7-14-2014 12:00 AM

# Study of high pressure steaming on lipid recovery from microalgae

Ana-Maria Aguirre, *The University of Western Ontario*

Supervisor: Dr. Amarjeet Bassi, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Chemical and Biochemical Engineering

STUDY OF HIGH PRESSURE STEAMING ON LIPID RECOVERY FROM MICROALGAE

(Format: Integrated Article)

by

Ana Maria Aguirre-Cardona

Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

**Abstract**

Sustainable and clean fuels are in demand due to the perceived negative effects on health and environment with current use of fossil fuels. Lipids from microalgae offer a potential approach to obtain sustainable biofuels. In this study a two step process was adopted: investigation of culture conditions to find optimal points for lipid productivity and cellulose content, followed by an investigation of microalgae disruption for lipid recovery.

In the first phase of the research the effect of culture conditions on *Chlorella vulgaris* biomass concentration and the ratio of lipid productivity/cellulose content were studied. Response surface methodology was applied to optimize the culture conditions. The response model for biomass concentration led to a predicted maximum of 1.12 g dw $L^{-1}$ when carbon dioxide and sodium nitrate concentrations were 2.33% $vv^{-1}$ and 5.77 mM, respectively. For lipid productivity/cellulose content ratio the maximum predicted value was 0.46 (mg lipid $L^{-1}d^{-1}$)(mg cellulose mg biomass$^{-1}$)$^{-1}$ when carbon dioxide concentration was 4.02% $vv^{-1}$ and sodium nitrate concentration was 3.21 mM. Also a common optimum point for both models was also found.

For the second phase of the study, the optimized *Chlorella vulgaris* microalgae obtained in the first phase was subjected to high pressure steaming as a hydrothermal treatment for recovery of bio-crude, and analysis by empirical modeling allowed finding operating points in terms of target temperature and microalgae concentration for high bio-crude and glucose yields. Within the range covered by these experiments the best conditions for high bio-crude yield were temperatures higher than 174˚C and low biomass concentrations (<5 g/L). For high glucose yield there were two suitable operating ranges, either low temperatures (<105˚C) and low biomass concentrations (<4 g/L); or low temperatures (<105˚C) and high biomass concentrations (<110 g/L).

To finalize this study, microalgae with different lipid and cellulose content was used to calculate the bio-crude recovery efficiency applying high pressure steaming. This thermal

treatment allowed extracting 97.94±8.26% of the total lipids. The biomass with the highest cellulose content was later subjected to high pressure steaming as a pre-treatment for glucose production via enzymatic hydrolysis, and the glucose yield for this process was 0.28 $g.g_{biomass}^{-1}$.

**Keywords**

High pressure steaming, biofuels, cellulose, microalgae, lipid extraction, enzymatic hydrolysis, response surface methodology.

**Co-Authorship Statement**

In the development of this work four papers were written and coauthored, the extent of the collaboration of the co-authors is stated below.

**Chapter 2**

| | |
|---|---|
| **Paper title** | Engineering challenges in biodiesel production from microalgae. |
| **Current status** | Published in Critical Reviews in Biotechnology, September 2013, Vol. 33, No. 3, pages 293-308. |
| **Ana Maria Aguirre:** Technical and theoretical advisor, literature review, writing and corrections of several drafts and final paper.<br>**Priyanka Saxena:** Technical and theoretical advisor, literature review, writing and corrections of several drafts and final paper.<br>**Amarjeet Bassi:** Corrections of several drafts and final paper. | |

**Chapter 3**

| | |
|---|---|
| **Paper title** | Investigation of Biomass Concentration, Lipid Production, and Cellulose Content in *Chlorella vulgaris* Cultures Using Response Surface Methodology. |
| **Current status** | Published in Biotechnology and Bioengineering, August 2013, Vol. 110, Issue 8, pages 2114-2122. |
| **Ana Maria Aguirre:** Experimental design, laboratory work, analysis of results, and paper writing.<br>**Amarjeet Bassi:** Technical and theoretical advisor and corrections of several drafts and final paper. | |

**Chapter 4**

| | |
|---|---|
| **Paper title** | Investigation of High Pressure Steaming as a thermal treatment for lipid extraction from *Chlorella vulgaris*. |

<table>
<tr><td>Current status</td><td>Accepted for publication in Bioresource technology, April 2014.</td></tr>
<tr><td colspan="2">Ana Maria Aguirre: Experimental design, laboratory work, analysis of results, and paper writing.<br>Amarjeet Bassi: Technical and theoretical advisor and corrections of several drafts and final paper.</td></tr>
</table>

**Chapter 5**

<table>
<tr><td>Paper title</td><td>Investigation of an integrated approach using high pressure steaming and enzymatic hydrolysis for glucose and bio-crude recovery from microalgae.</td></tr>
<tr><td>Current status</td><td>This paper is ready for submission to Industrial and engineering chemistry research.</td></tr>
<tr><td colspan="2">Ana Maria Aguirre: Experimental design, laboratory work, analysis of results, and paper writing.<br>Amarjeet Bassi: Technical and theoretical advisor and corrections of several drafts and final paper.</td></tr>
</table>

**Dedication**

To my mother Carmen Amanda Cardona and my father Jaime Aguirre for setting the best example, for their unconditional and endless love.

(A mi madre Carmen Amanda Cardona y mi padre Jaime Aguirre por ser el mejor ejemplo, por su incondicional e infinito amor.)

**Acknowledgements**

Foremost, I would like to express my sincere gratitude to my supervisor Professor Amarjeet Bassi for his continuous support, advice, trust, and motivation. Thanks for giving me this enriching opportunity.

I also want to thank my Ph.D advisory committee, Dr. Lars Rehmann and Dr. Sohrab Rohani for all the guidance and good advice received. Thanks to all the teachers and professors who have contributed to my professional development. They all have a special place in my life. Thanks to Brian Dennis and Souheil Afara for making research easier by having practical solutions to everyday problems.

Thanks to my laboratory and office mates Harpreet Kaur, Shreyas Yedahalli, Gureet Chandhok, Claudia Sacasa, Tulip Chakraborty, Mengyue Gong, Ahmed Alassuity, Roopa Devasya, Priyanka Saxena, Teresa Turnbull, Anam Kazim, Vahid Dehnavi, Isabela Reiniati, Reyna Gomez, Jun Zheng, Malihe Mehdizadeh, and Mahyar Javidi for the good times, refreshing work breaks and all the invaluable things I have learned from you.

I am also very thankful to my friends Sonia Gutierrez, Gabriela Navarro, Juan Manuel Restrepo, Natalia Lesmes, Diego Cufiño, Patricio Valadez, Jorge Garcia, and Martin Huard. They have contributed immensely to my personal development. They were always there for me when I needed them the most.

Thanks to Luis Carlos Luque Moreno for his support, understanding, and caring. It has been great to have someone close to me who shares my passion for chasing dreams.

Thanks with love to my family: My mother Carmen Amanda Cardona, my father Jaime Aguirre, my brother Juan Felipe, and my sister Laura Andrea. You are my strength, my biggest support and motivation. Your love has always been with me despite the thousands of miles between us. This would not be possible without you.

## Table of contents

## List of tables

## List of figures

## List of abbreviations

| | |
|---|---|
| **ACCase** | Acetyl-CoA carboxylase |
| **ANOVA** | Analysis of variance |
| **ASP** | Aquatic species program |
| **ASTM** | American Society for Testing and Materials |
| **CCD** | Central composite design |
| **D** | Desirability |
| **DOE** | Department of energy |
| **FAME** | Fatty acid methyl ester |
| **FFA** | Free fatty acids |
| **GC** | Gas cromatography |
| **HC** | High cellulose |
| **HPS** | High pressure steaming |
| **LC** | Low cellulose |
| **OD** | Optical density |
| $\mathbf{R^2}$ | Regression coefficient |
| **RSM** | Response surface methodology |
| **SEM** | Scanning electron microscopy |
| **TSS** | Total suspended solids |
| **UTEX** | University of Texas |

# 1. Chapter 1: Introduction

Lipids from microalgae are an attractive source of biofuels. However the implementation of this technology at industrial scale is challenging and need to address problems like low extraction efficiencies; even though different methods for breaking down the cell wall have been studied they are not efficient enough or they affect the lipids profile.

To help in the solution of this problem, this study proposes that if a successful process for lipid extraction is wanted; all aspects related with cell wall disruption should be taken into account. This means that not only the method for breaking down the cell plays an important role, but also the intrinsic characteristics of the cell wall. Thus, a culture containing cells with high lipid productivity and low cellulose content is ideally desired in a biodiesel from microalgae process. Therefore, treatments for breaking the cell wall would be less intensive and therefore more economically feasible and environmentally friendly. Even though cell wall plays a fundamental role on lipid extraction, only a few reports were found on the effect of culture conditions on cellulose content, making this area of high interest for research.

In this study, a holistic strategy to investigate the lipid recovery from microalgae is used, so manipulation of process variables will occur from culture conditions (to see their effect on lipid and cellulose content) to the disruption methods for breaking down the cell wall. In order to better understand the flow of ideas that were followed during this research, the next section summarizes the sequence of experiments conducted.

## 1.1. Research structure

This research study was divided into two main phases. The first phase involved an investigation of the culture conditions and their effect on lipid productivity and cellulose content, and the second on application of cell wall disruption methods for bio-crude recovery (See Figure 1.1).

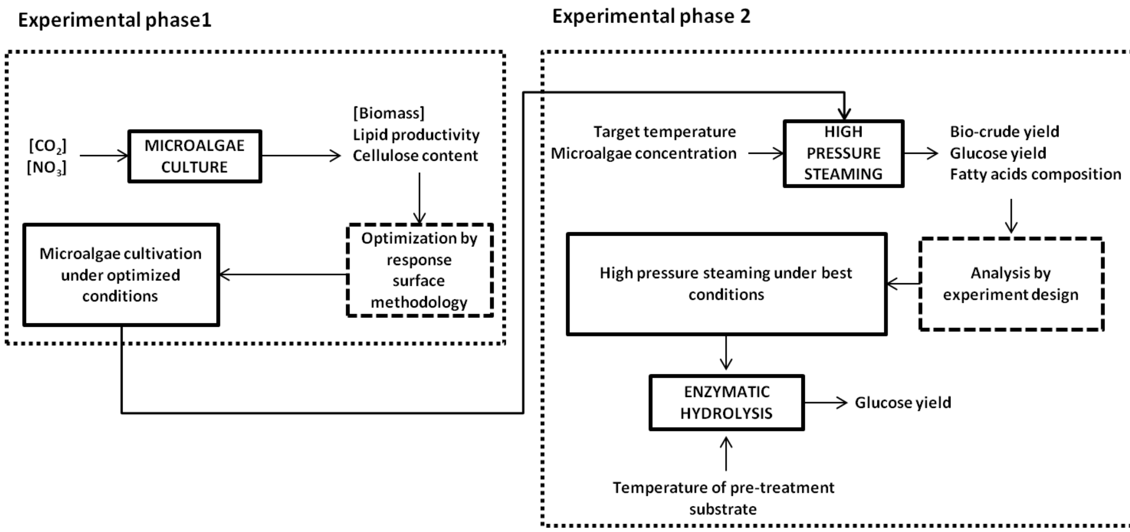



**Figure 1.1** General scheme of thesis structure.

In the first phase, carbon dioxide and nitrate concentrations in growth media were manipulated simultaneously in cultures of *Chlorella vulgaris*. The values for these variables were determined based on literature data and chosen based on the requirements of the central composite design (CCD) for response surface methodology (RSM). This kind of experiment design allows exploring the effect of some factors on a response variable in a determined region, which is chosen by the researcher as promissory. The main goal of the model obtained was to explore the region of interest for the response variable, and determine the location for its maximum or minimum according to research interest.

The model was used to study the effect of two factors, carbon dioxide and nitrate concentration, on lipid productivity and cellulose content. But additional variables were quantified in order to get information that helps to explain the results of the main response variables. Once the model was obtained, it was mathematically manipulated in order to find the operating point, in terms of carbon dioxide and nitrate concentration that gives the maximum lipid productivity with the lowest cellulose content. This point determines the conditions under which *C. vulgaris* cultures were established for experiments in the second stage of the research, where disruption methods were applied.

For the second phase of the research, cells of *C. vulgaris* were subjected to high pressure steaming (HPS). Simultaneous effect of target temperature and microalgae concentration was explored. In this case, a CCD with two factors was used. A model for bio-crude yield was obtained in terms of the variables mentioned and optimization was applied in order to find the conditions under which HPS allows the highest bio-crude yield. Additionally, measurement for glucose yield and fatty acids methyl esters (FAME) composition were made as a way of studying cellulose degradation and ensuring that HPS conditions do not affect significantly the lipids quality for biodiesel production in comparison with traditional solvent extraction.

Finally, enzymatic hydrolysis of biomass previously treated with HPS was proposed as a way of breaking down the remaining cellulose structures, allowing the production of glucose. In this case the effect of pre-treatment temperature and the nature of the substrate was study on glucose yield. The global idea for all the experiments was to provide a comprehensive analysis of lipid recovery from the very initial stages of any microalgae process (cultivation), to the application of extraction methods in order to increase extraction efficiency.

## 1.2. Objectives

Towards the completion of this study, one overall objective and several sub-objectives were proposed.

### 1.2.1. Overall objective

The overall objective of this study was to demonstrate the applicability of HPS treatment on *Chlorella vulgaris* for cell wall disruption by determining values for the main parameters of the process which lead to a high bio-crude recovery.

### 1.2.2. Specific objectives

The following were specific sub-objectives or milestones of this study.

**Objective 1: To study the effect of culture conditions on lipid production and cellulose content.** Effect of carbon dioxide and nitrate concentration on biomass concentration, lipid productivity and cellulose content were studied. Data obtained allowed to plot response surface graphics that gave the following information: Biomass concentration, lipid productivity and cellulose content at low and high $CO_2$ and nitrate concentrations.

**Objective 2: To develop an empirical model for optimization of culture conditions studied.** Statistical data obtained from RSM led to the development of an empirical model of the ratio lipid productivity/cellulose content as function of carbon dioxide and nitrate concentrations. This model was mathematically processed to obtain the culture conditions that produce the maximum (optimal point) for lipid productivity between the interval studied, and the minimum for cellulose content.

**Objective 3: To study the effect of high pressure steaming treatment on cell wall disruption and bio-crude recovery.** The effect of temperature and biomass concentration on bio-crude and glucose yields, and also FAME composition were studied. Analysis of Scanning Electron Microscopy (SEM) images allowed concluding on the physical effect of HPS on microalgae morphology.

**Objective 4: To find an operating area or point for high pressure steaming process.** Based on the empirical models obtained from RSM, an operating area in terms of target temperature and microalgae concentration was found. This operating area led to high bio-crude recovery yield between the intervals studied.

**Objective 5: To study the effect of algae composition on bio-crude recovery.** The effect of lipid and cellulose contents on the bio-crude recovery efficiency applying HPS was studied.

**Objective 6: Study of enzymatic hydrolysis of cellulose from *C. vulgaris* pre-treated with high pressure steaming.** The feasibility of the production of glucose from microalgae pre-treated with HPS was study using a cellulase. Data obtained provided information on the effect of pre-treatment temperature on glucose production as a possible source of fermentable sugars.

### 1.3. Thesis structure

The thesis was divided into 6 Chapters: Chapter 1 supplies an introduction to the research and thesis structure, Chapter 2 covers the literature review and gives the general background on biofuels production from microalgae. The following Chapters provide the background needed to explain and justify the experiments done. Chapter 3 presents the results on the first phase of experiments regarding the effect of culture conditions on lipid productivity, cellulose content and biomass concentration. Chapter 4 and 5 covers the experiments and results of the second phase of experiments mentioned above. Chapter 4 describes the effect of HPS on bio-crude and glucose yield as function of target temperature and microalgae concentration, while Chapter 5 shows the possibility of the use of HPS also as a pre-treatment for enzymatic hydrolysis of microalgae. Chapter 6 summarizes the conclusions of the study and provides some recommendations for future work.

### 1.4. Major contributions

The literature review done contributed to:

- Identify and describe challenging aspects of biofuels from microalgae; the bottlenecks in the implementation of microalgae technology at industrial scale were clearly stated.

The study of culture conditions on algae growth, lipid productivity and cellulose content contributed to:

- Be able to modulate the microalgae concentration, lipid productivity, and cellulose content to any wanted value (in the range of the study), by changing the carbon dioxide and nitrate concentration in the growth media using the empirical models obtained.
- Find optimal points for algae growth and the ratio lipid productivity/cellulose content in microalgae cultures.

The study of HPS as bio-crude recovery method contributed to:

- Test the feasibility of the application of HPS as disruption and bio-crude recovery method in microalgae systems. The process was efficient in extracting the lipids regardless of the algae composition, and it does not affect significantly the lipids profile.
- Find an operating area in terms of target temperature and microalgae concentration that lead to high bio-crude recovery yields.
- Identify operating areas in terms of target temperature and microalgae concentration for high glucose yield as by-product of the HPS process.

The study of HPS as pre-treatment for enzymatic hydrolysis contributed to:

- Show that the thermal pre-treatment aids the enzymatic hydrolysis of the cellulose allowing its conversion to glucose.
- Increases the viability of the bio-fuels from microalgae processes by showing the possibility of obtain two sources of biofuels, (bio-crude and glucose) by the implementation of only one method (HPS).

# 2. Chapter 2: Literature review: Engineering challenges in biodiesel production from microalgae

The information presented in this Chapter is based in the paper "Engineering challenges in biodiesel production from microalgae", published in Critical Reviews in Biotechnology, September 2013, Vol. 33, No. 3, pages 293-308.

## 2.1. Abstract

The combustion of fossil fuels produces several environmental intoxicants, contribute to emission of greenhouse gases and raise the concern for climate change and health problems. Production of biodiesel from microalgae represents an attractive solution to aforementioned problems, offers a renewable source of fuels and emits fewer pollutants. This literature review presents a compilation of engineering challenges related to microalgae as a source of biodiesel, advantages and current limitations for biodiesel production, and some aspects of microalgae cell biology. Also, recent advances in the different stages of the manufacturing process are included.

## 2.2. Introduction

Energy utilization in 2008 was equivalent to 11,295 million tons of oil, which will potentially rise by 60% in 2030, and China and India alone will account for 45% of this energy demand. Therefore, there is a requirement for adoption of global strategies for energy security, $CO_2$-energy reduction (Hoffert *et al*., 2002), and also the need for alternative sustainable fuels with high efficiency and low environmental impact.

To satisfy the increasing energy needs, all feasible alternative energies should be considered. Currently, many strategies are under investigation, among them are the use of (i) solar energy, in which the energy from the sun is converted into thermal or electrical energy through solar panels. It has low energy consumption, low maintenance requirements and allows the generation of energy in the same place of consumption. However, it also represents a high initial investment cost, requires large areas, and the efficiency depends on the sun location and intensity (Thirugnanasambandam *et al*., 2010). (ii) Hydroelectric energy: where the kinetic and potential energy accumulated in waterfalls are transformed into electrical energy. This process results in the production of moderate to high energy and it has low cost of operation and maintenance, but it implies high cost of infrastructure (Onat and Bayar, 2010). (iii) Geothermal energy: the heat accumulated inside the earth is converted by turbines or heat exchangers into useful energy. This requires low operating cost and low maintenance, but needs land suitable for plant installation, emission of some harmful gases is possible, and may cause landscape deterioration (Haehnlein *et al*., 2010). (iv) Tidal energy: this harnesses the kinetic energy of ocean currents through hydraulic turbines which convert this into electrical energy. This is, potentially, an inexhaustible energy source since there are no polluting by-products. However, it can be uneconomical, and may have high environmental impact during installation (Khan *et al*., 2009). (v) Wind energy: in this strategy the kinetic energy of air currents is transformed into electrical energy by wind turbines, it does not produce polluting compounds, and it is an inexhaustible energy source, but it depends of air currents, may has interference with communication systems, is detrimental to landscape quality, has negative effects on environment, and generates high noise levels (Saidur *et al*., 2010). (vi) Biofuels: they are a wide range of fuels which are in some way derived from biomass. The chemical energy stored in the molecules of the biomass is converted into other types of energy. For biofuels production, it is possible to use waste pollutants, as a way of energy recycling and producing less emission of gases as compared to the use of fossil fuels. Some biofuels have shown a strong negative impact on environment and food markets, sometimes the yields are low, and the land requirement is high.

Of all the sources listed above, in particular, the production of biofuels from microalgae is attracting a lot of interest as a potential transformational solution for the problems mentioned. Nevertheless, as a new technology, many engineering challenges must be overcome before the establishment of this process at industrial level. For example, the lipids from microalgae have a lower heating value when compared to regular diesel fuel, production of lipid from microalgae is unstable, the cost of production plants for microalgae's bio-oil remains higher than traditional oil crops (Huang *et al*., 2010), biomass concentration in reactors is low, and also supply, safety and policy barriers must be considered (Demirbas and Demirbas, 2011).

In this Chapter, the advantages, stages in the production process, and parameters affecting the production of biodiesel from microalgae are discussed, with special emphasis on lipid production and extraction as central topic of this thesis.

## 2.3. Biodiesel and its applications

Several fuels can be obtained from microalgae but discussion will focus on biodiesel from microalgae lipids. Biodiesel is a fuel comprised of mono-alkyl esters of long chain FAME derived from vegetable oils or animal fats, designated B100, and meeting the requirements of the American Society for Testing and Materials, ASTM D6751. The main use of biodiesel is as liquid fuel that can be pure or blended with petroleum in any percentage. Biodiesel has similar chemical and physical properties to fuels derived from petroleum. Some studies have shown that the use of biodiesel increases the engine performance in diesel cars (Atadashi *et al*., 2010).

Biodiesel can also be used for i) cleaning up oil spills: biodiesel promotes the biodegradation of aliphatic and aromatic fractions of the residual fuel oil (Fernández-Alvarez *et al*., 2007), ii) production of hydrogen: it has been proposed in autothermal reformers with high and low temperatures shift reactors, autothermal reformer with a single medium temperature shift reactor, and thermal cracker with high and low temperature shift reactors with high and low temperature shift reactor (Nahar, 2010), iii) heating oil in domestic and commercial boilers: studies have shown similar performance in boilers with biodiesel and petrodiesel (Bazooyar *et al*., 2011), among other uses.

## 2.4. Microalgae as engineering systems

Biofuels from microalgae have been suggested since the 1950s (Oswald and Goleeke, 1960). In particular, in the 1970s, the large scale cultivation of microalgae for production of sustainable liquid fuels was investigated (Lin *et al*., 2011; Sheehan *et al*., 1998). The process for producing biodiesel from microalgae generally comprises three stages. The first stage is the microalgae strain selection and the pretreatment of raw materials. The second stage comprises all the steps for biomass growth and production (the microalgae transform the nutrients present in the culture medium into new products such as biomass and fatty acids). The final stage consists of all the processes of separation and purification of fatty acids that are ultimately converted into biodiesel (FAME). Microalgae emerge as an attractive alternative due mainly to its high lipid content. Table 2.1 compares oil content and productivity of biodiesel per year from different feedstocks.

**Table 2.1** Comparison of different biodiesel feedstocks (Mata *et al*., 2010).

| **Plant source content** | **Seed oil** | **Oil yield** | **Land use** | **Biodiesel productivity** |
|---|---|---|---|---|
| | (% $ww^{-1}$) | (L oil/ha year) | ($m^2$ year/kg biodiesel) | (kg biodiesel/ha year) |
| Soybean (*Glycine max L*.) | 18 | 636 | 18 | 562 |
| Camelina (*Camelina sativa L*.) | 42 | 915 | 12 | 809 |
| Canola/Rapeseed (*Brassica napus L*.) | 41 | 974 | 12 | 862 |
| Sunflower (*Helianthus annuus L*.) | 40 | 1070 | 11 | 946 |
| Castor (*Ricinus communis*) | 48 | 1307 | 9 | 1156 |
| Palm oil (*Elaeis guineensis*) | 36 | 5366 | 2 | 4747 |
| Microalgae (low oil content) | 30 | 58,700 | 0.2 | 51,927 |
| Microalgae (medium oil content) | 50 | 97,800 | 0.1 | 86,515 |
| Microalgae (high oil content) | 70 | 136,900 | 0.1 | 121,104 |

Table 2.1 indicates that oil content in microalgae under conditions of environmental stress is 70% (by weight) of dry biomass versus values of 18% - 48% in plants. Therefore, the third-generation of biofuels derived from microalgae is considered as a technically viable energy source that overcomes the problems presented during the previous generation of biofuels (Goh and Lee, 2010). From previous and current research in production of biodiesel from microalgae the following advantages have been found (Costa and de Morais, 2011; Demirbas and Demirbas, 2011): Some of the crops from which biodiesel is traditionally produced cannot be grown continuously, especially in countries with extreme weather conditions. For their part, microalgae culture would be sustainable independent of the time of the year with high productivity of oil, since artificial conditions would be easier to implement. Microalgae are grown in aqueous media, the amount of water required is less than that used in traditional crops, which is an advantage in order to reduce fresh water consumption (Demirbas and Demirbas, 2011; Um and Kim, 2009). Microalgae can also grow in wastewater helping to control pollution not only by treating the water but also by fixing $CO_2$. The growth rate of microalgae in comparison with the growth rate of plants in a crop is much higher, so the processing time is significantly shortened promoting productivity. Furthermore, microalgae cultures can be used for simultaneous production of several products of interest, including biofuels and high value compounds, this would specially help to increase the economic feasibility. For control process, the manipulation of variables in bioreactors is easier than that in traditional crops. This facilitates the modulation of microalgae metabolism in order to increase the production of fatty acids or other compounds of interest. Never the less, there are still many barriers, some of them will be presented later.

## 2.5. Biology of microalgae

Understanding microalgae cell biology facilitates the development of strategies for biodiesel production at industrial level. Microalgae can be eukaryotic (Chlorophyta, Rhodophyta, Bacillariophyta) or prokaryotic (Cyanophyta) (Williams and Laurens, 2010), and can also be classified by pigmentation, product storage structures, cell wall composition, cycle life (eukaryotic), and basic cellular structure (Hoek *et al*., 1996; Khan *et al*., 2009). Table 2.2 presents some basic characteristics of different microalgae genera.

**Table 2.2** Some characteristics of algae (Hoek *et al*., 1996).

| Group | Type | Number of species | Positive or negative effects |
|---|---|---|---|
| Cyanobacteria | Prokaryote | 2000 species | Some produce toxins (e.i. cyclic peptide toxins) |
| | | | Can be used as dietary supplement (spirulina) |
| Dinoflagellates | Eukaryote | >2000 species | Responsible for “Red Tide” which destroys fishing |
| | | | Some produces potent toxins |
| Euglenoids | Eukaryote | ≈1000 species | Some species have been used for many years as experimental organism in biochemical and physiological investigations. |
| Diatoms | Eukaryote | ≈ 11000 species | Diatomaceous earth (made up of millions of diatoms skeletons) can be used as filter, insulator and bioindicator |
| | | | Important source of food and oxygen for heterotrophs |
| Red algae | Eukaryote | >5000 | Produce agar which is used commercially and in laboratory procedures |
| Brown algae | Eukaryote | 1500-2000 species | Include a number of edible seaweeds |
| | | | Some are used for the extraction of iodine and potash |
| | | | Extensively exploited for the extraction of alginic acid |
| Green algae | Eukaryote | 7500 species | *Chlorella* produces high levels of fatty acids, which are used for biodiesel production. |
| | | | *Dunaliella* produces compounds with aAnti-oxidative activity |

It can be seen that algae have a vast variety of species, each one with unique features and industrial applications. For the production of biodiesel, the green algae are of particular interest, as in this group have been found species with high fatty acid yields. In this research *Chlorella vulgaris* was selected. Details of its advantages for lipid production are presented in Chapter 3.

Based on energy and carbon source microalgae can be classified as autotrophic, heterotrophic or mixotrophic. Photoautotrophic growth involves only photosynthesis in the presence of light and carbon dioxide. Heterotrophic metabolism uses organic carbon as the source of energy. Mixotrophic growth occurs when the microorganisms utilize both mechanisms, i.e. phototrophy and heterotrophy for energy (Barsanti and Gualtieri, 2006). Microalgae have the ability to tolerate and survive even on harsh environmental conditions; this represents a positive feature for large scale process where control of process variables would be difficult.

### 2.5.1. Lipid Droplets

Lipid droplets are the main target to increase lipid productivity and content want to be increased, since they represent the lipid storage compartments in the cell. Lipid droplets are the reservoir of triglycerides (precursors of lipids), which primarily serves as a source of carbon and energy under deprived growth conditions. In response to environmental stress like salinity (Qin, 2005), nitrogen limitation (Weldy and Huesemann, 2007; Widjaja *et al*., 2009) or extreme temperature (Qin, 2005) some unicellular microalgae are able to accumulate high lipid content (30-70% dry weight). Wang *et al*., (2009) successfully demonstrated that genetic inhibition of starch biosynthesis in *C. reinhardtii* starchless (sta6 mutant), increased lipid bodies. After 48 h of nitrogen depletion, content of lipid droplet was increased by 15-fold in wild-type cells, but 30-fold increase in lipid droplets was observed in the sta6 starch-less mutant algae. Moreover, after 18 h of nitrogen starvation, on average 17 ng of triglycerides were accumulated in sta6 starchless mutant in comparison to 10 ng in the wild-type cells (Wang *et al*., 2009).

Li *et al*., (2008) demonstrated that alteration of physical parameters results in desirable changes for lipid production. Their results showed that more lipid accumulation occurs under high light and nitrogen-depleted conditions. They also registered a dramatically decrease in starch granules, and the lipid content increased to about 50% of cell dry weight ($gg^{-1}$) during the first ten days under high light and nitrogen-depleted conditions. Correspondingly, size of lipid droplets increased considerably. The C16 and C18 derivatives of total fatty acids accounted for 95% of the neutral acid (Li *et al*., 2008). For biodiesel production fatty acids from C14:0 to C20:0 are preferred since they have higher cetane numbers and are less prone to oxidation.

### 2.5.2. Microalgae cell wall

Increasing oil production in microalgae is not the only challenge imposed by microalgae biology. The extraction of the lipids also constitutes an important aspect of the process. In this case, cell wall represents the biggest barrier for lipid extraction. The cell wall is a set of layers that are located outside microalgae's membrane. The cell wall protects the contents of the cell, gives rigidity to the cell structure, and functions as a mediator in the relations of the cell with the environment. Cell wall composition is of great interest for the establishment of some strategies for cell disruption with the aim of liberating the fatty acids produced by cells (Arad and Levy-Ontman, 2010a).

Cell wall composition varies among different species of microalgae. Cyanobacteria are surrounded only by their cell wall, but some of them have an outer layer composed of mucilage. In other microalgae cell wall is made up of four layers; the innermost layer is composed of murein, in which small pores are usually seen as cytoplasm extensions. The remaining layers are comprised mainly of polysaccharides. In red, brown and green algae cell walls are composed of two fractions - the fibrillar fraction and the amorphous fraction. In red algae, the fibrillar fraction (consisting mainly of cellulose), is embedded in the second layer, and it gives the cell wall strength. This cellulose is arranged irregularly. In some species of red algae, it has been found that fibrillar fraction is made up of xylose or mannans. The amorphous fraction is composed of "slime" generally consisting of galactans like agar and carrageenan. In brown algae, the fibrillar fraction is composed of cellulose and reinforced with alginate, forming cross-linked structures. Alginate in the brown algae's wall could be of two kinds; the insoluble alginate is present in greater proportion in the fibrillar fraction, while the amorphous fraction can be formed by water-soluble alginate and/or fucoidan. In green algae (microalgae used in this study), the fibrillar fraction is embedded in the matrix or amorphous fraction. It is located at the inner side of the cell wall, arranged in parallel, while the amorphous fraction is the most external.

The cell wall composition of green algae species is quite variable, but cellulose is generally the main component (Hammed *et al*., 2013; Hoek *et al*., 1996). Figure 2.1 presents a general representation of microalgae cell wall. The main differences found in microalgae cell walls are in their composition and arrangement. According to Shefner *et al*., (1962), in *Chlorella* the cell wall is approximately 210 Å thick and contains polymers of glucose, galactose, mannose, arabinose, and rhamnose.

