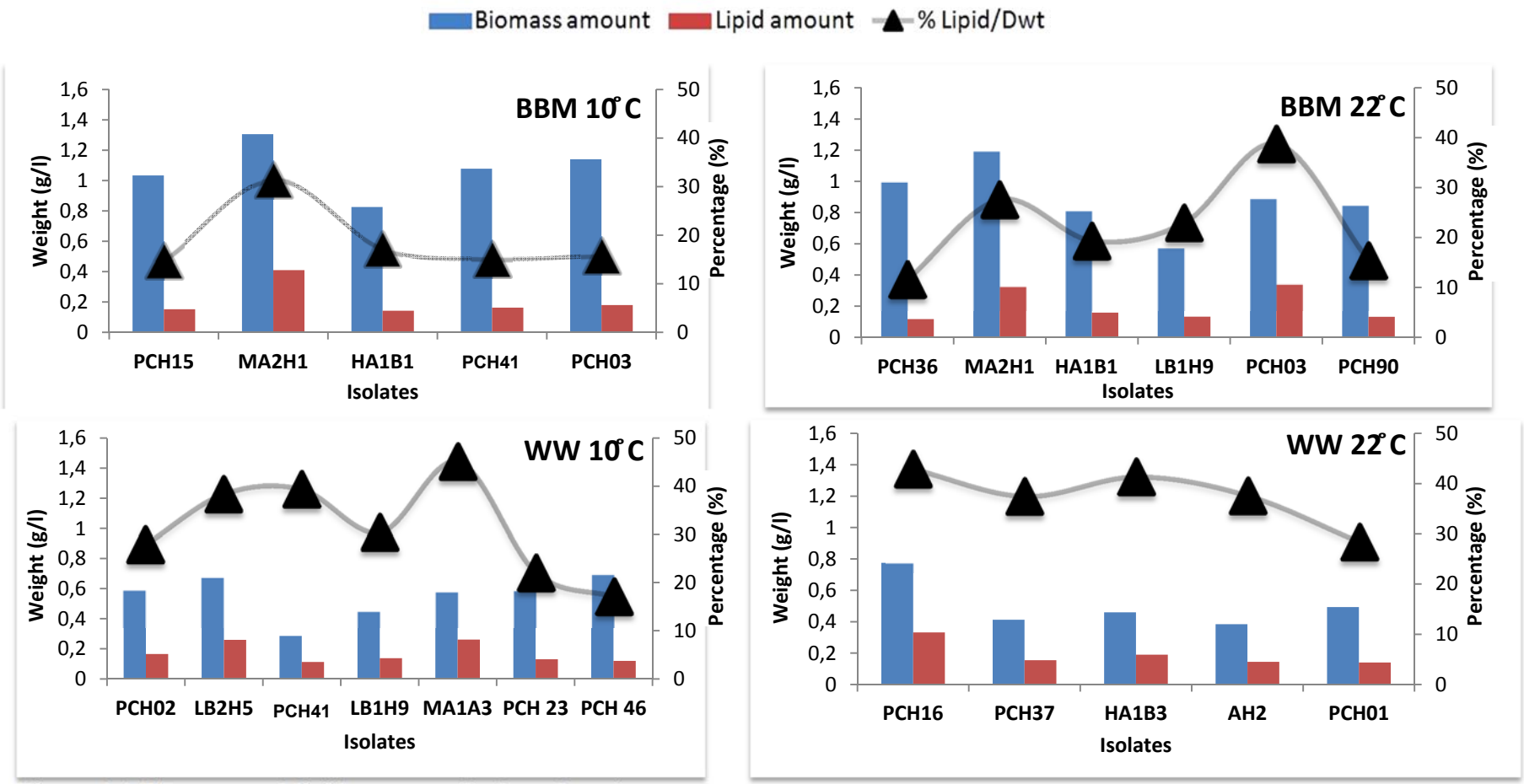

Figure 3.2 Biomass and lipid content of selected strains.

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 Materials and Methods. 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 $\cdot l^{-1}$ & 31.4 % respectively) in BBM at 10 °C and strain PCH03 with a biomass content of 889 mg $\cdot l^{-1}$ 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 $\cdot l^{-1}$ & 45% respectively) and at 22 °C, strain PCH16 gave 775 mg $\cdot l^{-1}$ and 43%.

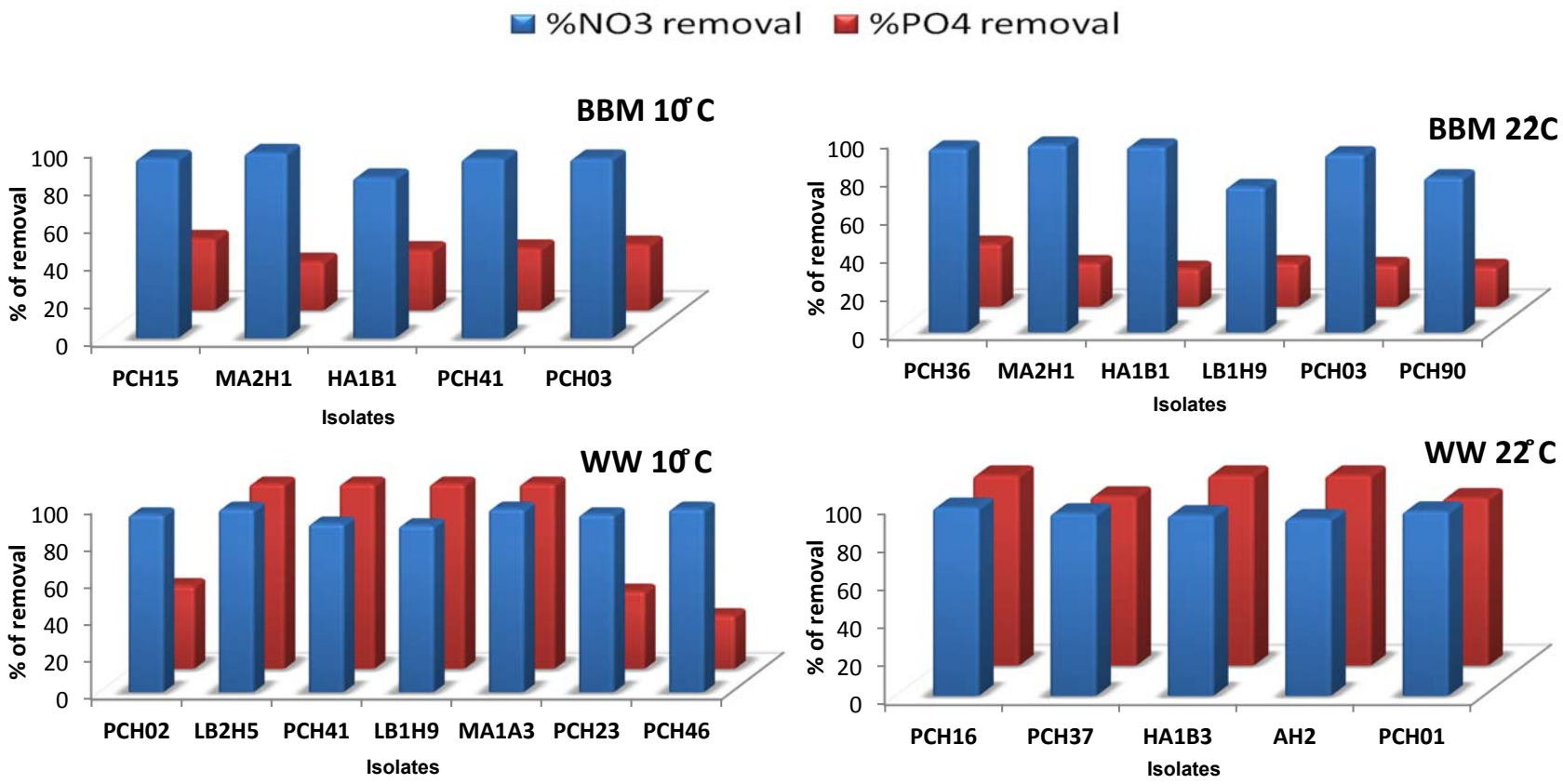

Figure 3.3 Nutrient (PO_4 & NO_3) removals by the selected strains under the different experimental conditions.

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℃) and PCH16 (grown in WW @22℃) 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.

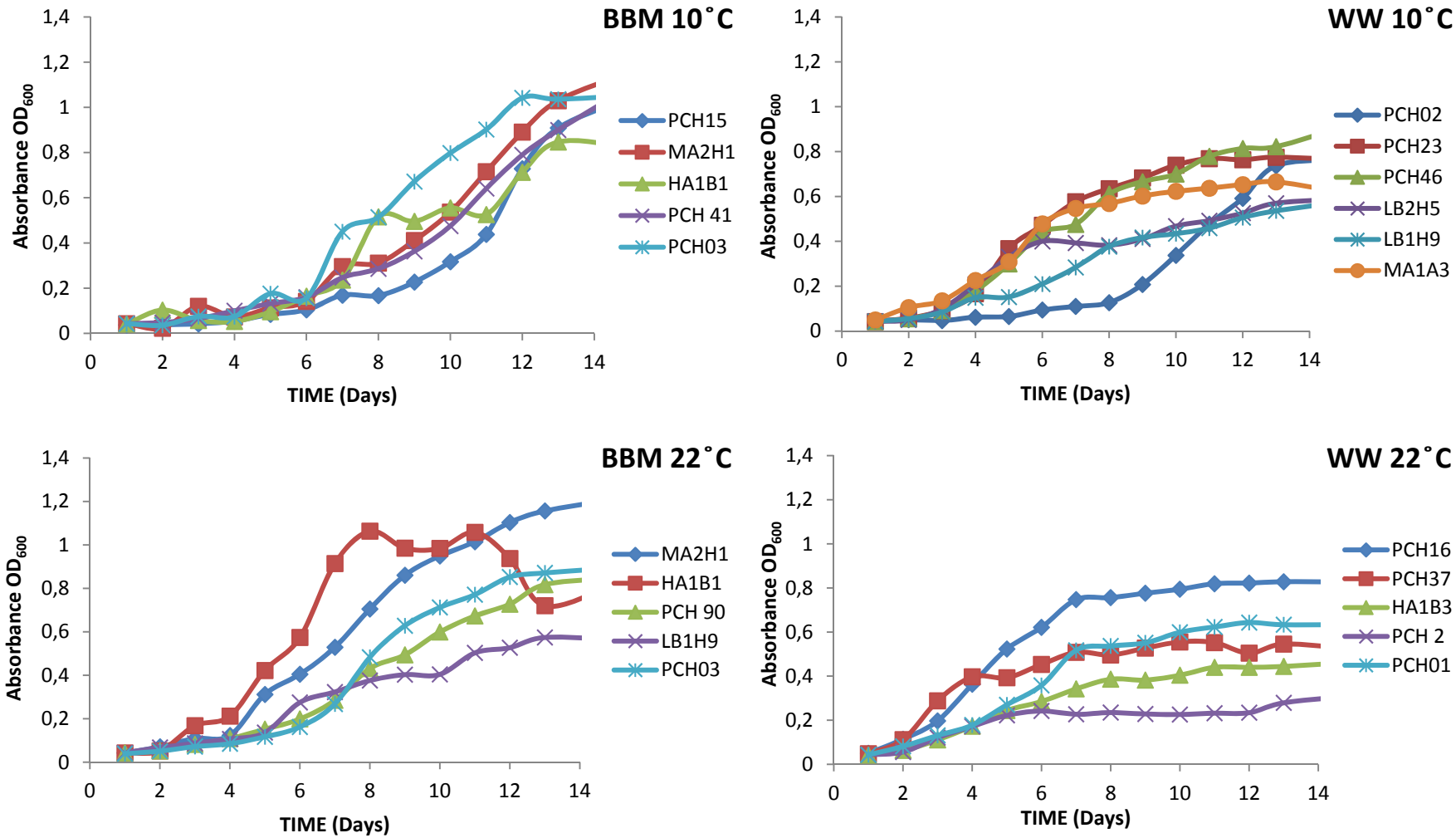

Figure 3.4 The growth curves for the selected strains under the different experimental conditions.

The results show that the strains grew more rapidly and robustly in synthetic medium (BBM) than in wastewater (WW).

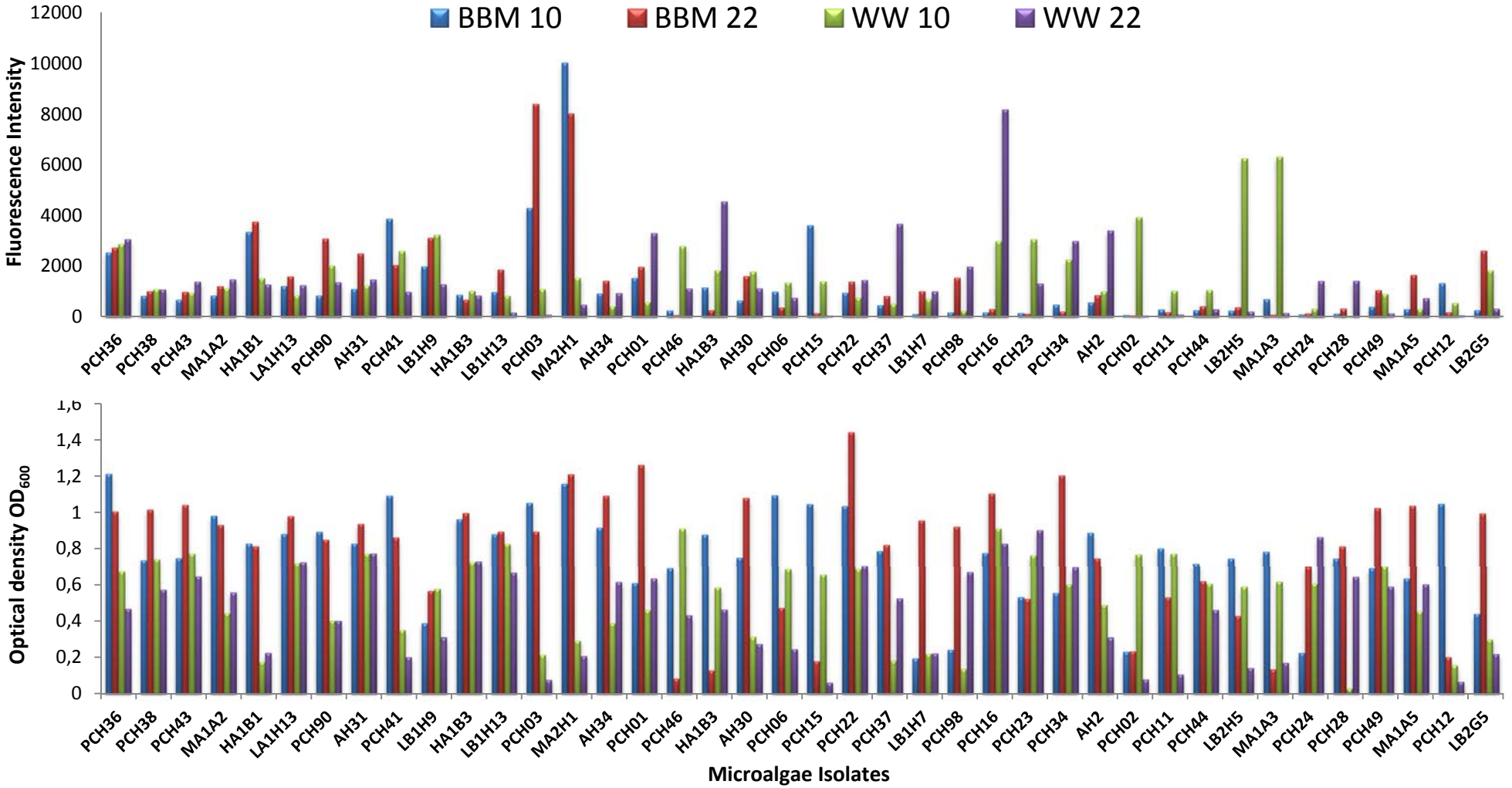

Figure 3.5 Nile red fluorescence and optical density measurements of the selected microalgae.

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

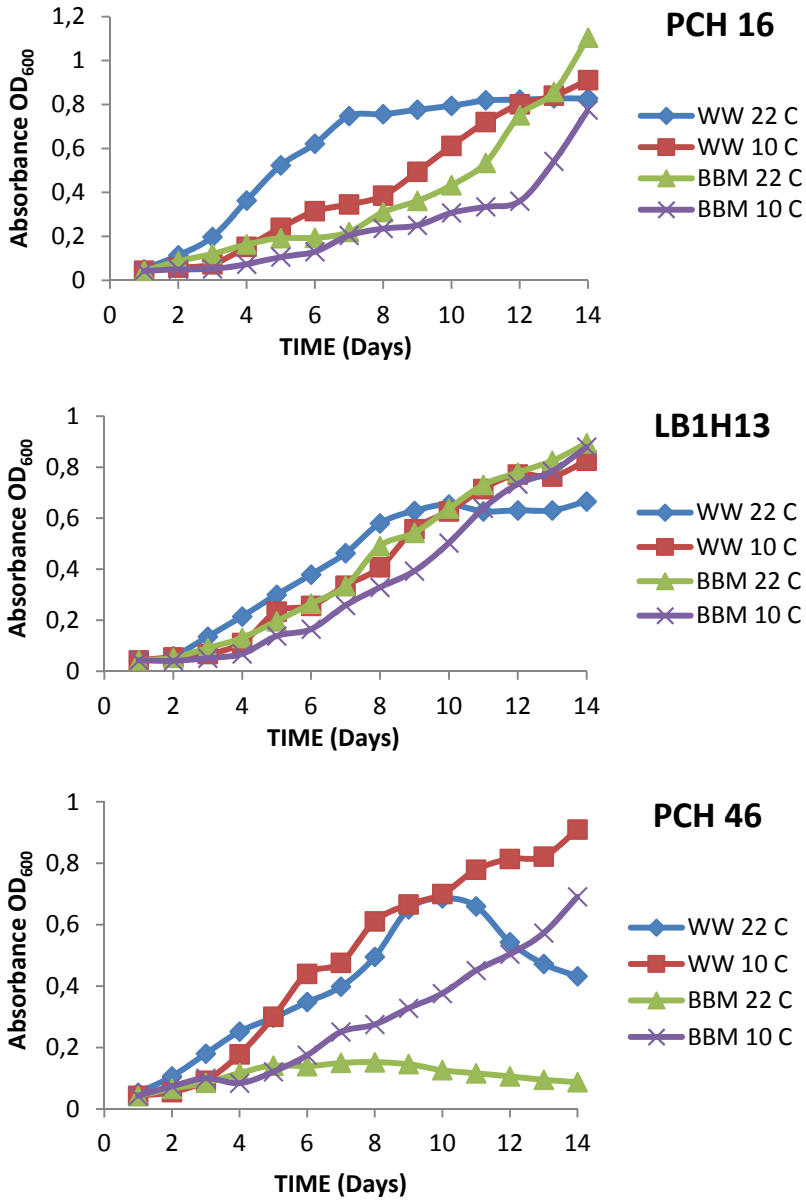

Figure 3.6 Examples of strains with better growth in wastewater than their growth in BBM regardless their lipid production efficiency.

The strain PCH 46 showed better growth at low temperature and WW compared to its growth in BBM while the strain LB1H3 revealed better growth at 22℃ in WW compared to its growth in BBM and low temperature. Strains PCH16 and LB1H13 showed almost similar growth curves under all the culturing conditions. Strain PCH23 showed better growth in WW than BBM.

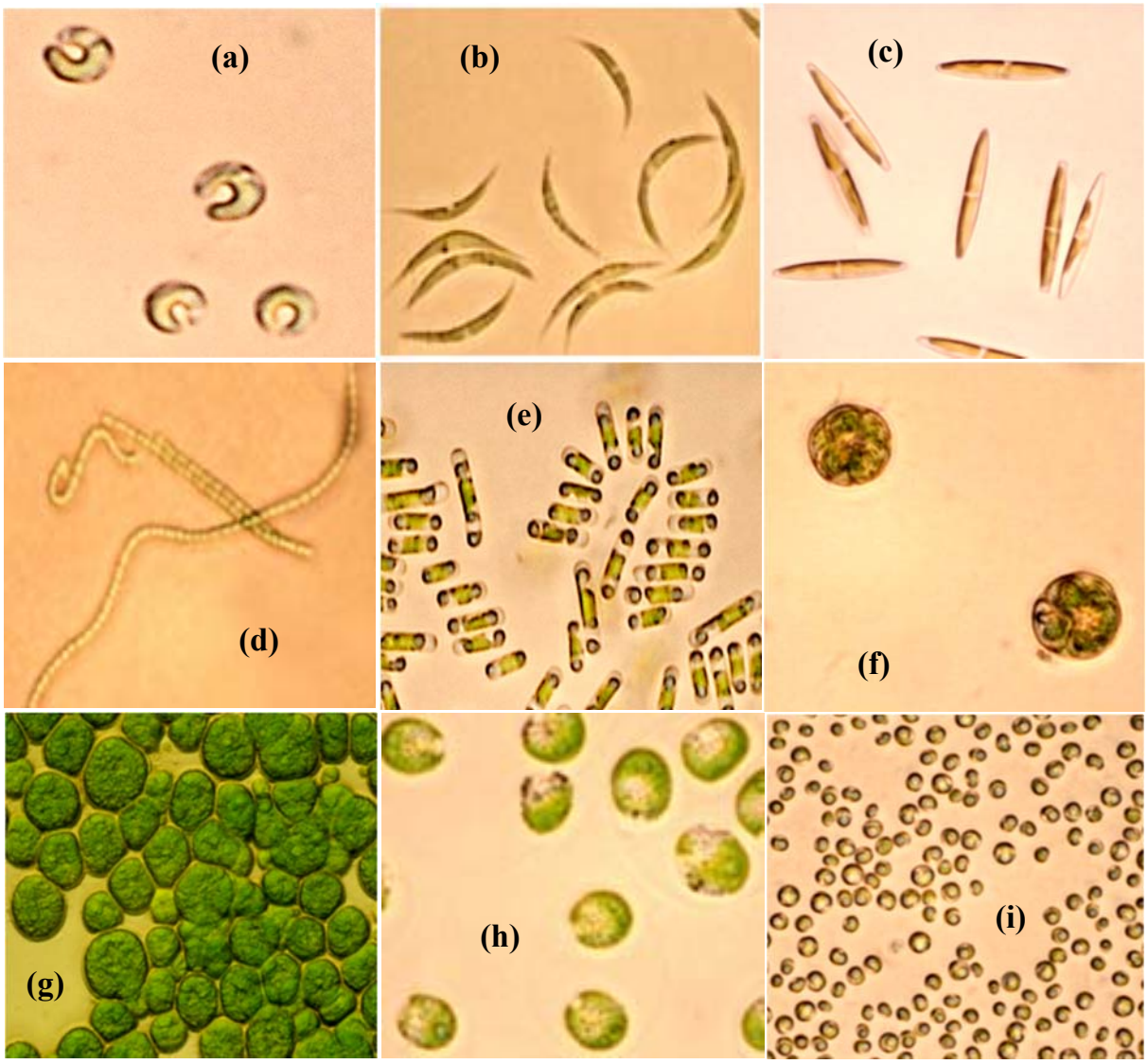

Figure S 3.1 Photomicrographs of representative microalgal strains isolated from different locations around Montreal, showing the wide diversity of algal classes found.

(a) Selenastrum capricornutum, (b) Ankistrodesmus sp., (c) Nitzschia sp., (d) Oscillatoria sp., (e) Stichococcus bacillaris, (f) Pandorina sp., (g) unidentified, (h) Gloeocystis sp., (i) Chlorella vulgaris.

Chapter 4: Characterization of growth and lipid production by *Chlorella sp* PCH90, a microalga native to Quebec

Ahmed Elsayed Mohamed Abdelaziz ¥, Dipankar Ghosh ¥ and Patrick C. Hallenbeck*

Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP6128 Succursale Centre-ville, Montréal, Québec H3C 3J7 Canada

Author contribution: ¥ Both authors contributed equally to this work. D.G. carried out the experimental design, genetic work and analysis. A.E.M.A. carried out the wet laboratory experiments and performed the dataset analysis. The manuscript has been written by D.G and assisted by A.E.M.A and all the work guided and revised by our supervisor, Prof. Patrick Hallenbeck.

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Chapter 4: Characterization of growth and lipid production by *Chlorella sp* PCH90, a microalga native to Quebec

Ahmed Elsayed Mohamed Abdelaziz ¥, Dipankar Ghosh ¥ and Patrick C. Hallenbeck*

Département de Microbiologie, Infectologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec H3C 3J7 Canada

*Corresponding Author

"This chapter examines the characterization of the novel microalga *Chlorella sp.* PCH90, one of the high-lipid producing selected strains from the results obtained from the previous chapter bioprospecting analysis. Its molecular phylogeny was established and lipid production studies as a function of the initial concentrations of nitrate, phosphate, and sodium chloride were carried out using Response Surface Methodology (RSM). Nitrogen and phosphorus depletion as well as salt sufficiency were found to maximize lipid accumulations in this microalga".

Keywords: Biodiesel; Lipid productivity; Biomass productivity; Lipid content; Phylogeny; Response Surface Methodology.

Abstract

Microalgae are being investigated as potential candidates for biodiesel production since they can be grown without competition with food production, have an inherently fast growth rate, and can have a high lipid content under different nutrient limiting conditions. However, large scale production will best be carried out with indigenous strains, well adapted to local conditions.

Here we report on the characterization of the novel microalga *Chlorella sp.* PCH90, isolated in Quebec. Its molecular phylogeny was established and lipid production studies as a function of the initial concentrations of nitrate, phosphate, and sodium chloride were carried out using Response Surface Methodology. Under the appropriate conditions this microalga could produce up to 36% lipid and grew well in both synthetic medium and secondary effluent from a wastewater treatment plant at both 22°C and 10°C. Thus, this strain is promising for further development as a potential biofuels producer under local climatic conditions.

1.0 Introduction

The need to develop renewable and sustainable sources of energy is driving R&D on various new energy sources including biofuels. In this scenario, there is a significant need to find a replacement for fossil-derived diesel as a significant source of mobile power for moving goods and people. Biodiesel production has ramped up over the past decade with billions of liters now being produced annually. Conventional biodiesel is made either from waste cooking oil or from soybean and other plant oils, which can lead to either competition with the food supply or at best land conversion in the case of palm oil. For these reasons, attention has turned to biodiesel production by microalgae, photosynthetic microorganisms capable of the conversion of sunlight, water and carbon dioxide to triacylglycerols (TAGs), a sustainable feedstock for biodiesel generation.