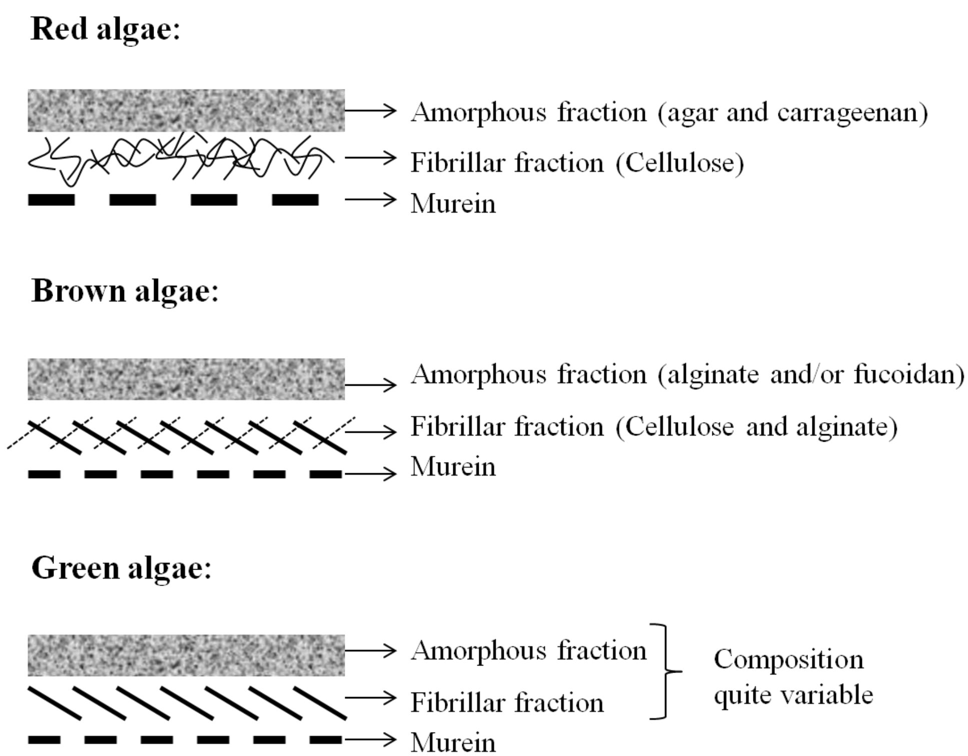



**Figure 2.1** General representation of microalgae cell wall structure.

Table 2.3 shows the cell wall composition for some representative microalgae (*Chlorella, Monoraphidium, Ankistrodesmus* and *Scenedesmus*) (Blumreisinger et al., 1983). For the different microalgae studied, neutral sugars were the main cell wall constituent; *C. vulgaris* K presented the highest value (74%) (Takeda, 1988). This information would be useful for the implementation of cell wall disruption techniques.

**Table 2.3** Cell wall composition for some microalgae* (Blumreisinger *et al*., 1983).

| **Algae** | **Neutral sugars** | **Uronic acids** | **Glucosamine** | **Protein** | **Unknown** |
|---|---|---|---|---|---|
| *C. vuulgaris* K | 74 | 4.1 | - | 3.9 | 18 |
| *C. vulgaris* 211-81 | 24 | 18 | 6.3 | 4.5 | 47 |
| *C. vulgaris* 211-llf | 44 | 24 | 15 | 3.9 | 13 |
| *C. saccharophila* 211-la | 54 | 14 | 0 | 1.7 | 30 |
| *C. fusca* 211-8c | 68 | 6.9 | 0 | 11 | 14 |
| *M. braunii* 202-7b | 47 | 6.1 | 0.4 | 16 | 31 |
| *A. densus* 202-l | 32 | 2.3 | - | 14 | 52 |
| *S. obliquus* 276-3a | 39 | 1.2 | 0 | 15 | 45 |

*(% dry wt).

The effect of light over cellulose content in the cell wall of *Chlorella pyrenoidosa* was studied by Makooi, (1976). Mixotrophic growth produces the highest amount of cellulose, followed by heterotrophic and photoautotrophic growth. The content of cellulose for mixotrophic growth was 2.25 times higher than in photoautrophic growth and, 2.76 times higher than in heterotrophic growth. Low content of cellulose in heterotrophic cultures may be due to the factor that cell growth and maintenance depends on an external source of glucose, which is used as a precursor for the synthesis of all cellular components. Thus, the cell would spend a minimum amount of energy in cell wall formation, giving priority to energetic metabolism. The cells in mixotrophic growth, on the other hand, have two sources of carbon ($CO_2$ and glucose), so the expense of this element would be less regulated (Makooi, 1976). The information on the effect of other culture conditions on cellulose content is very limited and insufficient.

As previously mentioned, cell wall composition may change between different strains of the same species. Takeda, (1988), performed a study over nineteen strains of *Chlorella*. The strains tested were divisible into two different groups; the first one was composed by cells with the presence of glucose and a smaller amount of mannose, and the second group had glucose and glucosamine.

## 2.6. Challenges in different stages of biodiesel production from microalgae

Microalgae technology has to overcome some limitations in order to become safe enough for investors. It is known that a big effort of research and development is needed to reduce the still high-risk level and uncertainty associated with this process. But not only technical issues must be taken into account, but also the regulations and standards in public and private sectors and, market analyses, including quality and safety trials to meet standards (Richardson *et al*., 2010). Intensive research has been conducted on carbon balance for biofuels from microalgae processes and many questions remain opened.

Simulations had shown the potential of microalgae as energy source, but for the establishment of this process at a large scale, there is a need for decreasing the energy and nutrients consumption by means of optimization of culture conditions, lipid extraction and the coupling of anaerobic digestion of oilcakes (Lardon *et al*., 2009). Other important issue on the establishment of biofuels from microalgae according to current policies is the estimation of the ecological impact of the process, for this it is necessary to calculate the ecological footprint of the products, the land area needed, promote studies on restoration of degraded areas and selection of microalgae species that do not affect the natural interaction between native species (Groom *et al*., 2008).

At present, microalgae have most potential as a source for biodiesel production. For this, product must meet ASTM standards with respect to diesel quality (Antolin, 2002; Demirbas and Demirbas, 2011). Table 2.4 compares some properties of biodiesel from microalgae, conventional diesel and the ASTM biodiesel standards (Xu *et al*., 2006).

**Table 2.4** Comparison of properties of biodiesel from microalgae oil, diesel fuel and ASTM biodiesel standard (Xu *et al*., 2006).

| **Properties** | **Biodiesel from microalgae oil** | **Diesel fuel** | **ASTM biodiesel standard** |
|---|---|---|---|
| Density (kg $L^{-1}$) | 0.864 | 0.838 | 0.86-0.90 |
| Viscosity ($mm^2$ $s^{-1}$, cSt at 40˚C) | 5.2 | 1.9-4.1 | 3.5-5.0 |
| Flash point (˚C) | 115 | 75 | Min 100 |
| Solidifying point (˚C) | -12 | -50 to -10 | - |
| Cold filter plugging point (◦C) | -11 | -3.0 (max -6.7) | Summer max 0 |
| | | | Winter max <-15 |
| Acid value (mg $KOHg^{-1}$) | 0.374 | Max 0.5 | Max 0.5 |
| Heating value (MJ $kg^{-1}$) | 41 | 40-45 | - |
| H/C ratio | 1.81 | 1.81 | - |

Figure 2.2 presents a general scheme of biodiesel production from microalgae and some engineering challenges that need to be overcome before industrial implementation (Amaro *et al*., 2011; U.S. DOE, 2010). The following sections will present some of the latest research and efforts made for these bottlenecks. Also, highlighted with dashes in Figure 2.2 are the topics where this research attempts to provide contributions.

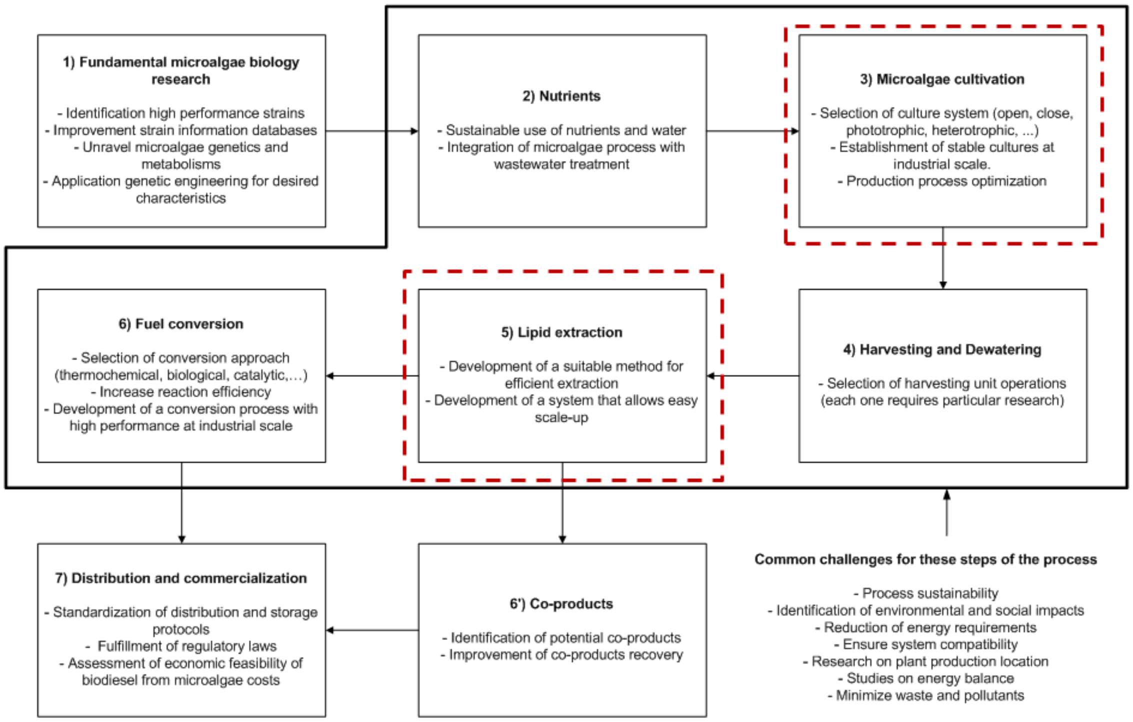



**Figure 2.2** Bottleneck in biodiesel production from microalgae.

## 2.7. Fundamental microalgae biology research

As mentioned before, microalgae comprise a wide range of species. In a process for the production of biodiesel from microalgae, the first bottleneck that can be found is in the identification of strains with high lipid content and productivity. The enormous diversity of environments where microalgae can be found makes hard tasks the steps related with screening and selection of microalgae (Doan *et al*., 2011; Sydney *et al*., 2011). When microalgae strains screening is performed for commercial production of microalgae the following aspects should be consider: microalgae (i) should have high lipid productivity without affecting cell division, (ii) must be able to grow under severe conditions (e.g. extreme temperatures), (iii) must be able to compete with local strains in open production systems (ponds and wastewater facilities), (iv) must uptake $CO_2$ effectively and, (v) should display self-flocculation capacity. It is important to consider the steps required for the product separation since it is a factor greatly affecting the final product cost. The strain allowing the separation of fatty acids with few economic steps is a strain with potential use in biodiesel production.

Nevertheless, no known microalgae strain has fulfilled all requirements to unlock the profitable commercialization of microalgae. The Aquatic Species Program (ASP), of the U.S. Department of Energy (DOE), suggested that exploitation of local microalgae strains could overcome the limitation of environmental factors in commercial microalgae production (Sheehan *et al*., 1998). To solve the limitation of low lipid productivity in dominant strains, genetic manipulation can be implemented. Hopefully, genetically modified strains can potentially reach and yield the theoretically achievable photosynthetic conversion efficiencies and accumulate high amounts of neutral lipids (Sheehan *et al*., 1998).

Furthermore, increasing lipid productivity cannot go beyond where microalgae biology allows, therefore genetic manipulation represent a way to solve this limitation but a bigger understanding of biochemical pathways is needed. Genetic engineering and manipulation of metabolic pathways can promote cellular synthesis of preferred lipids in microalgae. In this interest, the ASP attempted over-expression of acetyl-CoA carboxylase (ACCase), an enzyme that catalyzes the conversion of acetyl-coenzyme A (CoA) to malonyl- CoA in lipid biosynthesis (Radakovits *et al*., 2010). Even though, the over-expression of ACCase has been achieved, it did not result in a significant increased lipid synthesis (Sheehan *et al*., 1998). In addition, instability of genetically engineered strains limits the projections for industrial process with the current technology. Thus, in spite of potency of transgenic microalgae to produce sustainable biofuels and nutraceuticals, this area is still in its infancy. For research purposes, access to reliable information is fundamental, but there is still a lack of unified microalgae databases containing relevant, specific and detailed information about microalgae strains.

## 2.8. Nutrients source and microalgae cultivation

Challenges in nutrients source and microalgae cultivation are abundant, specially taking into account all the different possible approaches and the specie-specific behavior of microalgae strains under given conditions. For microalgae cultivation several factors should be taken into account simultaneously, i.e. light availability and intensity, land topography, climatic conditions, water supply and access to the carbon source and other nutrients (Demirbas, 2011; Mata *et al*., 2010). Furthermore, biomass also depends on the mode of cultivation including photoautotrophic, heterotrophic and mixotrophic production, types of culture (open and closed systems), culture strategies (batch or continuous culture), inhibitors concentration, mixing, dilution rate, depth and harvests frequency (Gallardo Rodríguez *et al*., 2010). Nevertheless, for all of them, big efforts should be done in order to optimize the main variables according to specific production plant location. This must be accompanied by the design of robust and stable cultures, able to mitigate changes in environmental conditions without affecting microalgae growth and lipid productivity.

Under favorable conditions of growth, the algae can double their biomass within 24 h (Chisti, 2008). The growth rate directly affects the concentration of the metabolites of interest; for metabolites associated with growth, increased cell concentration will lead to greater final concentration of the product. Moreover, the yield of fatty acids and their composition varies between different strains, so it is needed to select those that best correspond to established standards. If the microalgae will grow in culture with little control over environmental conditions, it is necessary to ensure that they can respond positively to these changes, so productivity would be not affected significantly (Doran, 1995).

Durability and life cycle studies are important for long term operating plants. On the other hand, if sustainability problems want to be avoided, a detailed study on use of land, water, and nutrients are required, for which the predictable risks and impacts must be identified (Campbell *et al*., 2011; Yang *et al*., 2011). The economics of microalgae technology is very dependent of the scale and scaling-up of the process is still a main issue. Conversion rates of lipids into biodiesel reduce when scale increases. If a technically successful scale-up is achieved, some other aspects related with plant construction would become of interest. For a big production plant, larger markets of raw materials and products are required, as well enough skilled personnel. This significantly reduces the plant location options, but also in case of a successful plant establishment bigger positive impacts would be expected.

### 2.8.1. Light and carbon source

Under natural growth conditions, photosynthetic yield a of microbial system is affected by sunlight exposure and $CO_2$ concentration (both natural free sources). The theoretical photosynthetic yield of microalgae is around 6%-7% of total solar energy. However, this yield is limited by availability of sunlight because of light-dark cycle and seasonal variations. This limitation can be overcome by artificial sunlight or fluorescent lamps implementation (Muller-Feuga *et al*., 1998; Yeh *et al*., 2010); or construction of microalgae-based industries in tropical countries where light is more stable over the year.

Fifty percent of biomass dry weight of microalgae is approximately carbon by weight, (generally derived from carbon dioxide). Most microalgae can tolerate high levels of $CO_2$ with a theoretical yield of roughly 513 tons of $CO_2$ to produce 280 tons of dry biomass per $ha^{-1}y^{-1}$. *Chlorococcum littorale*, a marine alga, can utilize up to 40 percent $CO_2$ concentration (Iwasaki *et al*., 1998). *Chlorella* strains from hot springs are used for biological fixation of carbon dioxide from industrial flue gases (Sakai *et al*., 1995). Therefore, in commercial scale, power plant exhaust can be applied for microalgae biomass production.

Metabolism of microalgae also determines the biomass concentration and cost of biodiesel production. As previously mentioned microalgae have several different modes of metabolisms (e.g. autotrophic, heterotrophic, mixotrophic, photoheterotrophic) and can make metabolic shift to cope with variable environmental conditions. Usually phototropic production is feasible for commercial production of microalgae biomass, and commonly deploys for open pond and closed photobioreactor system. However in autotrophic culture it is hard to attain high density of microalgae biomass and lipid content (Chen and Johns, 1991).

Heterotrophic growth of microalgae is independent of light and use organic substrate as carbon source thus offers the more opportunities to increase cell density, productivity of algal biomass, and cellular lipid content (Miao and Wu, 2004; Xu *et al*., 2006). This is noteworthy that heterotrophic production lowers the harvesting cost (Chen and Chen, 2006), but the use of glucose or acetate as carbon source is costly. To resolve the drawback of high carbon source cost, crude glycerol a cheap resource derived from biodiesel production processes, can be used as carbon substrate (Liang *et al*., 2009).

Mixotrophic microalgae have successful alliance of photosynthetic and heterotrophic metabolism. The capability of mixotrophs to process organic substrates or carbon dioxide as carbon source depends on several factors including the concentration of carbon substrates, and also light intensity in the growth medium. For example, *C. protothecoides*, a mixotropic microalgae shift its metabolic process from photoautotrophic to heterotrophic in response change of organic carbon source (glucose) and reduction of the inorganic nitrogen source in the medium (Miao and Wu, 2004). It infers that in mixotrophic cultivation, there is less loss of biomass during the dark phase.

### 2.8.2. Temperature and pH

Temperature is one of the most limiting factors among the environmental parameters governing the activities and growth rate of microalgae in open and closed system (Park *et al*., 2011). The optimal temperature range is generally between 25-35°C. Many microalgae can even tolerate temperatures around 15°C. Temperature affects, among others aspects, the types of fatty acids produced by these cells. Usually by lowering the temperature, the amount of saturated fatty acids increases, but it is not necessarily true for all species of microalgae (Renaud *et al*., 2002).

The pH of the algal system affects the biomass regulation, photosynthesis rate, availability of phosphorous to microalgae and species competition. pH influences toxicity of free ammonia to living algal cells by altering the ratio of free ammonia and ammonium ion (*Y*. Azov, 1982), and also directly influencing the metabolic rate of microalgae in open or closed system.

## 2.9. Harvesting and dewatering of microalgae biomass

As mentioned previously in this Chapter, downstream processes are responsible for a big portion of lipid cost. Therefore, to overcome the bottlenecks in these steps is critical for large-scale implementation of microalgae technology (Chen *et al*., 2011). All approaches for harvesting must be studied and those with the lowest energy requirements, capital, operating cost and higher efficiency would be good candidates. Although, the compatibility of these operations with other steps in the process should be also considered simultaneously.

In biotechnological processes, the product purification processes generally involve several steps that represent about 33% of the total production cost. For biodiesel production from microalgae, the scenario is quite similar. There is not a unique process for biomass harvesting and it is still an area of active research, where the method developed must be technically appropriate and economically favorable for any species of microalgae (Mata *et al*., 2010). The traditional process includes flocculation, filtration, flotation, and centrifugation, some of which consume large amounts of energy. The low cell densities and the small size of the cells make the biomass recovery process difficult.

The harvesting method selection depends on biomass characteristics (size, density and product value). This process can be divided into two stages: bulk harvesting and thickening. The aim of the bulk harvesting is the separation of biomass of the bulk suspension; the methods used are generally flocculation, flotation or gravity settling. For its part, thickening seeks to concentrate the slurry produced in the previous step. The unit operations of centrifugation, filtration and ultrasonic aggregation utilize higher energy as compared to bulk harvesting (Brennan and Owende, 2010). Additionally, the method employed should be able to process all the culture media produced (Molina Grima *et al*., 2003).

Flocculation aims to increase particle cell size by aggregation, and involves the use of multivalent cations to neutralize negative charges present on the surface of microalgae, preventing the adhesion of algal cells in suspension. This technique commonly use metal salts such as aluminum sulfate ($Al_2(SO_4)_3$), ferric chloride ($FeCl_3$), polyferric sulfate (PFS), ferric sulfate ($Fe_2(SO_4)_3$), cationic polymers and chitosan (Molina Grima *et al*., 2003). However, if the biomass is to be used in specific food related applications, flocculation by metal salts should be avoided.

Uduman *et al*., (2010) evaluated the effect of polyelectrolyte with different charges as flocculants for marine microalgae cells cultured in bioreactors. A flocculation efficiency of 89.9% was obtained with the cationic flocculant. This study also confirmed that the pH and temperature of the process affect the microalgae flocculation (Uduman *et al*., 2010).

Acoustic effects have been also evaluated on cell aggregation. Ultrasonic harvesting systems has some major advantages at laboratory and/or pilot-plant scale. First, this technology never gets blocked with cells (as it can happen when filters are used). Secondly, it does not cause shear stress on biomass even if the system is in continuous operation; this means the harvested biomass can be used as inoculum. Thirdly, the space needed for the complete system is very small. Also, when an organism excretes a high valuable secondary metabolite, this technique can be used as a retention system. The resonation chamber acts as a biological filter by rejecting the organisms and allowing the solubilized product to pass. Ultrasonic method can achieve 92% separating efficacy and a concentration factor of 20 times. Its main disadvantage is that it can destroy the metabolites of interest (Brennan and Owende, 2010).

In flotation, the objective is to reduce the density of suspended solids by trapping in a lower density gas. The gas used is generally micro-bubbles of air where the microalgae cells are trapped. Cheng *et al*., (2010) applied dispersed ozone gas to cultures of *Chlorella vulgaris*, the amount of ozone required to achieve an acceptable separation of biomass was <0.05 mg/g biomass. Similar results were obtained for *Scenedesmus obliquus* FSP-3 (Cheng *et al*., 2010).

Sedimentation is the process in which a solid material carried by flowing water, is deposited on a surface, since it requires large space and recovery is time dependent would not be a convenient option for biodiesel production (Al-Sammarraee *et al*., 2009). Gravity sedimentation is most commonly used for concentrating algal biomass in wastewater treatment, since it allows treatment of large volumes. Centrifugation makes use of rotational force which produces a force greater than gravity, allowing a faster sedimentation. Harvesting of the biomass by centrifugation depends on biomass characteristics, settling depth and the residence time in the centrifuge. Its main disadvantage is the high cost of operation and the need for constant maintenance of equipment (Brennan and Owende, 2010). Possibly, efficiency of sedimentation can be increase by the use of flocculants (Chen *et al*., 2011).

Conventional filtration processes are most appropriate for the recollection of microalgae with relatively large sizes (>70µm) (Brennan and Owende, 2010). For the recollection of smaller cells (<30 microns), it is possible to use microfiltration- generally employed for fragile cells-, or ultrafiltration. Because of the cost of replacing membranes and large-scale pumping, this may be an expensive method for biomass harvesting (Brennan and Owende, 2010; Molina Grima *et al*., 2003).

Rossignol *et al*., (1999) compared performances of eight commercial membranes for recovery of two types of marine microalgae (*Haslea ostrearia* and *Skeletonema costatum*). They found that the ultrafiltration membrane of 40 kDa was optimum for recovering of the cells at commercial production (e.g., >20 $m^3 day^{-1}$). Membrane filtration processes is not economical method because of high cost of membrane replacement and pumping (Rossignol *et al*., 1999).

Dehydration and drying are used to prolong the viability. Among the most common methods are low-pressure shelf, direct sun, and use of rotating drums, spray dryers, freeze dryers or fluidized beds. The sun drying is understandably inexpensive; however, it is time consuming, requires large surfaces and, due to high water content in cells, it is not very efficient (Mata *et al*., 2010). For biofuels extraction, it is important to consider efficiency and cost of drying-effectiveness with the objective of maximizing the net production of biofuels. The drying temperature affects lipids extraction, either its composition or yield (Brennan and Owende, 2010; Molina Grima *et al*., 2003).

## 2.10. Lipid extraction

Even though there has been a considerable amount of research on lipid extraction, there is still not a suitable method that satisfies all the requirements for an efficient and economical feasible method. This represents a main bottleneck in biodiesel production from microalgae, since it does not matter if lipid content has increased when there is not a good method to extract them from the cell to convert these lipids into biodiesel. Some methods have shown acceptable efficiency at laboratory scale, but when the technique is scaled-up, the efficiency reduces and lipids remain trapped in the cell.

Following the processes presented in Figure 2.2, once the biomass is concentrated and dried, the next step is the extraction of fatty acids. There are basically three ways to achieve the extraction: the first option consists of a biomass pretreatment which seeks to disrupt the cell wall, followed by an extraction process with solvents. The second approach involves solvent extraction of the fatty acids without prior cell disruption, and the third option is the spontaneous release of components of interest from the cell into the culture medium (U.S. DOE, 2010). In terms of safety and energy consumption the ideal system should allow the use of wet cells and reduce or eliminate the need of solvents (Horst *et al*., 2012).

### 2.10.1. Cell wall disruption

Although all the above-mentioned harvesting processes are important for the extraction of oil from microalgae, cell disruption is a key step since it determines the yield of lipids obtained after disruption method (Araujo *et al*., 2013). Therefore, the development of an appropriate method and device for cell disruption is essential. Despite all the research in this field, the most efficient method for microalgae has not yet been obtained (Lee *et al*., 2010). The variables that affect the extraction of lipids are still not well known, making the process scale-up for commercial purposes difficult (Halim *et al*., 2011).

Most of the methods to break microalgae cell walls were adapted from methods used in other cell types. Among the most used are high-pressure homogenizers, autoclaving, ultrasound, microwaves, freezing, enzyme reactions, and acid or alkaline hydrolysis. The cell wall properties, mentioned earlier in this Chapter, play a crucial role in the extraction of oil, as it may hinder direct contact between the solvent and solute (Brennan and Owende, 2010; Mata *et al*., 2010). The ideal system for cell wall disruption should maximize the yield of the product without contamination or degradation of the target compounds. It also has to be efficient at an industrial scale and it should not conduct to any complication in farther steps of the process (Goettel *et al*., 2013).

Lee *et al*., (2010) compared the efficiency of bead-beating, autoclaving, sonication, microwaves and 10% sodium chloride (NaCl) solution on microalgae disruption. The research results indicated that the efficiency of extraction of lipids from microalgae differ according to species and the method used (Lee *et al*., 2010).

Fu *et al*., (2010), reports the first article that used immobilized cellulase to degrade microalgae cell walls. Under the best conditions tested, the immobilized cellulase reached 62% conversion and the yield of hydrolysis remained above 40% after 5 reuses. Additionally, the extraction of lipids from microalgae increased from 32% to 56% after enzyme treatment (Fu *et al*., 2010).

Some heat treatments have been employed on microalgae in order to facilitate lipid extraction. Kita *et al*., (2010) reported the use of thermal pre-treatment. The microalgae cells were suspended in water and subjected to heating at temperatures from 75-120°C in a reactor for 10 minutes, and then hexane extraction was used. The results showed that the recollection of hexane-soluble materials substantially improved around 90% or more (85°C) when heat pre-treatment was applied at very low cell concentrations. Additionally it was not necessary to apply biomass dehydration or drying, which is very advantageous for the process economy. In this research a hydrothermal treatment is studied (Chapter 4).

### 2.10.2. Extraction methods

There are three main methods for extracting oil from microalgae: solvent extraction, expeller/press and supercritical fluid extraction. Table 2.5 summarizes some advantages and disadvantages for different harvesting, extraction, purification, and cell wall disruption methods. For lipid extraction, solvent is usually applied directly to dry biomass. In solvent extraction of bio-oil from microalgae the lipids are transferred from one phase (microalgae biomass) to a second phase (solvent). The solubility of the lipids in the solvent is governed by the Gibbs free energy of the dissolution process, which is related to the equilibrium constant that fixes the concentration of the lipids in either phase (Cooney *et al*., 2009); a detailed description of the extraction process of lipids from inside microalgae cells is presented by Halim *et al*., (2011).

**Table 2.5** Some advantages and disadvantages for different harvesting, extraction, purification and cell wall disruption methods (Brennan and Owende, 2010; Fu *et al*., 2010; Halim *et al*., 2011; Kita *et al*., 2010; Lee *et al*., 2010).

| **Method** | **Advantages** | **Disadvantages** |
|---|---|---|
| **Harvesting and dewatering** | | |
| Flocculation | It can be operated in continuous | It is necessary to add flocculants |
| | No shear stress on biomass | |
| Flotation | It does not require chemicals addition | Limited evidence of its technical and economic feasibility |
| Centrifugation | It allows treatment of large volumes | High cost of operation |
| | | Need for constant maintenance |
| Filtration | Effective | High cost of replacing membranes and large-scale pumping |
| Sun drying | It is cheap | It is time consuming |
| | | It requires large surfaces |
| Other drying methods cited in text | Effective | They are expensive |

| | | |
|---|---|---|
| **Cell wall disruption** | | |
| Microwaves | Effective | High cost of operation |
| Cellulase treatment | Low energy required | High cost of the cellulase |
| | High selectivity | |
| | Few side products | |
| Heat pre-treatment | Improve the recovery rate | Energy demanding |
| **Lipid extraction** | | |
| Supercritical carbon dioxide | Low toxicity | High cost of infrastructure and operation |
| | Favorable mass transfer equilibrium | |
| | Solvent-free extract | |

Ranjan *et al.*, (2010) evaluated Soxhlet extraction method, Bligh and Dyer method and sonication with two solvents for lipid extraction of *Scenedesmus* sp cells. The response variables were cell disruption, lipid diffusion, bulk convection, and solvent selectivity. Complete cell wall disruption was not achieved with any of the pre-mentioned methods. Results also confirmed that selection of the solvent was a dominating factor in the overall lipid extraction in comparison to intensity of bulk convection in the medium under these test conditions. Supercritical fluids have been studied; among them is carbon dioxide (SC-$CO_2$) extraction, which has several advantages, e.g. low toxicity, favorable mass transfer, and production of a solvent-free extract. Its main disadvantage is the high costs associated with the process. Experimental results have shown that for SC-$CO_2$ extraction, lipid yield decreases with increasing temperature and pressure (Halim *et al.*, 2011).

### 2.11. Conversion of lipids into biodiesel

As presented in Figure 2.2, the main challenges in conversion of lipids into biodiesel are the finding of a suitable method that allows high conversion yields reducing the amount of lipids that remain without reacting. This method should be also versatile and easily enough to scale-up, so its implementation at large scale becomes reliable.

Conversion of algal biomass to biofuel depends on the sources and types of biomass, conversion option and final product. On the basis of cost and project specificity, thermochemical liquefaction, pyrolysis and transesterification have been used for this purpose.

The transesterification process comprises conversion of algal lipids into biodiesel in the presence of a catalyst; usually methanol (methanolysis), to yield the corresponding FAME and glycerol. Depending on the phase of catalysis, transesterification can be homogeneous (same phase) or heterogeneous (different phases) (Lam *et al*., 2010).

Homogeneous catalysts are categorized into acid and base type. Homogeneous catalyst increase the reaction rate for biodiesel production since catalyst are in constant contact with the reaction mixture. The principal variables in the reaction are alcohol quantity, reaction time, reaction temperature and catalyst concentration (Leung *et al*., 2010).

Acid catalysts are corrosive, and they often results in damages to reactors. They also require high temperatures and pressure conditions. Therefore, the use of the basic catalysts like potassium hydroxide and sodium hydroxide are commercially more acceptable relative to acidic catalysts (Leung *et al*., 2010). Moreover, basic catalysts have low cost and more than 98% conversion yield (Canakci and Sanli, 2008). However, this method is not favorable when free fatty acids (FFA) content is over 0.1–0.5% in the oil source, because of formation of metal soaps hinders final purification of biodiesel (Marchetti and Errazu, 2008). Basic catalysts increase the production cost of biodiesel since several washings using hot (distilled) water are required for removal of basic catalyst (Janaun and Ellis, 2010; Sharma *et al*., 2010).

Ehimen *et al*., (2010) studied the production of biodiesel from lipids of microalgae using the in-situ acid-catalyze transesterification process. The results confirmed that increase in volume of alcohol and temperature has a direct correlation with the production of FAME. They also observed that the reactor mixing positively affects the production of biodiesel, while increasing the biomass water content leads to a reduction in reaction yield.

Efficient heterogeneous (solid) catalysts have economic benefits for production of biofuels, since they catalyze materials with high FFA content (>0.1–0.5%) (Leung *et al.*, 2010). Commercially used heterogeneous catalysts include alumina, zirconia, titania, ion-exchange resins and strong acid zeolites. Recently, Park *et al*., (2010) have examined $WO_3/ZrO_2$ for FFA conversion to biodiesel and the yield of conversion was approximately 93%. Also, Feng *et al*., (2010) reported 90% conversion yield by NKC-9. Heterogeneous catalysts are economical (Di Serio *et al*., 2008), noncorrosive and environmental friendly thus considerably decrease over all production cost of biodiesel (Marchetti and Errazu, 2008).

A more recent idea is the use of enzymes (lipases) as reaction catalysts. By their use, the process could be conducted at moderate conditions, and it does not produce pollutant co-products. However, some drawbacks must be overcome as some compounds in the reaction medium can act as inhibitors of the reaction, and the cost of enzymes are high.

### 2.12. Microalgae industries and Economics

In recent years, microalgae are commercially exploited for the production of biofuels, nutritional supplements, drug screening and waste water treatment. For all these activities, more than $7.5x10^6$ tons of algae are harvested every year representing a world market of US$ $6X10^9$/year. Economic viability of the process for the production of biodiesel from microalgae is the main bottleneck for development and establishment of this technology at industrial level. There are many factors affecting the economy of the process.

Chisti (2008), provides a compilation of information about economics of biodiesel production. The production of one kilogram of biomass in raceways is $ 0.85 more expensive than in bioreactors, but by increasing biomass production capacity this difference in costs is reduced. The step that most contributes in increasing the production cost of biodiesel is the oil collection, it represents about 50% of the final cost of oil. A liter of oil from microalgae costs 5.3 times more than a liter of palm oil. Economic feasibility of biodiesel production can be enhanced by the use of the by- products of the process, by optimizing the process parameters, by the design of more efficient bioreactors and/or by increasing the oil content in the cells, photosynthetic efficiency and growth rate.