Microalgae are proposed to offer a number of advantages including a much higher biomass and lipid productivity, and the lack of competition with food crops for land and fresh water resources since they can be cultivated on non-arable land using waste or saline water (Abdelaziz et al., 2013a; Abdelaziz et al., 2013b; Amaro et al., 2011; Leite et al., 2013) . Amongst different microalgae, *Chlorella* sp. have attracted particular attention. Some have been shown to produce TAGs heterotrophically (Xu et al., 2006) and autotrophic lipid production has been shown to be stimulated by a variety of factors (Li et al., 2013; Liang et al., 2013; Menon et al., 2013; Nigam et al., 2011). A recent study has suggested that stresses have in common the generation of specific intracellular reactive oxygen species (siROS) as a signal which controls different downstream pathways, including lipid biosynthesis pathway (Menon et al., 2013). In the future, genetic manipulation to increase lipid production may even be possible since the complete genome sequence as well as electrophoretic karyotyping and chromosomal gene mapping has been established for one strain (Higashivama & Yamada, 1991).

Previous studies on the optimization of lipid production by *Clorella* have relied on the "single-factor-at-a-time" approach. Such conventional processes are time-consuming and

incapable of reaching the true optimum since potential interactions among process variables are ignored. Therefore, an experimental design based on statistical modeling can be a very useful tool for evaluating the interactions between a set of independent experimental factors and observed responses, while at the same time reducing the number of experiments required to determine the optimal condition. Response surface methodology (RSM) is a mathematical approach that can be used for multiprocess parameter optimization, especially where the intermediate mechanisms are not known. Amongst the variety of designed statistical tools that are available, Box-Behnken design is the most efficient design model for three independent process variables (Box & Behnken, 1960).

In the present study, we isolated a new *Chlorella sp* strain from fresh water (St. Laurence River, Quebec, Canada) and subjected it to morphological and molecular characterization. We also investigated, using response surface methodology, the interactive effects between the initial sodium nitrate concentration ($NaNO_3$), initial phosphate concentration (K_2HPO_4) and initial sodium chloride concentration (NaCl) in order to improve lipid and biomass productivity as well as the lipid content. Finally, we determined the effect of light intensity at the central point of this initial study.

2.0 Material and Methods

2.1 Microalgal strain, Culture Conditions and Experimental setup

A newly isolated and characterized a fresh water *Chlorella sp.* strain PCH90 (from the St. Laurence River, Quebec, Canada), isolated as part of a larger project to establish a culture collection of local microalgal strains, was used. The alga was grown in Bold Basal Medium (BBM) (Bold, 1949) amended with 0.1 X (v/v) BME vitamin (Sigma-Aldrich) and 20 mM MES (Bioshop Canada) buffer, sterilized by autoclaving. For the optimization studies, cultivation was in 250 ml conical flasks with cotton plugs which were placed in an orbital shaker (150 rpm) at an illumination intensity of 16 $W \cdot m^{-2}$ and a temperature of 22 (± 3)˚C with an initial pH of 6.8.

Cultures (75 ml total liquid volume) were inoculated (5%) with washed cultures that had been pre-grown for 16 days using the same medium. The effect of different light intensities was determined using 500 ml cylindrical transparent glass reactors (200 ml working volume) placed inside a water bath system equipped with a stirring unit and a thermostat for temperature control (22˚C) at the indicated light intensities. Algal morphology was determined through light microscopy (Nikon Eclipse 80i microscope equipped with a Nikon Digital Sight DS-U1 camera (Nikon, Japan) with immersion oil. Algal samples (novel isolate *Chlorella sp* PCH90) were taken from active exponential phase cultures for microscopic observations.

2.2 Analytical methods

2.2.1 Growth

Growth was measured using a spectrophotometer (Shimadzu UV-VIS Spectrophotometer) at $O.D_{680}$ and the algal biomass determined using a calibration curve of dry weight versus optical density (OD). The lipid present in terms of percentage (%) dry weight was calculated from the dry weight of biomass.

2.2.2 Lipid determination using Nile red

Algal cells (80 µL) were placed into 1 ml tubes, treated using a microwave oven for 50 – 60 seconds, and then mixed with 20 µl of DMSO 25 % (v/v) and again subjected to a microwave oven for the same period of time (50 - 60 sec.). The microwave power was set at high power level for both processes. 1 µL of Nile red solution (250 µg/ml in acetone) was added and the solution incubated for 10 minutes in darkness before being pipetted into 96 well plates. The fluorescence intensity (excitation, 535nm; emission, 580 nm) was measure using a Packard Fusion Spectrophotometer (Chen et al., 2009). The relative fluorescence intensity of Nile red was calculated by subtracting the fluorescence intensity from both the auto-fluorescence of the algal cells and Nile red alone. Relative fluorescence intensity was converted to lipid content using triolein (Fischer Scientific, USA) as a standard.

2.3 Design of Experiments study of operational parameters

A 3^K factorial Box–Behnken model was used as the experimental design model to investigate the key process parameters. For three factors, this design requires fewer experimental runs. The 3^K factorial design also allows efficient estimation of second degree quadratic polynomials and gives the combination of values that optimizes the response within the region of the three dimensional observation space (Annadurai et al., 1999). In developing the regression equation, the relation between the coded values and actual values are described according to the following equation 1:

$$\boldsymbol{xi} = (\boldsymbol{Xi} - \boldsymbol{Xi}*)/\boldsymbol{\Delta Xi} \qquad \text{(Equation 1)}$$

Where x_i is the coded value of the i^{th} independent variable; X_i is the uncoded value of the i^{th} independent variable; X_i* is the uncoded value of the i^{th} independent variable at the center point, and ΔX_i is the step change value. The levels of the variables and the experimental design are shown in Table 4.1. Lipid and biomass production were associated with simultaneous changes in initial $NaNO_3$ concentration (0.85, 11.48, and 22.1 mM), initial

K_2HPO_4 concentration (5, 32.5, and 60 μM) and initial concentration of NaCl (1, 10.5 and 20 mM). A total of 15 experimental runs decided by the 3^K factorial Box–Behnken design were carried out, and the center point was replicated three times to estimate experimental errors. For predicting the optimal condition, the quadratic polynomial equation was fitted to correlate the relationship between independent variables and responses, total biomass production and total lipid production and estimated with the following Equation 2:

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ij} X_i^2 + \sum_{i=1}^{3} \sum_{i<j=2}^{3} \alpha_{ij} X_i X_j \quad \text{(Equation 2)}$$

Where X_i are the input variables, which influence the response variable Y; α_0 is the offset term; α_i is the i^{th} linear coefficient; α_{ij} is the ij^{th} interaction coefficient. The input values of X_1, X_2 and X_3 corresponding to the maximum value of Y were solved by setting the partial derivatives of the functions to zero. The appropriate program from Design Expert 8 (Stat-Ease) was used.

2.4 PCR amplification and sequencing

Cells were harvested by centrifugation (6000 rpm for 5 min) and total DNA extracted from liquid cultures using a modified Doyle & Doyle method (Doyle, 1987) followed by RNaseA treatment. DNA fragments (18s 23s rRNAs and *rbcL* (ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo)) were amplified by PCR using the following primers: 5'-CCTGGTTGATCCTGCCAG-3' and 5'- (A/T)TGATCCTTC(T/C)GCAGGTTCA -3' (18s rRNA gene) (Wan et al., 2011); 5'-AGGGGTARAGCA CTGYTTYG-3' and 5'-CCTTCTC CCGAAGTTACG-3' (for 23s rRNA gene (del Campo et al., 2010)); 5'-GCGGGTGTTAAAGACTACCG-3' and 5'-CCTAAAGTACCACCGCCAAA-3' (for *rbcL* gene) (Wan et al., 2011). 1μl (≈70 ng template) total DNA samples were used in a 50 μl PCR reaction with 1X Q5 reaction buffer; 200 μM dNTPs; 0.5 μM primers; 0.02 U/ μl Q5 high fidelity DNA polymerase; and 5% (w/v) DMSO or 1X Q5 GC enhancer (New England Biolabs).

PCR reaction conditions were: 98^{o}C for 30 sec; 98^{o}C for 10 sec; 63^{o}C for 30 sec; 72^{o}C for 1 min 30 sec; 72^{o}C for 2 min; for a total of 35 reaction cycles in a thermal cycler (Biometra). PCR amplified products were gel eluted and subjected to purification (Geneaid Biotech Ltd, Canada) prior to sequencing which was performed with a ABI 3730 using the original PCR primers.

2.5 Phylogenetic analysis

Sequence alignments for molecular phylogenetic analysis were generated using ClustalW (Thompson et al., 1994) and optimized using MEGA5 (Tamura et al., 2011). Neighbor-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) statistical models were used to infer the phylogenetic relationship among strains of closely associated green algae based on *rbcL* gene sequences. A substitution model for phylogenetics of each nucleotide sequence data set was selected using the jModel Test (Posada, 2008) with model selection in MEGA5. Representative phylogenetic trees were drawn using the NJ, ML methods. Evolutionary analysis and following analysis were all conducted in MEGA5.

3.0 Results and Discussions

3.1 Growth characteristics and phylogenetic analysis of the strain used (PCH90)

As part of a survey of microalgal strains native to Quebec, we identified one, PCH90, which showed interesting growth characteristics (Table 4.2). Not only did it grew well in both synthetic medium (BBM) and in secondary effluent from local wastewater treatment plant (WW), it showed a relatively high growth rate at 10° C, equally to (BBM) or only lightly lower (WW) than that observed at 22° C. Moreover, under most conditions the lipid content was relatively high (15.6-27.6 %) suggesting that this strain showed potential promise as a biofuel producer and merited further detailed study.

Microscopic observation of the new microalgal isolate under bright field with oil immersion at 100X magnification found unicellular, coccoid cells, of an average size of 12 μM (Fig. S 4.1). It would be impossible to place this alga taxonomically based on microscopic observation alone so we carried out molecular phylogenetic analysis using an amplified RuBisCo DNA sequence (*rbcL*). Phylogenetic analysis was performed on a concatenated data set obtained from a NCBIBLAST search using the translated *rbcL* nucleotide sequence of PCH90. The fifty sequences showing the highest homology (96-99%) were subjected to multiple sequence alignment using ClustalW and MEGA5. Finally, the aligned translated protein sequences were subjected to phylogenetic tree construction, estimation and validation using neighbor joining (NJ) and Mwith.

Evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 4.1). This phylogenetic analysis shows that the newly isolated strain is in a deeply rooted branch, distant from the main *Chlorella* species. It is grouped with a number of other strains of uncertain taxonomic status. Therefore, more work on the molecular and physiological level would be required to firmly establish the taxonomy

of this strain. For the purposes of this article this strain will be referred to as *Chlorella sp.* PCH90.

3.2 Modeling of lipid and biomass production using DOE

It was of interest to examine the effects of variation in salt, phosphate and nitrate concentrations on biomass and lipid production by strain PCH90. The method used here was a Design of Experiments approach where a statistical design is used to choose a series of experimental conditions such that a minimum number will give a robust description and model verification (Hanrahan & Lu, 2006). This technique, often used in engineering and manufacturing, can be advantageously applied to biological systems, especially when a bioprocess, such as in this study, is involved. We wished to assess the effects of three independent variables; [NaCl], [$NaNO_3$], and [K_2HPO_4], on biomass and lipid yields as well as the degree, if any, of their interaction. We used a Box–Behnken design since it uses a minimum of tests, is robust, and can be applied when values at the extremes are uninteresting (Whittinghill, 1998). 15 experimental sets were run at the different parameter values indicated by the design and the resultant lipid and biomass production measured. The statistical treatment of the test variables along with the measured response values, expressed as final lipid and biomass concentrations (g/l) corresponding to each combination, are summarized in Table 4.1**.** The summary of the analysis of variance (ANOVA) of the results of the quadratic model fitting are shown in Table S 4.1 and Table S 4.2. ANOVA is essential to test the significance and adequacy of the model and the various parameters indicating the quality of the fit are discussed as foot notes to these tables. The three and two dimensional contour plots for biomass production (Y1) with test variables initial [NaCl] (X1), initial [K_2HPO_4] (X2) and initial [$NaNO_3$] (X3) are shown in Figure 4.2 and Figure S 4.2. These figures clearly show that total biomass (g/l) after 16 days of growth is influenced by all three parameters. In figure 4.2A, biomass production (Y1) and initial NaCl concentration (X1), initial K_2HPO_4 concentration (X2) had a flat nature and an unclear elongated running diagonal, indicating a non-significant interactive effect on biomass production (Y1) between these two independent variables. As well, Figures 4.2B and 4.2C shows that there is very little interactive effect on

biomass production (Y1) between initial NaCl concentration (X1) and initial $NaNO_3$ concentration (X3), or initial $NaNO_3$ concentration and initial K_2HPO_4 concentration. Plots of residuals versus responses predicted by the model were randomly distributed around zero without any trends (not shown). This indicates good prediction of the response along with constant variance and adequacy of the quadratic models. In fact, this, taken together with the residual analysis, validates the model in general. On the other hand, analysis suggests that at a given fixed concentration of both K_2HPO_4 and $NaNO_3$, biomass yields are increased by higher initial concentrations of NaCl with a possible maximum outside the range of this initial study.

The three dimensional contour plots for the total amount of lipid produced by strain PCH90 after sixteen days of growth (Y_2) with test variables initial [NaCl] (X1), initial [K_2HPO_4] (X2) and initial [$NaNO_3$] (X3) are shown in Figure 4.3. Once again, variations in initial [NaCl] and initial [K_2HPO_4] appeared to have little overall effect and very little interactive effect on lipid production (Y_2) (Fig. 4.3A). On the other hand, initial [$NaNO_3$] strongly influenced final lipid yields when either [NaCl] or [K_2HPO_4] were varied (Figures 4.3B and 4.3C respectively). However, the response contour plots did not have clear maxima, suggesting that maximal lipid production (Y_2) lies outside the design boundaries chosen for this initial analysis. Indeed, analysis of the data obtained from the present range strongly suggests that increasing the concentration of NaCl at the lowest level of nitrate used here would lead to higher lipid production (Fig. 4.3B & Fig. S 4.3).

As generally acknowledged (Leite & Hallenbeck, 2012) and as can be ascertained from the results presented here (Figures 4.2 and 4.3), there is in general a trade-off between growth (biomass production) and lipid production. Of prime importance in developing biodiesel production from microalgae is the percent lipid content and therefore it is critical to understand the factor(s) that can influence this. In addition, this parameter integrates biomass and lipid production into a single parameter. When the RSM experiments are analyzed for the effects of variation of variables initial [NaCl], initial [K_2HPO_4], and initial [$NaNO_3$] on the percent lipid content (Fig. S 4.4 and 4.4) under conditions where the responses were maximum, three conclusions are apparent. At the lowest [$NaNO_3$] examined (0.83 mM), the

percent lipid can be increased by increasing both initial [NaCl] and initial [K_2HPO_4] (Figures S 4.3 and 4.4A). At the highest initial [K_2HPO_4] examined (60 μM), the percent lipid can be increased by decreasing the initial [$NaNO_3$] and increasing the initial [NaCl] (Fig. 4.4B). Finally, at the highest initial [NaCl], the percent lipid can be increased by increasing initial [K_2HPO_4] and decreasing initial [$NaNO_3$] (Fig. 4.4C). However, since both nitrate and phosphate are required for supporting maximum biomass production at a given percent lipid content, the easiest way to increase the percent lipid content without sacrificing total lipid production would be by increasing the initial [NaCl]. Therefore, future studies should examine the effect of NaCl levels higher than those examined in the present study.

3.3 Effect of different light intensities on biomass and lipid production by strain PCH90

Another important factor in microalgal lipid production is, of course, light intensity. Although we did not extensively study the effects of this parameter on growth and lipid production of strain PCH90, we compared the effects of lower (8 $W \cdot m^{-2}$) and higher (30 $W \cdot m^{-2}$) light intensities to what was found with the DOE experiments detailed above at the intermediate concentrations of NaCl (10.5 mM), $NaNO_3$ (11.5 mM), and K_2HPO_4 (32.5 μM) and a light intensity of 16 $W \cdot m^{-2}$. Under both conditions, production of both biomass and lipid were decreased with a biomass and lipid content at the lower light intensity that was only 12.2 and 12.8 % respectively of that at the intermediate light intensity (Fig. 4.5, Fig. S 4.5). Likewise, at the higher light intensity, both biomass and lipid were decreased with a biomass and lipid content at the lower light intensity that was only 49 and 25 % respectively of that at the intermediate light intensity.

3.4 Lipid and biomass productivities as a function of growth phase

Samples were taken for lipid and biomass analysis on days 2, 6, 10, 14, and 16, roughly corresponding to different points in the growth curve: day 2, lag phase; days 6 and 10, exponential phase; and finally, days 14 and 16, stationary phase. In general, maximum lipid productivity ($mg \cdot l^{-1} \cdot d^{-1}$) was obtained at stationary phase, days 14 to 16 whereas maximum

biomass productivity ($mg \cdot l^{-1} \cdot d^{-1}$) was found at the beginning of growth, days 0 to 6, when the cultures were in exponential growth (Fig. 4.6 & Fig. S 4.6). Nitrate concentration had a great influence on maximum biomass productivity with the highest growth rates being supported by intermediate (11.5 mM, runs 1, 2, 4, 13, 14 and 15) or high (22.1 mM, runs 8, 11 and 12) levels.

On the other hand, lipid productivity was highest at intermediate (11.5 mM, runs 2, 13, and 14) or low (0.85 mM, runs 6 and 10) levels of nitrate.

4.0 Conclusions

In the present study a novel microalgal isolate native to Quebec, PCH90, related to the *Chlorella* group, was shown to have a number of interesting characteristics, including the ability to grow at low temperatures (10 °C) on secondary effluent from a wastewater treatment plant. Under the appropriate conditions, partially defined here using DOE analysis to examine the effects of variation in NaCl, nitrate and phosphate concentrations, this strain also produces high concentrations of lipid, suggesting that it might be suitable for biofuel production. Future work on *Chlorella sp.* PCH90 could lead to improved lipid productivity and lipid content

5.0 Acknowledgements

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Table 4.1 Box-Behnken experimental design with three independent variables

Run	NaCl (mM)		K_2HPO_4 (µM)		$NaNO_3$ (mM)		BP (g/L)	LP (g/L)
	X_1	Code	X_2	Code	X_1	Code		
1	1.00	-1	5.00	-1	11.48	0	1.12	0.08
2	20.00	+1	5.00	-1	11.48	0	1.19	0.14
3	1.00	-1	60.00	+1	11.48	0	1.09	0.06
4	20.00	+1	60.00	+1	11.48	0	1.23	0.15
5	1.00	-1	32.50	0	0.85	-1	0.65	0.17
6	20.00	+1	32.50	0	0.85	-1	0.60	0.24
7	1.00	-1	32.50	0	22.1	+1	1.14	0.07
8	20.00	+1	32.50	0	22.1	+1	1.15	0.05
9	10.50	0	5.00	-1	0.85	-1	0.63	0.19
10	10.50	0	60.00	+1	0.85	-1	0.62	0.22
11	10.50	0	5.00	-1	22.1	+1	1.18	0.05
12	10.50	0	60.00	+1	22.1	+1	1.11	0.05
13[a]	10.50	0	32.50	0	11.48	0	1.18	0.12
14[a]	10.50	0	32.50	0	11.48	0	1.19	0.19
15a	10.50	0	32.50	0	11.48	0	1.17	0.11

a The center point was replicated three times

Table 4.2 Growth Characterisitics of PCH90

Medium	Final OD	Lipid (mg/l)	Dry wt (mg/l)	% lipid	Growth rate (μ day^{-1})
BBM 10	0.893	42	888	4.6	0.53
BBM 22	0.85	132	846	15.6	0.46
WW 10	0.42	89	322	27.6	0.71
WW 22	0.41	63	322	19.5	0.97

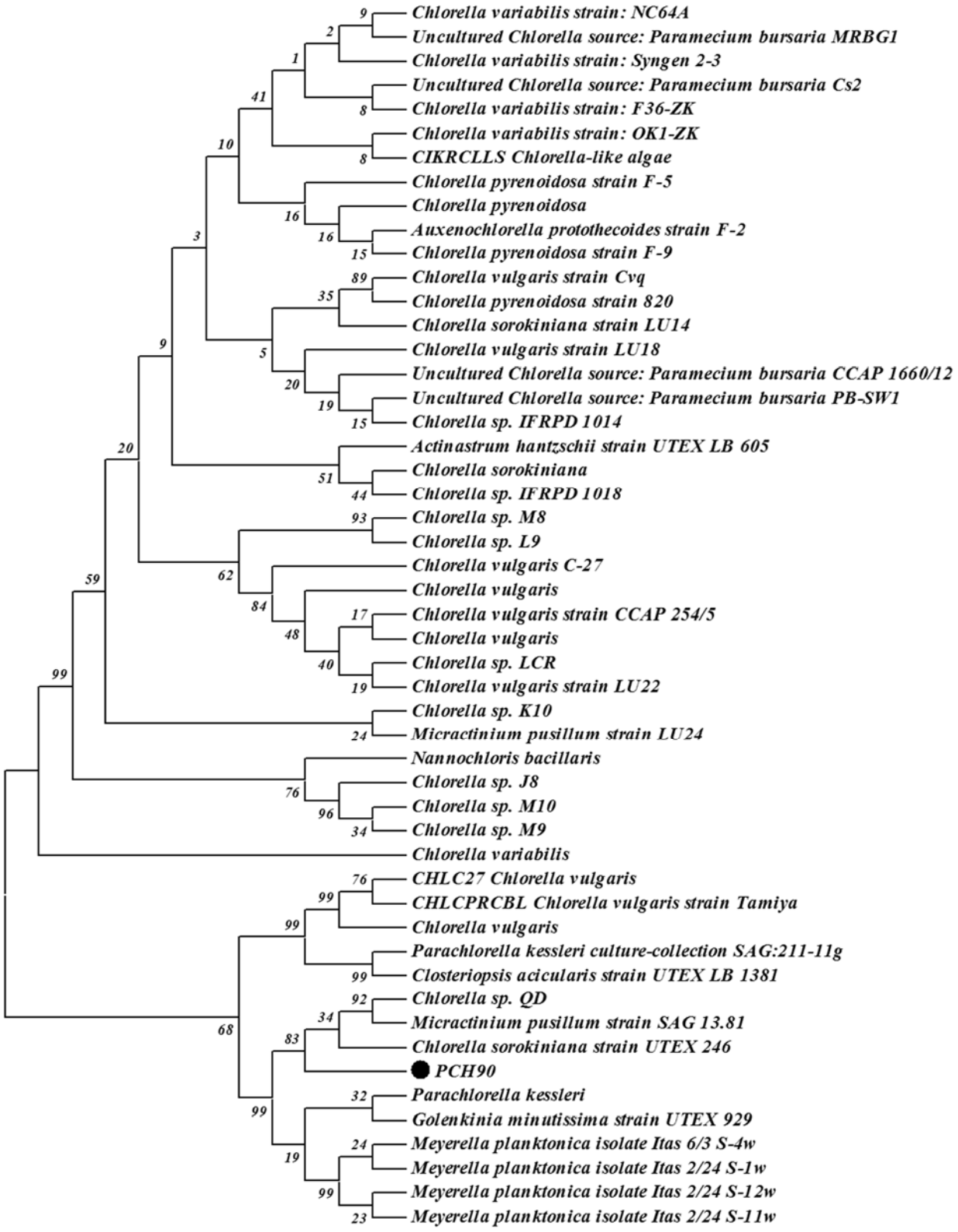
Chlorella variabilis strain: NC64A
Uncultured Chlorella source: Paramecium bursaria MRBG1
Chlorella variabilis strain: Syngen 2-3
Uncultured Chlorella source: Paramecium bursaria Cs2
Chlorella variabilis strain: F36-ZK
Chlorella variabilis strain: OK1-ZK
CIKRCLLS Chlorella-like algae
Chlorella pyrenoidosa strain F-5
Chlorella pyrenoidosa
Auxenochlorella protothecoides strain F-2
Chlorella pyrenoidosa strain F-9
Chlorella vulgaris strain Cvq
Chlorella pyrenoidosa strain 820
Chlorella sorokiniana strain LU14
Chlorella vulgaris strain LU18
Uncultured Chlorella source: Paramecium bursaria CCAP 1660/12
Uncultured Chlorella source: Paramecium bursaria PB-SW1
Chlorella sp. IFRPD 1014
Actinastrum hantzschii strain UTEX LB 605
Chlorella sorokiniana
Chlorella sp. IFRPD 1018
Chlorella sp. M8
Chlorella sp. L9
Chlorella vulgaris C-27
Chlorella vulgaris
Chlorella vulgaris strain CCAP 254/5
Chlorella vulgaris
Chlorella sp. LCR
Chlorella vulgaris strain LU22
Chlorella sp. K10
Micractinium pusillum strain LU24
Nannochloris bacillaris
Chlorella sp. J8
Chlorella sp. M10
Chlorella sp. M9
Chlorella variabilis
CHLC27 Chlorella vulgaris
CHLCPRCBL Chlorella vulgaris strain Tamiya
Chlorella vulgaris
Parachlorella kessleri culture-collection SAG:211-11g
Closteriopsis acicularis strain UTEX LB 1381
Chlorella sp. QD
Micractinium pusillum strain SAG 13.81
Chlorella sorokiniana strain UTEX 246
PCH90
Parachlorella kessleri
Golenkinia minutissima strain UTEX 929
Meyerella planktonica isolate Itas 6/3 S-4w
Meyerella planktonica isolate Itas 2/24 S-1w
Meyerella planktonica isolate Itas 2/24 S-12w
Meyerella planktonica isolate Itas 2/24 S-11w

Figure 4.1 Phylogenetic tree of PCH90 and related algal *rbcL* sequences.