Norsker *et al*., (2011) conducted a comparative study of costs of biodiesel production from microalgae in either a tubular photobioreactor or a flat-panel photobioreactor versus open ponds. The cost of producing a kilogram of biomass including the cost of dewatering was €4.15, €5.96 and €4.95 respectively. They also identified the factors that most influence these costs for each of these processes, these were respectively: the centrifuge, the culture circulation pump and the blower/paddle wheel.

As mentioned before many economical constrains need to be overcome. The latest studies on the topic have shown that even small changes in technological aspects of the process may improve economic viability which increases the potential of microalgae technology in the long term (Richardson *et al*., 2010; Stephens *et al*., 2010). According to Schulz, (2006), the best way to achieve the potential of microalgae industry is by the development of a large scale demonstration plant so more reliable result would be found. Richardson *et al*., (2010) provides a complete economic study; according to their research carbon dioxide cost ranges from 0.0035 to 0.2 $/kg microalgae biomass, water cost from 0.01 to 0.26 $/ m$^3$, media cost is around 0.02 to 0.59 $/kg microalgae biomass, and labor 0.006 to 0.39 $/kg microalgae biomass, differences in costs depend of culture and operation conditions.

Currently there are many industries dedicated to the cultivation of microalgae with different objectives, e.g. some industries produce microalgae biomass as a final product, others take biomass to obtain high value products such as proteins or pigments. Many industries are focusing their research on the production of biodiesel and bioethanol, while others are developing equipment for laboratory, pilot and industrial scale cultivation and harvesting of microalgae. Table 2.6 presents a list of some industries around the world currently working with microalgae.

**Table 2.6** Some microalgae industries (All the websites were visited on 08-05-2014).

| Company name | Description | Country | Website |
|---|---|---|---|
| **Microalgae biomass and derivate production** | | | |
| Algae food and Fuel | Production and commercialization of microalgae in fluid, pasta dried and freeze-dried forms. | Netherlands | http://www.algaefoodfuel.com/english/home/ |
| Algaltech | Developing and commercialization of microalgae derived products for the nutraceutical and cosmetic industries | Israel | http://www.algatech.com |
| Aquacarotene Ltd. | Growing of *Dunaliella salina* for production of dry marine algae | Australia | http://www.aquacarotene.com.au |
| Astaxa | Microalgae biomass production at industrial scale, production of fresh or frozen microalgae of different genera | Germany | http://www.algae-biotech.com/ |
| Bioprodukte Prof. Steinberg GmbH | Research and production of *Chlorella vulgaris* tablets, powder, and organic ribbons | Germany | http://www.algomed.de/ |
| Earthrise Nutritional | Production of *Chlorella* and Spirulina based products | United States | http://www.earthrise.com/ |
| Easy algae | Microalgae production for aquaculture, aquarium and cosmetic markets | Spain | http://www.easyalgae.com |
| Far East Microalgae Ind Co., Ltd | Preparation of dietary supplements, aqua culture feeds, and skin care products from algae | China | http://www.femico.com.tw/eng/algaeintro.html |
| Nutrimed Group | Raw materials from microalgae for food, pharmaceutical and nutraceutical industries | Australia | http://www.nutrimedgroup.com/ingredients.htm |
| Parry Nutraceuticals | Spirulina, carotenoids, and astaxanton production | India | http://www.parrynutraceuticals.com/ |
| Solarium Biotechnology | Spirulina production as food supplements | Chile | http://www.spirulina.cl/ |
| Subitec | Microalgae biomass production at industrial scale | Germany | http://www.subitec.com/ |
| Tianjin Norland Biotech Co., Ltd. | Spirulina and *Chlorella* production | China | http://www.norlandbiotech.com/ |
| **Biofuels from microalgae (Biodiesel or bioethanol)** | | | |
| A2Be Carbon Capture | Currently developing a system for biodiesel production | United States | http://www.algaeatwork.com/ |

| | | | |
|---|---|---|---|
| Algae floating systems | Production of biodiesel in algae floating systems | United States | http://www.algaefloatingsystems.com/ |
| Algaenergy | Research and production of biomass, oil and biofuels from microalgae | Spain | http://www.algaenergy.es/ |
| Aurora Algae | Pilot plant for the production of fuel, pharmaceutical and food products from algae | United States | http://www.aurorabiofuels.com/ |
| Biofuel systems | Pilot plant for the production of biofuels from microalgae | Spain | http://www.biopetroleo.com/ |
| Breen biotec | Microalgae cultures for the production of biodiesel and other bioresuorces | Germany | http://www.breen-biotec.de/ |
| Petroalgae | Production of biodiesel from microalgae in photosynthetic micro-crops | United States | http://www.parabel.com/ |
| Pond biofuels | Biodiesel production from microalgae employing $CO_2$ | Canada | http://www.pondbiofuels.com |
| Seambiotic | Utilization of flue gases from coal burning power stations for the production of biodiesel from microalgae | Israel | http://www.seambiotic.com/ |
| Solazyme | Algal biotechnology for the production of fuels, chemicals, food and health science products | Unites States | http://www.solazyme.com/ |
| **Bioreactors and Harvesting systems** | | | |
| Algae link | Photobioreactor for controlled, large-scale production of microalgae | Netherlands | http://www.algaelink.com/ |
| Algasol technologies | Commercialization of floating photobioreactor | Spain | http://www.algasolrenewables.com/en/ |
| Culturing Solutions Inc. | Tubular photobioreactor, software and extraction systems | United States | http://www.culturingsolutions.com/ |
| Diversified Energy Corporation | Developing and commercializing algal biomass production system | United States | http://www.diversified-energy.com/ |
| Evodos | Microalgae harvesting system | Netherlands | www.evodos.eu |
| Origin Oil | Research and development of bioreactors and harvesting systems | United States | http://www.originoil.com/ |
| Phyco Biosciences | Algae production system and commercial scale harvester for dewatering and drying algae biomass | United States | http://www.xlrenewables.com/ |
| Solix Biofuels | Oil extraction technology from microalgae in | United States | http://www.solixbiof |

| | | | |
|---|---|---|---|
| | a continuous process | | uels.com/ |
| **Pollution control with microalgae** | | | |
| Hydromentia | Water pollution control | United States | http://www.hydromentia.com |
| Kent Seatech | Water pollution remediation and $CO_2$ capture | United States | http://www.kentbioenergy.com/page5/page5.html |
| **Microalgae research and development** | | | |
| AlgoSource technologie | Services in conceptual Engineering and Process development for photosynthesis and bio-refining of microalgae and $CO_2$ sequestration | France | www.algosource.com |
| Chevron Corpotation | Research and development for the production of liquid transportation fuels using algae | United States | http://www.chevron.com/News/Press/Release/?id=2007-10-31 |
| Cyano Biofuels | Company focused on the biology research of microalgae for the production of biofuels and chemical feedstock | Germany | http://www.cyano-biofuels.com/ |
| Phytolutions | Research, monitoring, analysis and disposal for microalgae industry | Germany | www.phytolutions.com |

### 2.13. Current research directions

Microalgae technology is getting stronger every day due to significant support from a diversity of companies and governmental institutions. The Air Force Office of Scientific Research (AFOSR) in United States has started the algal bio-jet program (AFOSR, 2008). This is a long term, basic research funding program that is interested in facilitating the production of bio-based jet fuel using oil derived from microalgae. Research supported comprises identification of specific hurdles that must be overcome in order to achieve cost-effective production of algae oil for jet fuel conversion and address the basic science research requirements needed to overcome these drawbacks as well as to elucidate various novel scientific approaches that will be needed for developing a fundamental understanding of algal lipid biosynthesis and biomass cultivation principles. The Advanced Research Projects Agency-Energy of the DOE, Office of Science, Office of fossil Energy, and Biomass Program are all founding research activities that include investigating microalgae.

Topics under current major research are sources of microalgae, biochemistry, genetic and biotechnology of microalgae, photobioreactor design, manufacture and microalgae culture systems; mass production of microalgae for different applications and optimization; downstream processing; sustainable development of microalgae activities; biofuels production; wastewater treatment; and $CO_2$ capture (S. Carlsson, J.B. van Beilen, R. Moller, 2007).

### 2.14. Conclusions

The production of biodiesel from microalgae is an attractive alternative because it provides a renewable source of fuel and helps to reduce the pollution problems. Microalgae cultivation for the production of biodiesel has major advantages over other biofuels production, although it is necessary to overcome some problems before full scale implementation. Each of process stages should be improved in order to increase the process economic viability. The extraction of lipids from the cell is a fundamental step and represents a bottleneck for the production of biodiesel, so it is necessary to develop technologies that allow the release of lipids within the cell in an efficient and economical way.

## References

AFOSR, 2008. Algal Oil for Jet Fuel Production, in: National Renewable Energy Laboratory.

Al-Sammarraee, M., Chan, A., Salim, S.M., Mahabaleswar, U.S., 2009. Large-eddy simulations of particle sedimentation in a longitudinal sedimentation basin of a water treatment plant. Part I: Particle settling performance. Chem. Eng. J. 152, 307–314.

Amaro, H.M., Guedes, A.C., Malcata, F.X., 2011. Advances and perspectives in using microalgae to produce biodiesel. Appl. Energy 88, 3402–3410.

Antolin, G., 2002. Optimisation of biodiesel production by sunflower oil transesterification. Bioresour. Technol. 83, 111–114.

Arad, S.M., Levy-Ontman, O., 2010. Red microalgal cell-wall polysaccharides: biotechnological aspects. Curr. Opin. Biotechnol. 21, 358–64.

Araujo, G.S., Matos, L.J.B.L., Fernandes, J.O., Cartaxo, S.J.M., Gonçalves, L.R.B., Fernandes, F.A.N., Farias, W.R.L., 2013. Extraction of lipids from microalgae by ultrasound application: prospection of the optimal extraction method. Ultrason. Sonochem. 20, 95–8.

Atadashi, I.M., Aroua, M.K., Aziz, A.A., 2010. High quality biodiesel and its diesel engine application: A review. Renew. Sustain. Energy Rev. 14, 1999–2008.

Barsanti, L., Gualtieri, P., 2006. Algae: Anatomy, Biochemistry, and Biotechnology, Journal of Phycology. CRC Press, Boca Raton.

Bazooyar, B., Ghorbani, A., Shariati, A., 2011. Combustion performance and emissions of petrodiesel and biodiesels based on various vegetable oils in a semi industrial boiler. Fuel 90, 3078–3092.

Blumreisinger, M., Meindl, D., Loos, E., 1983. Cell wall composition of chlorococcal algae. Phytochemistry 22, 1603–1604.

Brennan, L., Owende, P., 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. 14, 557–577.

Campbell, P.K., Beer, T., Batten, D., 2011. Life cycle assessment of biodiesel production from microalgae in ponds. Bioresour. Technol. 102, 50–6.

Canakci, M., Sanli, H., 2008. Biodiesel production from various feedstocks and their effects on the fuel properties. J. Ind. Microbiol. Biotechnol. 35, 431–41.

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., Chang, J.-S., 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. Bioresour. Technol. 102, 71–81.

Chen, F., Johns, M.R., 1991. Effect of C/N ratio and aeration on the fatty acid composition of heterotrophicChlorella sorokiniana. J. Appl. Phycol. 3, 203–209.

Chen, G.-Q., Chen, F., 2006. Growing phototrophic cells without light. Biotechnol. Lett. 28, 607–16.

Cheng, Y.-L., Juang, Y.-C., Liao, G.-Y., Ho, S.-H., Yeh, K.-L., Chen, C.-Y., Chang, J.-S., Liu, J.-C., Lee, D.-J., 2010. Dispersed ozone flotation of Chlorella vulgaris. Bioresour. Technol. 101, 9092–6.

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126–31.

Cooney, M., Young, G., Nagle, N., 2009. Extraction of Bio- oils from Microalgae. Sep. Purif. Rev. 38, 291–325.

Costa, J.A.V., de Morais, M.G., 2011. The role of biochemical engineering in the production of biofuels from microalgae. Bioresour. Technol. 102, 2–9.

Demirbas, A., Demirbas, M.F., 2011. Importance of algae oil as a source of biodiesel. Energy Convers. Manag. 52, 163–170.

Demirbas, M.F., 2011. Biofuels from algae for sustainable development. Appl. Energy 88, 3473–3480.

Di Serio, M., Tesser, R., Pengmei, L., Santacesaria, E., 2008. Heterogeneous Catalysts for Biodiesel Production. Energy & Fuels 22, 207–217.

Doan, T.T.Y., Sivaloganathan, B., Obbard, J.P., 2011. Screening of marine microalgae for biodiesel feedstock. Biomass and Bioenergy 35, 2534–2544.

Doran, P.M., 1995. Bioprocess Engineering Principles. Academic Press, London.

Ehimen, E.A., Sun, Z.F., Carrington, C.G., 2010. Variables affecting the in situ transesterification of microalgae lipids. Fuel 89, 677–684.

Feng, Y., He, B., Cao, Y., Li, J., Liu, M., Yan, F., Liang, X., 2010. Biodiesel production using cation-exchange resin as heterogeneous catalyst. Bioresour. Technol. 101, 1518–21.

Fernández-Alvarez, P., Vila, J., Garrido, J.M., Grifoll, M., Feijoo, G., Lema, J.M., 2007. Evaluation of biodiesel as bioremediation agent for the treatment of the shore affected by the heavy oil spill of the Prestige. J. Hazard. Mater. 147, 914–22.

Fu, C.-C., Hung, T.-C., Chen, J.-Y., Su, C.-H., Wu, W.-T., 2010. Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction. Bioresour. Technol. 101, 8750–4.

Gallardo Rodríguez, J.J., Sánchez Mirón, A., García Camacho, F., Cerón García, M.C., Belarbi, E.H., Molina Grima, E., 2010. Culture of dinoflagellates in a fed-batch and continuous stirred-tank photobioreactors: Growth, oxidative stress and toxin production. Process Biochem. 45, 660–666.

Goettel, M., Eing, C., Gusbeth, C., Straessner, R., Frey, W., 2013. Pulsed electric field assisted extraction of intracellular valuables from microalgae. Algal Res. 2, 401–408.

Goh, C.S., Lee, K.T., 2010. A visionary and conceptual macroalgae-based third-generation bioethanol (TGB) biorefinery in Sabah, Malaysia as an underlay for renewable and sustainable development. Renew. Sustain. Energy Rev. 14, 842–848.

Groom, M.J., Gray, E.M., Townsend, P.A., 2008. Biofuels and biodiversity: principles for creating better policies for biofuel production. Conserv. Biol. 22, 602–9.

Haehnlein, S., Bayer, P., Blum, P., 2010. International legal status of the use of shallow geothermal energy. Renew. Sustain. Energy Rev. 14, 2611–2625.

Halim, R., Gladman, B., Danquah, M.K., Webley, P.A., 2011. Oil extraction from microalgae for biodiesel production. Bioresour. Technol. 102, 178–85.

Hammed, A.M., Jaswir, I., Amid, A., Alam, Z., Asiyanbi-H, T.T., Ramli, N., 2013. Enzymatic Hydrolysis of Plants and Algae for Extraction of Bioactive Compounds. Food Rev. Int. 29, 352–370.

Hoek, C. van den, Mann, D., Jahns, H.M., 1996. Algae: An Introduction to Phycology. Cambridge University Press.

Hoffert, M.I., Caldeira, K., Benford, G., Criswell, D.R., Green, C., Herzog, H., Jain, A.K., Kheshgi, H.S., Lackner, K.S., Lewis, J.S., Lightfoot, H.D., Manheimer, W., Mankins, J.C., Mauel, M.E., Perkins, L.J., Schlesinger, M.E., Volk, T., Wigley, T.M.L., 2002. Advanced technology paths to global climate stability: energy for a greenhouse planet. Science 298, 981–7.

Horst, I., Parker, B.M., Dennis, J.S., Howe, C.J., Scott, S.A., Smith, A.G., 2012. Treatment of Phaeodactylum tricornutum cells with papain facilitates lipid extraction. J. Biotechnol. 162, 40–9.

Huang, G., Chen, F., Wei, D., Zhang, X., Chen, G., 2010. Biodiesel production by microalgal biotechnology. Appl. Energy 87, 38–46.

Iwasaki, I., Hu, Q., Kurano, N., Miyachi, S., 1998. Effect of extremely high-CO2 stress on energy distribution between photosystem I and photosystem II in a “high-CO2” tolerant green alga, Chlorococcum littorale and the intolerant green alga Stichococcus bacillaris. J. Photochem. Photobiol. B Biol. 44, 184–190.

Janaun, J., Ellis, N., 2010. Perspectives on biodiesel as a sustainable fuel. Renew. Sustain. Energy Rev. 14, 1312–1320.

Khan, M.J., Bhuyan, G., Iqbal, M.T., Quaicoe, J.E., 2009. Hydrokinetic energy conversion systems and assessment of horizontal and vertical axis turbines for river and tidal applications: A technology status review. Appl. Energy 86, 1823–1835.

Kita, K., Okada, S., Sekino, H., Imou, K., Yokoyama, S., Amano, T., 2010. Thermal pre-treatment of wet microalgae harvest for efficient hydrocarbon recovery. Appl. Energy 87, 2420–2423.

Lam, M.K., Lee, K.T., Mohamed, A.R., 2010. Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: a review. Biotechnol. Adv. 28, 500–18.

Lardon, L., Hélias, A., Sialve, B., Steyer, J.-P., Bernard, O., 2009. Life-cycle assessment of biodiesel production from microalgae. Environ. Sci. Technol. 43, 6475–81.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M., 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. 101 Suppl, S75–7.

Leung, D.Y.C., Wu, X., Leung, M.K.H., 2010. A review on biodiesel production using catalyzed transesterification. Appl. Energy 87, 1083–1095.

Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C.Q., 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. Appl. Microbiol. Biotechnol. 81, 629–36.

Liang, Y., Sarkany, N., Cui, Y., 2009. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol. Lett. 31, 1043–9.

Lin, L., Cunshan, Z., Vittayapadung, S., Xiangqian, S., Mingdong, D., 2011. Opportunities and challenges for biodiesel fuel. Appl. Energy 88, 1020–1031.

Makooi, M., 1976. Effects of glucose and light on cellulose content of Chlorella pyrenoidosa. Phytochemistry 15, 367–369.

Marchetti, J.M., Errazu, A.F., 2008. Esterification of free fatty acids using sulfuric acid as catalyst in the presence of triglycerides. Biomass and Bioenergy 32, 892–895.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy Rev. 14, 217–232.

Miao, X., Wu, Q., 2004. High yield bio-oil production from fast pyrolysis by metabolic controlling of Chlorella protothecoides. J. Biotechnol. 110, 85–93.

Molina Grima, E., Belarbi, E.-H., Acién Fernández, F.., Robles Medina, A., Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol. Adv. 20, 491–515.

Muller-Feuga, A., Guédes, R. Le, Hervé, A., Durand, P., 1998. Comparison of artificial light photobioreactors and other production systems using Porphyridium cruentum. J. Appl. Phycol. 10, 83–90.

Nahar, G.A., 2010. Hydrogen rich gas production by the autothermal reforming of biodiesel (FAME) for utilization in the solid-oxide fuel cells: A thermodynamic analysis. Int. J. Hydrogen Energy 35, 8891–8911.

Norsker, N.-H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H., 2011. Microalgal production- a close look at the economics. Biotechnol. Adv. 29, 24–7.

Onat, N., Bayar, H., 2010. The sustainability indicators of power production systems. Renew. Sustain. Energy Rev. 14, 3108–3115.

Oswald, W.J., Goleeke, C.G., 1960. Biological transformation of solar energy. Adv. Appl. Microbiol. 2, 223–62.

Park, J.B.K., Craggs, R.J., Shilton, A.N., 2011. Wastewater treatment high rate algal ponds for biofuel production. Bioresour. Technol. 102, 35–42.

Park, Y.-M., Lee, J.Y., Chung, S.-H., Park, I.S., Lee, S.-Y., Kim, D.-K., Lee, J.-S., Lee, K.-Y., 2010. Esterification of used vegetable oils using the heterogeneous WO3/ZrO2 catalyst for production of biodiesel. Bioresour. Technol. 101 Suppl, S59–61.

Qin, J., 2005. Bio - hydrocarbons from algae : impacts of temperature, light and salinity on algae growth : a report for the Rural Industries Research and Development Corporation, RIRDC publ. ed. Barton ACT.

Radakovits, R., Jinkerson, R.E., Darzins, A., Posewitz, M.C., 2010. Genetic engineering of algae for enhanced biofuel production. Eukaryot. Cell 9, 486–501.

Ranjan, A., Patil, C., Moholkar, V.S., 2010. Mechanistic Assessment of Microalgal Lipid Extraction. Ind. Eng. Chem. Res. 49, 2979–2985.

Renaud, S.M., Thinh, L.-V., Lambrinidis, G., Parry, D.L., 2002. Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture 211, 195–214.

Richardson, J.W., Outlaw, J.L., Allison, M., 2010. The economics of microalgae oil. AgBioForum 13, 119–130.

Rossignol, N., Vandanjon, L., Jaouen, P., Quéméneur, F., 1999. Membrane technology for the continuous separation microalgae/culture medium: compared performances of cross-flow microfiltration and ultrafiltration. Aquac. Eng. 20, 191–208.

S. Carlsson, J.B. van Beilen, R. Moller, D.C., 2007. Micro and macro algae: Utility for industrial applications. CPL Press. University of York, Newbury.

Saidur, R., Islam, M.R., Rahim, N.A., Solangi, K.H., 2010. A review on global wind energy policy. Renew. Sustain. Energy Rev. 14, 1744–1762.

Sakai, N., Sakamoto, Y., Kishimoto, N., Chihara, M., Karube, I., 1995. Chlorella strains from hot springs tolerant to high temperature and high CO2. Energy Convers. Manag. 36, 693–696.

Schulz, T., 2006. The economics of micro-algae production and processing into biofuel.

Sharma, Y.C., Singh, B., Korstad, J., 2010. High yield and conversion of biodiesel from a nonedible feedstock (Pongamia pinnata). J. Agric. Food Chem. 58, 242–7.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae.

Shefner, A.M., King, M.E., Kohn, B., 1962. Enzymatic digestion of algal cells.

Stephens, E., Ross, I.L., King, Z., Mussgnug, J.H., Kruse, O., Posten, C., Borowitzka, M.A., Hankamer, B., 2010. An economic and technical evaluation of microalgal biofuels. Nat. Biotechnol. 28, 126–8.

Sydney, E.B., da Silva, T.E., Tokarski, A., Novak, A.C., de Carvalho, J.C., Woiciecohwski, A.L., Larroche, C., Soccol, C.R., 2011. Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. Appl. Energy 88, 3291–3294.

Takeda, H., 1988. Classification of Chlorella strains by cell wall sugar composition. Phytochemistry 27, 3823–3826.

Thirugnanasambandam, M., Iniyan, S., Goic, R., 2010. A review of solar thermal technologies. Renew. Sustain. Energy Rev. 14, 312–322.

U.S. DOE, 2010. National Algal Biofuels Technology Roadmap. U.S. Dep. Energy, Off. Energy Effic. Renew. Energy, Biomass Progr.

Uduman, N., Qi, Y., Danquah, M.K., Hoadley, A.F.A., 2010. Marine microalgae flocculation and focused beam reflectance measurement. Chem. Eng. J. 162, 935–940.

Um, B.-H., Kim, Y.-S., 2009. Review: A chance for Korea to advance algal-biodiesel technology. J. Ind. Eng. Chem. 15, 1–7.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S., Goodenough, U., 2009. Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless Chlamydomonas reinhardtii. Eukaryot. Cell 8, 1856–68.

Weldy, Huesemann, 2007. Lipid Production by Dunaliella salina in Batch Culture: Effects of Nitrogen Limitation and Light Intensity. J. Undergrad. Res. 7, 115–122.

Widjaja, A., Chien, C.-C., Ju, Y.-H., 2009. Study of increasing lipid production from fresh water microalgae Chlorella vulgaris. J. Taiwan Inst. Chem. Eng. 40, 13–20.

Williams, P.J. le B., Laurens, L.M.L., 2010. Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. Energy Environ. Sci. 3, 554.

Xu, H., Miao, X., Wu, Q., 2006. High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters. J. Biotechnol. 126, 499–507.

Y. Azov, J.C.G., 1982. Free Ammonia Inhibition of Algal Photosynthesis in Intensive Cultures. Appl. Environ. Microbiol. 43, 735–739.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y., 2011. Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. Bioresour. Technol. 102, 159–65.

Yeh, K.-L., Chang, J.-S., Chen, W., 2010. Effect of light supply and carbon source on cell growth and cellular composition of a newly isolated microalga Chlorella vulgaris ESP-31. Eng. Life Sci. 10, 201–208.

# 3. Chapter 3: Investigation of biomass concentration, lipid production and cellulose content in *Chlorella vulgaris* cultures using response surface methodology

The information presented in this Chapter is based in the paper "Investigation of Biomass Concentration, Lipid Production, and Cellulose Content in *Chlorella vulgaris* Cultures Using Response Surface Methodology.", published in Biotechnology and Bioengineering, August 2013, Vol. 110, Issue 8, pages 2114-2122. The sections in Chapter 3 present the results towards the completion of objectives 1 and 2 of the thesis (see section 1.2.2).

## 3.1. Abstract

The microalgae *Chlorella vulgaris* produce lipids that after extraction from cells can be converted into biodiesel. However, these lipids cannot be efficiently extracted from cells due to the presence of the microalgae cell wall, which acts as a barrier for lipid removal when traditional extraction methods are employed. Therefore, a microalgae system with high lipid productivity and thinner cell walls could be more suitable for lipid production from microalgae. This Chapter addresses the effect of culture conditions, specifically carbon dioxide and sodium nitrate concentrations, on biomass concentration and the ratio of lipid productivity/cellulose content. Optimization of culture conditions was done by RSM. The empirical model for biomass concentration ($R^2$=96.0%) led to a predicted maximum of 1123.2 mg dw $L^{-1}$ when carbon dioxide and sodium nitrate concentrations were 2.33% $vv^{-1}$ and 5.77 mM, respectively. For lipid productivity/cellulose content ratio ($R^2$=95.2%) the maximum predicted value was 0.46 (mg lipid $L^{-1}d^{-1}$)(mg cellulose mg biomass$^{-1}$)$^{-1}$ when carbon dioxide concentration was 4.02% $vv^{-1}$ and sodium nitrate concentration was 3.21 mM. A common optimum point for both variables (biomass concentration and lipid productivity/cellulose content ratio) was also found, predicting a biomass concentration of 1119.7 mg dw $L^{-1}$ and lipid productivity/cellulose content ratio

of 0.44 (mg lipid $L^{-1}d^{-1}$)(mg cellulose mg $biomass^{-1})^{-1}$ for culture conditions of 3.77% $vv^{-1}$ carbon dioxide and 4.01 mM sodium nitrate. The models were experimentally validated and results supported their accuracy. This study shows that it is possible to improve lipid productivity/cellulose content by manipulation of culture conditions, which may be applicable to any scale of bioreactors.

## 3.2. Introduction

Microalgae are known for producing high levels of lipids that after extraction from the cells can be converted into biodiesel. Among the different microalgae species, *Chlorella vulgaris* is one of the most studied due to its high lipid content reaching up to 50% $ww^{-1}$ (Costa and de Morais, 2011), and biological characteristics which makes it easier to culture (Aguirre *et al*., 2013; Fu *et al*., 2010). Lipids from *C. vulgaris* cannot be efficiently extracted due to the presence of a rigid cell wall. This cell wall represents a barrier for lipids diffusion when traditional extraction methods with solvents are employed. It reduces the yield product/biomass (in this case lipids/biomass) of the process, and consequently the amount of biodiesel that can be further produced. A culture containing microalgae cells with high lipid productivity and low cellulose content (as an indicator of cell wall thickness) is ideally desired in a biodiesel from microalgae process. So, treatments for breaking the cell wall would be less intensive and therefore more economically feasible and environmentally friendly, since less solvents or energy would be needed. A first attempt to understand lipid extraction consists in the study of culture conditions on cellulose content in microalgae.

Previous studies show that cell wall permeability depends on the size of the molecule that is being extracted (Skene, 1943; Sokolnicki *et al*., 2006) and cell wall thickness. It is speculated that when cellulose content increases the difficulty for mass transfer (lipid extraction from inside the cell) potentially can also increase, since the cell wall acts as a barrier where only diffusional processes take place. On the other hand, when cell wall disruption techniques are employed, the thicker the cell wall the more intensive the potential treatment needed (Van Hee *et al*., 2004), especially if microalgae cell size is considered.

Cellulose content is of great interest in the study of some strategies for cell disruption aiming to release the lipids produced by cells (Arad and Levy-Ontman, 2010b; Barbir *et al*., 1990). The cellulose content in sea-weed species was studied and found to be 1-20% of the algae biomass and in filamentous green algae as high as 20-45% (Mihranyan, 2011; Siddhanta *et al*., 2009). The composition of the cell wall varies among different species of microalgae, but in the case of *C. vulgaris* cellulose is the main polymer in the cell wall comprising around 70-80% dw (Abo-Shady *et al*., 1993; Preston, 1974). Even though the cell wall plays a fundamental role on lipid extraction, few reports have been found on the effect of culture conditions on cellulose content, making this area of high interest for research (Adda *et al*., 1986). For example, the effect of light over cellulose content in the cell wall of *Chlorella pyrenoidosa* was studied by Makooi, (1976) and their results showed that mixotrophic growth produces the highest amount of cellulose, followed by heterotrophic and photoautotrophic growth, as detailed in section 2.5.2.

The production of carbohydrates in microalgae has two purposes, first they are structural components in the cell wall, and second they provide storage of energy inside the cell (Markou *et al*., 2012). The composition of microalgae can be manipulated by modifying the cultivation conditions including for instance nutrients, light, and temperature. It results in one affordable way to change the amount of carbohydrates and lipids produce by the cell.

It is known that carbon dioxide and nitrate concentration have a significant effect on microalgae growth and lipid production, but little information is available on their effects on cellulose content. The effect of $CO_2$ concentration on microalgae growth was first shown by Briggs and Whittingham, (1952) in cultures of *Chlorella* (Briggs and Whittingham, 1952; Tsuzuki and Miyachi, 1989). Tang *et al*., (2011), evaluated a wide range of $CO_2$ concentrations (from 0.03% to 50%) on *Scenedesmus obliquus* and *Chlorella pyrenoidosa*; for both species, best growth was observed at 10% $CO_2$, but higher concentrations were favorable for lipid accumulation, reaching lipid content values of 24.4% and 26.8%, respectively. In cultures of *C. vulgaris* the increase in $CO_2$ concentration did not increase the biomass growth until the later stages of the batch culture, however changes in lipid content were significant (Lv *et al*., 2010). Optimal values for $CO_2$ concentration not only changes among different microalgae strains, but also for the same strain growing under slightly different conditions.

Under unfavorable environmental conditions for growth (e.g. nitrogen depletion or high temperature), microalgae change their biosynthetic pathways towards the formation and storage of neutral lipids, especially triacylglycerols and hydrocarbons (Guschina and Harwood, 2006). Illman *et al*., (2000) found that the reduction of nitrogen in the medium increases the lipid content in five *Chlorella* strains. Specifically for *C. vulgaris* it was found that nitrogen reduction increased lipid content in biomass to 40%. Xin *et al*., (2010), performed a study where cells of *Scenedesmus* sp. were subjected to nitrogen and phosphorus limitation, the results showed that even though lipid content was increased, lipid productivity was not enhanced. This suggests that nitrogen source not only increases lipid production but also reduces biomass growth. Tam and Wong (1996), found that cultures containing either very low (10 $mgL^{-1}$) or very high (1000 $mgL^{-1}$) nitrogen concentrations have less growth. In cultures of *Chlorella sorokiniana* and *Oocystis polymorpha* grown in batch reactors, nitrogen could be reduced to 3% of dry weight, causing a remarkable increase in total fatty acids and changes in their composition (Richardson *et al*., 1969). In the case of *Nannochloropsis oculata*, a 75% reduction of the nitrogen concentration in media (compared with optimal values for biomass production), increased the lipid content from 7.90% to 15.31%; and in cultures of *C.vulgaris* from 5.90% to 16.41% (Converti *et al*., 2009). For these reasons, an optimal value for high lipid productivity, that implies high biomass productivity and lipid content, must be found.

RSM is a statistical and mathematical tool used for optimization processes. Especially in those cases where the underlying mechanisms are not completely known, and therefore a mechanistic model is not easy to obtain. The nature of the model is usually a first or second order polynomial from where a response surface is plot. In biochemical processes, non-linear behaviors are common and a second-order model will likely be required to better represent and fit experimental data. The use of second order equations for empirical modeling has several advantages, among them are flexibility in providing good approximations to the true response surface, the regression of data for coefficients estimations is easy, and it has shown to provide feasible results in many applications. There are many statistical design approaches available, the most used one is the CCD that includes the use of a two-level factorial, axial and central points; each one providing information about the existence and estimation of terms in the second order equation (Box and Wilson, 1992).