The final phylogenetic tree presented here was developed as described in Materials and Methods through using MEGA5 with 1000 times bootstrap replication and a substitution model p-distance (substitution type amino acids, complete deletion of gaps/missing data treatments). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 2.76751904 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

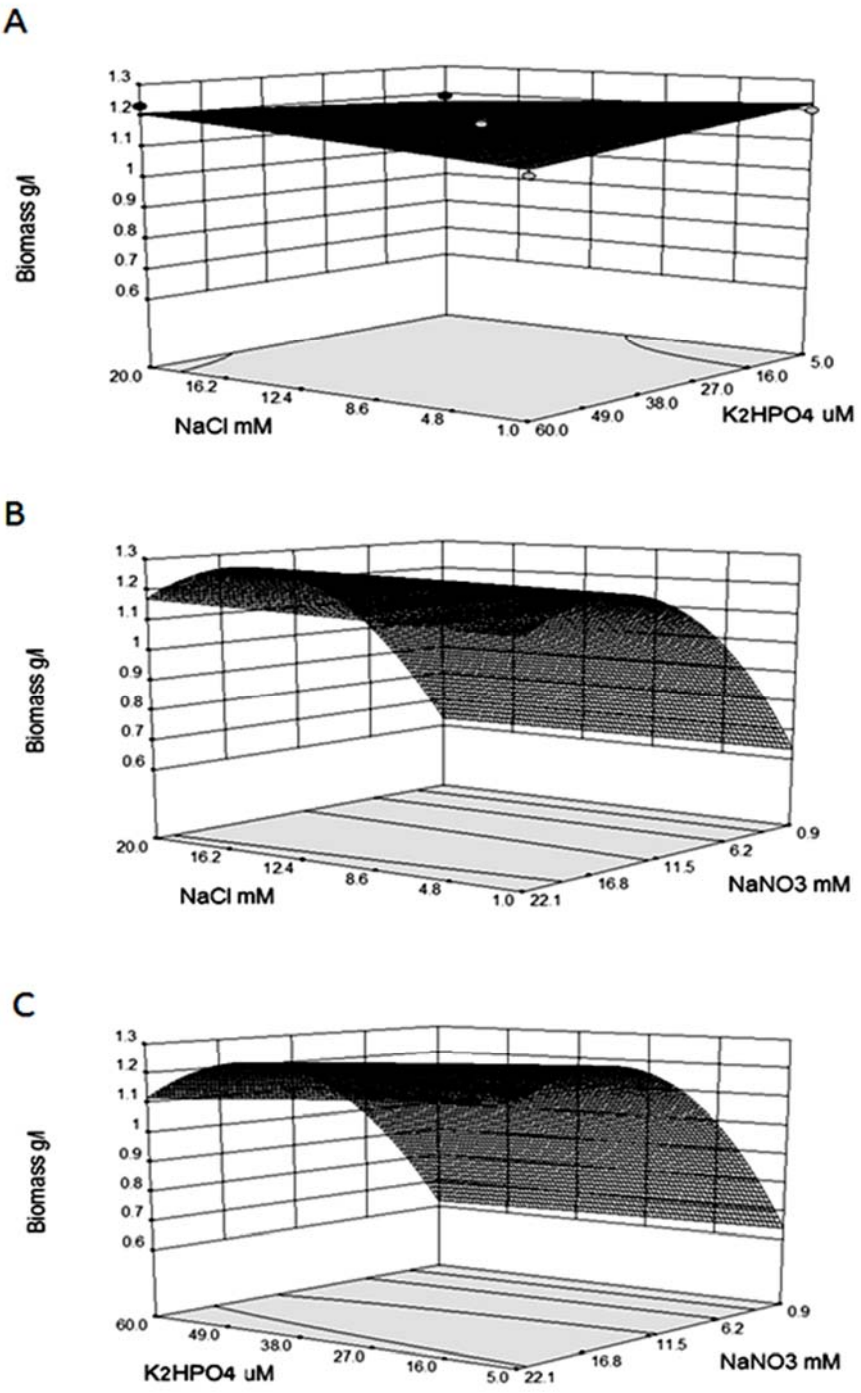

Figure 4.2 Three dimensional contour plots of the effects of variation in the concentrations of NaCl, $NaNO_3$ and K_2HPO_4 on biomass production.

Biomass production (g/l) by strain PCH90 after sixteen days of growth is shown as a function of different initial concentrations of: A) NaCl and K_2HPO_4 ([$NaNO_3$]=11.5 mM), B) NaCl and $NaNO_3$ ([K_2HPO_4]=32.5 μM), C) K_2HPO_4 and $NaNO_3$ ([NaCl]=10.5 mM).

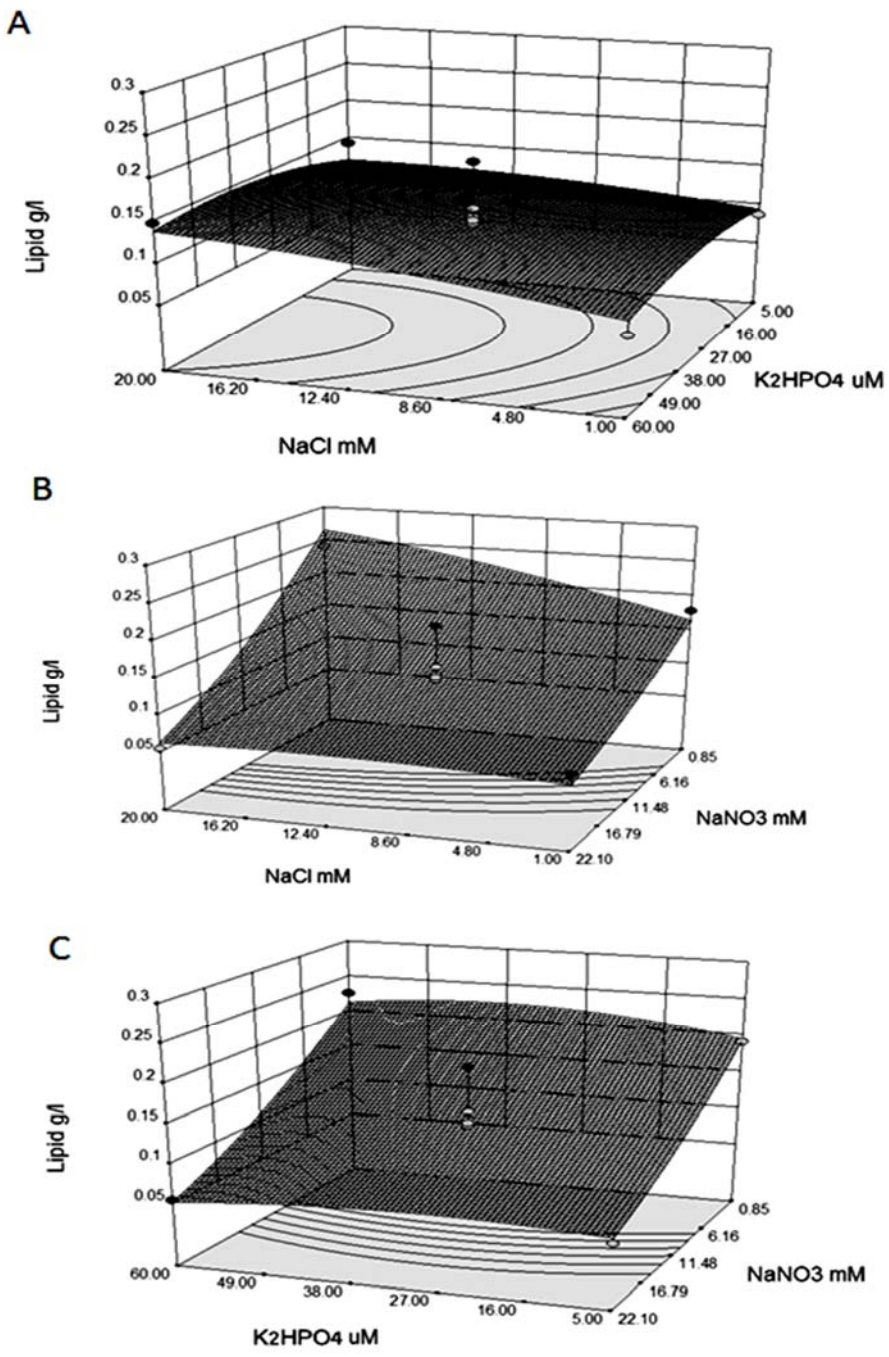

Figure 4.3 Three dimensional contour plots of the effects of variation in the concentrations of NaCl, $NaNO_3$ and K_2HPO_4 on lipid production.

Lipid production (g/l) by strain PCH90 after sixteen days of growth is shown as a function of different initial concentrations of: A) NaCl and K_2HPO_4 ([$NaNO_3$]=11.5 mM), B) NaCl and $NaNO_3$ ([K_2HPO_4]=32.5 μM), C) K_2HPO_4 and $NaNO_3$ ([NaCl]=10.5 mM).

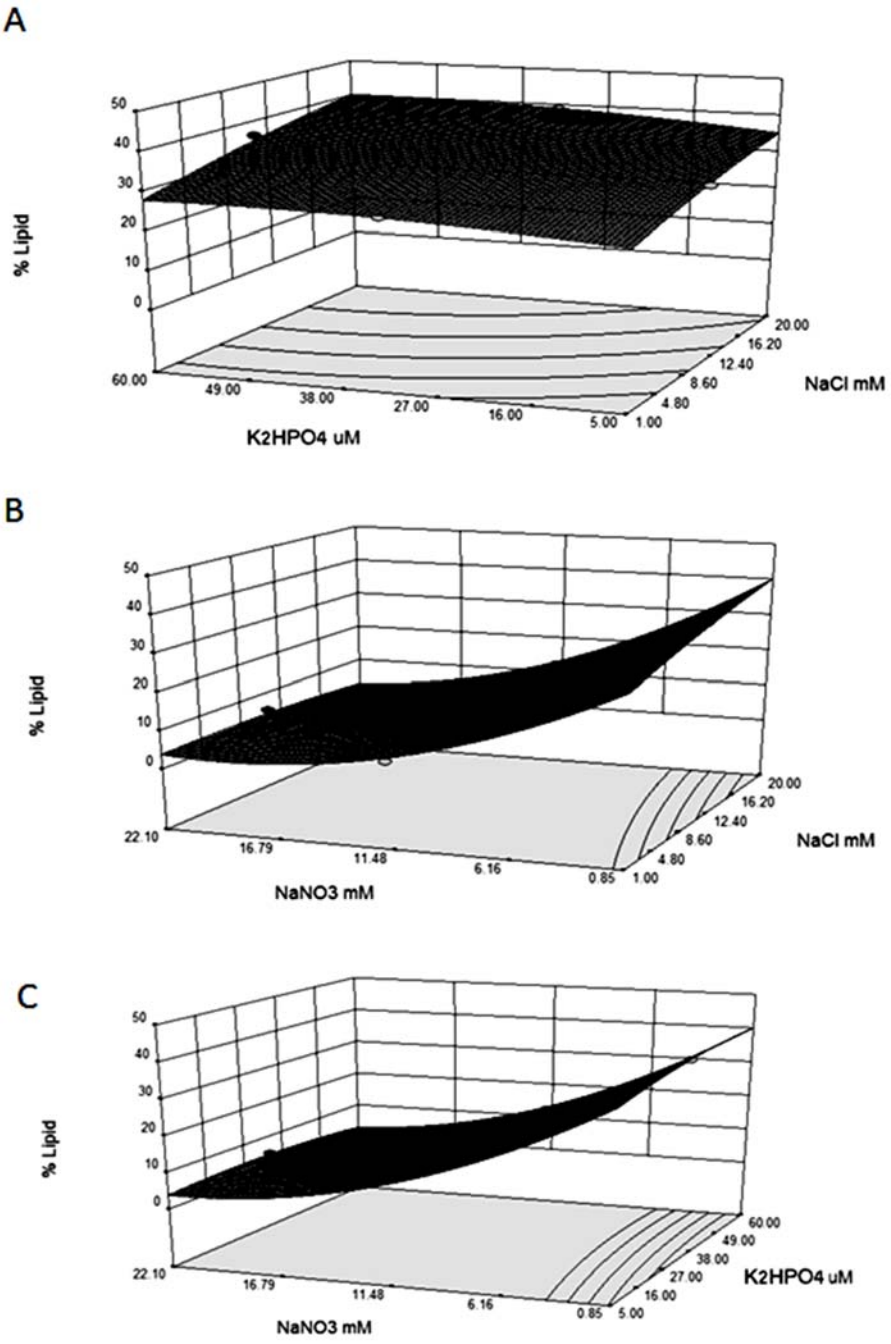

Figure 4.4 Three dimensional contour plots of the effects of variation in the concentrations of NaCl, $NaNO_3$ and K_2HPO_4 on the percent lipid content after sixteen days of growth.

Lipid content (%) of strain PCH90 after sixteen days of growth is shown as a function of different initial concentrations of: A) NaCl and K_2HPO_4 ([$NaNO_3$]=0.85 mM), B) NaCl and $NaNO_3$ ([K_2HPO_4]=60 μM), C) K_2HPO_4 and $NaNO_3$ ([NaCl]=20 mM).

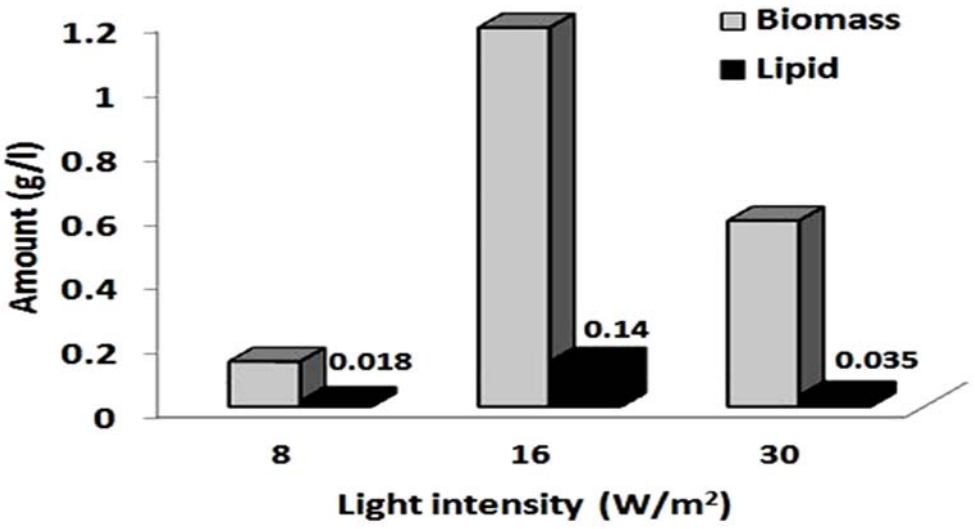

Figure 4.5 Effect of different light intensities on biomass and lipid production by strain PCH90.

Cultures were grown in small diameter 500 ml cylindrical transparent glass reactors (200 ml working volume) placed inside a water bath system equipped with a stirring unit and a thermostat for temperature control (22˚C) at the indicated light intensities.

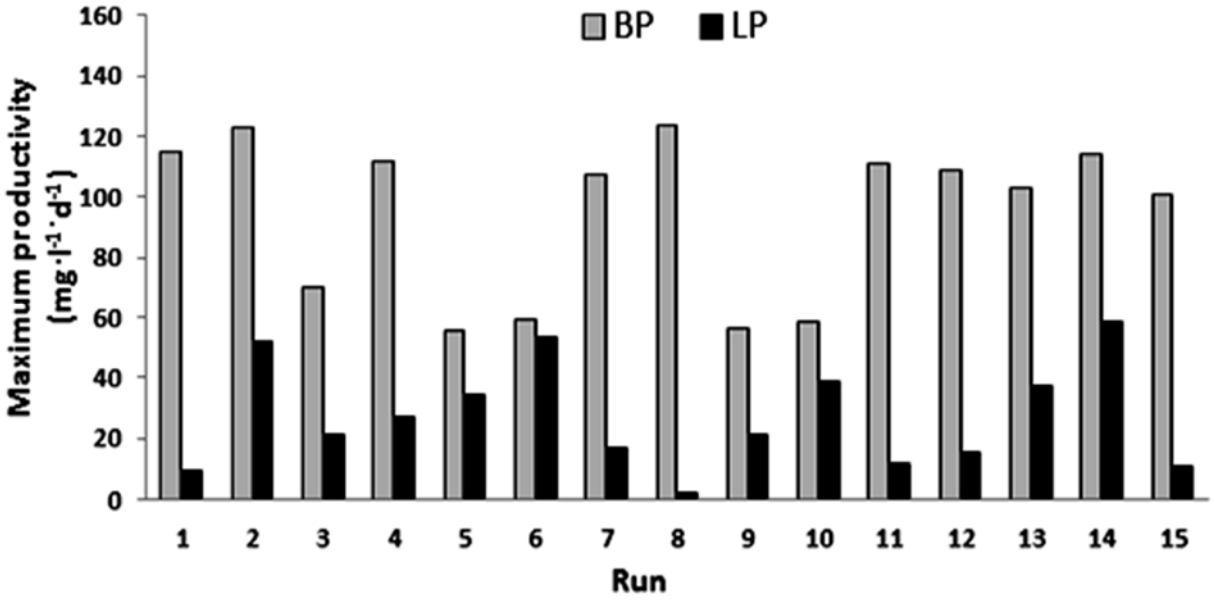

Figure 4.6 Maximum lipid and biomass productivities of strain PCH90 as a function of growth phase.

Maximum lipid and biomass productivities for the different experimental runs given in Table 4.1 were calculated according to changes occurring at the appropriate point in the growth phase using the analytical methods described in Materials and Methods. For lipid productivity, the difference in lipid amount between days 14 and 16 (stationary growth phase) were used. For biomass, the difference in dry weights between days 0 and 6 (exponential growth phase) were used.

Table S 4.1 ANOVA for Total Biomass by Chlorella sp. PCH90					
Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	**p-value**
Model	0.90	9	0.1	150.82	<0.0001
X_1	0.000903	1	0.000903	1.36	0.2825
X_2	0.00263	1	0.00263	3.94	0.0874
X_3	0.54	1	0.54	806.91	<0.0001
$X_1 X_2$	0.00533	1	0.00533	8.00	0.0255
$X_1 X_3$	0.000552	1	0.000552	0.83	0.3929
$X_2 X_3$	0.000506	1	0.000506	0.76	0.4123
X_1^2	0.0000136	1	0.0000136	0.020	0.8903
X_2^2	0.000022	1	0.000022	0.033	0.8601
X_3^2	0.35	1	0.35	531.59	<0.0001
Residual	0.00466	7	0.00066		
Lack of Fit	0.0035	3	0.00117	**4.05**	0.1050
Pure Error	0.00116	4	0.000289		
Cor Total	0.91	16			

The Model *F-value* of 150.82 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case X_3 [$NaNO_3$], X_1X_2 [NaCl][K_2HPO_4] and X_3^2 [$NaNO_3$]^2 are significant model terms. The "Lack of Fit F-value" of 4.05 implies the Lack of Fit is not significant relative to the pure error. There is a 10.5% chance that a "Lack of Fit *F-value*" this large could occur due to noise. The "Pred R-Squared" of 0.9363 is in reasonable agreement with the "Adj R-Squared" of 0.9883. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 30.224 indicates an adequate signal. The regression equation **3a** and **3b** show Total biomass (Y_1) as a function of the test variables X_1[NaCl], X_2 [K_2HPO_4] and X_3 [$NaNO_3$].

Equation 3a

$(Y_1)_{CODED}=1.18+0.011X_1-0.018X_2+0.26X_3+0.036X_1X_2+0.012X_1X_3-0.011X_2X_3-0.0018X_1^2-0.0023X_2^2-0.29X_3^2$

Equation 3b

$(Y_1)_{ACTUAL}=0.62-0.00434[NaCl]-0.00149[K_2HPO_4]+0.0834[NaNO3]+0.000140[NaCl][K_2HPO_4]+0.32[NaCl][NaNO3]-0.11[K_2HPO_4][NaNO3]-0.008[NaCl]^2-0.003[K_2HPO_4]^2-4.89[NaNO3]^2$

The first of these (**3a**) is the equation actually used in development of the response curves, thus is valid for the coded values, i.e.,-1, 0, 1, of the variables shown in Table 4.1. Actual values can be calculated from the second equations (**3b**).

Table S 4.2 ANOVA for Total Lipid by Chlorella sp. PCH90					
Source	**Sum of Squares**	**df**	**Mean Square**	***F-Value***	***p-value***
Model	0.13	9	0.014	**7.27**	0.0080
X_1	0.0089	1	0.0089	4.68	0.0673
X_2	0.000033	1	0.000033	0.018	0.8980
X_3	0.11	1	0.045	53.05	0.0080
X_1X_2	0.018	1	0.018	0.12	0.7453
X_1 X_3	0.099	1	0.099	2.39	0.1830
X_2 X_3	0.04	1	0.04	0.27	0.6285
X12	0.118	1	0.118	0.77	0.4193
X22	0.06	1	0.06	1.46	0.2805
X32	0.055	1	0.055	0.37	0.5717
Residual	0.013	7	0.0019		
Lack of Fit	0.00545	3	0.082	**0.92**	0.5084
Pure Error	0.0079	4	0.07		
Cor. Total	0.14	16			

The Model F-value of 7.27 implies the model is significant. There is only a 0.8% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case X_3 ($NaNO_3$ concentration) is borderline in terms of significance (Values greater than 0.1 indicate the model terms are not significant.) The "Pred R-Squared" of 0.51 is in reasonable agreement with the "Adj R-Squared" of 0.79. This may an indicative of partial block effect. The ‘‘Lack of fit F-value” of 0.92 implies that the lack of fit is not significant relative to the pure error. There is an 50.84% chance that a ‘‘Lack of fit *F-value*” this large could occur due to noise" Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 9.316 indicates an adequate signal. Thus, the following regression equations, analogous to the **Equation 4a** and **Equation 4b** shows the relative lipid productivity (Y_1) as a function of the test variables X_1 (NaCl concentration), X_2 (K_2HPO_4 concentration) and X_3 ($NaNO_3$ concentration).

Equation 4a

$(Y_2)_{CODED} = 0.37 + 0.033X_1 + 0.00205X_2 - 0.12X_3 + 0.012X_1X_2 - 0.00908X_1X_3 - 0.013X_2X_3 - 0.013X_1^2 - 0.024X_2^2 + 0.00418X_3^2$

Equation 4b

$(Y_2)_{ACTUAL} = 0.386 + 0.00825\ [NaCl] + 0.00198[K_2HPO_4] - 0.0079[NaNO3] + 0.0000454[NaCl][K_2HPO_4] - 0.000278[NaCl]\ [NaNO3] - 0.000031[K_2HPO_4]\ [NaNO3] - 0.000144[NaCl]^2 - 0.0000311[K_2HPO_4]^2 + 0.000037\ [NaNO3]^2$

The first of these (4a) is the equation actually used in development of the response curves, thus is valid for the coded values, i.e.,-1, 0, 1, of the variables shown in Table 4.1**.** Actual values can be calculated from the second equations (4b).

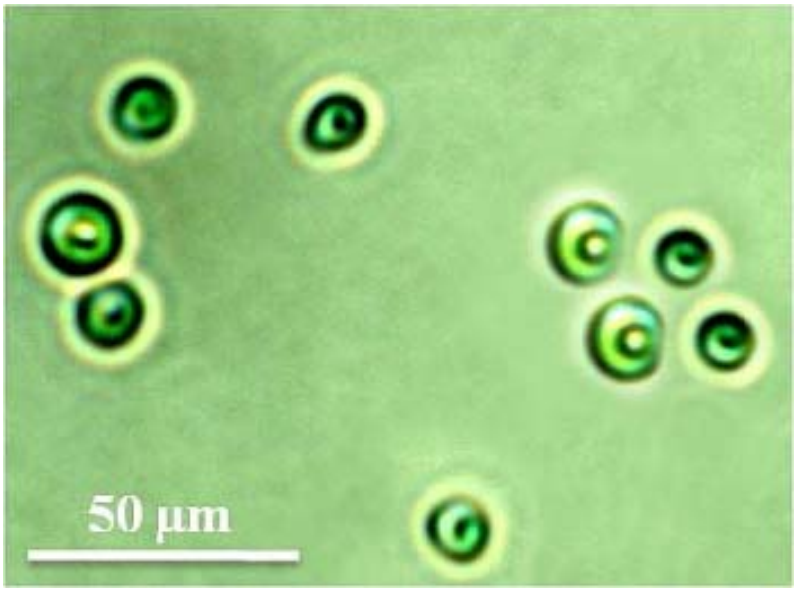

Figure S 4.1 Photomicrograph of strain PCH90 taken with an optical microscope at 100X.

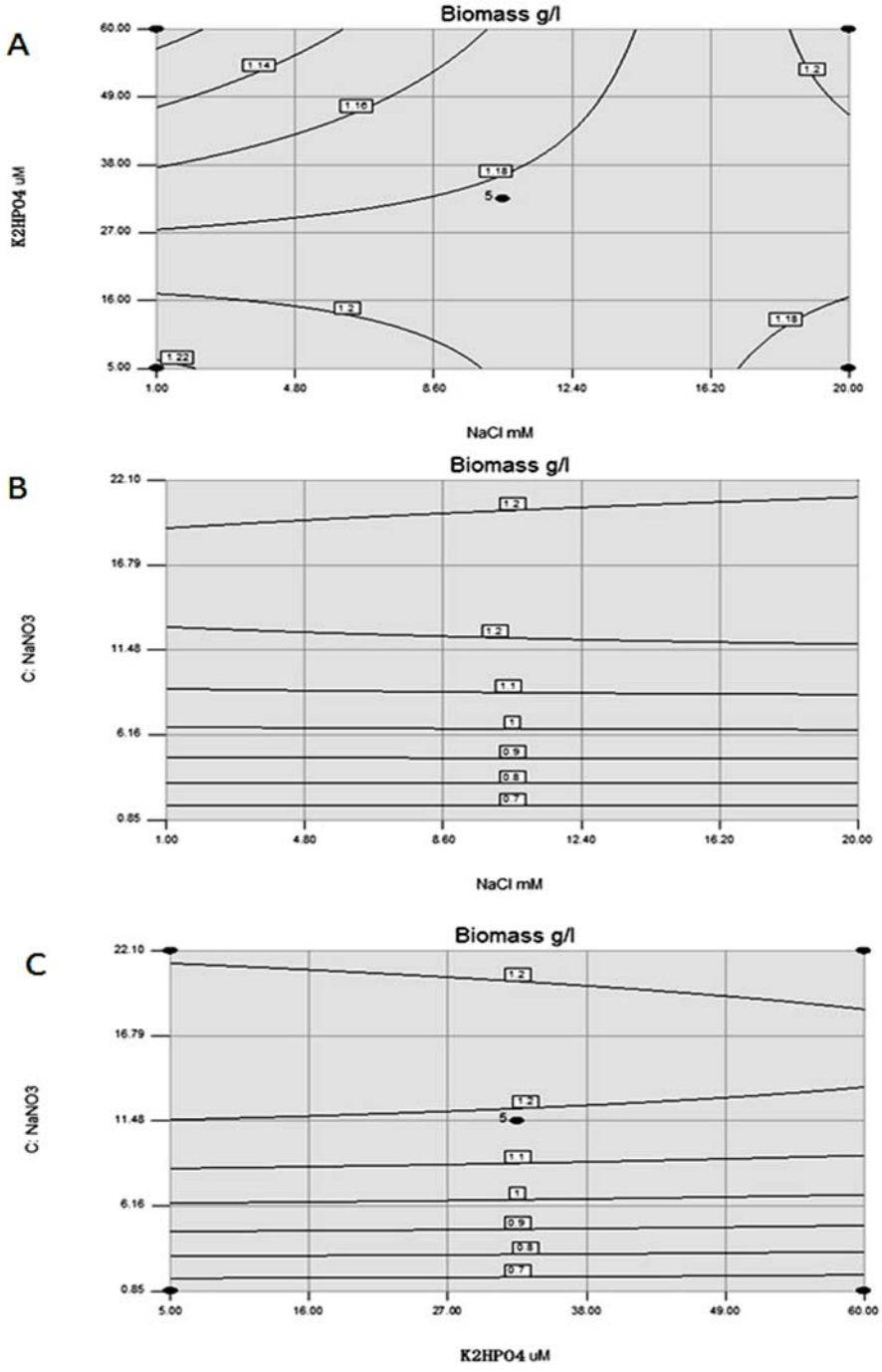

Figure S 4.2 Two dimensional contour plots of the effects of variation in the concentrations of NaCl, NaNO3 and K_2HPO_4 on biomass production.