CCD also provides the base for optimization of several parameters simultaneously, which is a common need in industrial processes since operating condition must satisfied different restrictions. Once an experiment to fit response models has been conduct, the optimization of multiple responses can be performed. There are some well-known methods for multiple response optimizations, but the desirability (D) approach is the most used one in industry to solve this kind of problems. In this method a D function is assigned for each response variable, each of them must have a value between 0 and 1, being 0 a completely undesirable value and 1 a completely desirable response. The D functions for each response are combined by means of the geometric mean, which provides the overall D that is maximized with respect to the controllable factors (Myers *et al*., 2004).

In this Chapter a study on the simultaneous effect of carbon dioxide ($CO_2$) and sodium nitrate ($NaNO_3$) concentrations on *C. vulgaris* biomass concentration (β), lipid productivity and cellulose content was carried out, the last two by means of the ratio lipid productivity/cellulose content (Θ) (See Table 3.1 for nomenclature). The term Θ (lipid productivity/cellulose content ratio) is applied as the parameter for optimization because the ratio between these variables  has a more practical meaning than their independent study, since the value found would be that one leading to an operating point where there is an equilibrium between the optimal for lipid productivity and the optimal for cellulose content. Optimization of these parameters under the conditions studied was performed by RSM that led to $CO_2$ (*X*) and $NaNO_3$ (*Y*) concentrations that produce the highest β and Θ ratio in the range covered by the experimental design. In this Chapter the Θ ratio is introduced as a way to compare the amount of lipids produced on a known period of time in relation with the amount of cellulose in the same culture. Therefore, a culture with a high Θ ratio could be suitable for production of biodiesel with a potentially easier extraction step. In a production system, the optimization of the parameters β and Θ would help to find the operating point where the biomass concentration and the lipid productivity are high, while the cellulose content remains low. Therefore the amount of lipids produced would be higher and the product separation would be easier, both features affecting the process feasibility in a positive way.

**Table 3.1** Nomenclature used in Chapter 3

| Symbol | Name | Units |
|---|---|---|
| *X* | Carbon dioxide concentration | % $vv^{-1}$ |
| *Y* | Sodium nitrate concentration | mM |
| β | Biomass concentration | mg dw $L^{-1}$ |
| Θ | Lipid productivity/cellulose content ratio | (mg lipid $L^{-1}d^{-1}$)(mg cellulose mg $biomass^{-1}$)$^{-1}$ |

## 3.3. Materials and methods

To obtain the information needed the following protocols were implemented.

### 3.3.1. Microalgae strain and culture media

*C. vulgaris* UTEX 2714 was used for this study. The microalgae were originally isolated from a wastewater-treatment stabilization pond in Bogota, Colombia. The strain was transferred from Proteose Medium agar slant to liquid Bold's modified media (0.25 $gL^{-1}$ $NaNO_3$, 0.025 $gL^{-1}$ $CaCl_2.2H_2O$, 0.075 $gL^{-1}$ $MgSO_4.7H_2O$, 0.075 $gL^{-1}$ $K_2HPO_4$, 0.175 $gL^{-1}$ $KH_2PO_4$, 0.025 $gL^{-1}$ NaCl, 63.9 $mgL^{-1}$ $Na_2EDTA$, 4.98 $mgL^{-1}$ $FeSO_4.7H_2O$, 11.42 $mgL^{-1}$ $H_3BO_3$, 8.82 $mgL^{-1}$ $ZnSO_4.7H_2O$, 1.44 $mgL^{-1}$ $MnCl_2.4H_2O$, 1.57 $mgL^{-1}$ $CuSO_4.5H_2O$), pH of the media was adjusted to 6.6 and sterilized in autoclave at 121°C, 21 psig for 15 minutes. Cultures were incubated at room temperature (23±2°C) with continuous air bubbling (7 $Lmin^{-1}$) and sub-cultured every 2 weeks until they reached 380 mg dw biomass per liter of media and were used as inoculum for experiments.

### 3.3.2. Experimental set-up

Microalgae were cultured in Bold's modified media with the same composition described above, but sodium nitrate ($NaNO_3$) concentrations changed according to experiment design (SeeTable 3.2 column 3).

**Table 3.2** Variables and experimental CCD levels for RSM.

| Treatment | $[CO_2]$ (*X*) (% $vv^{-1}$) | $[NaNO_3]$ (*Y*) (mM) | β (mg dw $L^{-1}$) | | Θ ratio (mg lipid $L^{-1}d^{-1}$)(mg cellulose mg $biomass^{-1})^{-1}$ | |
|---|---|---|---|---|---|---|
| | | | **Measured** | **Model** | **Measured** | **Model** |
| T1 | 1.50 (-1.41) | 3.77 (0) | 1075.4 | 1063.4 | 0.19 | 0.22 |
| T2 | 2.33 (-1) | 5.77 (1) | 1110.4 | 1123.2 | 0.20 | 0.19 |
| T3 | 2.33 (-1) | 1.77 (-1) | 808.8 | 826.0 | 0.32 | 0.30 |
| T4 | 4.33 (0) | 0.94 (-1.41) | 606.9 | 618.4 | 0.34 | 0.32 |
| T5'(replicate 1) | 4.33 (0) | 3.77 (0) | 1044.4 | 1082.4 | 0.50 | 0.45 |
| T5'(replicate 2) | 4.33 (0) | 3.77 (0) | 1121.4 | 1082.4 | 0.40 | 0.45 |
| T5'(replicate 3) | 4.33 (0) | 3.77 (0) | 1081.4 | 1082.4 | 0.44 | 0.45 |
| T6 | 4.33 (0) | 6.60 (1.41) | 1094.9 | 1038.7 | 0.13 | 0.16 |
| T7 | 6.33 (1) | 1.77 (-1) | 651.2 | 630.9 | 0.17 | 0.21 |
| T8 | 6.33 (1) | 5.77 (1) | 848.3 | 928.1 | 0.13 | 0.10 |
| T9 | 7.16 (1.41) | 3.77 (0) | 820.3 | 787.5 | 0.09 | 0.09 |

Several 4 L flasks containing 3 L of media and 0.5 L of inoculum with a biomass concentration of 380 mg dw biomass per liter of media were used. Air enriched with carbon dioxide ($CO_2$) was injected to media and flow was controlled with rotameters (Multi-tube rotameters and gas mixer, Omega, Stamford, USA) (for calibration curves of gas mixers and rotameters refer to Figure 7.1, Figure 7.2 and Figure 7.3). The $CO_2$ concentration in air varied according to experimental design (See Table 3.2 column 2). Light was provided with fluorescent lamps (T5) (see Figure 7.4 for lamp spectrum) and photo-period of 12 h light: 12 h dark. From previous experiments on *C. vulgaris* growth was seen that after 16 days of culture biomass had reached the stationary phase of growth

and therefore the experiments were conducted for that period of time. The experimental set-up is presented in Figure 3.1.

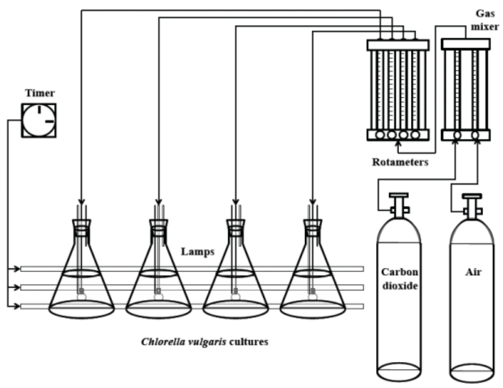


**Figure 3.1** Schematic diagram and picture of experimental set-up used for *C. vulgaris* cultivation.

### 3.3.3. Variables measurement

Following are the methodology used to measure the response variables.

#### *3.3.3.1. Biomass concentration (β)*

β was calculated by Optical Density (OD) measurements at 686 nm (Bhola *et al*., 2011) using a spectrophotometer (DR 2800 portable spectrophotometer, HACH.). A standard curve was done to correlate dry biomass concentration with OD. The equation for β (mg dw $L^{-1}$)=250.1*$OD_{686}$ ($R^2$=0.99) was obtained by linear regression of data (see Figure 7.5). Measurements were done every other day during culture time. Cultures were periodically monitored by observing samples under the microscope to avoid contaminated cultures.

#### *3.3.3.2. Nitrate concentration*

Nitrate concentration was measured by OD after reaction with chromotropic acid under acidic conditions (sulfuric acid). This reaction produces a yellow product with a maximum absorbance at 410 nm. The kit Nitrate High range Test 'N Tube (0 to 30.0 $mgL^{-1}$ $NO_3$-N) from HACH was used for this purpose and sample dilutions were done when needed (DR 2800 portable spectrophotometer, HACH, Loveland, USA).

#### *3.3.3.3. Lipid content*

The measurement of lipid content (lipid mass (biomass $mass^{-1}$) *100) was carried out by following a modified version of Folch's method (Folch *et al*., 1957; Krienitz and Wirth, 2006; Wahlen *et al*., 2011). At day 16 biomass was harvested by centrifugation (Sorvall ST 40R, Thermo Scientific) for 20 minutes at 3400 rpm and 4°C, pellet was collected on aluminum pans and dried at 105°C for 40 h (Converti *et al*., 2009). Dried biomass was pulverized to improve lipid extraction. For each treatment a known amount of dried biomass was homogenized with chloroform: methanol 2:1 ($vv^{-1}$) to a final dilution 20 fold the volume of the sample. Samples were placed for 15 minutes in a sonicator (UP400S, Hielscher, Teltow, Germany) to break the cell wall down. Extraction was allowed to take place overnight (12 h). The sample was vacuum filtered and the extract was mixed in a vortex with 0.2 times its volume of water, the mixture was allowed to separate into two phases by centrifugation, the upper phase was removed and the lower phase was placed in pre-weighed aluminum pans for evaporation of solvent and the lipids remained on the pans. Lipid content was then calculated gravimetrically (Wahlen *et al*., 2011).

#### *3.3.3.4. Cellulose content*

The Updegraff method (Updegraff, 1969) was used to extract and quantify the cellulose content. At day 16 biomass was harvested using the same conditions previously described. To each sample 3 ml of acetic-nitric reagent (150 ml 80% of acetic acid and 15 ml concentrated nitric acid) was added and mixed. The samples were placed in a boiling water bath for 30 minutes and then centrifuged 5 minutes at 3500 rpm. The supernatant was discarded and the pellet was washed with distilled water and centrifuged to remove water. Ten milliliters of 67% ($vv^{-1}$) sulfuric acid was added and samples were left to stand for 1 h and then diluted with water according to original protocol. Anthrone reagent (0.2 g anthrone in 100 ml concentrated $H_2SO_4$ and chilled for 2 h in refrigerator prior to use) was added and diluted samples mixed with a vortex. The reaction took place in a boiling water bath for 16 minutes. The samples were cooled down to room temperature and OD was measured at 620 nm. The cellulose content and $OD_{620}$ was correlated with a standard curve previously obtained for pure cellulose, following the protocol presented by Updegraff, (1969) for this purpose (see Figure 7.6).

#### *3.3.3.5. Experimental design*

RSM was employed to optimize the concentrations of $CO_2$ and $NaNO_3$ that leads to the highest values for β and Θ ratio. To fit experimental data to mathematical model, the CCD $2^2$ + star was used with 2 factors and 5 levels. The CCD consisted of 9 experiments with 3 replicates for the central point and $\alpha=\pm1.41$., Table 3.2 in columns 2 and 3, presents the codified (terms in parenthesis) and actual values for each treatment. The experiments appear in the table in order they were performed. Mathematical models describing the relationship between response variables (β and Θ ratio) and manipulated variables ($CO_2$ ($X$) and $NaNO_3$ ($Y$) concentrations) were developed by finding the coefficients of a second order equation.

All the calculations were done using the data for day 16 (last day of the culture). The accuracy of the model was calculated by the regression coefficients $R^2$ and adjusted $R^2$ (adj $R^2$). To identify the statistically significant terms the analysis of variance (ANOVA) was employed. Significance of regression coefficients was determined with a confidence level of 95%. The statistical analysis and the optimum values for each response variable were found based on mathematical models using Statgraphics Centurion XVI (StatPoint Technologies, Inc, Warrenton, USA).

## 3.4. Results and discussion

### 3.4.1. Codified and actual values for central composite design

Table 3.2 shows the CCD for treatments evaluated. The $CO_2$ and $NaNO_3$ concentrations ranged from 1.50 % $vv^{-1}$ to 7.16 % $vv^{-1}$ and from 0.94 mM to 6.60 mM, respectively. The area of study covered by these concentrations was selected according to previous literature information about best $CO_2$ and $NaNO_3$ concentrations found for *C. vulgaris* growing under different conditions (Converti *et al*., 2009; Illman *et al*., 2000; Lv *et al*., 2010; Tang *et al*., 2011).

### 3.4.2. Biomass concentration as a function of carbon dioxide and sodium nitrate concentration

Figure 3.2 shows the growth curves for all treatments. As it can be seen all cultures followed a similar pattern; no adaptation phase was observed for any treatment, meaning that cells were well adapted to media and operating conditions. Based in all the points the growth is likely linear. Cultures grew in a linear fashion way up to the point where the nutrients are depleting. For all treatments the growth rate in this stage was quite similar (average value for all treatments was 0.38±0.03 $gL^{-1}d^{-1}$, and $R^2$=0.99±0.01). After day 4, the growth rate was reduced for all treatments, especially for that one with the lowest $NaNO_3$ concentration (T4). The time where growth rate is reduced corresponds to that where $NaNO_3$ concentration is depleting.

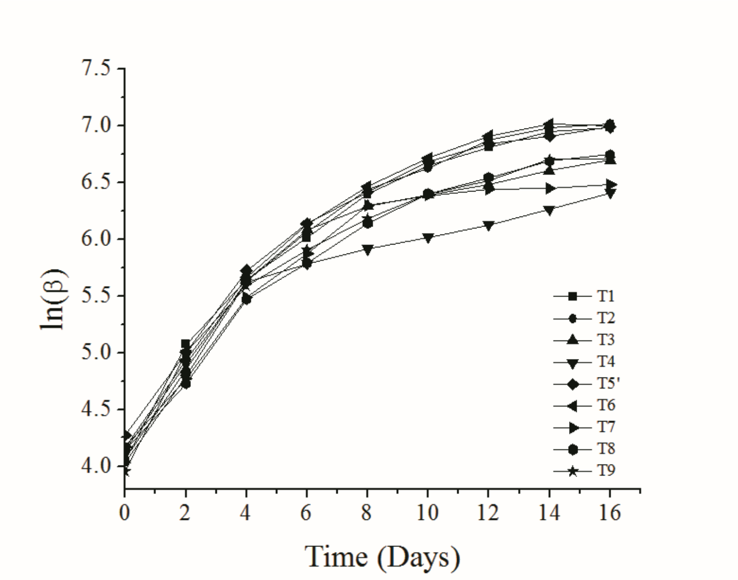



**Figure 3.2** *C. vulgaris* growth curves under different $CO_2$ (*X*) and $NaNO_3$ (*Y*) concentrations.

Figure 3.3 presents nitrate consumption profile for treatments T4, T5’ and T6 (all at 4.33% $vv^{-1}$ $CO_2$) where the behavior mentioned is clearly depicted. Treatment T4 had the lowest initial $NaNO_3$ concentration (0.94 mM), for this treatment nitrates were completely depleted at day 4 which corresponds to a remarkable reduction in biomass growth (See Figure 3.2). Treatment T6 had the highest $NaNO_3$ concentration and the biomass growth rate was higher for longer period of time reaching one of the highest β values in the rage studied. This suggests that $NaNO_3$ played a fundamental role in *C. vulgaris* growth (Mahboob *et al*., 2011; Shi *et al*., 2000).

The simultaneous effect of $CO_2$ (*X*) and $NaNO_3$ (*Y*) concentration on β was studied. The experimental CCD matrix is presented in Table 3.2. β ranged from 606.9 mg dw $L^{-1}$ to 1121.4 mg dw $L^{-1}$ which correspond to treatments T4 and T5’, respectively. Table 3.3 shows the ANOVA that partitions the variability in β into separate pieces for each of the effects. In this case only the significant regression coefficients having *P*-values less than 0.05 (indicating that they are significant different from zero) were considered into the model. The $R^2$ coefficient indicates that the model as fitted explains 96.01% of the variability in β; the adj $R^2$, which is more used to compare different models, was 93.35% (For statistical software outputs refer to section 7.2.1).

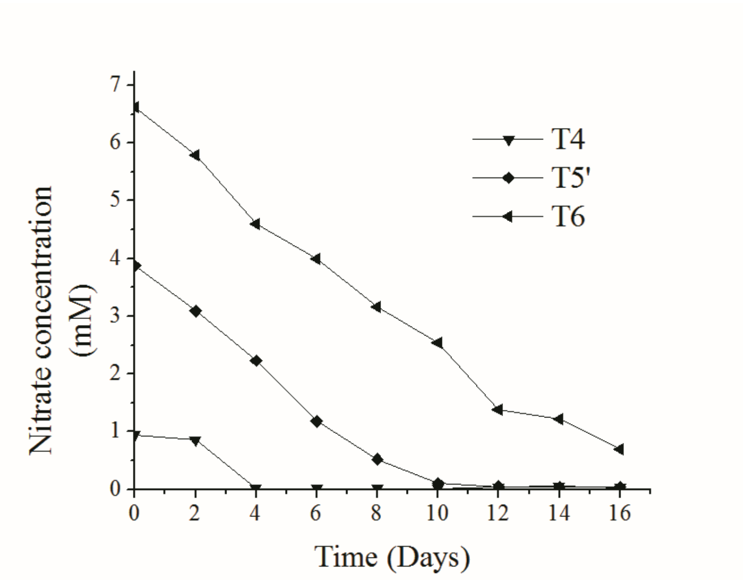



**Figure 3.3** $NaNO_3$ consumption profile in *C. vulgaris* cultures under same $CO_2$ (*X*) concentration.

**Table 3.3** Analysis of variance for β model

| **Source** | **Sum of Squares** | **Degree of freedom** | **Mean Square** | **F-Ratio** | ***P*-Value** |
|---|---|---|---|---|---|
| *X* | 76131.9 | 1 | 76131.9 | 31.78 | 0.0024 |
| *Y* | 176650. | 1 | 176650. | 73.74 | 0.0004 |
| $X^2$ | 34758.1 | 1 | 34758.1 | 14.51 | 0.0125 |
| *XY* | 2732.15 | 1 | 2732.15 | 1.14 | 0.3344 |
| $Y^2$ | 90955.1 | 1 | 90955.1 | 37.97 | 0.0016 |
| Total error | 11978.0 | 5 | 2395.6 | | |
| Total (corr.) | 368903. | 10 | | | |

$R^2$:96.0125%, Adj $R^2$:93.3541%

These results indicate good accuracy of the model. The regression coefficients of the second order equation were calculated using the designed experimental data leading to the following model of β as a function of $CO_2$ (*X*) and $NaNO_3$ (*Y*) concentrations (Equation 3.1):

$$\beta=194.84+121.08X+313.53Y-19.61X^2-31.73Y^2 \quad \textbf{Equation 3.1}$$

Equation 3.1 indicates that coefficients of the linear terms, *X* and *Y*, have positive effect by increasing β. However, quadratic terms ($X^2$ and $Y^2$) have negative effects. Figure 3.4 shows the response surface and contours plot for β. From this figure it can be seen that $NaNO_3$ has a stronger effect than $CO_2$ concentration.

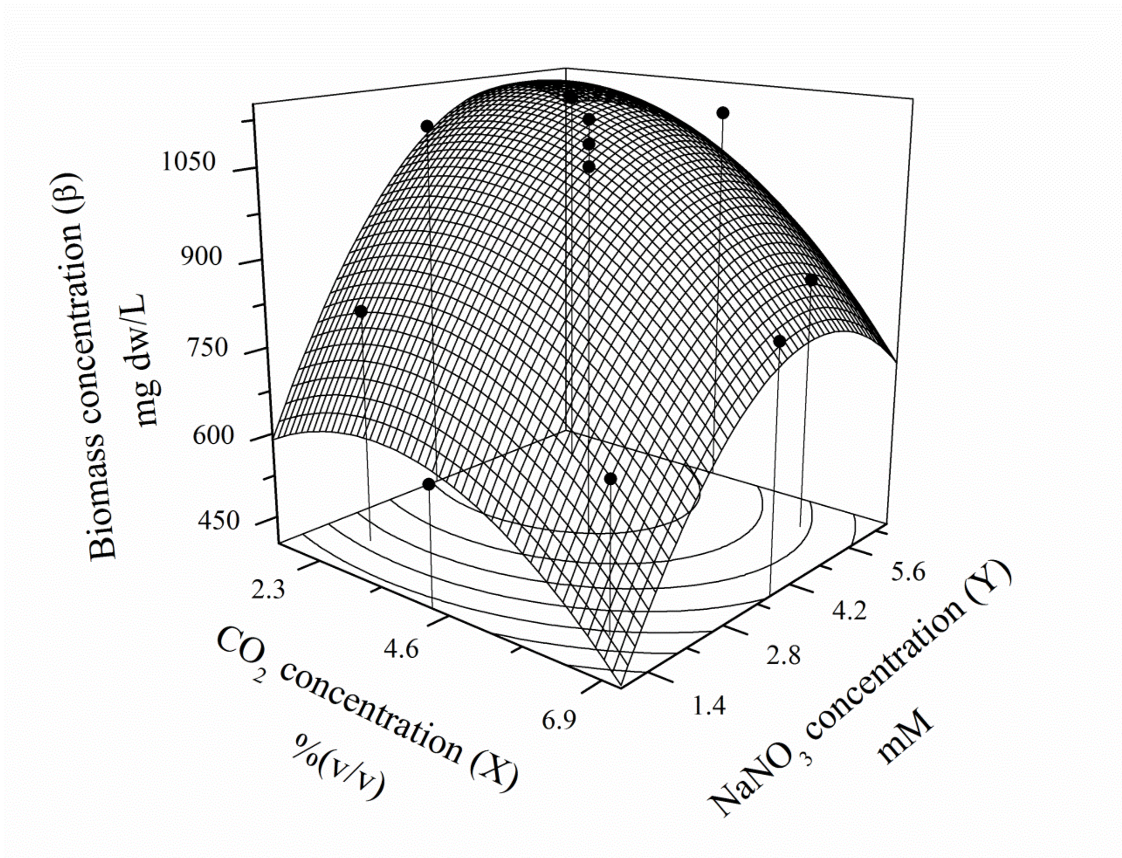



**Figure 3.4** Response surface and contour lines indicating the effect of $CO_2$ (*X*) and $NaNO_3$ (*Y*) on *C. vulgaris* biomass concentration (β).

When the $CO_2$ concentration is set at a high value, β increases ($NaNO_3$ concentrations from 0.94 mM to around 4.5 mM), and then slightly decreases ($NaNO_3$ concentrations from 4.5 mM to 5.77 mM). This effect is enhanced for lower $CO_2$ concentrations, where biomass increases ($NaNO_3$ concentrations from 0.94 mM to around 5.3 mM) and the reduction in β occurring for the higher $NaNO_3$ concentration is practically negligible. The effect of $CO_2$ seems to be the opposite; for the lower values of $NaNO_3$, β slightly increases ($CO_2$ concentrations from 2.33% to around 3.5%) and then strongly decreases ($CO_2$ concentrations from 3.5% to 7.16%). Table 3.2, in columns 4 and 5, compares the observed experimental data with model predicted results for β.

### 3.4.3. Lipid productivity/cellulose content as a function of carbon dioxide and sodium nitrate concentration

Lipid content and cellulose content were measured for each treatment at last day of culture. Figure 3.5 present these results in order of increasing lipid content.

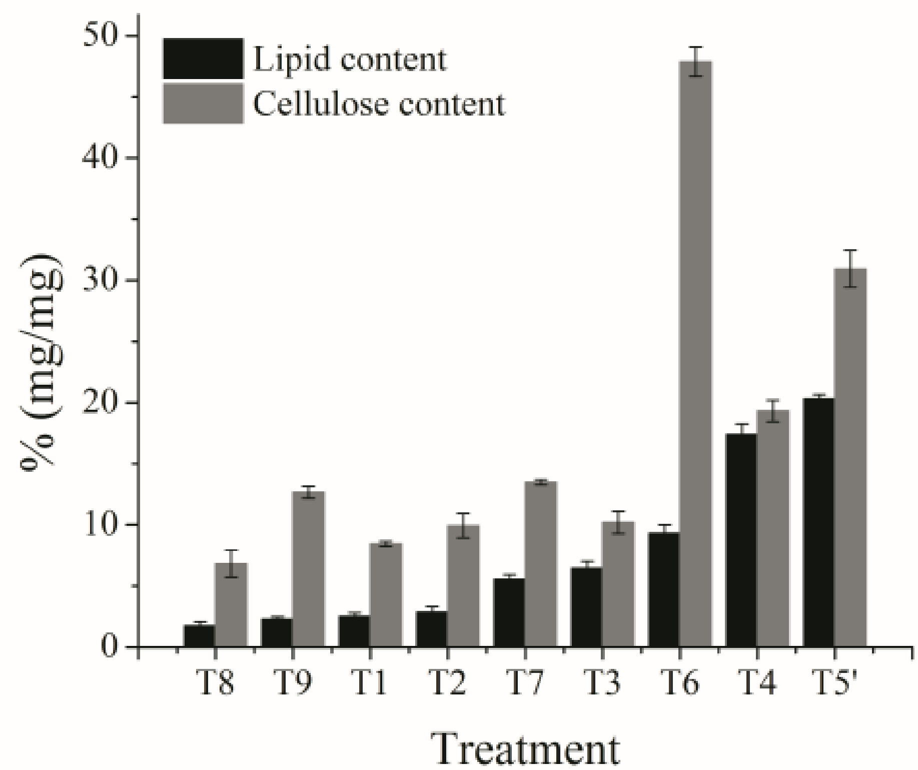



**Figure 3.5** Lipid and cellulose content in *C. vulgaris* cultures under different $CO_2$ ($X$) and $NaNO_3$ ($Y$) concentrations.

Treatments T4 and T5' had the highest lipid content with values of 17.39 % $ww^{-1}$ and 20.31 % $ww^{-1}$, respectively. Their nitrate concentrations belong to the lowest values tried in this experiment. Nitrogen deprivation has a strong effect on amino acid synthesis, reducing protein availability and production, and therefore mitigating the rate of growth and photosynthesis. As response to these conditions the cell produces lipids that act as carbon and energy reservoir (Falkowski *et al*., 1989). According to Gerken *et al*., (2013) nitrogen depletion not only changes the lipid composition of the cell but also the morphology of the cell wall in *C. vulgaris* by reducing the hair-like fibers on the surface of the cell.

The data in Figure 3.5 were plotted in 3D in order to better understand the behavior of results (figures not shown), from this was seen that lipid content increases when $CO_2$ concentration increases until it reaches a value close to 4.3% ($vv^{-1}$) from this $CO_2$ value lipid content reduces; this pattern was observed for all $NaNO_3$ concentrations. For cellulose content results, it was seen that the cellulose content is quite constant for the lowest and highest $CO_2$ concentrations, but for 4.3% $CO_2$ ($vv^{-1}$) cellulose content increases when $NaNO_3$ increases. The treatment leading to the highest lipid content/cellulose content was T4 with a value of 0.9 mg $mg^{-1}$. Treatment T6 had the highest cellulose content in relation with the amount of lipid produced. This treatment had the highest initial $NaNO_3$ concentration and relative high $CO_2$ concentration, so no limitations for growth were imposed over cells and probably they could produce more cellulose. Even though, lipid content is an important factor in lipid production from microalgae, at industrial scale lipid productivity plays an even more important role, since industry is more interested in the amount of lipids that can be obtained in a known period of time which have a strong effect on economic feasibility of the process. It is for that reason that in this study a model which includes the lipid productivity term as part of the response variable was considered.

With the aim of finding the $CO_2$ and $NaNO_3$ concentrations that lead to the point where the $\Theta$ ratio is maximum (indicating cultures where cells produce more lipids in a shorter period of time and have low cellulose content) an optimization based on RSM was performed. The same levels and factors in the study of β were used (see Table 3.2). The lowest Θ ratio was obtained when cultures were subjected to treatment T9 and the highest ratio corresponds to treatment T5'. Treatment T5' had $CO_2$ (4.3% $vv^{-1}$) and $NaNO_3$ (3.77 mM) concentrations, so enough nutrients were provided during the first stage of growth (biomass productivity of 67.65 $mgL^{-1}d^{-1}$), therefore a high β value was present when $NaNO_3$ was depleted at day 10 (see Figure 3.3); and microalgae cultures remained under nitrate starvation conditions for 6 days, inducing cells towards the production of lipids.

Table 3.4 shows the ANOVA for Θ ratio study. Once again, only statistically significant terms (*P*-value<0.05) were taken into account for model fitting. Regression coefficients had values of $R^2$: 95.17% and Adj $R^2$: 91.95%, which indicates a good fitting of experimental data to second order model.

**Table 3.4** Analysis of variance for Θ ratio model.

| Source | Sum of Squares | Degree of freedom | Mean Square | F-Ratio | *P*-Value |
|---|---|---|---|---|---|
| $X$ | 0.0168886 | 1 | 0.0168886 | 11.31 | 0.0200 |
| $Y$ | 0.0253594 | 1 | 0.0253594 | 16.98 | 0.0092 |
| $X^2$ | 0.121515 | 1 | 0.121515 | 81.39 | 0.0003 |
| $XY$ | 0.00189305 | 1 | 0.00189305 | 1.27 | 0.3113 |
| $Y^2$ | 0.0576065 | 1 | 0.0576065 | 38.58 | 0.0016 |
| Total error | 0.00746538 | 5 | 0.00149308 | | |
| Total (corr.) | 0.193814 | 10 | | | |

$R^2$:95.1714%, Adj $R^2$:91.9524%

Regression coefficients were calculated and an empirical model describing the effect of $CO_2$ (*X*) and $NaNO_3$ (*Y*) concentration on Θ ratio was obtained (Equation 3.2). From Equation 3.2 it can be concluded that linear terms have a positive effect on increasing Θ ratio while quadratic terms have a negative effect (For statistical software outputs refer to section 7.2.2).

$$\Theta=-0.39+0.29X+0.16Y-0.04X^2-0.03Y^2 \qquad \textbf{Equation 3.2}$$

Figure 3.6 shows the response surface and contour plot for Θ ratio. For high and low $CO_2$ concentrations, as $NaNO_3$ concentration increases the Θ ratio increases up to 3.7 mM of $NaNO_3$ where this ratio started to decrease. On the other hand, the $CO_2$ concentration showed that, independently of $NaNO_3$ concentration, for values higher than 4.0% $vv^{-1}$ the Θ ratio decreases abruptly. Table 3.2, in columns 6 and 7, compares the observed experimental data with model predicted results for Θ ratio; for this model similar amount of sub and over-estimated points were obtained.

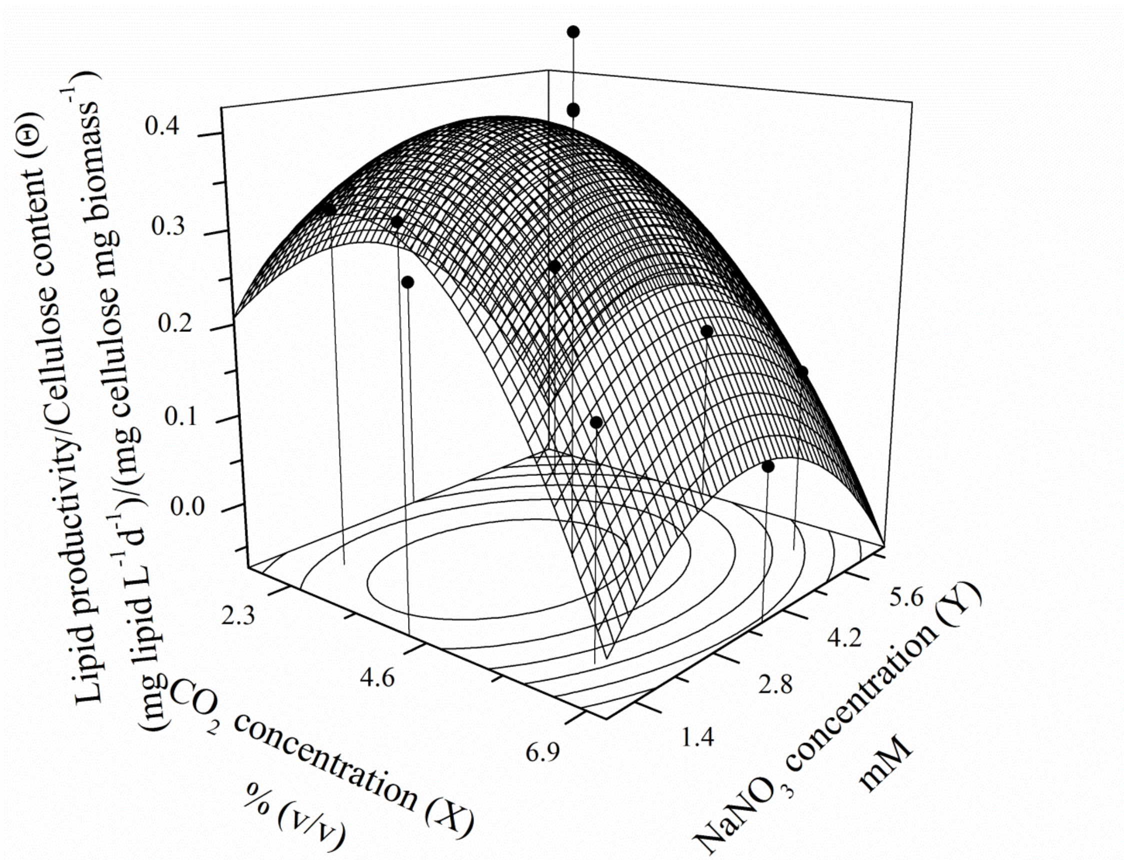



**Figure 3.6** Response surface and contour lines indicating the effect of $CO_2$ (*X*) and $NaNO_3$ (*Y*) on *C. vulgaris* lipid productivity/cellulose content ratio (Θ).