Biomass production (g/l) by strain PCH90 after sixteen days of growth is shown as a function of different initial concentrations of: A) NaCl and K_2HPO_4 ([NaNO3]=11.5 mM), B) NaCl and $NaNO_3$ ([K_2HPO_4]=32.5 μM), C) K_2HPO_4 and $NaNO_3$ ([NaCl]=10.5 mM).

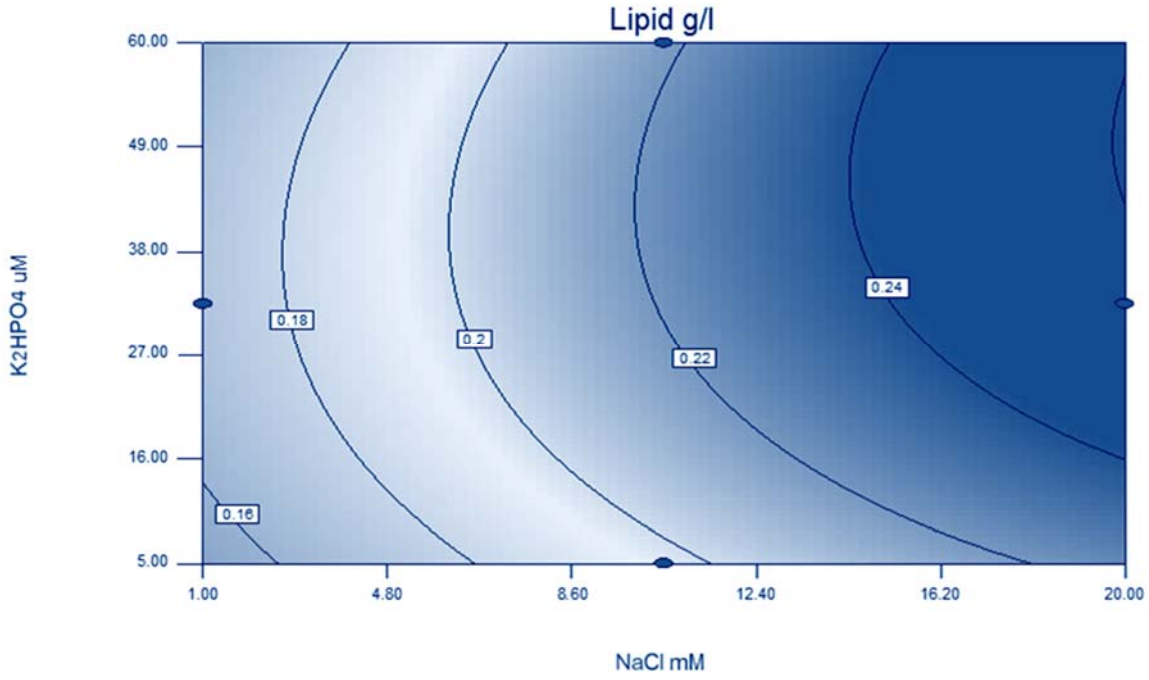

Figure S 4.3 Two dimensional contour plot showing the effect of different concentrations of NaCl on lipid content of PCH90 at the lowest [$NaNO_3$] tested.

The lipid content (g/l) of PCH90 is shown after sixteen days of growth at varying [NaCl] and [K_2HPO_4] conditions. [$NaNO_3$] was constant at 0.83 mM.

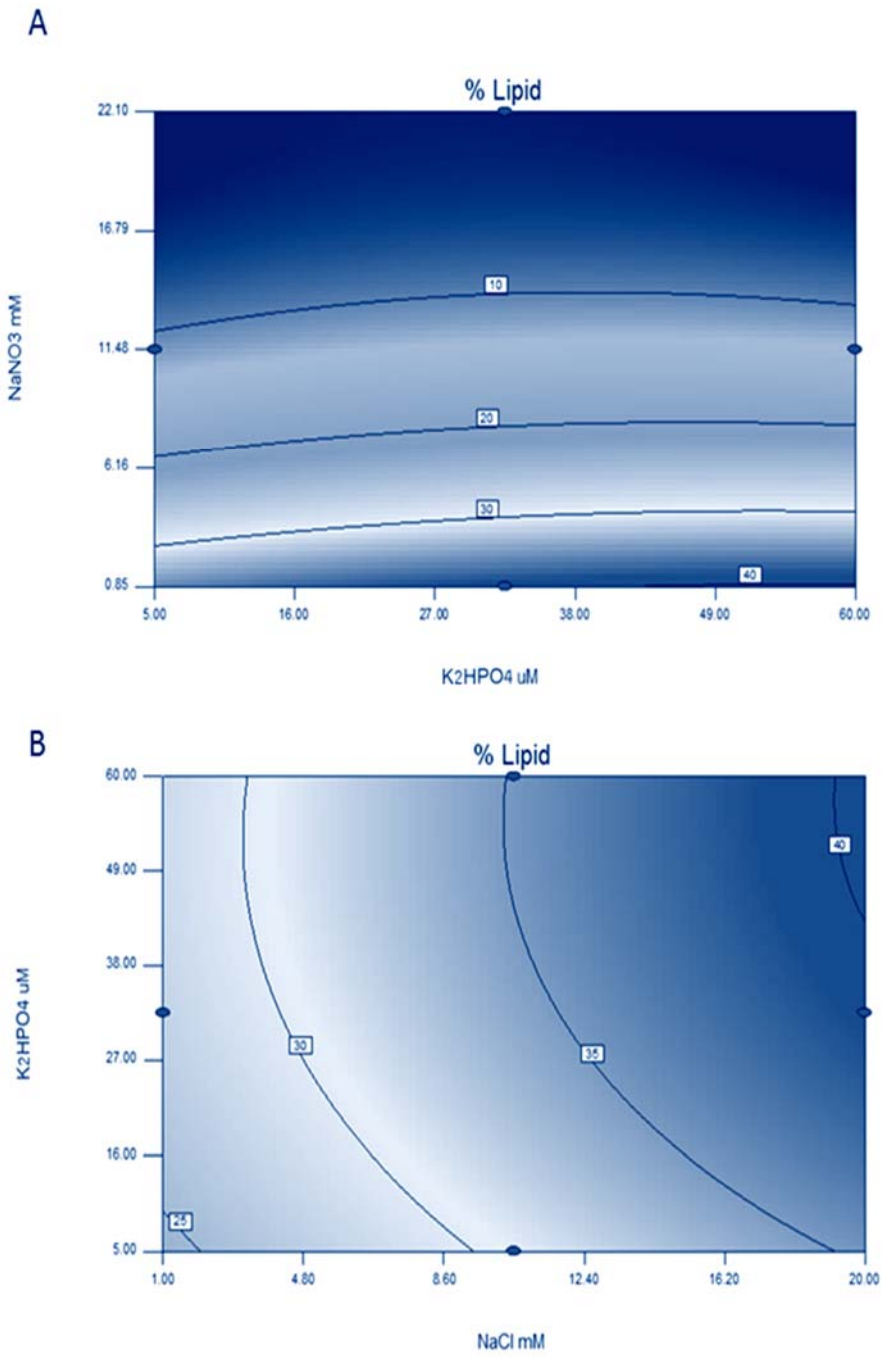

Figure S 4.4 Two dimensional contour plots of the effects of variation in the concentrations of NaCl, $NaNO_3$ and K_2HPO_4 on the percent lipid of strain PCH90.

The lipid content (percent) of strain PCH90 after sixteen days of growth is shown as a function of different initial concentrations of: A) $NaNO_3$ and K_2HPO_4 ([NaCl]=20 mM) and B) NaCl and K_2HPO_4 ([NaNO3]=0.85 mM)

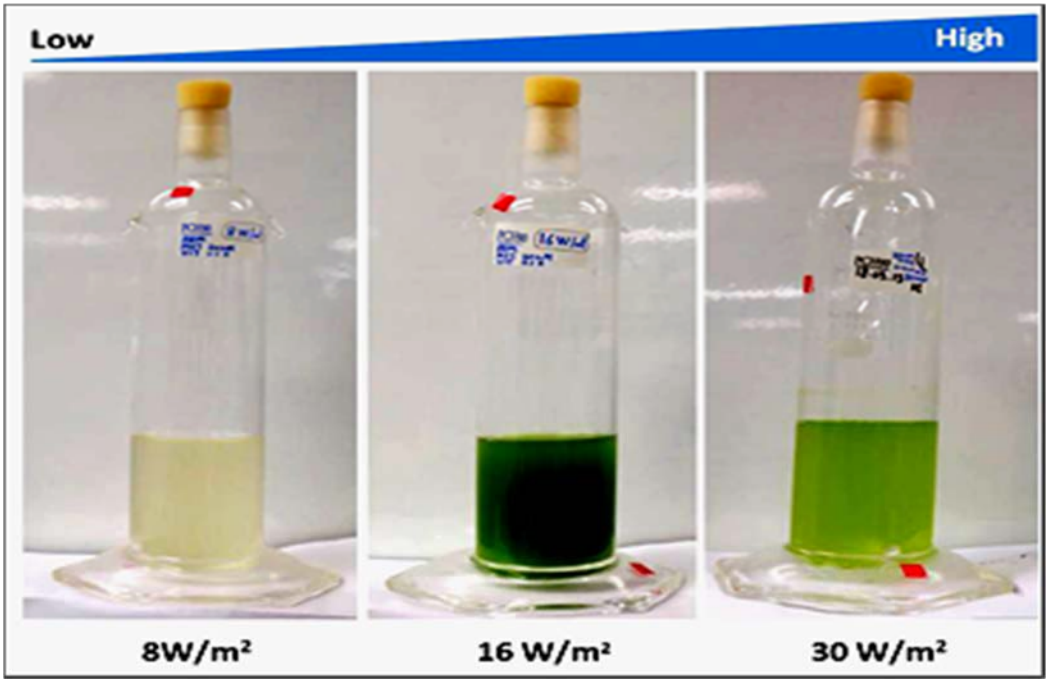

Figure S 4.5 Effect of different light intensities on biomass and lipid production by strain PCH90.

Photograph of cultures were grown in small diameter 500 ml cylindrical transparent glass reactors (200 ml working volume) placed inside a water bath system equipped with a stirring unit and a thermostat for temperature control (22˚C) at the indicated light intensities.

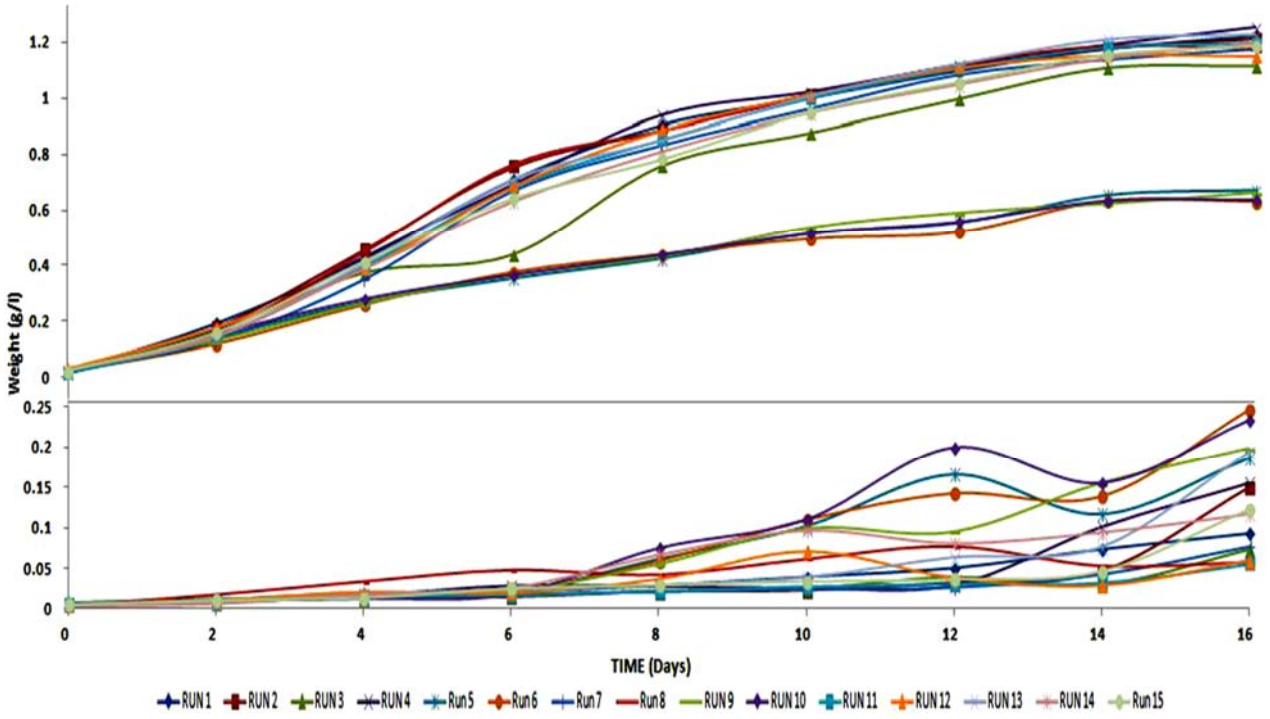

Figure S 4.6 Growth and lipid accumulation of strain PCH90 over time.