### 3.4.4. Optimization and model validation

Optimization was performed for the empirical mathematical models. Two different approaches were used and each one would be useful under different conditions. The first approach involved the independent optimization of each response variable. It means that one optimum point, in terms of $CO_2$ and $NaNO_3$ concentration, was found for β and other different point was found for Θ ratio, each one based on its own mathematical model. The results are presented in Table 3.5.

**Table 3.5** Optimized values and model validation results for β and Θ ratio

| **Optimization approach** | **Response variable** | **$CO_2$ concentration (*X*) (% $vv^{-1}$)** | **$NaNO_3$ concentration (*Y*) (mM)** | **Lower 95.0% limit** | **Upper 95.0% limit** | **Observed** | **Model result** |
|---|---|---|---|---|---|---|---|
| Individual optimization | β | 2.33 | 5.77 | 1049.01 | 1197.4 | 1069.0±7.33 | 1123.2 |
| | Θ ratio | 4.02 | 3.21 | 0.40 | 0.51 | 0.50±0.09 | 0.46 |
| Simultaneous optimization | β | 3.77 | 4.01 | 1051.6 | 1187.7 | 1102.9±33.8 | 1119.7 |
| | Θ ratio | | | 0.38 | 0.49 | 0.45±0.05 | 0.44 |

Optimized D for simultaneous approach: 0.92

According to Equation 3.1 the highest β value in the range covered by the experimental design would be obtained when cultures are submitted to 2.33 % $vv^{-1}$ $CO_2$ and 5.77 mM $NaNO_3$. The optimal point was located very close to central point of the experiment design. On the other hand, optimization based on Equation 3.2 leads to the point where Θ ratio is a maximum, this point corresponds to 4.02 % $vv^{-1}$ $CO_2$ and 3.21 mM $NaNO_3$. This optimization approach would be useful when only one of the variables is of interest for researchers. The second approach involves the simultaneous optimization of both response variables. It means that conditions found are those leading to maximize β and Θ ratio at the same time. This point was found at 3.77 % $vv^{-1}$ $CO_2$ and 4.01 mM $NaNO_3$ (see Table 3.5).

Experimental validation of the models was done under the conditions leading to the optimal results (see Table 3.5). Independent experiments to those for the CCD were done. The accuracy of the model was validated with triplicate experiments. For individual optimization of β the optimal point was found to be 2.33 % $vv^{-1}$ of $CO_2$ and 5.77 mM of $NaNO_3$. The model predicted a β value of 1123.2 mg dw $L^{-1}$ and the experimental result obtained for these conditions was 1069.0±7.33 mg dw $L^{-1}$; this β value is in the range of the confidence interval. As a result, the model was considered to be accurate and reliable for predicting β. Also positive results were obtained for individual optimization of Θ ratio; the optimal culture conditions were found to be 4.02 % $vv^{-1}$ of $CO_2$ and 3.21 mM of $NaNO_3$. The optimal value from the model was 0.46 (mg lipid $L^{-1}d^{-1}$)(mg cellulose mg biomass$^{-1}$)$^{-1}$ which agreed with the experimental value 0.50±0.09 (mg lipid $L^{-1}d^{-1}$)(mg cellulose mg biomass$^{-1}$)$^{-1}$ well.

In the case of simultaneous optimization (experiments conducted at 3.77 % $vv^{-1}$ of $CO_2$ and 4.01 mM of $NaNO_3$) there was also agreement between predicted and experimental results for the response variables $\beta$ and $\Theta$ ratio. Therefore, this approach for the optimization is also accurate and reliable.

## 3.5. Conclusions

Cells of *Chlorella vulgaris* growing under the studied conditions were well adapted, all treatments followed a similar growing profile and no adaptation phase was observed. Sodium nitrate was a determining factor for cell growth; its depletion considerable reduced the growth rate. Empirical models obtained for biomass concentration and lipid/content ratio applying the RSM had good accuracy explaining the 96.01% and 93.35% of the response variables respectively. Differences on cellulose content in cells of *Chlorella vulgaris* were obtained when subjected to different culture conditions. The location of an optimal point in the range of study, where lipid productivity is high and cellulose content is low, was possible by means of the CCD. Experimental validation confirmed de accuracy and feasibility of the models. The main goal of this Chapter was to show the effect of culture conditions on cellulose content. The results of this study could be applied in further experiments about cell wall disruption, where the cellulose content could play an important role in the intensity of the treatment needed. Any small increases in efficiency can translate into potentially large savings at high volumes.

## References

Abo-Shady, A.M., Mohamed, Y.A., Lasheen, T., 1993. Chemical composition of the cell wall in some green algae species. Biol. Plant. 35, 629–632.

Adda, M., Merchuk, J.C., (Malis) Arad, S., 1986. Effect of nitrate on growth and production of cell-wall polysaccharide by the unicellular red alga Porphyridium. Biomass 10, 131–140.

Aguirre, A.-M., Bassi, A., Saxena, P., 2013. Engineering challenges in biodiesel production from microalgae. Crit. Rev. Biotechnol. 33, 293–308.

Arad, S.M., Levy-Ontman, O., 2010. Red microalgal cell-wall polysaccharides: biotechnological aspects. Curr. Opin. Biotechnol. 21, 358–64.

Barbir, F., Veziroglu, T., Plassjr, H., 1990. Environmental damage due to fossil fuels use. Int. J. Hydrogen Energy 15, 739–749.

Bhola, V., Desikan, R., Santosh, S.K., Subburamu, K., Sanniyasi, E., Bux, F., 2011. Effects of parameters affecting biomass yield and thermal behaviour of Chlorella vulgaris. J. Biosci. Bioeng. 111, 377–82.

Box, G.E.P., Wilson, K.B., 1992. On the Experimental Attainment of Optimum Conditions, in: Breakthroughs in Statistics. Springer Series in Statistics, pp. 270–310.

Briggs, G.E., Whittingham, C.P., 1952. Factors affecting the rate of photosynthesys of Chlorella at low concentrations of carbon dioxide and in high illumination. New Phytol. 51, 236–249.

Converti, A., Casazza, A.A., Ortiz, E.Y., Perego, P., Del Borghi, M., 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. Chem. Eng. Process. Process Intensif. 48, 1146–1151.

Costa, J.A.V., de Morais, M.G., 2011. The role of biochemical engineering in the production of biofuels from microalgae. Bioresour. Technol. 102, 2–9.

Falkowski, P.G., Sukenik, A., Herzig, R., 1989. Nitrogen limitation in Isochrysis galbana (Haptophyceae). II. Relative abundance of chloroplast protein. J. Phycol. 25, 471–478.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.

Fu, C.-C., Hung, T.-C., Chen, J.-Y., Su, C.-H., Wu, W.-T., 2010. Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction. Bioresour. Technol. 101, 8750–4.

Gerken, H.G., Donohoe, B., Knoshaug, E.P., 2013. Enzymatic cell wall degradation of Chlorella vulgaris and other microalgae for biofuels production. Planta 237, 239–53.

Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog. Lipid Res. 45, 160–86.

Illman, A.., Scragg, A.., Shales, S.., 2000. Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol. 27, 631–635.

Krienitz, L., Wirth, M., 2006. The high content of polyunsaturated fatty acids in Nannochloropsis limnetica (Eustigmatophyceae) and its implication for food web interactions, freshwater aquaculture and biotechnology. Limnol. - Ecol. Manag. Inl. Waters 36, 204–210.

Lv, J.-M., Cheng, L.-H., Xu, X.-H., Zhang, L., Chen, H.-L., 2010. Enhanced lipid production of Chlorella vulgaris by adjustment of cultivation conditions. Bioresour. Technol. 101, 6797–804.

Mahboob, S., Rauf, A., Ashraf, M., Sultana, T., Sultana, S., Jabeen, F., Rajoka, M.I., Al-Balawi, H.F.A., Al-Ghanim, K.A., 2011. High-density growth and crude protein productivity of a thermotolerant Chlorella vulgaris: production kinetics and thermodynamics. Aquac. Int. 20, 455–466.

Makooi, M., 1976. Effects of glucose and light on cellulose content of Chlorella pyrenoidosa. Phytochemistry 15, 367–369.

Markou, G., Angelidaki, I., Georgakakis, D., 2012. Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Appl. Microbiol. Biotechnol. 96, 631–45.

Mihranyan, A., 2011. Cellulose from cladophorales green algae: From environmental problem to high-tech composite materials. J. Appl. Polym. Sci. 119, 2449–2460.

Myers, R.H., Montgomery, D.C., Vining, G.G., Borror, C.M., Kowalski, S.M., 2004. Response surface methodology: A retrospective and literature survey. J. Qual. Technol. 36, 53–77.

Preston, R.D., 1974. The physical biology of plant cell walls. Chapman and Hall, London.

Richardson, B., Orcutt, D.M., Schwertner, H.A., Martinez, C.L., Wickline, H.E., 1969. Effects of nitrogen limitation on the growth and composition of unicellular algae in continuous culture. Appl. Microbiol. 18, 245–50.

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

Siddhanta, A.K., Prasad, K., Meena, R., Prasad, G., Mehta, G.K., Chhatbar, M.U., Oza, M.D., Kumar, S., Sanandiya, N.D., 2009. Profiling of cellulose content in Indian seaweed species. Bioresour. Technol. 100, 6669–73.

Skene, M., 1943. The Permeability of the Cellulose Cell Wall. Ann. Bot. 7, 261–273.

Sokolnicki, A.M., Fisher, R.J., Harrah, T.P., Kaplan, D.L., 2006. Permeability of bacterial cellulose membranes. J. Memb. Sci. 272, 15–27.

Tam, N.F.Y., Wong, Y.S., 1996. Effect of ammonia concentrations on growth of Chlorella vulgaris and nitrogen removal from media. Bioresour. Technol. 57, 45–50.

Tang, D., Han, W., Li, P., Miao, X., Zhong, J., 2011. CO2 biofixation and fatty acid composition of Scenedesmus obliquus and Chlorella pyrenoidosa in response to different CO2 levels. Bioresour. Technol. 102, 3071–6.

Tsuzuki, M., Miyachi, S., 1989. The function of carbonic anhydrase in aquatic photosynthesis. Aquat. Bot. 34, 85–104.

Updegraff, D.M., 1969. Semimicro determination of cellulose in biological materials. Anal. Biochem. 32, 420–4.

Van Hee, P., Middelberg, A.P.J., Van Der Lans, R.G.J.M., Van Der Wielen, L.A.M., 2004. Relation between cell disruption conditions, cell debris particle size, and inclusion body release. Biotechnol. Bioeng. 88, 100–10.

Wahlen, B.D., Willis, R.M., Seefeldt, L.C., 2011. Biodiesel production by simultaneous extraction and conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures. Bioresour. Technol. 102, 2724–30.

Xin, L., Hu, H., Ke, G., Sun, Y., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. Bioresour. Technol. 101, 5494–500.

# 4. Chapter 4: Investigation of high pressure steaming as a thermal treatment for lipid extraction from *Chlorella vulgaris*

The information presented in this Chapter is based in the paper "Investigation of High Pressure Steaming as a thermal treatment for lipid extraction from *Chlorella vulgaris*", published in Bioresource technology. July 2014, Vol. 164, pages 136-142. The sections in Chapter 4 present the results towards the completion of objectives 3 and 4 of the thesis (see section 1.2.2).

## 4.1. Abstract

In this part of the research HPS was studied as a hydrothermal treatment for extraction of lipids from *Chlorella vulgaris,* and analysis by RSM allowed finding operating points in terms of target temperature and microalgae concentration for high lipid and glucose yields. Within the range covered by these experiments the best conditions for high bio-crude yield are temperatures higher than 174˚C and low biomass concentrations (<5 g/L). For high glucose yield there are two suitable operating ranges, either low temperatures (<105˚C) and low biomass concentrations (<4 g/L); or low temperatures (<105˚C) and high biomass concentrations (<110 g/L). HPS is a good hydrothermal treatment for lipid recovery and does not significantly change the FAME profile for the range of temperatures studied.

## 4.2. Introduction

Traditionally oil has been extracted from plant biomass, and although the oil contents are similar between seed plants and microalgae (when they are grown under optimized conditions the lipid content can be higher), there are significant variations in the overall biomass productivity, resulting in an oil and biodiesel productivity with a clear advantage for microalgae (Mata *et al*., 2010). As mentioned, biofuels derived from microalgae are considered as a technically viable energy source which overcomes the problems associated with the previous generation of biofuels (Goh and Lee, 2010; Naik *et al*., 2010). Several researchers have reported the step of cell wall disruption (method or process for releasing biological molecules from inside a cell) as particularly important for establishing microalgae processes at an industrial scale, since the amount of lipids obtained from biomass depends to a large extent on the disruption method used. For this reason, different methods have been studied; among them are maceration, supercritical fluid extraction, osmotic shock, microwave, freezing, French press, ultrasound, bead-beating, enzymatic extractions, and thermal treatments. Despite the large list, more research in microalgae cell wall disruption is needed (Lee *et al*., 2010). The objective of the following experiments is to approach the inefficient lipid extraction by using thermal treatment as a cell wall disruption method.

Thermal treatments comprise any kind of technology involving heat in the processing of a substrate. Some previous works have shown good results for microalgae (Chen, 1998; Chow *et al*., 2013a). For these kinds of treatments, wet microalgae can be used before conversion of lipids to biodiesel, decreasing considerably the amount of energy required for the overall process. According to Minowa and Sawayama (1999), for every 1 kg of microalgae, about 3 MJ of energy are required only for centrifugation to produce an microalgae paste with 90% water by weight, and another 20 MJ are needed to decrease the water content to 10% water by weight using conventional drying, which represent an enormous amount of energy at an industrial scale. High extraction efficiencies have been reported for hydrothermal treatment, for instance Kita *et al*., (2010) reported a hydrocarbon recovery of 97.8% when using water at 90°C as thermal treatment. Their findings suggest that drying steps could be possibly bypassed if not avoided.

One of the most popular thermal treatments for biomass is thermochemical liquefaction. In this process, microalgae biomass is added to water and subjected to high temperatures and pressures; also, some chemicals (mainly alkaline compounds) can be added as catalysts. This treatment produces bio-crude, water, gas and a solid fraction. It has attracted much interest due to its many advantages, including relative stable oil product and high energy recovery. Unfortunately, this treatment still has drawbacks, such as equipment corrosion and the requirement of expensive process devices to reach temperature and pressure inputs (Toor *et al*., 2011). At this point the term bio-crude is introduced and it refers to all the lipid fraction that is soluble in organic solvents including those that are not convertible to biodiesel including some hydrocarbons, sterols, ketones, and pigments (carotenes and chlorophylls) (Halim *et al*., 2011).

Another recognized thermal treatment for biomass is HPS. This process differs from thermochemical liquefaction in the use of lower temperatures, generally in the range of subcritical water (water between 100˚C and 300˚C); which is an effective solvent for polar and non-polar compounds since the polarity of water changes with temperature. For example, when water is heated above 100˚C its dielectric constant becomes like dimethyl sulfoxide at ambient conditions (Carr *et al*., 2011). HPS is currently used in industry for the fractioning of wood, and many kinds of industrial HPS boilers are commercially available. This is an important advantage for the implementation of this technology for microalgae lipid extraction, since the technology is already available and only adaptation to microalgae feedstock and specific operating points should be found. HPS can be followed by a rapid decompression (also referred to as explosion), or by slowly decreasing the pressure to atmospheric (no explosion). The products after HPS include a bio-crude with a dark brown color and biomass with a modified cell wall structure; this modified cell wall may allow the extraction of the lipids remaining inside the microalgae easily.

Many advantages have been observed in processes using HPS for other feedstocks; among them are the high recovery yield and better substrate quality for further hydrolysis processes (e.g. thermal hydrolyzed carbohydrates can be used as substrate for enzymatic hydrolysis). In HPS, as in thermochemical liquefaction, it is also possible to add exogenous catalyst, but when no catalyst is added the process is referred as autohydrolysis, and the breakdown of the cellulose glycosidic linkages in the cell wall of the biomass depends on the acids naturally present in the microalgae biomass. The temperatures required for HPS usually range between 140 to 240˚C, with a wide residence time distribution generally extending from 2 up to 6000 seconds (Ramos, 2003). Thus, optimization of process variables for the application of this technology to microalgae processing is fundamental; however, the optimal values may change according to process specifications (i.e. complete cellulose degradation vs. cell wall disruption, being the later the objective of this research). The use of thermal treatments may lead to oil contents higher than the lipid content of microalgae (10-15% higher), due to the polymerization of proteins and carbohydrates into oily composites (Toor *et al*., 2011).

According to Biller and Ross (2011), the formation of bio-crude follows the trend lipids > proteins > carbohydrates, meaning that lipids and proteins are converted to bio-crude more efficiently when no catalyst is added. Particle size has also shown to be a significant variable when steam treatments are used (Liu *et al*., 2013), since the size of the feedstock may impose heat transfer problems; larger particles are susceptible to overcook in the surface and have low steam access to the inner part. When microalgae are used, the particle size (cell size) is rather homogenous and it can range from a few micrometers to a few hundreds of micrometers, they are considerable smaller particles when compared with other biomass feedstocks.

In this Chapter, a study on the simultaneous effect of target temperature ($T_t$), and microalgae concentration (β) (see Table 4.1 for nomenclature and units) on *C. vulgaris* bio-crude and glucose yields after HPS was carried out. The main goal was to find operating points for cell wall disruption to ensure an improved bio-crude recovery yield. A study of these parameters was performed by RSM.

**Table 4.1** Nomenclature used in Chapter 4.

| Symbol | Name | Units |
|---|---|---|
| $T_t$ | Target temperature | ˚C |
| β | Microalgae concentration | g/L |
| $T$ | Temperature | ˚C |
| $P$ | Pressure | psi |
| Ψ | Bio-crude yield | mg bio-crude/g microalgae |
| α | Glucose yield | mg glucose/g microalgae |

## 4.3. Materials and methods

To obtain the information needed the following protocols were implemented.

### 4.3.1. Microalgae strain and culture media

The microalgae strain *Chlorella vulgaris* UTEX 2714 was used in this study. Cultures were kept in Bold's modified media (section 3.3.1) with 3.21 mM of sodium nitrate and 4.02% ($vv^{-1}$) of carbon dioxide. These culture parameters were found to be the optimum for *C. vulgaris* cultures producing high lipid productivity and low cellulose content, as previously reported (Aguirre and Bassi, 2013) (section 3.4.4). After 15 days of culture, biomass was harvested by centrifugation at 3400 rpm for 20 minutes, and washed 3 times with distilled water to remove culture media and extracellular components. The microalgae paste was freeze dried for 24 h and stored at -20˚C until it was used in HPS experiments. To ensure biomass homogeneity for all the experiments, the freeze dried biomass produced in different batches was mixed together before experiments.

### 4.3.2. Experimental set-up

HPS experiments were conducted in a custom made and laboratory-scale device at Western University Machine Services (London, ON, Canada) (See Figure 4.1). Detailed information of device configuration is presented in Appendix 3. The equipment has one steam chamber (120 mL) and one expansion chamber (460 mL) separated by one ball valve which allows fast decompression of the sample. Close to the ball valve there is a nozzle where sample was forced to pass through during decompression. This process increases the shearing action and helps in the cell wall disruption by homogenization of the sample (Samarasinghe *et al*., 2012).

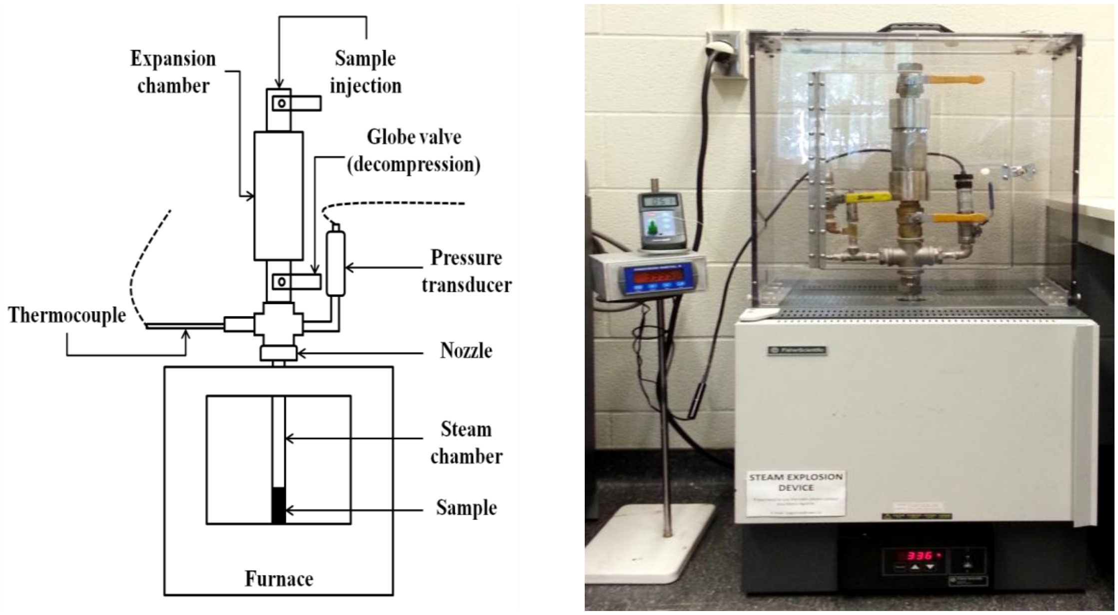



**Figure 4.1** Schematic diagram and picture of experimental set-up used for high pressure steaming of *C. vulgaris* biomass.

Experiments were done in the following steps. First, a 20 mL sample consisting of freeze dried microalgae and distilled water was introduced into the steam chamber (still at room temperature) by the upper part of the device using a long needle to reach the bottom of the steam chamber (the needle was wide enough to ensure that no disruption of the cells was happening at this point), the amount of microalgae in each sample was calculated according to experimental design (Table 4.2, column 3); then all the valves in the system were closed. The device, charged with the sample, was inserted into the furnace (pre-heated at 800˚C) by an upper hole in the furnace wall. Readings of temperature ($T$) and pressure ($P$) were taken every minute until the target temperature was reached. At this point the decompression valve was rapidly opened to allow a fast pressure drop of the system due to a sudden total volume increase.

The device was removed from the furnace and cooled with tap water for 3 minutes and then allowed to naturally cool down until the temperature in steam chamber was 25˚C. At this temperature the sample was completely condensed, and the aqueous sample was removed from inside the device.

**Table 4.2** Variables and experimental CCD levels for RSM.

| Experiment | Target temperature $T_t$ (˚C) | Microalgae concentration $\beta$ (g/L) | Crude yield ($\Psi$) (mg bio-crude/g microalgae) | | Glucose yield ($\alpha$) (mg glucose/g microalgae) | | Cell fraction | Extraction efficiency (%) |
|---|---|---|---|---|---|---|---|---|
| | | | Measured | Model result (Equation 1) | Measured | Model result (Equation 2) | | |
| E1 | 104.47 | 60.00 | 49.40 | 67.43 | 8.06 | 8.86 | 1.72E-01 | 24.34 |
| E2 | 210.53 | 60.00 | 157.47 | 133.34 | 0.80 | 0.82 | 6.89E-02 | 77.60 |
| E3* (replicate 1) | 157.50 | 60.00 | 83.45 | 94.82 | 3.76 | 2.70 | 1.18E-01 | 41.12 |
| E3' (replicate 2) | 157.50 | 60.00 | 79.34 | 94.82 | 2.92 | 2.70 | 1.61E-01 | 39.10 |
| E3'' (replicate 3) | 157.50 | 60.00 | 69.12 | 94.82 | 2.73 | 2.70 | 1.10E-01 | 34.06 |
| E4 | 157.50 | 3.43 | 198.54 | 147.35 | 14.23 | 11.11 | 4.80E-01 | 97.84 |
| E5 | 157.50 | 116.57 | 66.32 | 61.02 | 1.94 | 1.84 | 2.79E-01 | 32.68 |
| E6 | 195.00 | 20.00 | 127.86 | 164.80 | 3.63 | 4.63 | 5.70E-02 | 63.01 |
| E7 | 120.00 | 100.00 | 69.07 | 54.56 | 6.58 | 6.98 | 2.97E-01 | 34.04 |
| E8 | 195.00 | 100.00 | 98.53 | 88.36 | 0.44 | 0.49 | 7.07E-02 | 48.55 |
| E9 | 120.00 | 20.00 | 136.64 | 101.77 | 7.72 | 9.39 | 3.48E-01 | 67.33 |

*E3 is the central point of the CCD.

### 4.3.3. Analytical techniques

#### *4.3.3.1. Temperature and pressure*

Temperature ($T$) during HPS and target temperature ($T_t$) were measured with a thermocouple. Pressure was measured with a pressure transducer with an operating pressure range of 0-500 psi (1 psi is equivalent to 6.8948 kPa) (see Figure 7.7).The thermocouple and pressure transducer were both directly connected to the steam chamber. Even though temperature and pressure are correlated for steam, both readings were taken independently in order to ensure reliable and accurate experimental data. The HPS device was insulated with fiberglass to ensure stability of these measurements, and to reduce heat losses to its surroundings. For the target temperatures used in these experiments the steam produced was in the wet steam region (also known as the two-phase region). Due to equipment and sample size, both water and steam phases were always present but in different ratios for each experiment because of the different microalgae concentrations.

#### *4.3.3.2. Bio-crude yield*

The low value for the sample volume to internal surface area ratio of the device represents a challenge at small scale. The bio-crude obtained can easily stick to the walls of the device and therefore washing the device with a solvent was necessary (the use of solvent at large scale could be reduced or avoided). After HPS the aqueous sample was removed and the device was washed 4 times with 30 mL of hexane (30 minutes each). All the used hexane was mixed with the aqueous sample and stirred for 1 h to allow transfer of the bio-crude to the hexane phase. This step was done separately for each experimental sample. The hexane phase was filtered and transferred to pre-weighed aluminum pans. Bio crude recovery was achieved via evaporation of the hexane. Total extracted bio-crude for each experiment was measured gravimetrically. The bio-crude yield ($\Psi$) was calculated as the ratio between the mass of bio-crude produced over the dried mass of microalgae used in each experiment.

#### *4.3.3.3. Glucose concentration*

To quantify glucose concentration after HPS, aliquots of 1 mL from the aqueous phase after HPS were removed and filtered using 0.2 µm syringe filters. Samples were analyzed using an Agilent 1260 Infinity series high performance liquid chromatography device equipped with an Agilent Hi-Plex H column at 60˚C, using 0.005 M $H_2SO_4$ as the mobile phase at a flow rate of 0.7 mL/min. Injection volume was 20µL, and the refractive index detector was kept at 55˚C (Ewen, 2009). The glucose yield ($\alpha$) was calculated as the ratio between the mass of glucose produced over the dried mass of microalgae used in each experiment.

#### 4.3.3.4. *Cell fraction and scanning electron microscope*

Aliquots of 100 µL were taken before and after HPS, and cell counting under an optical microscope (40x) was performed using a haemacytometer. The intact "cell fraction" after treatment was calculated using the equation proposed by Samarasinghe *et al*., (2012) (cell fraction=cell density in sample after treatment*cell density in sample before treatment$^{-1}$). The lower the cell fraction value the more effective the cell wall disruption method.

SEM was used to see the effect of HPS treatment on microalgae morphology and surface. A standard preparation for biological samples was followed including microalgae fixation in 3% glutaraldehyde in 0.1 M phosphate buffer. Samples were later washed 3 times in the same buffer. Post-fixation was done with 2% osmium tetraoxide in phosphate solution. Dehydration was achieved by consecutive immersions of the sample in increasing concentration solutions of ethanol (Karcz, 2008). Samples were dried in the critical point for ethanol and attached to a paper filter for SEM imaging.

#### 4.3.3.5. *Fatty acid methyl esters profile*

Gas chromatography (GC) analysis was performed to determine the FAME profiles. The total bio-crude obtained from each experiment was dissolved in 20 mL of methanol. For GC analysis, the FFA must be converted into FAME, for which 1 mL of bio-crude-methanol solution was mixed with 1 mL of methylene chloride, 50 µL of internal standard (methyl nonadecanoate, 74208 Fluka) and 16.5 µL of pure sulfuric acid. Each sample was introduced in sealed high pressure test tubes and transesterification reaction of FFA into FAME was allowed for 3 hours at 100˚C in a water bath. FAME were analyzed by injecting 2 µL samples into an Agilent 7890A GC-flame ionization detector equipped with a 30 m *X* 0.32 mm *X* 0.25 µm J&W HP-5 column. Oven temperature was kept at 80˚C for 2 minutes, then heated up to 140˚C at the rate of 20˚C/min, and then to 260˚C at the rate of 4˚C/min. Temperature was maintained at 260˚C for 10 minutes (Kim *et al*., 2012).

### 4.3.4. Experimental design

The manipulated variables in this study were target temperature (final temperature reached in the experiment) and microalgae concentration. A statistical approach was used to study the simultaneous effect of multiple variables. RSM was used to fit experimental data to a mathematical model by means of the CCD $2^2$ + star with 2 factors (target temperature and microalgae concentration) and 5 levels. Again, the CCD consisted of nine experiments with three replicates for the central point and alpha value of ±1.41. Table 4.2, in columns 2 and 3, presents the actual values for each experiment. The accuracy of the model was calculated by the regression coefficients $R^2$ and adjusted $R^2$ (adj $R^2$). To identify the statistically significant terms the ANOVA was employed. Significance of regression coefficients was determined with a confidence level of 95%. The models were obtained after several trials for best accuracy and fitting of experimental data. These analysis included analysis of variables transformations with different functions and inclusion and exclusion of no statistically significant terms. The model with the highest regression coefficient ($R^2$) and only statistical significant terms was selected. The statistical analysis was done using the software Statgraphics Centurion XVI (StatPoint Technologies, Inc., Warrenton, VA).

Target temperature and microalgae concentrations ranged from 104.47 to 210.53˚C and from 3.43 to 116.57 g/L, respectively. This range of temperature was wide enough to ensure the formation of steam to different extents in the system and create different thermal environments. In terms of the effect of temperature on biomass it is known that hot compressed water at temperatures close to 100˚C may cause the extraction of the aqueous soluble fraction. When temperature is above 150˚C, hydrolysis of polymers like cellulose starts producing shorter polymers and monomers. Finally, when temperature is around 200˚C and pressure near 145 psi, the biomass is transformed into a slurry in a process known as liquidization (The-Japan-institute-of-Energy, 2008). The temperatures studied in this set of experiments covered all the conditions mentioned above. On the other hand, biomass concentration may have a role on the efficiency of cell breakage

(Samarasinghe *et al*., 2012). Accordingly, a range of biomass as wide as possible was used.

## 4.4. Results and discussion

Next sections present the results for the experiments conducted.

### 4.4.1. Temperature and pressure profiles

Figure 4.2 shows the profiles of temperature with respect to time for all experiments. Temperature profiles followed a similar behavior (logarithm-like), where the temperature increased quickly in the first 120 seconds, and after that time the rate of heating was 5.5 times slower. Heat transfer in a vessel initially filled with water, which is the case of the device used, is in the first stage by conduction (Brownell *et al*., 1986). The heat increases the internal energy by means of molecular agitation. Heat transfer by convection occurs later, when the heated water expands and becomes more buoyant; cooler hence denser water descends and patterns of circulation are formed. The energy transferred to biomass reduces the stability of the cell wall and makes it more susceptible to break.

The similar profiles of temperature for all the treatments indicate that the presence of microalgae in the suspension did not affect significantly the heating process. Experiment 1 (E1) had the lowest target temperature (104.47˚C), reached after 67 seconds, which corresponds to the experiment with the lowest bio-crude yield (see Table 4.2). Experiment 2 (E2) had the highest target temperature of 210.53˚C, which was reached after 706 seconds. In this case E2 did not have the highest bio-crude yield, which implies that temperature is not the only variable playing an important role in bio-crude recovery, as discussed later.

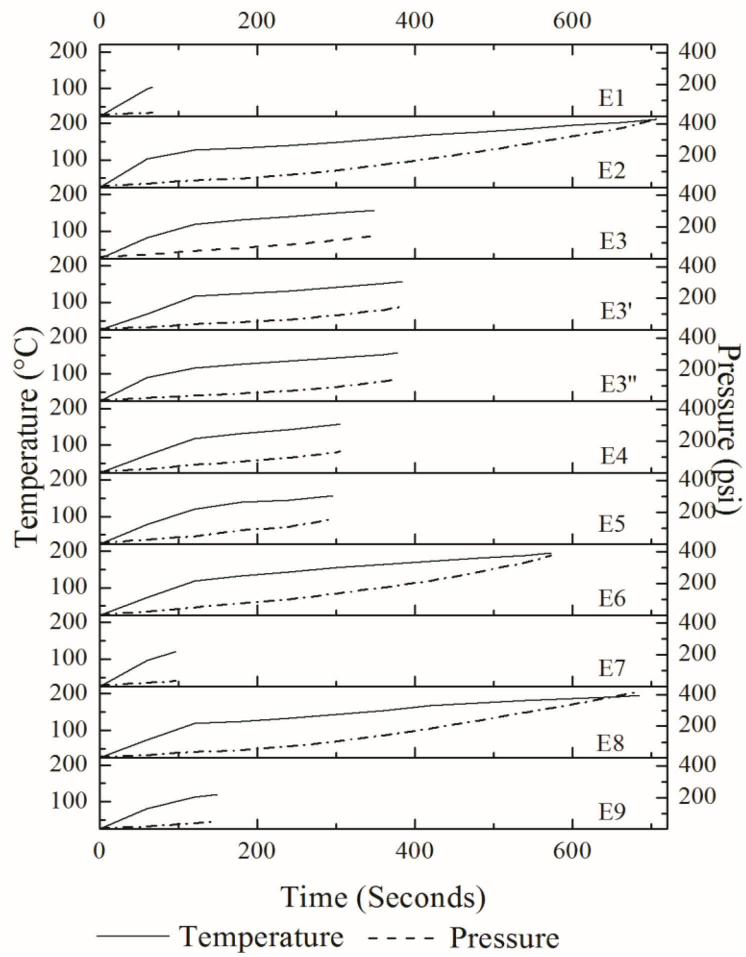



**Figure 4.2** Profile of temperature (*T*) and pressure (*P*) during high pressure steaming of *C. vulgaris*.

Pressure (Figure 4.2) also followed similar profiles for all treatments (exponential-like). Experiment 2 (E2) (as expected for the given target temperature), reached the highest pressure (432 psi) and the lowest pressure of 22.179 psi corresponded to Experiment 1 (E1). For HPS, not only the final pressure reached at the target temperature accounts for cell wall disruption, but also the pressure drop after decompression of the system. When a gas-liquid system (air-water) is subjected to high pressure and then the pressure drops rapidly, the gas dissolved in the liquid is released causing cavitation bubbles that aid to lyse the cells. In addition to this, pressure within the cell drops at a slower rate than the pressure outside. This pressure difference is mainly responsible for the cell wall breakage along with cavitation bubble effects. Samarasinghe *et al*., (2012) reported that one of the most significant parameters for rupture of *N. oculata* cell wall is pressure differential when cells go across a nozzle.

Based on the data for each experiment (before and after decompression) and using RSM, a correlation between pressure drop ($\Delta P$) and target temperature was found: $\Delta P$ = 494.04-8.49$T_t$+0.04$T_t^2$, ($R^2$=0.98), where, $\Delta P$ is measured in psi and $T_t$ in ˚C. This correlation can be used to predict any pressure drop wanted in the system within the range of temperatures covered by the experiments. Microalgae concentration did not have a statistically significant effect on the pressure drop. From this equation it is deduced that the pressure drop increases with the target temperature, but with a higher effect for higher temperatures due to the effect of the quadratic term of temperature ($T_t^2$). At higher temperatures more of the liquid mass of water is transformed into steam, after decompression the density of this steam drastically increases (condensation), leading to higher pressure drops. The experiments with the highest pressure drops correspond to E2 ($\Delta P$=357.61 psi), E6 ($\Delta P$=221 psi), and E8 ($\Delta P$=266 psi), accounting for some of the treatments with the highest bio-crude yields (Table 4.2).

Even though, pressure drop does not appear as one term in the models proposed in the next sections, this variable is implicit in the equations (and linked to target temperature term, $T_t$), due to the relationship between pressure and temperature for saturated steam in the system. Each decompression starts at one different final pressure for each experimental treatment and this final pressure is the saturation pressure at temperature $T_t$. Some researchers have introduced the term pressure drop in their models directly, but it is important to notice that the goal of most of those works is the thermo-mechanical disruption of biomass for depolymerisation of lignocellulosic components, and therefore complete degradation of these polymers is wanted, hence the inclusion of the term pressure drop into those models is practical. The main polymer found in *C. vulgaris* cell wall is cellulose comprising around 80% by dry weight of the cell wall (Abo-Shady *et al*., 1993), making this polymer the main target in cell wall disruption techniques. In this case, the goal is not the complete depolymerisation of the cellulose but the release of lipids from inside the cell, which can be achieved by breaking the cellulose in several points but not necessarily completely.

When bio-crude samples for all the experiments were compared, a stark visual difference in color was observed. Pictures of all samples were taken and it was clear that as the target temperature or microalgae concentration increased the bio-crude became darker. Target temperature seems to have a stronger effect on bio-crude color than microalgae concentration (see Figure 4.3). This could have some implications at industrial scale purification of the bio-crude.

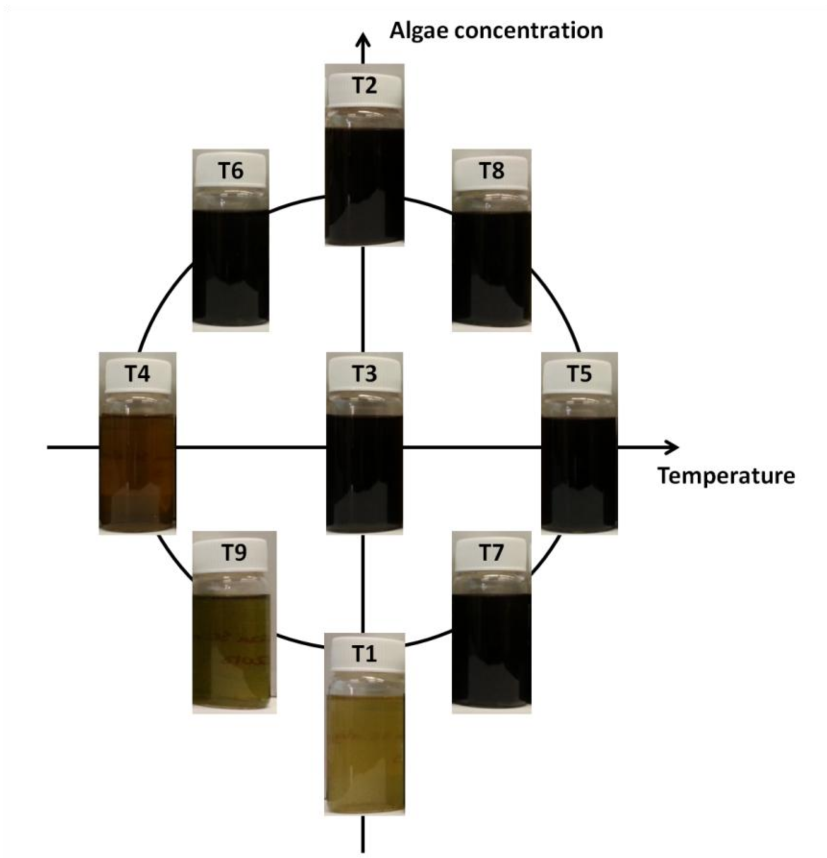



**Figure 4.3** Pictures of bio-crude dissolved in methanol for each of the treatments in this study.

### 4.4.2. Cell breakage and scanning electron microcopy images

The ratio between the amount of undisrupted cells before and after HPS is an indicator of the extent of the cell disruption treatment. According to Samarasinghe *et al*., (2012) as the intensity of the treatment increases the number of intact cells disappears in the sample matrix because the disrupted particles are smaller than the resolution of the microscope used for counting the intact cells.

The intact cell fraction remaining after HPS (referred as cell fraction) was analyzed using RSM (Table 4.2 shows the cell fraction for each treatment). According to data fitting the equation describing the relationship between cell breakage and target temperature is: (1/Cell fraction)=28.6497-0.410514*$T_t$+0.0017027*$T_t^2$ ($R^2$=75.50%), which means that as the temperature increases the cell fraction decreases. Figure 4.4g shows how cell fraction is reduced (for a constant microalgae concentration of 60 g/L) as temperature increases.

Figure 4.4 shows SEM images of microalgae before and after HPS. Microalgae cells before thermal treatment had a spherical shape and smooth surface (4.3a, 4.3b, and 4.3c); after HPS the microalgae cell collapses, some microalgae break into pieces and cell debris appears, the cell surface becomes rough, and some small pores on the cell wall are visible (4.3d, 4.3e, and 4.3f). It is clear from these images that the HPS treatment has a very strong effect on microalgae integrity.

### 4.4.3. Bio-crude yield as a function of target temperature and microalgae concentration

RSM was used to evaluate the effect of target temperature and microalgae concentration on bio-crude yield. Logarithmic transformation of data was applied to improve the fit of the model to data and only statistically significant terms were taken into account for the second order mathematical model.

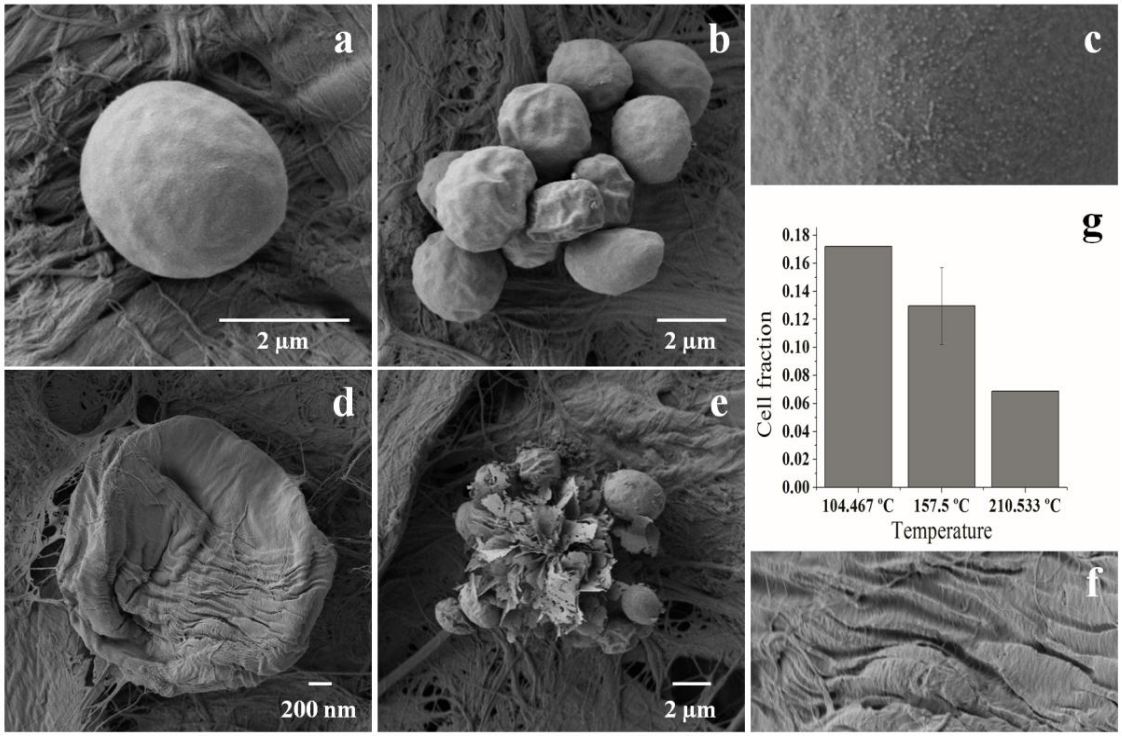



**Figure 4.4** SEM images of *C. vulgaris* microalgae. (Images a and b show microalgae before HPS. Images d and e show microalgae after HPS. Images c and f show microalgae surface details before and after HPS, respectively. Image g shows the effect of target temperature on cell fraction).

The ANOVA (Table 4.3) partitions the variability in LN(Ψ) into separate pieces for each of the effects. It tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. The model as fitted accounted for 67.89% of the variability in LN(Ψ). Equation 4.1 describes the response surface for this variable (For statistical software outputs refer to section 7.2.3).

**Table 4.3** Analysis of variance for LN(Ψ) model.

| Source | Sum of Squares | Degree of freedom | Mean Square | F-Ratio | *P*-Value |
|---|---|---|---|---|---|
| $T_t$ | 0.464778 | 1 | 0.464778 | 6.33 | 0.0360 |
| β | 0.777191 | 1 | 0.777191 | 10.59 | 0.0116 |
| Total error | 0.587251 | 8 | 0.0734064 | | |
| Total (corr.) | 1.82922 | 10 | | | |

$R^2$: 67.8961%; adj $R^2$:59.8701%.

$$\mathbf{LN(\Psi) = 4.00721+0.00642757}\boldsymbol{Tt}\mathbf{-0.00779216\ \beta} \quad \textbf{Equation 4.1}$$

Figure 4.5, shows the estimated response surface and the points represent the experimental data. For a given value of microalgae concentration, the results show that the bio-crude yield increased linearly with temperature. Conversely, for constant values of temperature, as microalgae concentration increases, the bio-crude yield decreases.

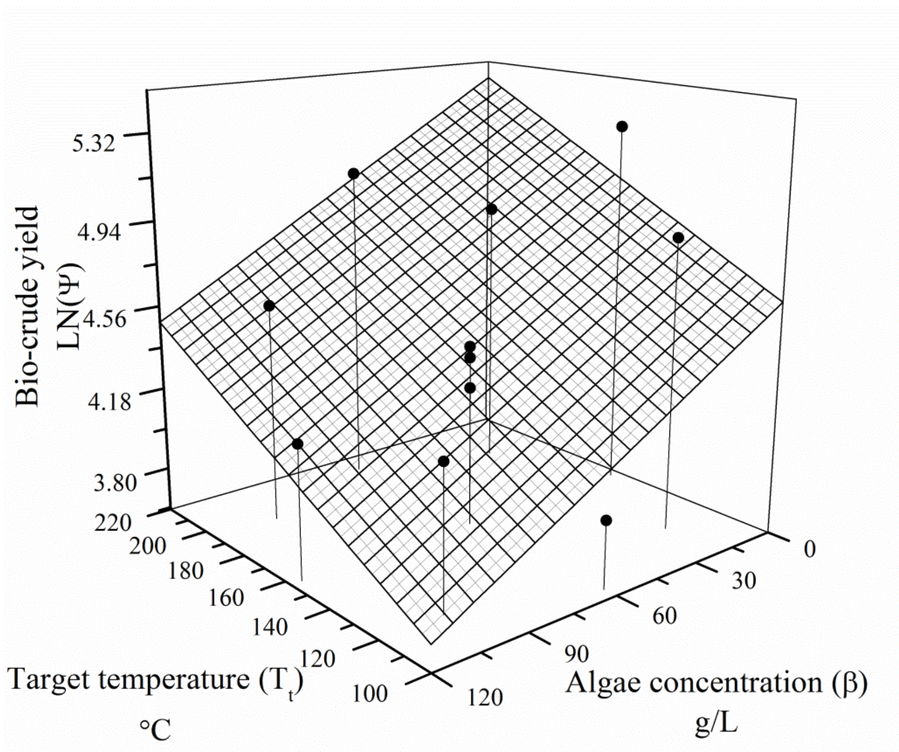



**Figure 4.5** Response surface of the effect of target temperature (*Tt*) and microalgae concentration (β) on LN(Ψ). Black dots represent experimental results data.

If the equation for cell fraction and bio-crude yield are overlapped, it is noticed that for a given microalgae concentration, as the cell fraction decreases the bio-crude yield increases, meaning that cell wall disruption and lipid recovery are linked.

According to Equation 4.1 for LN(Ψ), within the range covered by these experiments the best conditions for high bio-crude yield are temperatures higher than 174˚C, and low biomass concentrations (<5.90 g/L). One possible explanation to why low microalgae concentrations are beneficial for bio-crude recovery is that the ratio "solid (microalgae) concentration/steam mass" is higher. I.e. given an amount of steam in the system and as the microalgae concentration decreases, the total area of the cell walls exposed to that steam increases, more steam is available for each microalgae and the processes of extraction is improved. Figure 4.6 shows the bio-crude obtained after microalgae are subjected to HPS.

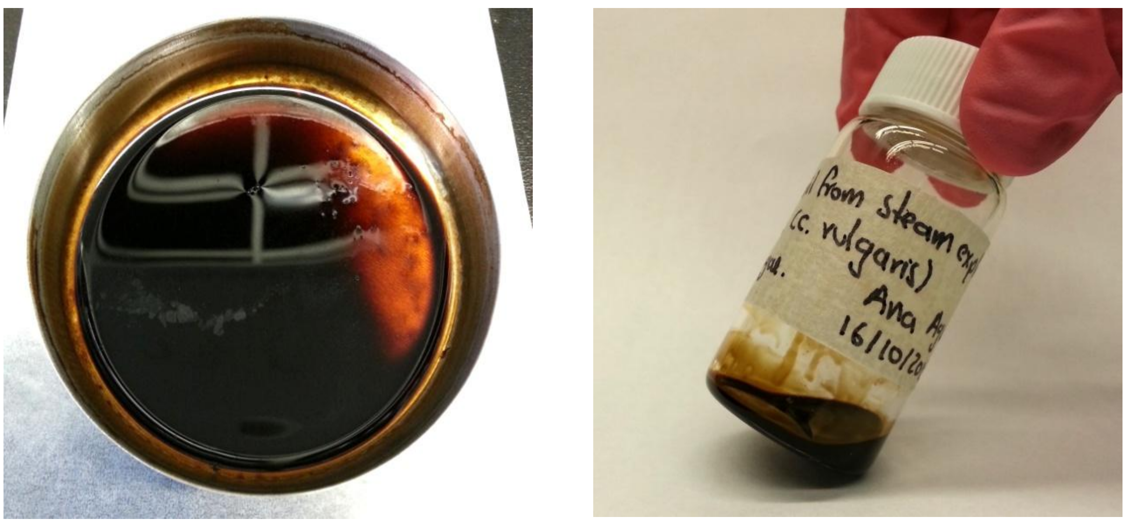



**Figure 4.6** Picture of the bio-crude obtained using high pressure steaming.

Table 4.2 also presents the extraction efficiency for each treatment. The total lipid content in the biomass used for all the experiments was 20.29% ($ww^{-1}$) and was calculated applying the Folch's modified method described in section 3.3.3.3 (in this case cells were freeze dried), this percentage was compared to the amount of bio-crude recovered using HPS for each treatment, and the efficiency of the extraction was calculated. The treatment with the highest bio-crude recovery efficiency was E4 (97.84%) where almost all the lipids from the cell were recovered; while the treatment with the lowest bio-crude recovery efficiency was E1 (24.34%).

**Table 4.4** Comparison of bio-crude recovery efficiency.

| Algae strain | Extraction conditions | Bio-crude recovery efficiency | Reference |
|---|---|---|---|
| *Botryococcus braunii* | Thermal pre-treatment 90˚C 10 minutes | 97.8% Compared with solvent extraction | (Kita *et al*., 2010) |
| *Scenedesmus obliquus* | resonant continuous microwave processing system 95˚C 30 min | 76–77% Compared with hexane:ethanol extraction | (Balasubramanian *et al*., 2011) |
| *Nannochloropsis oceanica* | hydrothermal liquefaction 300˚C 0.5 h | 67.73% Of total biomass energy | (Cheng *et al*., 2014) |
| *Nannochloropsis* sp. | continuous flow lipid extraction system 100 °C 50 psi | 100% Compared with soxhlet extraction | (Iqbal and Theegala, 2013) |
| *Chlorella* (KAS603) | Solvent extraction with 2-ethoxyethanol (2-EE) 60˚C 30 min | 150–200 % Compared to extraction solvents with chloroform:methanol or hexane | (Jones *et al*., 2012) |
| *Chlorella vulgaris* (This study) | High pressure steaming 157.5 3.43 g/L | 97.84% Compared with Folch's method | (Aguirre and Bassi, 2014) |

The average extraction efficiency for all the treatments was 50.88%. Chow, Jackson, Chaffee, & Marshall, (2013) present a comprehensive review on thermal treatment of microalgae for production of biofuels; comparison of the results summarized by them for *Chlorella* species with this study's results, show that the bio-crude recovered applying HPS are consistent with the results obtained by other researchers.

### 4.4.4. Glucose yield as a function of target temperature and microalgae concentration

The amount of glucose produced after HPS is not only one indicator of the extent of cellulose degradation, but also this glucose can be used as substrate for production of other bio-fuels, as ethanol or butanol, via fermentation. Glucose in the aqueous phase after HPS was measured and analyzed as function of target temperature and microalgae concentration. Logarithmic transformation of data was applied again. Table 4.5 shows the ANOVA.

**Table 4.5** Analysis of variance for LN(α) model.

| **Source** | **Sum of Squares** | **Degree of freedom** | **Mean Square** | **F-Ratio** | ***P*-Value** |
|---|---|---|---|---|---|
| $T_t$ | 5.66497 | 1 | 5.66497 | 111.91 | 0.0000 |
| β | 3.23496 | 1 | 3.23496 | 63.91 | 0.0002 |
| $T_t$ β | 0.951266 | 1 | 0.951266 | 18.79 | 0.0049 |
| $\beta^2$ | 0.412667 | 1 | 0.412667 | 8.15 | 0.0290 |
| Total error | 0.303717 | 6 | 0.0506195 | | |
| Total (corr.) | 10.5676 | 10 | | | |

$R^2$: = 97.1259%; adj $R^2$: 95.2099%.

In this case, four effects have *P*-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. Only statistically significant terms were taken into account for the second order mathematical model. Therefore, the term $T_t^2$ was excluded of the final analysis. The model as fitted was able to explain 97.13% of the variability in LN(α). The equation describing the surface is Equation 4.2 (For statistical software outputs refer to section 7.2.4).

$$\mathbf{LN(\alpha) = 2.98877 - 0.00293341\mathit{T_t} + 0.0159297\,\beta - 0.00032511\mathit{T_t}\,\beta + 0.00016148\,\beta^2} \qquad \textbf{Equation 4.2}$$

Figure 4.7 presents the estimated response surface for LN(α). For temperatures between 104˚C and 150˚C as the microalgae concentration increases the glucose yield slightly increases as well. However, at higher temperatures the behavior seems to change and as the microalgae concentration increases the glucose yield decreases. For lower values of microalgae concentration (3.43 g/L to 40 g/L), the glucose yield decreases slowly, but for values of biomass higher than 80 g/L and one temperature of 200˚C, the glucose yield decreases rapidly.

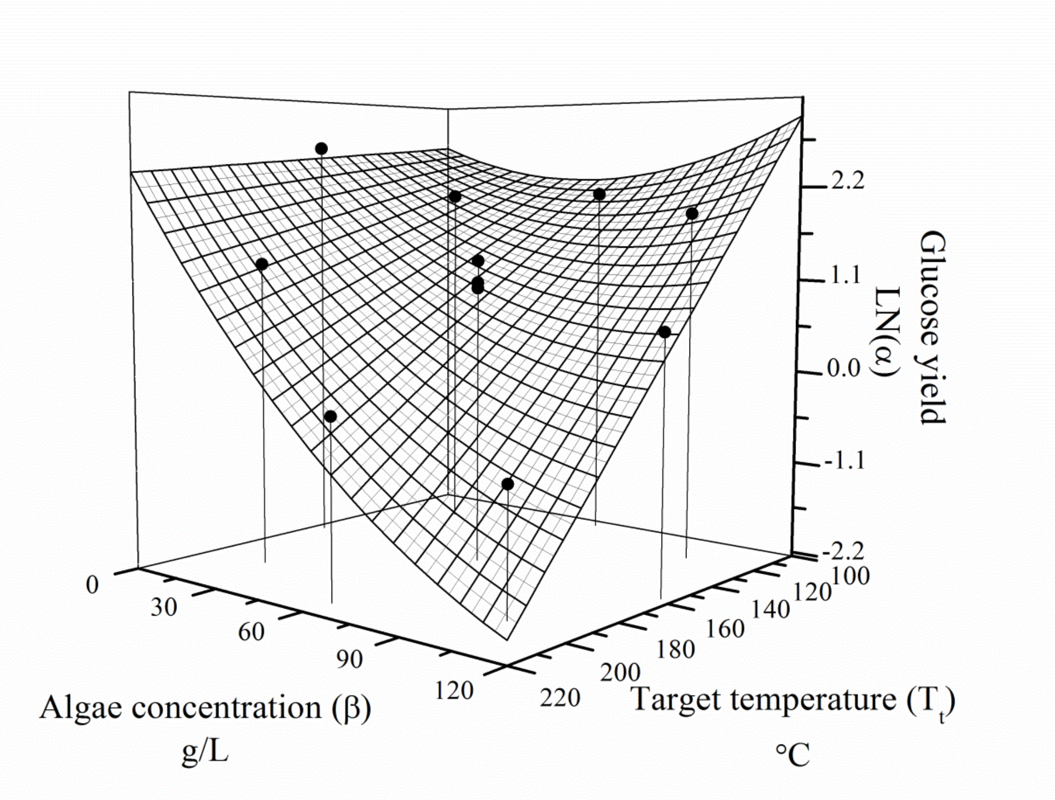



**Figure 4.7** Response surface of the effect of target temperature ($T_t$) and microalgae concentration (β) on LN(α). Black dots represent experimental results data.

Glucose can be transformed into 5-Hydroxymethylfurfural (5-HMF) at temperatures in the range of 150–250 °C (de Souza *et al.*, 2012) which could explain the reduction in glucose concentration in the aqueous phase after HPS. According to Equation 4.2, for LN(α), within the range covered by these experiments there are two possible operating points for high glucose yield. They can be either low temperatures (<105˚C) and low biomass concentrations (<4 g/L), or low temperatures (<105˚C) and high biomass concentrations (<110 g/L).

Although the glucose yield is low it does not affect the bio-crude recovery from microalgae biomass; the long cellulose fibers could be broken allowing the release of the intracellular compounds. At this point, if higher glucose yields are needed, enzymatic hydrolysis of the cellulose after HPS can be applied (see Chapter 5 for experiments on this topic). The results of higher glucose concentrations at lower temperatures are in accordance to the results obtained by Cara *et al*., (2006) when olive tree wood was treated with steam explosion.

### 4.4.5. FAME profile

GC analysis of the FAME was done in order to see if differences in the HPS conditions had any effect on the FAME composition. A pool of 37 different FAMEs was analyzed but only those found in higher concentrations are highlighted in this study, while the others were grouped in one set called “other FAMEs”. There was no statistical difference (*T*-test, *P*-value=0.05) between the FAME compositions among the HPS treatments in the range of temperature studied, meaning that the range of conditions studied did not affect FAME composition. Hence, an average for all the HPS treatments was found and plotted in Figure 4.8.

The FAMEs found in larger percentages were palmitoleic acid methyl ester (C16:1), palmitic acid methyl ester (C16:0), linoleic acid methyl ester (C18:2n6C), oleic acid methyl ester (C18:1n9c), linolelaidic acid methyl ester (C18:2n6t), α-linolenic acid methyl ester (C18:3n3), stearic acid methyl ester (C18:0), elaidic acid methyl ester (C18:1n9t), and cis-8,11,14-eicosatrienoic acid methyl ester (C20:3n6). The composition of FAME from *C. vulgaris* in this study was similar to that found by Kim *et al*., (2012), Lohman *et al*., (2013), and Ryckebosch *et al*., (2011).

The percentage of FAME after HPS was also compared to the percentage of FAME extracted using only hexane (no thermal treatment was applied) in order to see how the thermal treatment was affecting the percentage of FAME composition in comparison to no thermal treatments. There were statistically significant differences (*T*-test, *P*-value=0.05) for C16:1, C16:0, C18:2n6c, and C18:1n9t. Tyagi and Vasishtha (1996), reported the changes in specific gravity, saponification value, color, refractive index, viscosity, and FFA composition of soybean oil when was subjected to temperatures from 170 to 190˚C. These differences are expected when thermal treatments are applied but from Figure 4.8 it can also be concluded that even if there are statistically significant differences for some FAME, they are not extreme and some of them favor the HPS treatment. Santana *et al*., (2012) observed similar behavior comparing the profile of fatty acids extracted from *Botryococcus braunii* with supercritical carbon dioxide and traditional solvent extraction.

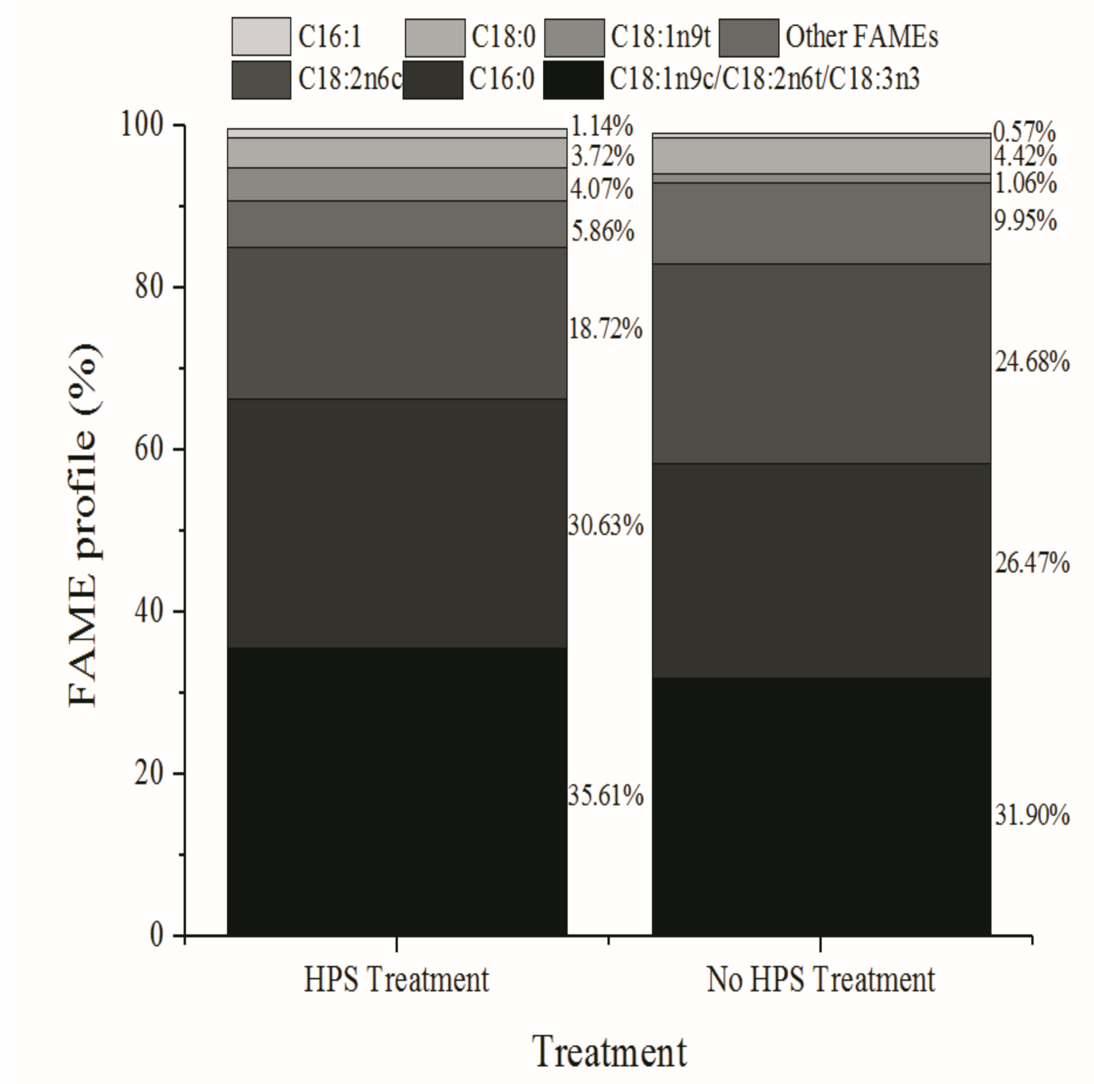



**Figure 4.8** Comparison of fatty acid methyl esters in microalgae using high pressure steaming and solvent extraction

## 4.5. Conclusions

RSM allows the study of the effect of simultaneous variables (target temperature and microalgae concentration) on HPS by empirical modeling. Also, target temperature and microalgae concentrations play significant roles on lipid and glucose yields. It was found that to achieve high bio-crude yields, low microalgae concentrations and high temperatures are favorable; meanwhile, for high glucose yields, low temperatures and microalgae concentrations produce better results. From SEM images it was evident that HPS modifies cell morphology and surface; cell breakage and formation of pores was observed.

## References

Abo-Shady, A.M., Mohamed, Y.A., Lasheen, T., 1993. Chemical composition of the cell wall in some green algae species. Biol. Plant. 35, 629–632.

Aguirre, A.-M., Bassi, A., 2013. Investigation of biomass concentration, lipid production, and cellulose content in Chlorella vulgaris cultures using response surface methodology. Biotechnol. Bioeng. 110, 2114–22.

Aguirre, A.-M., Bassi, A., 2014. Investigation of High Pressure Steaming as a thermal treatment for lipid extraction from Chlorella vulgaris. Bioresour. Technol. Accepted.