Growth (above) and lipid accumulation (below). The figure shows that maximum biomass production was in the exponential phase (from day0 up to day 6) while the lipid accumulation increased in the stationary phase (from day 14- day 16).

Discussions

The overall objective of this research was ultimately to assess the potential of naturally occurring highly diverse species of algae derived from local habitats in Quebec for their potential as a biofuel feedstock. This was accomplished by first isolating and purifying algae from five different locations around Montréal, followed by analysis of their growth patterns under different media (Municipal wastewater and BBM synthetic media) and temperature conditions along with assessing the growth and lipid yield under these varying growth parameters.

The needs for renewable and sustainable energy sources like biodiesel increased significantly due to the increasing concerns related to the long term availability and prices of the non renewable sources of energy due to the large utilization of fossil fuels coupled with concerns over atmospheric greenhouse gas levels such as CO_2. Algae introduce a promising feedstock for production of such biofuels and help to mitigate the reduction in CO_2 concentration in the atmosphere. They can be grown on marginal lands using poor quality water (e.g. wastewater) in addition, they do not compete with, and potentially produce more biomass and lipid than, the conventional agricultural resources required for food production.

Biodiesel is a clean renewable and biodegradable fuel source. Current study has focused on screening native Québec algal strains as potential source of biofuel coupled with their potential in wastewater treatment. The commercial success of using microalgae for biofuel production is mainly depends on identification and selection of high lipid producing microalgae. A strain which produce high lipid content, able to respond to accumulating lipids under nutrient deficiency and well adapted to unavoidable changes in the environment like pH, temperature and light intensity would be an ideal candidate for growing on a large scale for biofuel production.

The discharge of wastewater that contains excess amounts of N and P in the water streams can lead to downstream eutrophication, ecosystem damage, and affect human health. Chemical and/or physical-based technologies used to remove these nutrients are expensive tools as they often consume high amounts of energy and chemicals, which increase the treatment costs. Algae introduce a treatment promising solution as they are capable of removing these pollutants and produce biomass that can be harvested and used as a feedstock for biofuel production.

Microalgae are high diverse group of photosynthetic microorganisms that exist in almost all kind of environments including fresh, brackish and marine water. In recent years, microalgae have attracted much attention for biofuel production, CO_2 fixation and wastewater treatment. They are photosynthetically convert CO_2 and nutrients in the presence of light into biomass, and at much higher rates than the traditional oil-producing crops. Biofuel production by microalgae can be made more sustainable through coupling microalgal biomass production with wastewater treatment.

Chapter Three in this thesis demonstrates a study of high throughput microalgae screening method, using 12 well microplates, for hundred Québec native microalgae isolates. The method included screening for their growth potential in wastewater and biofuel production at two different temperature conditions and results were compared with their growth in the synthetic medium. Highly efficient strains in producing high lipid content were selected. Several isolates carried out the nearly complete removal of nitrate and phosphorus from municipal wastewaters and had a lipid content and productivity as high as 45% and 29 mg l^{-1} d^{-1}, respectively. Interestingly, a number of strains showed good growth at low temperature (10 °C) and might be useful in a waste-to-biofuel that would provide wastewater treatment and lipid production.

The isolated strains were tested for their potential to produce biodiesel through estimating the neutral lipid content that present in their cells. Relatively simple, rapid and less time consuming methods were used to screen this high number of strains for the presence of lipids.

The strains were screened after 14th days of growth using the Nile red method. Previous studies showed that lipid accumulations typically increased at the end of the logarithmic phase and early in the stationary phase, for example an increase in lipid yield was observed in the marine dinoflagellate *Gymnodium sp.,* from 1.3 mg L^{-1} during the logarithmic phase to 30.1 mg L^{-1} during the stationary phase, in the same manner, *C.vulgaris* reached the highest lipid content of 56% by day 12 (Mansour et al. 2003; Chen et al. 2009). Therefore, we chose to measure the amount of neutral lipids on the 15th day assuming that the cultures would have reached the stationary phase. TAGs (neutral lipids) are primarily serving as a storage form of carbon and energy and are the important substrate of interest for biodiesel production therefore it was important to measure the amount of TAGs in the microlagal cells to determine the potential strains (Illman et al., 2000). TAGs accumulation in the microalgal cells increases as the age of the cultures increases or when cells undergo stressful conditions as because the cells shift their lipid metabolism from membrane lipid synthesis into storage of neutral lipid (Smith et al., 1995; Hu et al., 2008)

Temperature is an important environmental parameter for algal growth. The province of Québec is distinguished by low temperature conditions for long period of the year giving the native flora the well adaptability to tolerate and grow in this certain conditions. Temperatures range between 15℃ –25℃ is usually considered optimal for algae growth and lower temperatures result in decreased the growth rates however, these temperature specific effects are most likely different from species to species (Goldman and Carpenter, 1974) Although, it is expected that nutrient uptake and photosynthesis might be lower at low temperatures, the use of algal strains that are native and adapted to cold climates as previously indicated, might still achieve higher growth rates, lipid production and nutrient treatment goals (Powell et al., 2008). *Nitzschia paleacea* grew at 10˚C and gave its highest lipid content compared to *N. closterium* that didn't grow outside the temperature range of 20–30˚C (Renaud et al., 1995). *Trachydiscus minutes* showed its maximum lipid content (35% w/w) when grown at 15˚C (Gigova et al., 2012). *Ettlia oleoabundans* highest biomass concentration of 2.2 g l^{-1} was achieved at 10˚C (and at light intensity of 70 μmol m^{-2} s^{-1}) and that was twice of cultures grown at 15˚C or 25˚C (Ying et al., 2013). Recently, a mature culture of *Heterococcus* DN1,

novel yellow green cold tolerant species recovered from snowfields in Colorado, USA, revealed to have composed of 55±3% lipids by dry weight (Nelson et al., 2013). The study gave a promise for the cold climates native algae to be a strong candidate for biofuel production. Several studies demonstrated that there is an interactive effect between temperature and light, for examples, *Chlorella vulgaris* showed successful growth at 5˚C under excess of light, with lower chlorophyll content, compared to the cultures grown at 27˚C where cells adjusted their photosynthetic apparatus via the excitation pressure on the photosystem II which caused by the excess of light (Denis et al., 1994). Temperature and light intensity appeared to work interactively where the effect of either one of them on the algal growth rate is strongly influenced by the level of the other (Halldal & French, 1958; Raven & Geider, 1988). To this end, the use of strong light intensity (40 Wm^{-2}) was assumed to be helpful and favourable for cultivation of our strains.

Our results clearly showed that several strains were rapidly and effectively able to take up and remove >95 % of the nutrients from the municipal wastewater under all the different experimental growth conditions, more interestingly, in the low temperatures cultures. In addition to reclamation of municipal wastewater, algae help to mitigate CO2 sequestration, and produce biofuel production. Several isolates were able to produce lipid yield of more than 45% of dry weight biomass in both of the temperature conditions. In terms of growth, 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 ºC 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 ºC of > 1 day^{-1}. As far as we are aware no comparable studies have been done at low temperatures like this.

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.

To this end, in Chapter Four, we have chosen one of the high lipid producing selected isolates, PCH90, related to the *Chlorella* group, to setup the effect of three different nutrient stress conditions including the effect of nitrogen, phosphorus and salt in biomass and lipid production. This strain was used for further studies as it shown to have a number of interesting characteristics, including the ability to grow at low temperatures (10 °C) on secondary effluent from a wastewater treatment plant. The strain was identified using the genetic tools and phylogenetic analysis that was performed on a concatenated data set obtained from a NCBI BLAST search using the translated *rbcL* nucleotide sequence of PCH90.

Previous studies on the optimization of lipid production by *Chlorella* have relied on the "single-factor-at-a-time" approach which is time-consuming and incapable of reaching the true optimum since potential interactions among process variables are ignored. Therefore, Response surface methodology (RSM), an experimental design based on statistical modeling was chosen and used to evaluate the interactions between a set of independent experimental factors and observed responses, while at the same time reducing the number of experiments required to determine the optimal condition, can be used for multiprocess parameter optimization, especially where the intermediate mechanisms are not known. Two and three dimensional contour plots were able to explain the interactive effects between the three initial variables concentrations (N, P and NaCl) in the biomass and lipid production of the *Chlorella* PCH90 strain.

Under the appropriate conditions using DOE analysis to examine the effects of variation in NaCl, nitrate and phosphate concentrations, and this strain was found to produce high concentrations of lipid content of up to 36% of dry weight compared to only 16% at nutrient sufficient cultivation conditions, suggesting that it might be suitable for biofuel production. In particular, Nitrogen, phosphorus depletion, and salt sufficiency were found to maximize lipid accumulation in *Chlorella sp.* PCH90, ensuring that nutrient limitations induce the microalgae metabolic pathways towards more lipid storage and accumulations.

Conclusions and Future prospectives

This research investigated the potential of hundred native microalgae strains, isolated from five different areas around Montreal, for producing biodiesel and wastewater treatment at two different temperature conditions (10°C and 22°C). The use of Nile red stain and cultivation in 12 wells plates provided a high throughput, rapid and reliable method for quantification of the neutral lipids and growth pattern in these native microalgae. The method is distinguished by requiring very low quantities of samples and is rapidly screens large number of strains. Our bioprospecting procedures involved collecting algae samples from local water habitats, then screening and acclimation using different culture media and temperature conditions resulted in a number of algal strains having high biomass and lipid productivities and high wastewater nutrient removal efficiency. Out of the 100 isolates screened, there were at least about five strains in each condition which showed substantially a high lipid accumulations and potential ability to remove more than 90% of P and N from the used media. Interestingly, strains such as MA1A3 and LB2H5 were able to produce up to 30-45% lipid content and remove >95 % of nutrient from the municipal wastewater medium at low temperatures. These strains are promising well adapted Québec native algal species that can be used for biodiesel production and wastewater treatment.

Oil-accumulating fast-growing microalgae have the potential to enable large-scale biodiesel production without competing for arable land or effecting on the food prices or availability. Under optimal growth conditions algae tend to produce large amounts of biomass, but with relatively low lipid contents. Attempts to improve the algal lipid productivity can be performed through the induction of lipid biosynthesis (for example by environmental stresses). Lipids (mainly in the form of triacylglycerides) are storage cell components that enable microalgae to endure the adverse environmental conditions. Both algal biomass and lipid synthesis are competing for photosynthetic assimilate and manipulations in the cell metabolic pathways or by genetic tools are typically required to stimulate lipid biosynthesis. To this end, *Chlorella sp.* PCH90 was selected and subjected to further studies to improve their lipid production via examining the interactive effects of initial concentrations of three selected

variables; nitrogen, phosphorus and NaCl using RSM. It has been revealed that nitrogen and phosphorus limitations were able to increase the lipid content of *chlorella* PCH90 to up to 36% of dry weight compared to 16% lipid content under the optimum growth conditions.

The algae-based fuel science is a very complex technology which requires continuing efforts and investments towards enhancing the biofuel economy, commercial use and production. A considerable investment is still needed by R&D in technological, methodological and technical development to close the gap between micro-algae derived fuels and other fuel sources.

The isolation and characterization of well adapted native microalgae from different kinds of environments should be a continuing effort. The algal strains that were examined for lipid production only represent a small portion of the over 40,000 identified species that are available in nature. Thereby more research on additional organisms is required. The continuing research investigations will provide novel insights into the unique mechanisms and pathways that algae use for more lipid production. Additionally, new approaches of biomass harvesting technology and lipid extraction methods from algal is required to economize the algal production related costs and this still represents a major task. More importantly, the use of genetic manipulation through metabolic engineering represents another promising strategy for more efficient strains for algal oil. A complete understanding of the mechanisms that control the relationship between the cell cycle and lipid production (mainly TAG) will allow genetic manipulation of the rapidly growing and high TAG accumulation selected algal strain simultaneously to ensure highest sustainable biodiesel production.

It is recommended that the lipid extracted from the selected high lipid producing strains should further be subjected to GC-MS analysis for the determination of FAME and biodiesel yield and quality.

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