Balasubramanian, S., Allen, J.D., Kanitkar, A., Boldor, D., 2011. Oil extraction from Scenedesmus obliquus using a continuous microwave system--design, optimization, and quality characterization. Bioresour. Technol. 102, 3396–403.

Biller, P., Ross, A.B., 2011. Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. Bioresour. Technol. 102, 215–25.

Brownell, H.H., Yu, E.K., Saddler, J.N., 1986. Steam-explosion pretreatment of wood: Effect of chip size, acid, moisture content and pressure drop. Biotechnol. Bioeng. 28, 792–801.

Cara, C., Ruiz, E., Ballesteros, I., Negro, M.J., Castro, E., 2006. Enhanced enzymatic hydrolysis of olive tree wood by steam explosion and alkaline peroxide delignification. Process Biochem. 41, 423–429.

Carr, A.G., Mammucari, R., Foster, N.R., 2011. A review of subcritical water as a solvent and its utilisation for the processing of hydrophobic organic compounds. Chem. Eng. J. 172, 1–17.

Chen, P., 1998. Thermochemical treatment for algal fermentation. Environ. Int. 24, 889–897.

Cheng, J., Huang, R., Yu, T., Li, T., Zhou, J., Cen, K., 2014. Biodiesel production from lipids in wet microalgae with microwave irradiation and bio-crude production from algal residue through hydrothermal liquefaction. Bioresour. Technol. 151, 415–8.

Chow, M.C., Jackson, W.R., Chaffee, A.L., Marshall, M., 2013a. Thermal Treatment of Algae for Production of Biofuel. Energy & Fuels 27, 1926–1950.

Chow, M.C., Jackson, W.R., Chaffee, A.L., Marshall, M., 2013b. Thermal Treatment of Algae for Production of Biofuel. Energy & Fuels 27, 1926–1950.

De Souza, R.L., Yu, H., Rataboul, F., Essayem, N., 2012. 5-Hydroxymethylfurfural (5-HMF) Production from Hexoses: Limits of Heterogeneous Catalysis in Hydrothermal Conditions and Potential of Concentrated Aqueous Organic Acids as Reactive Solvent System. Challenges 3, 212–232.

Ewen, 2009. Analysis of byproducts in fermentation liquids using an Agilent Hi-Plex H column. Application Note. Agil. Technol. (Prüf- und Forschungsinstitut) 8–9.

Goh, C.S., Lee, K.T., 2010. A visionary and conceptual macroalgae-based third-generation bioethanol (TGB) biorefinery in Sabah, Malaysia as an underlay for renewable and sustainable development. Renew. Sustain. Energy Rev. 14, 842–848.

Halim, R., Gladman, B., Danquah, M.K., Webley, P.A., 2011. Oil extraction from microalgae for biodiesel production. Bioresour. Technol. 102, 178–85.

Iqbal, J., Theegala, C., 2013. Optimizing a continuous flow lipid extraction system (CFLES) used for extracting microalgal lipids. GCB Bioenergy 5, 327–337.

Jones, J., Manning, S., Montoya, M., Keller, K., Poenie, M., 2012. Extraction of Algal Lipids and Their Analysis by HPLC and Mass Spectrometry. J. Am. Oil Chem. Soc.

Karcz, J., 2008. Scanning Electron Microscopy technique: Standard preparation of biological material for SEM analysis. Univ. Silesia SEM-lab.

Kim, Y.-H., Choi, Y.-K., Park, J., Lee, S., Yang, Y.-H., Kim, H.J., Park, T.-J., Hwan Kim, Y., Lee, S.H., 2012. Ionic liquid-mediated extraction of lipids from algal biomass. Bioresour. Technol. 109, 312–5.

Kita, K., Okada, S., Sekino, H., Imou, K., Yokoyama, S., Amano, T., 2010. Thermal pre-treatment of wet microalgae harvest for efficient hydrocarbon recovery. Appl. Energy 87, 2420–2423.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M., 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. 101 Suppl, S75–7.

Liu, Z.-H., Qin, L., Pang, F., Jin, M.-J., Li, B.-Z., Kang, Y., Dale, B.E., Yuan, Y.-J., 2013. Effects of biomass particle size on steam explosion pretreatment performance for improving the enzyme digestibility of corn stover. Ind. Crops Prod. 44, 176–184.

Lohman, E.J., Gardner, R.D., Halverson, L., Macur, R.E., Peyton, B.M., Gerlach, R., 2013. An efficient and scalable extraction and quantification method for algal derived biofuel. J. Microbiol. Methods 94, 235–44.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy Rev. 14, 217–232.

Minowa, T., Sawayama, S., 1999. A novel microalgal system for energy production with nitrogen cycling. Fuel 78, 1213–1215.

Naik, S.N., Goud, V. V., Rout, P.K., Dalai, A.K., 2010. Production of first and second generation biofuels: A comprehensive review. Renew. Sustain. Energy Rev. 14, 578–597.

Ramos, L.P., 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Quim. Nova 26, 863–871.

Ryckebosch, E., Muylaert, K., Foubert, I., 2011. Optimization of an Analytical Procedure for Extraction of Lipids from Microalgae. J. Am. Oil Chem. Soc. 89, 189–198.

Samarasinghe, N., Fernando, S., Lacey, R., Faulkner, W.B., 2012. Algal cell rupture using high pressure homogenization as a prelude to oil extraction. Renew. Energy 48, 300–308.

Santana, A., Jesus, S., Larrayoz, M.A., Filho, R.M., 2012. Supercritical Carbon Dioxide Extraction of Algal Lipids for the Biodiesel Production. Procedia Eng. 42, 1755–1761.

The-Japan-institute-of-Energy, 2008. The Asian biomass handbook. Aguide for biomass production and utilization.

Toor, S.S., Rosendahl, L., Rudolf, A., 2011. Hydrothermal liquefaction of biomass: A review of subcritical water technologies. Energy 36, 2328–2342.

Tyagi, V.K., Vasishtha, A.K., 1996. Changes in the characteristics and composition of oils during deep-fat frying. J. Am. Oil Chem. Soc. 73, 499–506.

# 5. Chapter 5: Investigation of an integrated approach for bio-crude recovery and enzymatic hydrolysis of microalgae cellulose for glucose production

The information presented in this Chapter is based in the paper "Investigation of an integrated approach for bio-crude recovery and enzymatic hydrolysis of microalgae cellulose for glucose production", ready to be submitted to Industrial and engineering chemistry research. The sections in Chapter 5 present the results towards the completion of objectives 5 and 6 of the thesis (see section 1.2.2).

## 5.1. Abstract

Microalgae cellulose offers potential value as a source of fermentable sugars; this cellulose can be used after the extraction of other valued products in the biomass such as bio-crude. In Chapter 4, the bio-crude recovery efficiency using HPS was calculated for different target temperatures and biomass concentrations; in this Chapter the same efficiency was calculated for microalgae cultures with different lipid and cellulose contents, and integrated to the obtainment of fermentable sugars via enzymatic hydrolysis. The efficiency of the extraction was 97.94±8.26% for the algae with the lowest cellulose content. Later, the algae with the highest cellulose content was pre-treated with HPS and hydrolyzed with cellulase, and the glucose yields after both treatments was 0.28 $g.g_{biomass}^{-1}$ at 210˚C.

## 5.2. Introduction

Biofuels from microalgae are an attractive alternative as a sustainable energy source. They are renewable, can use waste as substrate for microalgae growth and they have less carbon emissions than regular fossil fuels. Biofuels from microalgae include lipids that can be converted into biodiesel, the microalgae biomass can be transformed into bio-char (Chaiwong *et al.*, 2013) and bio-crude, and the remaining carbohydrates can be hydrolyzed for the production of fermentable sugars for the production of methanol or methane. In terms of energy density microalgae lipids are 37.6 $kJg^{-1}$, followed by proteins with 16.7 $kJg^{-1}$, and carbohydrates with 15.7 $kJg^{-1}$ (Wilhelm and Jakob, 2011). Even though this is a promising technology, more studies are needed to be done for integration of all these processes. One of the alternatives for application of microalgae feedstock is the initial use of microalgae biomass for lipid recovery and the further utilization of the remaining biomass debris for glucose production from the remaining carbohydrates.

Different approaches have been previously tried for using microalgae as a source of lipids. The most traditional method, but difficult to implement at large scale due to environmental challenges, is solvent extraction. This method requires dried biomass which is expensive since dewatering of microalgae is highly energy consuming, also organic solvent extraction is slow and requires considerable amounts of toxic and expensive solvents (Halim *et al.*, 2011). To avoid this step other researchers have applied hydrothermal treatment, where wet microalgae are utilized directly for cell wall disruption and lipid extraction. The hydrothermal treatment makes use of water at temperatures usually between 100-600˚C and the equipment can be pressurized to increase mechanical stress on microalgae cells. At these conditions, water decreases its dielectric constant (also known as relative permittivity) and behaves similar to organic solvents (Carr *et al*., 2011). Hence, the solubility of the hydrophobic components is improved. The products obtained and the distribution of these compounds is however a characteristic of each microalgae strain, but there are some general groups of compounds present in most of them including lipids (oils). The bio-crude obtained may potentially meet market demands or it could be further upgraded to desired quality standards.

The components remaining in the biomass, after bio-crude recovery through hydrothermal treatment, can further be utilized for other processes or can be re-circulated (as supplement in the culture media) thus increasing the economic feasibility of the overall process. The use of mild conditions (≈200˚C) for hydrothermal treatments is very attractive, since the production of unwanted by-products is reduced. If the temperature for lipid recovery is increased (e.g. 15 min at 250˚C) the process leads to charring and degradation of some biopolymers (e.g. proteins and carbohydrates) that may contaminate the oil in a negative way (Roussis *et al*., 2012). Using hydrothermal treatment, investigators have shown it is possible to extract most of the lipid content of the cell (around 30% ($ww^{-1}$). The amount of lipids that can be recovered depends on microalgae strain, culture and extraction conditions. But efficiencies of extraction of lipids as high as 95% have been reported, using milder thermal conditions (80-90˚C) for microalgae biomass (Chow *et al*., 2013a). The operating points for high bio-crude yield were previously presented in Chapter 4.

The same hydrothermal processing of microalgae can also be implemented as pre-treatment step for enzymatic processes, allowing the use of biomass for the production of sugars (including low cellulose content biomass) due to the increased accessibility of the enzyme to the remaining cellulose. Many hydrothermal treatments including uncatalyzed steam explosion, liquid hot water, pH controlled hot water, and flow-through liquid hot water have shown to increase the accessible surface area (Mosier *et al.*, 2005) of polymers to enzymes. Other advantages of the use of hydrothermal pre-treatment is the significantly lower environmental impact, lower capital investment and application of less hazardous process chemicals (Cara *et al*., 2006). *Chlorella* species have cell walls with up to 80% carbohydrates (Rodrigues and da Silva Bon, 2011), including cellulose, that produces glucose monomers after hydrolysis. The high carbohydrate content of *Chlorella* biomass makes these green microalgae of particular interest as source of fermentable sugars through degradation of their cell walls.

As mentioned, enzymatic hydrolysis is one of the ways to produce fermentable sugars from cellulose originally present in the cells. The enzymes involved in this reaction are cellulases that generally consist of one catalytic domain and one carbohydrate binding module. Cellulases catalyze the reaction via acid catalysis, and this reaction requires the addition of water to break the cellulose bonds (Alvira *et al*., 2010; Zverlov *et al*., 1998). Accessibility of the enzyme to the cellulose fibers is fundamental to increase the efficiency of the reaction; otherwise the hydrolysis will not proceed. This accessibility is function of the specific surface area, crystallinity of the substrate, particle size, porosity and the presence of other compounds associated to the cellulose (Alvira *et al*., 2010; Fan *et al*., 1981). When enzymatic hydrolysis is selected for the production of sugars, the process requires specific conditions for the enzyme. These conditions are optimum pH, temperature, enzyme concentration, hydrolysis time, and agitation speed. Some of these conditions have interactive effects on enzyme performance (Hammed *et al.,* 2013). Therefore, multivariable optimization is generally preferred for accurate and reliable results.

In some cases the production of inhibitory compounds has been reported after the use of hydrothermal pre-treatment on biomass, especially in the cases where the feedstock contains lignin (not in the case of microalgae biomass (Markou *et al*., 2012)). After hydrothermal treatment the produced compounds may include phenolic compounds which have in many processes an inhibitory or toxic effect on enzymes, bacteria, yeast and methanogens (Hendriks and Zeeman, 2009). As mentioned previously, some reports apply in conjunction thermal treatments and enzymatic hydrolysis for production of a wide variety of compounds (from oil extraction to high value chemicals). Grala *et al*., (2012) studied the effect of the use of hydrothermal depolymerisation as pre-treatment for enzymatic hydrolysis of microalgae for the production of methane and concluded that the application of these two processes contributed to increase the quantity and qualitative composition of biogas produced.

The objective of this Chapter was to investigate if the microalgae composition, in terms of cellulose and lipid content, affects the bio-crude recovery efficiency using HPS. Later, the same hydrothermal process was implemented as pre-treatment for enzymatic hydrolysis of the microalgae for glucose production.

## 5.3. Materials and methods

This study was divided into two stages. For the first stage, microalgae were cultivated under different carbon dioxide ($CO_2$) and sodium nitrate ($NaNO_3$) concentrations to produce biomass with different cellulose content, following the approach presented in Chapter 3. The microalgae with the lowest and the highest cellulose content were denoted as LC and HC, respectively. This biomass was used to analyze the effect of cellulose and lipid content on bio-crude recovery efficiency using HPS. For the second stage, the microalgae biomass identified in the first stage to have the highest cellulose content was later used to study the feasibility of HPS as pre-treatment for enzymatic hydrolysis for glucose production.

### 5.3.1. Microalgae strain and culture media

*C. vulgaris* UTEX 2714 was used for this study. The strain was cultivated in liquid Bold's modified media with the same composition described in previous Chapters. For LC content cultures the $CO_2$ concentration in the air was adjusted to 1.5 % ($vv^{-1}$) and $NaNO_3$ concentration to 3.77 mM; for HC content cultures the $CO_2$ concentration was adjusted to 2.33 % ($vv^{-1}$) and $NaNO_3$ concentration to 1.77 mM. These values were obtained from previous studies on the effect of culture conditions on cellulose content and microalgae growth (Aguirre and Bassi, 2013), see section 3.4.3. For all the cultures the pH of the media was adjusted to 6.6 and sterilized in an autoclave at 121˚C, 21 psig for 15 min. Cultures were incubated at room temperature (23-25˚C) with continuous bubbling (7 $Lmin^{-1}$), according to the $CO_2$ concentration specified by each treatment.

### 5.3.2. Analysis of the effect of cellulose and lipid contents on bio-crude recovery efficiency using high pressure steaming

Each microalgae culture (LC and HC) consisted of 3 flasks containing 3 L of culture media and 0.5 L of inoculum with a biomass concentration of 380 mg dw biomass per liter of media. Experimental setup for microalgae cultivation was described in detail in section 3.3.2. Biomass was harvested after 16 days of culture by centrifugation and then freeze dried for 24 hours. To quantify the cellulose content after cultivation, the Updegraff method (Updegraff, 1969) was used (protocol presented in section 3.3.3.4)

After finding the cellulose content in each of the cultures, samples consisting of freeze dried microalgae (LC or HC) with a concentration 4.04 $gL^{-1}$ in distilled water (volume of 20 mL) were subjected to HPS. This thermal treatment was conducted in a custom made device at Western University Machine Services (London, ON, Canada). Details on device operation and configuration are provided in section 4.3.2. Readings of temperature ($T$) and pressure ($P$) were taken every minute until 180˚C were reached inside the HPS device. The selection of temperature and biomass concentration was based on high bio-crude recovery yields obtained in previous experiments at high temperatures (>174˚C) and low biomass concentrations (<5 g/L) (section 4.4.3). At this point the decompression valve was rapidly opened to allow a fast pressure drop of the system due to a sudden total volume increase. After cooling the device to 25˚C the sample was removed from inside the device.  Bio-crude quantification was done following the same protocol in section 4.3.3.2.

Extraction efficiency was calculated according to equation 5.1. The lipid content was determined applying a modified version of Folch's method (Folch *et al.*, 1957) (protocol in section 3.3.3.3), and calculated using equation 5.2.

$$\textbf{Extraction efficiency} = \frac{\textbf{Extracted bio-crude yield using HPS}}{\textbf{Total lipid content}} \times \mathbf{100\%} \qquad \textbf{Equation 5.1}$$

$$\textbf{Total lipid content} = \frac{\textbf{Lipid mass}}{\textbf{Microalgae mass}} \times \mathbf{100} \qquad \textbf{Equation 5.2}$$

### 5.3.3. Enzymatic hydrolysis of microalgae pre-treated with high pressure steaming

After determining the effect of lipid and cellulose contents on bio-crude recovery, the microalgae with the highest cellulose content (HC) was used to study the production of glucose as by-product of the bio-crude recovery process using enzymatic hydrolysis. In this case 5 different treatments were studied: Control (pure cellulose without enzyme), pure cellulose, NoHPS-algae (biomass no pre-treated with HPS), microalgae subjected to HPS at 104˚C (HPS-algae 104C) and microalgae subjected to HPS at 210˚C (HPS-algae 210C). For the enzymatic reaction 3 ml of each sample were introduced in 20 ml glass vials. The pH was adjusted to 5.0 with sodium hydroxide and 50 mM Sodium acetate buffer (pH: 5.0) was added to increase volume to 7.5 ml. Cellulase from *Aspergillus niger* (Sigma C1148-100KU) was hydrated with the same buffer and added to each vial ($7.5x10^{-3}$ $gvial^{-1}$). Enzymatic hydrolysis took place for 8 hours at 50˚C in a water bath. Samples were taken at times 0, 0.5, 1, 2, 6, and 8 hours.

Glucose production was measured following the protocol proposed by Wood et al., (2012), which is a rapid quantification method for reducing sugars. To remove the solids, samples were centrifuged at 4000 rpm for 5 minutes and supernatant was collected. In PCR plates (Fisher Brand) 9 µL of sample are mixed with 171 µL DNS solution. The samples were placed in a PCR thermocycler (Touchgene Gradient. Techne.) at 100˚C for 1 minute, and then held for 2 minutes at 20˚C. A 90 µL aliquot of this mixture was transferred to 96-well flat transparent microplates (Corning Costar), and absorbance was read at 540 nm (see Figure 7.8).

Silva *et al.*, (2011) reported solubilization of components of sugarcane bagasse after hydrothermal pre-treatment processing (e.g. 23% of the cellulose was solubilized at 185˚C while 26.5% was solubilized at 195˚C). Then, the total suspended solids (TSS) were calculated for all the samples prior to enzymatic hydrolysis, since the amount of solids is reduced in those treatments subjected to HPS. To calculate the TSS after HPS, samples of 5 ml were filtrated with pre-weighted filters and rinsed with distilled water. The samples were dried in oven for 1 hour at 105˚C and place in desiccator for 30 minutes prior to final weighing.

### 5.3.4. Experimental design

All experiments were conducted by triplicate. An ANOVA was done for each experiment, and treatments were considered to be statistically significantly different when the *P*-value of the F-test was less than 0.05. To determine which means were significantly different from others a multiple range test was done.

## 5.4. Results and discussion

### 5.4.1. Efficiency of HPS on bio-crude recovery

The amount of cellulose in biomass may affect the lipid recovery due to the presence of thicker cell walls. In this Chapter the main goal was to calculate the efficiency of extraction of lipids as function of cellulose content in biomass. When *C. vulgaris* microalgae was grown at different $CO_2$ and $NaNO_3$ concentrations, the biomass obtained after 16 days of culture had different cellulose and lipid content. Microalgae cultured at 1.55% ($vv^{-1}$) $CO_2$ and 3.77 mM $NaNO_3$ had cellulose content of 9.53±0.13%, while biomass grown at 2.33% ($vv^{-1}$) $CO_2$ and 1.77 mM $NaNO_3$ produced biomass with 42.21±0.04% cellulose (Figure 5.1). These results are in accordance with previous experiments were the effect of $CO_2$ and $NaNO_3$ was studied (see Figure 3.5) (Aguirre and Bassi, 2013). Therefore, the treatments were effective for the production of biomass with different cellulose content, and the biomass obtained was suitable for the study of the effect of cellulose content on lipid recovery.

It is important to notice that culture conditions may not only affect cellulose content but also the percentage of lipids inside the cell. For more accurate calculations of the lipid extraction efficiency, the total lipid content in LC and HC content microalgae was quantified independently. As expected lipid contents were statistically different for both treatments; for LC content microalgae the lipid content was 22.97±1.94% while in HC content microalgae was 15.96±0.11% (Figure 5.1).

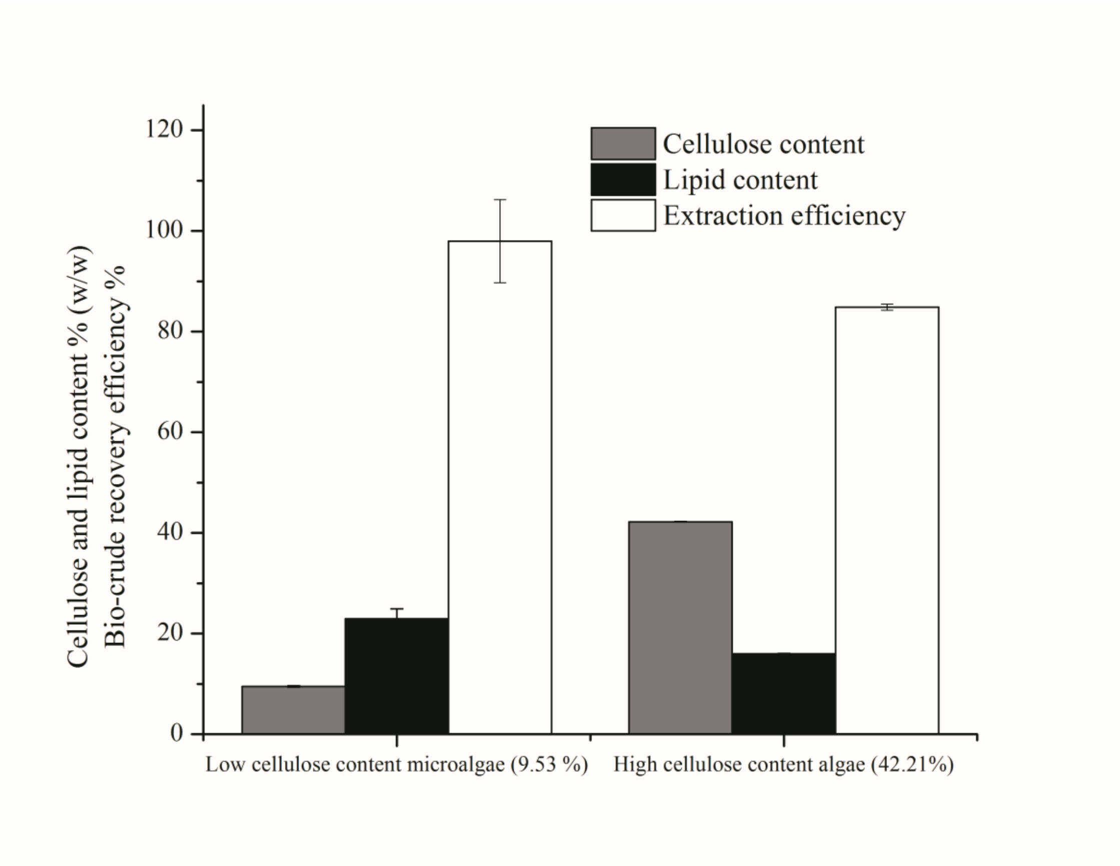



**Figure 5.1** Microalgae composition and bio-crude recovery efficiency.

After characterizing the cellulose and lipid content in LC and HC content microalgae, the biomass was subjected to HPS as thermal treatment for lipid recovery. From LC content biomass the bio-crude yield after HPS was 229.66±19.38 mg.g$^{-1}$, and in HC content microalgae the same yield was 159.64±1.13 mgg$^{-1}$. The bio-crude yield obtained from microalgae grown under different $CO_2$ and $NaNO_3$ concentrations were statistically different.

The bio-crude yield after HPS was very similar to the amount of total lipids quantified by Folch's method and mentioned above. When extraction efficiency was calculated, the values obtained for both treatments (HC and LC) were 97.94±8.26% and 84.84±0.60 for LC and HC content biomass, respectively (Figure 5.1). Both efficiencies are high.

Mercer and Armenta (2011), present a comprehensive review on extraction efficiencies from different algae species and extraction methods. Nagle and Lemke (1990), obtained extraction efficiencies of 90%, 73%, and 78%, when using 1-butanol, ethanol, and hexane/propanol, respectively from *Chaetoceros muelleri*. According with (Lee et al., 1998), solvent extraction may not lead to the highest lipid recovery; they obtained more lipids from *Botrycoccus braunni* using bead-beater extraction, then it can explain why in some cases the extraction efficiency calculations are higher that 100%. Comparison of the extraction efficiency for both biomass compositions (LC and HC), in this experiment, showed that there is not statistical difference; it means that HPS is able to efficiently extract lipids from microalgae with different biomass compositions.

### 5.4.2. Enzymatic production of glucose from HPS pre-treated microalgae

Knowing that HPS steaming is an efficient method for lipid recovery, the next step was to study the possible use of the biomass obtained after this process for the production of glucose which can be a by-product of the overall microalgae process, and it can be later used as fermentable sugar. The aim of this experiment was not the optimization of the enzymatic process but the study of its technical feasibility. Some papers have reported the production of enzymatic inhibitory compounds after thermal treatment; hence, it was wanted to know if the cellulose remaining after HPS can be used for enzymatic processes.

The biomass selected in the previous experiment as high cellulose (HC) content was used for the enzymatic hydrolysis experiments. Glucose production was measured during 8 hours. For all the treatments the initial glucose concentration was very close to zero. Most of the glucose was produced during the first 2 hours of the enzymatic reaction. There was no difference between the treatments evaluated in terms of glucose yield, calculated as gram of glucose per gram of biomass before HPS (Figure 5.2).

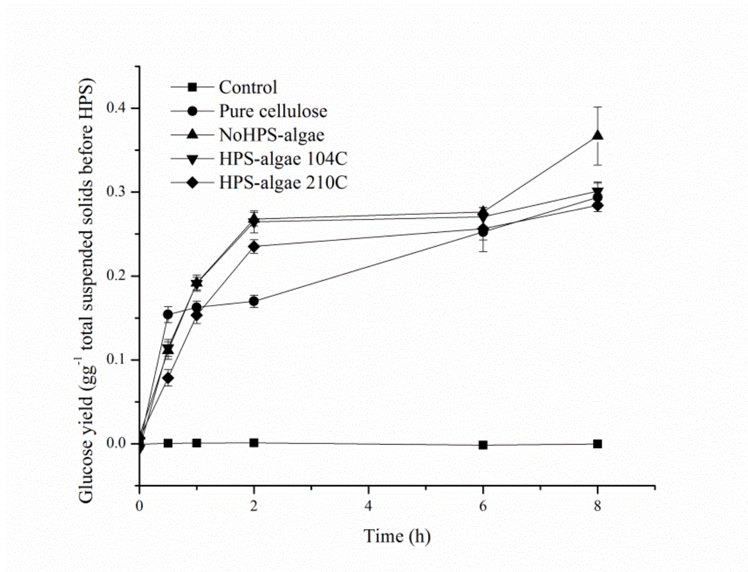



**Figure 5.2** Glucose yield calculated based on total suspended solids before HPS.

When microalgae is subjected to HPS the amount of TSS is reduced, therefore glucose yield was also calculated based on the TSS after HPS, which is the actual amount of solids in the enzymatic reaction as a way of finding the effect of cellulose solubilization on enzymatic reaction. Table 5.1 shows the total suspended solids before enzymatic hydrolysis.

**Table 5.1** Total suspended solids after HPS and dilution with buffer.

| **Treatment** | **TSS (gl$^{-1}$)** | **TSS after dilution with buffer (gl$^{-1}$)** |
|---|---|---|
| HPS 104˚C | 2.04±0.03 | 0.816 |
| HPS 210˚C | 0.84±0.01 | 0.336 |
| NoHPS-algae | 4±0.04 | 1.6 |
| NoHPS Pure cellulose | 4.2±0.01 | 1.68 |
| Control | 4.2±0.02 | 1.68 |

If glucose yields are calculated based on the amount of TSS after HPS there is significant difference between some of the treatments (Figure 5.3).

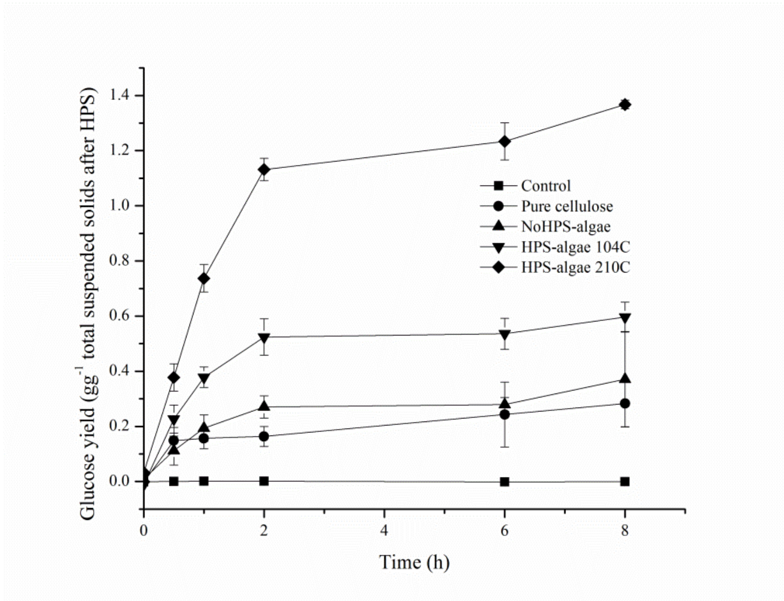



**Figure 5.3** Glucose yield calculated based on total suspended solids after HPS.

In this case, microalgae subjected to HPS at 210˚C had the highest yield of glucose (1.37 grams of glucose per gram of TTS or 0.28 grams of glucose per gram of biomass before pre-treatment), followed by the treatment of HPS-microalgae 104˚C, meaning that solubilization of components due to HPS has a positive effect on glucose production. In subcritical water the cellulose is rapidly solubilized (Toor *et al*., 2011), and also the smaller the substrate size, the higher the enzyme degradation (Hammed *et al*., 2013), explaining the higher yields for pre-treated microalgae. There was no difference between the microalgae untreated and pure cellulose, probably due to difficult access of the enzyme to cellulose and the insolubility of cellulose in water. Additionally, the enzyme is much bigger than the cellobiose in the cellulose chain, as consequence the enzyme covers numerous bonds making them even more unavailable (Andersen, 2007). Comparison of sugar yields from other research with this study is presented in Table 5.2.

**Table 5.2** Comparison of sugar yields for different pre-treatments of biomass.

| **Feedstock** | **Pre-treatment** | **Enzyme** | **Sugars yield** | **Reference** |
|---|---|---|---|---|
| Olive tree wood | Steam explosion 190˚C | Celluclast | 0.288 $g.g_{biomass}^{-1}$ | (Cara *et al.*, 2006) |
| Sunflower stalks | Steam explosion 220 ◦C 5 minutes | Celluclast 1.5 L | 0.167 $g.g_{biomass}^{-1}$ | (Ruiz *et al.*, 2008) |
| Ulva pertusa kjellman | High pressure steaming 180˚C 8 min | Cellulase | 0.7 $g.g_{cellulose}^{-1}$ | (Choi *et al.*, 2013) |
| Seaweed Ulva | Pre-heat treatment 120˚C 1 hour | Cellulase 22119 | 0.207 $g.g_{biomass}^{-1}$ | (Trivedi *et al.*, 2013) |
| *Chlorella homosphaera* | Cells were washed with chilled ethanol, cold dried, and grounded | Cellulases, xylanases, and amylases blend | 0.245 $g.g_{biomass}^{-1}$ | (Rodrigues and da Silva Bon, 2011) |
| *Chlorella zofingiensis* | Cells were washed with chilled ethanol, cold dried, and grounded | Cellulases, xylanases, and amylases blend | 0.193 $g.g_{biomass}^{-1}$ | (Rodrigues and da Silva Bon, 2011) |
| *Monostroma nitidum* Wittrock | Hydrothermal fractional 150C | Cellulosin T2) | 0.107 $g.g_{biomass}^{-1}$ | (Okuda *et al.*, 2008) |
| *Chlorella vulgaris* | High pressure steaming 210˚ 4.04 $gL^{-1}$ | Cellulase from *Aspergillus niger* | 0.28 $g.g_{biomass}^{-1}$ | (This study) |

## 5.5. Conclusions

From the results presented in this chapter it is concluded that regardless of the microalgae composition in terms of cellulose and lipid content, HPS was able to extract most of the bio-crude in the biomass leading to extraction efficiencies as high as 97.94±8.26%. Also HPS was a suitable pre-treatment to increase accessibility of the cellulase to microalgae cellulose and helped in the solubilization of the substrate. The reductions of the TSS after this hydrothermal treatment aimed the enzymatic hydrolysis, with glucose yield of 0.28 $g.g_{biomass}^{-1}$for microalgae subjected at 210˚C.

The possible use of HPS as a thermal treatment for bio-crude recovery and as a pre-treatment for enzymatic hydrolysis, favors the use of this technology on microalgae integrated processes, where the economical feasibility and sustainability may be increased. The good glucose yields obtained using this technology under non-optimized conditions motivates the further study where even better production could be reached.

**References**


Aguirre, A.-M., Bassi, A., 2013. Investigation of biomass concentration, lipid production, and cellulose content in Chlorella vulgaris cultures using response surface methodology. Biotechnol. Bioeng. 110, 2114–22.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour. Technol. 101, 4851–61.

Andersen, N., 2007. Enzymatic Hydrolysis of Cellulose. Experimental and Modeling Studies. Technical University of Denmark.

Cara, C., Ruiz, E., Ballesteros, I., Negro, M.J., Castro, E., 2006. Enhanced enzymatic hydrolysis of olive tree wood by steam explosion and alkaline peroxide delignification. Process Biochem. 41, 423–429.

Carr, A.G., Mammucari, R., Foster, N.R., 2011. A review of subcritical water as a solvent and its utilisation for the processing of hydrophobic organic compounds. Chem. Eng. J. 172, 1–17.

Chaiwong, K., Kiatsiriroat, T., Vorayos, N., Thararax, C., 2013. Study of bio-oil and bio-char production from algae by slow pyrolysis. Biomass and Bioenergy 56, 600–606.

Choi, W.-Y., Kang, D.-H., Lee, H.-Y., 2013. Enhancement of the saccharification yields of Ulva pertusa kjellmann and rape stems by the high-pressure steam pretreatment process. Biotechnol. Bioprocess Eng. 18, 728–735.

Chow, M.C., Jackson, W.R., Chaffee, A.L., Marshall, M., 2013. Thermal Treatment of Algae for Production of Biofuel. Energy & Fuels 27, 1926–1950.

Fan, L.T., Lee, Y.-H., Beardmore, D.R., 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. Biotechnol. Bioeng. 23, 419–424.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.

Grala, A., Zieliński, M., Dębowski, M., Dudek, M., 2012. Effects of Hydrothermal Depolymerization and Enzymatic Hydrolysis of Algae Biomass on Yield of Methane Fermentation Process. Pol. J. Environ. Stud 21, 363–368.

Halim, R., Gladman, B., Danquah, M.K., Webley, P.A., 2011. Oil extraction from microalgae for biodiesel production. Bioresour. Technol. 102, 178–85.

Hammed, A.M., Jaswir, I., Amid, A., Alam, Z., Asiyanbi-H, T.T., Ramli, N., 2013. Enzymatic Hydrolysis of Plants and Algae for Extraction of Bioactive Compounds. Food Rev. Int. 29, 352–370.

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–8.

Lee, S.J., Yoon, B.-D., Oh, H.-M., 1998. Rapid method for the determination of lipid from the green alga Botryococcus braunii. Biotechnol. Tech. 12, 553–556.

Markou, G., Angelidaki, I., Georgakakis, D., 2012. Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Appl. Microbiol. Biotechnol. 96, 631–45.

Mercer, P., Armenta, R.E., 2011. Developments in oil extraction from microalgae. Eur. J. Lipid Sci. Technol. 113, 539–547.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673–86.

Nagle, N., Lemke, P., 1990. Production of methyl ester fuel from microalgae. Appl. Biochem. Biotechnol. 24-25, 355–361.

Okuda, K., Oka, K., Onda, A., Kajiyoshi, K., Hiraoka, M., Yanagisawa, K., 2008. Hydrothermal fractional pretreatment of sea algae and its enhanced enzymatic hydrolysis. J. Chem. Technol. Biotechnol. 83, 836–841.

Rodrigues, M.A., da Silva Bon, E.P., 2011. Evaluation of Chlorella (Chlorophyta) as Source of Fermentable Sugars via Cell Wall Enzymatic Hydrolysis. Enzyme Res. 2011, 405603.

Roussis, S.G., Cranford, R., Sytkovetskiy, N., 2012. Thermal Treatment of Crude Algae Oils Prepared Under Hydrothermal Extraction Conditions. Energy & Fuels 26, 5294–5299.

Ruiz, E., Cara, C., Manzanares, P., Ballesteros, M., Castro, E., 2008. Evaluation of steam explosion pre-treatment for enzymatic hydrolysis of sunflower stalks. Enzyme Microb. Technol. 42, 160–6.

Silva, V.F.N., Arruda, P. V, Felipe, M.G.A., Gonçalves, A.R., Rocha, G.J.M., 2011. Fermentation of cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse pretreated by hydrothermal processing. J. Ind. Microbiol. Biotechnol. 38, 809–17.

Toor, S.S., Rosendahl, L., Rudolf, A., 2011. Hydrothermal liquefaction of biomass: A review of subcritical water technologies. Energy 36, 2328–2342.

Trivedi, N., Gupta, V., Reddy, C.R.K., Jha, B., 2013. Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga Ulva fasciata Delile. Bioresour. Technol. 150, 106–12.

Updegraff, D.M., 1969. Semimicro determination of cellulose in biological materials. Anal. Biochem. 32, 420–4.

Wilhelm, C., Jakob, T., 2011. From photons to biomass and biofuels: evaluation of different strategies for the improvement of algal biotechnology based on comparative energy balances. Appl. Microbiol. Biotechnol. 92, 909–19.

Wood, I.P., Elliston, A., Ryden, P., Bancroft, I., Roberts, I.N., Waldron, K.W., 2012. Rapid quantification of reducing sugars in biomass hydrolysates: Improving the speed and precision of the dinitrosalicylic acid assay. Biomass and Bioenergy 44, 117–121.

Zverlov, V., Mahr, S., Riedel, K., Bronnenmeier, K., 1998. Properties and gene structure of a bifunctional cellulolytic enzyme (CelA) from the extreme thermophile "Anaerocellum thermophilum" with separate glycosyl hydrolase family 9 and 48 catalytic domains. Microbiology 144 ( Pt 2, 457–65.

# 6. Chapter 6: Conclusions and recommendations

In this Chapter the main conclusions of this study are presented; also some recommendations for future work are suggested.

## 6.1. Conclusions

Current limitations in biodiesel production from microalgae were identified in the earliest stages of the research, and the low lipid extraction efficiency from the cell was highlighted as one of the principal bottlenecks of this process, leading to the development of this research where the culture conditions and extraction methods were integrated towards one objective: increasing lipid extraction efficiency.

Differences on cellulose and lipid contents in cells of *Chlorella vulgaris* were obtained when cultures were subjected to different culture conditions in terms of carbon dioxide and sodium nitrate concentrations. The empirical models obtained for biomass concentration and lipid/content ratio applying the RSM had good accuracy explaining the 96.01% and 93.35% of the response variables respectively. The location of an optimal point in the range of study, where lipid productivity is high and cellulose content is low, was possible by means of the CCD.

The models in Chapter 3 can be used as a tool when algae with specific characteristics are needed. For instance, if the objective is only to produce biomass Equation 3.1 can be maximized to lead to the highest biomass concentration within the range covered by the variables. Similarly, if a culture with high lipid productivity is wanted, Equation 3.2 can be maximized, granting a good amount of lipids in a shorter period of time. But also if a culture with high cellulose content is required (for instance for the further production of fermentable sugars), Equation 3.2 can be minimized. So cultures can be adjusted to specific needs.

Microalgae cultures under the optimal conditions for high lipid productivity and low cellulose content were used for studies of HPS as bio-crude recovery method in Chapter 4. The manipulated variables, target temperature and microalgae concentrations, played both significant roles on bio-crude and glucose yields. From SEM images it was evident that HPS modifies cell morphology and surface, cell breakage and formation of pores in the microalgae surface was observed. RSM allowed the study of the effect of target temperature and microalgae concentration on HPS. It was found that to achieve high bio-crude yields, low microalgae concentrations and high temperatures are favorable; meanwhile, for high glucose yields, low temperatures and microalgae concentrations produce better results.

Once again the empirical models obtained allow manipulating the process towards a wanted output. If high bio-crude yield is the objective of the process, then according to Equation 4.1 the system should operate at low microalgae concentrations and high temperatures; meanwhile, for high glucose yields, Equation 4.2 suggest that low temperatures and microalgae concentrations produce better results.

One important annotation on the significance of the operating areas obtained in this research is that both, target temperature and microalgae concentration, are intensive properties, therefore they do not depend of the system size. Then, the results presented in this research can be used as a start point in scale-up studies.

It was also concluded that regardless of the microalgae composition, in terms of cellulose and lipid content, HPS was able to extract most of the bio-crude in the biomass leading to extraction efficiencies as high as 97.94±8.26%.

Finally, the possible use of the same HPS process for the production of glucose via enzymatic hydrolysis was investigated, and results showed that the thermal treatment aid the solubilisation of the remaining biomass after bio-crude recovery, leading to higher glucose yields. This means that the thermal treatment proposed for bio-crude recovery can be integrated with enzymatic processes to increase the global process feasibility. The higher glucose yield obtained without any optimization was 0.28 $g.g_{biomass}^{-1}$ for microalgae subjected at 210˚C.

## 6.2. Recommendations

From the experience obtained after the completion of this thesis the following suggestions are done for future work:

- In Chapter 4 the effect of pressure in the system was indirectly study by the relationship between temperature and pressure for saturated steam, but it would be interesting to study the independent effect of pressure without increasing the target temperature in the system. This experiment could be done by injecting nitrogen in the system until the target pressure is reached. It could possible reduce the change in color of the bio-crude and the degradation of other by-products.

- As stated several times in this thesis, one of the advantages of the use of thermal treatments for lipid recovery is the possible use of wet microalgae, which reduces the cost of drying the biomass. It is suggested to investigate the feasibility of the direct injection of microalgae culture (algae+media), after growth, in the HPS device. It would not only reduce the cost of drying but also it would avoid any step to dilute or concentrate the microalgae, since the algae concentration after growing usually belongs to the values found to produce high bio-crude recovery yields.

- The operating points for high bio-crude recovery can be used at a start point in scaling-up studies of HPS as mentioned in the conclusion. The scale-up of the process would allow obtaining larger quantities of the bio-crude and therefore its characterization would be easier. HPS devices for larger volumes already exist in the market, so adaptation of this technology to microalgae process would be the main objective.

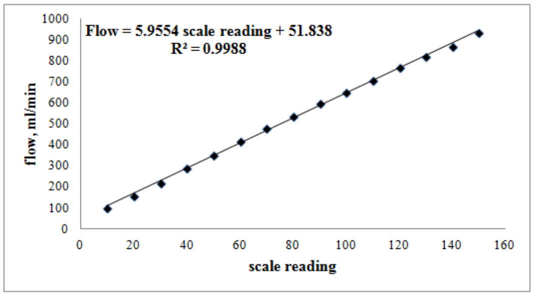
- If the same HPS device presented in Figure 4.1 is going to be used in other experiments the following modification are suggested:
    - Increase the expansion chamber volume to allow bigger pressure drops, or adapt the system to decompression to atmosphere; this theoretically would increase the cell wall breakage.
    - If the volume of the steam chamber is increased then all the water in the sample could be transformed into vapor without bursting the safety valve. This can create the conditions needed for a steam explosion which is known to be an effective treatment of breaking polymers such as cellulose.
    - The current geometry of the device does not allow the easy removal of the sample, so solvents are needed to wash all the lipids from the walls. A wider or disassemble system would facilitate the removal of the sample and shorten the experimental times.

- HPS showed to be an efficient method for recovery of bio-crude from algae with different cellulose concentrations, partly due to the physical disruption and formation of pores in the call wall. It would be interesting to study the effect of cellulose content in milder extraction treatments where the cell wall may impose diffusional limitations for lipid extraction.

- Chapter 5 briefly explored the possible use of HPS as pre-treatment for the production of fermentable sugars using cellulases. This can be studied by multivariable optimization of the enzymatic process parameter such as temperature, pH, substrate-enzyme ratio, and the possible use of multiple enzymes.

## 7. Appendices

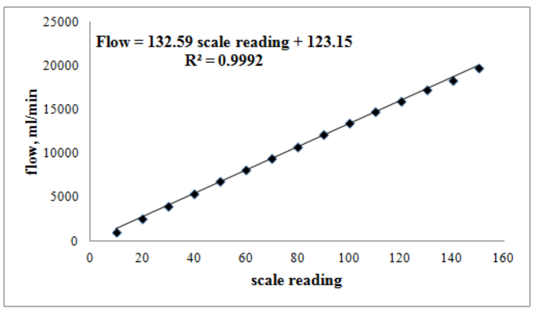
### 7.1. Appendix 1: Calibration and standard curves.




**Equipment:**

Flowmeter N034-39 (G, S, ST, C), Omega.

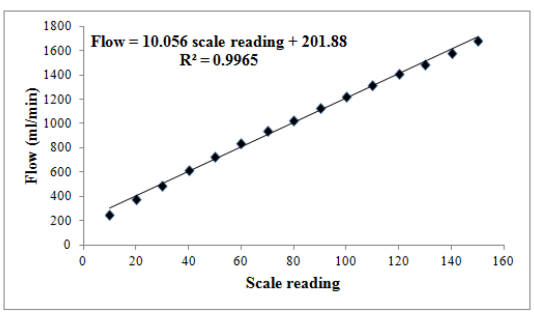
**Figure 7.1** Calibration curve for carbon dioxide flow in mixer (N112-02).




**Equipment:**

Flowmeter N034-39 (G, S, ST, C), Omega.

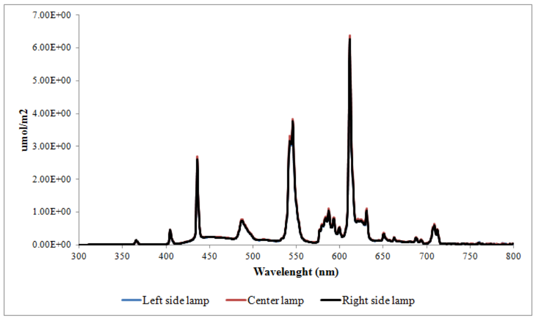
**Figure 7.2** Calibration curve for air flow in mixer (N034-39).




**Equipment:**

Flowmeter 082-03 (GL,SA, ST, CA, TA), Omega.

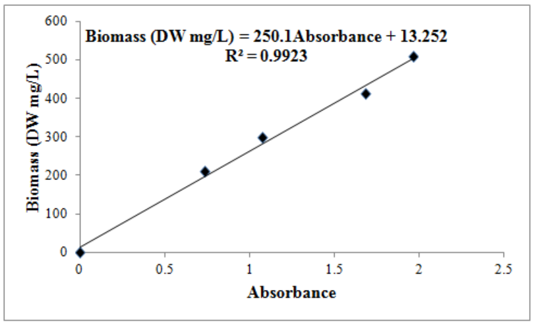
**Figure 7.3** Calibration curve for rotameters.




**Equipment:**

39’’ T5 fluorescent lamp, Illume.

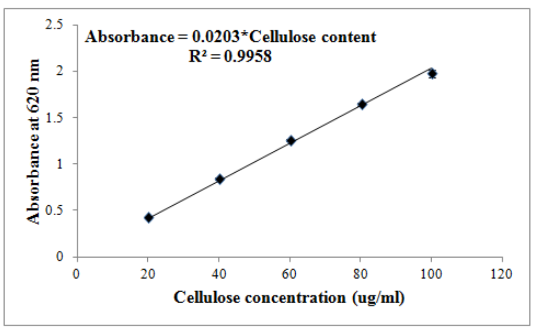
**Figure 7.4** Lamp spectrum.




**Equipment:**

DR 2800 portable spectrophotometer, HACH.

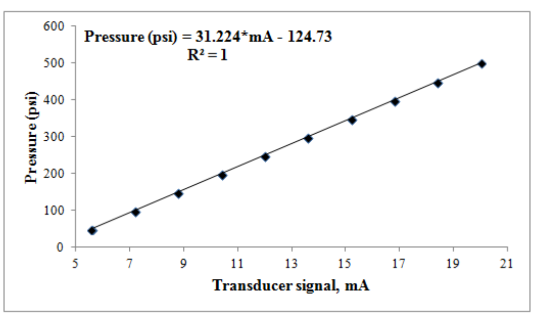
**Figure 7.5** Standard curve for dry biomass concentration.




**Equipment:**

DR 2800 portable spectrophotometer, HACH.

**Figure 7.6** Standard curve for cellulose concentration.




**Equipment:**

Model A2 Heavy Industrial PressureTransmitter, Ashcroft.

**Figure 7.7** Standard curve for pressure transducer.

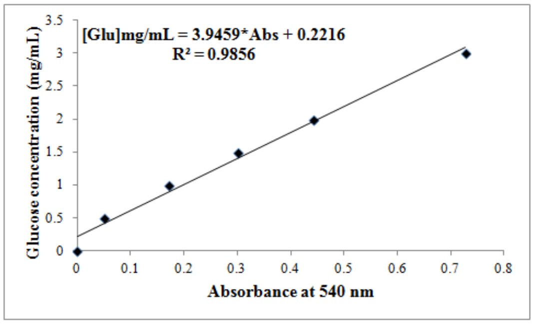



**Figure 7.8 Calibration curve for glucose concentration**

## 7.2. Appendix 2: Statistical results from software.

Following are the outputs from the statistical software "Statgraphics centurion" for all the empirical models presented in this study.

### 7.2.1. Biomass concentration model.

**Table 7.1** Estimated effects for Biomass concentration.

| Effect | Estimate | Stnd. Error | V.I.F. |
|---|---|---|---|
| average | 1082.42 | 28.5873 | |
| A:Carbon dioxide concentration | -195.105 | 35.0121 | 1.0 |
| B:Nitrate concentration | 297.195 | 35.0121 | 1.0 |
| AA | -156.908 | 41.6727 | 1.0947 |
| BB | -253.824 | 41.6727 | 1.0947 |

Standard errors are based on total error with 6 d.f.

**The StatAdvisor:** This table shows each of the estimated effects and interactions. Also shown is the standard error of each of the effects, which measures their sampling error. Note also that the largest variance inflation factor (V.I.F.) equals 1.0947. For a perfectly orthogonal design, all of the factors would equal 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects.

**R-squared = 96.0125 percent**

**R-squared (adjusted for d.f.) = 93.3541 percent**

**Standard Error of Est. = 49.5146**

**Mean absolute error = 29.138**

**Durbin-Watson statistic = 1.91941 (*P*=0.4625)**

**Lag 1 residual autocorrelation = -0.00173548**

**The StatAdvisor:** The R-Squared statistic indicates that the model as fitted explains 96.0125% of the variability in Biomass concentration. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 93.3541%. The standard error of the estimate shows the standard deviation of the residuals to be 49.5146. The mean absolute error (MAE) of 29.138 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the *P*-value is greater than 5.0%, there is no indication of serial autocorrelation in the residuals at the 5.0% significance level.

**Table 7.2** Regression coefficients for Biomass concentration.

| **Coefficient** | **Estimate** |
|---|---|
| constant | 194.836 |
| A:Carbon dioxide concentration | 121.077 |
| B:Nitrate concentration | 313.528 |
| AA | -19.6136 |
| BB | -31.728 |

**The StatAdvisor:** This pane displays the regression equation which has been fitted to the data. The equation of the fitted model is

Biomass concentration = 194.836 + 121.077*Carbon dioxide concentration + 313.528*Nitrate concentration - 19.6136*Carbon dioxide concentration^2 - 31.728*Nitrate concentration^2

where the values of the variables are specified in their original units.

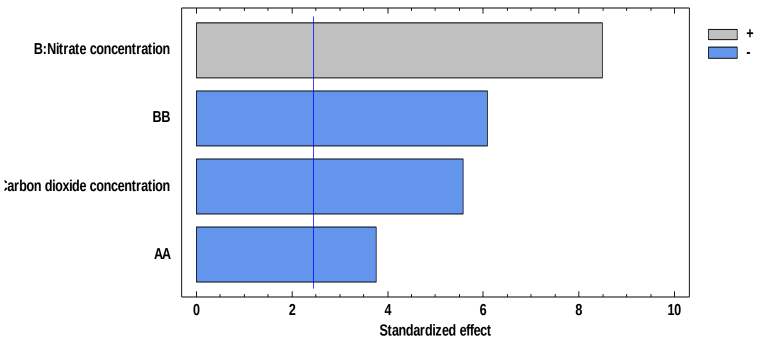



**Figure 7.9** Standarized pareto chart for biomass concentration.

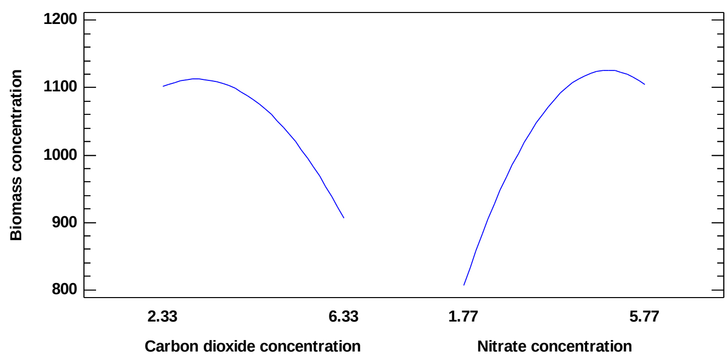



**Figure 7.10** Main effects plot for biomass concentration.

### 7.2.2. Lipid productivity over cellulose content model

**Table 7.3** Estimated effects for LP/CC.

| Effect | Estimate | Stnd. Error | V.I.F. |
|---|---|---|---|
| average | 0.446005 | 0.0228016 | |
| A:Carbon dioxide concentration | -0.0918927 | 0.0279261 | 1.0 |
| B:Nitrate concentration | -0.112604 | 0.0279261 | 1.0 |
| AA | -0.293382 | 0.0332387 | 1.0947 |
| BB | -0.202001 | 0.0332387 | 1.0947 |

Standard errors are based on total error with 6 d.f.

**The StatAdvisor:** This table shows each of the estimated effects and interactions. Also shown is the standard error of each of the effects, which measures their sampling error.

Note also that the largest variance inflation factor (V.I.F.) equals 1.0947. For a perfectly orthogonal design, all of the factors would equal 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects.

**R-squared = 95.1714 percent**

**R-squared (adjusted for d.f.) = 91.9524 percent**

**Standard Error of Est. = 0.0394935**

**Mean absolute error = 0.0259922**

**Durbin-Watson statistic = 2.42667 (*P*=0.7838)**

**Lag 1 residual autocorrelation = -0.220734**

**The StatAdvisor:** The R-Squared statistic indicates that the model as fitted explains 95.1714% of the variability in LP/CC. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 91.9524%. The standard error of the estimate shows the standard deviation of the residuals to be 0.0394935. The mean absolute error (MAE) of 0.0259922 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the *P*-value is greater than 5.0%, there is no indication of serial autocorrelation in the residuals at the 5.0% significance level.

**Table 7.4** Regression coefficients for LP/CC.

| **Coefficient** | **Estimate** |
|---|---|
| constant | -0.394844 |
| A:Carbon dioxide concentration | 0.294613 |
| B:Nitrate concentration | 0.162235 |
| AA | -0.0366727 |
| BB | -0.0252502 |

**The StatAdvisor:** This pane displays the regression equation which has been fitted to the data. The equation of the fitted model is

LP/CC = -0.394844 + 0.294613*Carbon dioxide concentration + 0.162235*Nitrate concentration - 0.0366727*Carbon dioxide concentration^2 - 0.0252502*Nitrate concentration^2

where the values of the variables are specified in their original units.

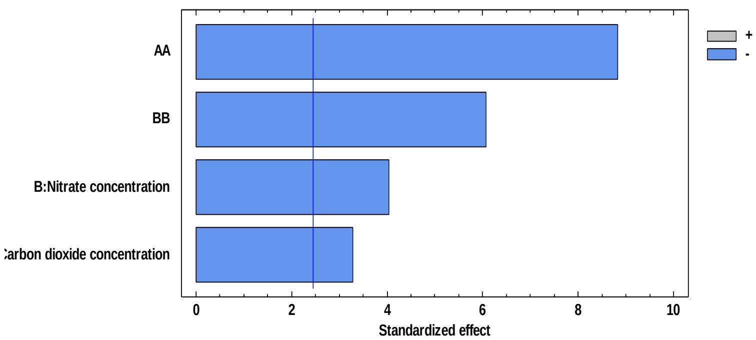



**Figure 7.11** Standarized pareto chart for LP/CC.

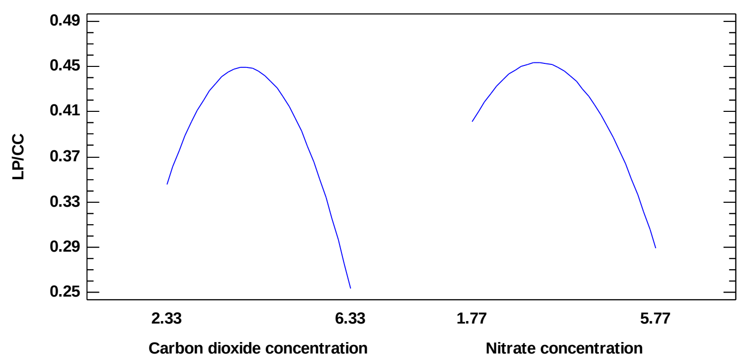



**Figure 7.12** Main effects plot for LP/CC.

### 7.2.3. Bio-crude yield model

**Table 7.5** Estimated effects for LOG(Bio-crude yield).

| **Effect** | **Estimate** | **Stnd. Error** | **V.I.F.** |
|---|---|---|---|
| average | 4.55202 | 0.0816903 | |
| A:Temperature | 0.482067 | 0.191581 | 1.0 |
| B:Biomass concentration | -0.623373 | 0.19158 | 1.0 |

Standard errors are based on total error with 8 d.f.

**The StatAdvisor:** This table shows each of the estimated effects and interactions. Also shown is the standard error of each of the effects, which measures their sampling error. Note also that the largest variance inflation factor (V.I.F.) equals 1.0. For a perfectly orthogonal design, all of the factors would equal 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects.

**R-squared = 67.8961 percent**

**R-squared (adjusted for d.f.) = 59.8701 percent**

**Standard Error of Est. = 0.270936**

**Mean absolute error = 0.215875**

**Durbin-Watson statistic = 2.49868 (P=0.7861)**

**Lag 1 residual autocorrelation = -0.310086**

**The StatAdvisor:** The R-Squared statistic indicates that the model as fitted explains 67.8961% of the variability in LOG(Bio-crude yield). The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 59.8701%. The standard error of the estimate shows the standard deviation of the residuals to be 0.270936. The mean absolute error (MAE) of 0.215875 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the P-value is greater than 5.0%, there is no indication of serial autocorrelation in the residuals at the 5.0% significance level.

**Table 7.6** Regression coefficients for LOG(Bio-crude yield).

| **Coefficient** | **Estimate** |
|---|---|
| constant | 4.00721 |
| A:Temperature | 0.00642757 |
| B:Biomass concentration | -0.00779216 |

**The StatAdvisor:** This pane displays the regression equation which has been fitted to the data. The equation of the fitted model is

LOG(Bio-crude yield) = 4.00721 + 0.00642757*Temperature - 0.00779216*Biomass concentration

where the values of the variables are specified in their original units.

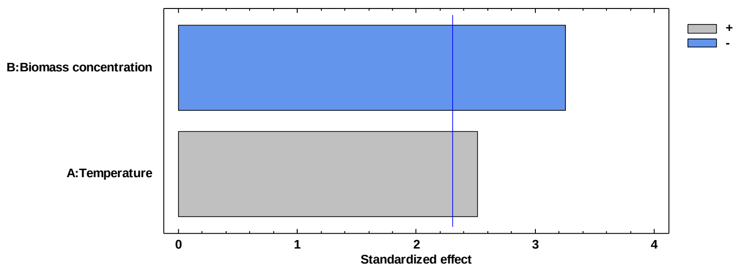



**Figure 7.13** Standarized pareto chart for bio-crude yield.

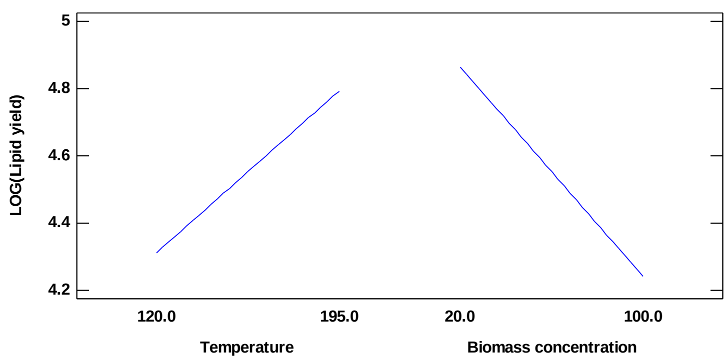



**Figure 7.14** Main effects plot for bio-crude yield.

### 7.2.4. Glucose yield model

**Table 7.7** Estimated effects for LOG(Glucose yield).

| Effect | Estimate | Stnd. Error | V.I.F. |
|---|---|---|---|
| average | 0.991576 | 0.0945137 | |
| A:Temperature | -1.683 | 0.15909 | 1.0 |
| B:Biomass concentration | -1.2718 | 0.15909 | 1.0 |
| AB | -0.975329 | 0.224988 | 1.0 |
| BB | 0.516736 | 0.180979 | 1.0 |

Standard errors are based on total error with 6 d.f.

**The StatAdvisor:** This table shows each of the estimated effects and interactions. Also shown is the standard error of each of the effects, which measures their sampling error. Note also that the largest variance inflation factor (V.I.F.) equals 1.0. For a perfectly orthogonal design, all of the factors would equal 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects.

**R-squared = 97.1259 percent**
**R-squared (adjusted for d.f.) = 95.2099 percent**
**Standard Error of Est. = 0.224988**
**Mean absolute error = 0.132297**
**Durbin-Watson statistic = 2.30259 (P=0.7647)**
**Lag 1 residual autocorrelation = -0.15743**

**The StatAdvisor:** The R-Squared statistic indicates that the model as fitted explains 97.1259% of the variability in LOG(Glucose yield). The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 95.2099%. The standard error of the estimate shows the standard deviation of the residuals to be 0.224988. The mean absolute error (MAE) of 0.132297 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the P-value is greater than 5.0%, there is no indication of serial autocorrelation in the residuals at the 5.0% significance level.

**Table 7.8** Regression coefficients for LOG(Glucose yield).

| **Coefficient** | **Estimate** |
|---|---|
| constant | 2.98877 |
| A:Temperature | -0.00293341 |
| B:Biomass concentration | 0.0159297 |
| AB | -0.00032511 |
| BB | 0.00016148 |

**The StatAdvisor:** This pane displays the regression equation which has been fitted to the data.  The equation of the fitted model is

LOG(Glucose yield) = 2.98877 - 0.00293341*Temperature + 0.0159297*Biomass concentration - 0.00032511*Temperature*Biomass concentration + 0.00016148*Biomass concentration^2

where the values of the variables are specified in their original units.

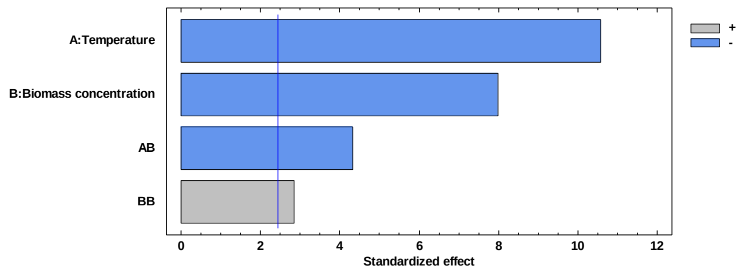



**Figure 7.15** Standarized pareto chart for glucose yield.

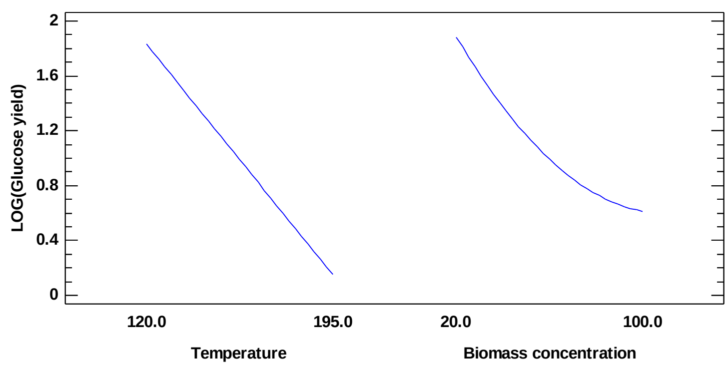



**Figure 7.16** Main effects plot for glucose yield.

### 7.3. Appendix 3: High pressure steaming device and pre-assays.

The first step was to adapt the HPS device to project requirements. Initially this equipment was a cylindrical device with one steam chamber and one expansion chamber. The chambers were separated by one ball valve for sample decompression. With this initial design the control and measurement of process variables was null. To increase the reliability of the data obtained from the steam explosion device the following changes were implemented:

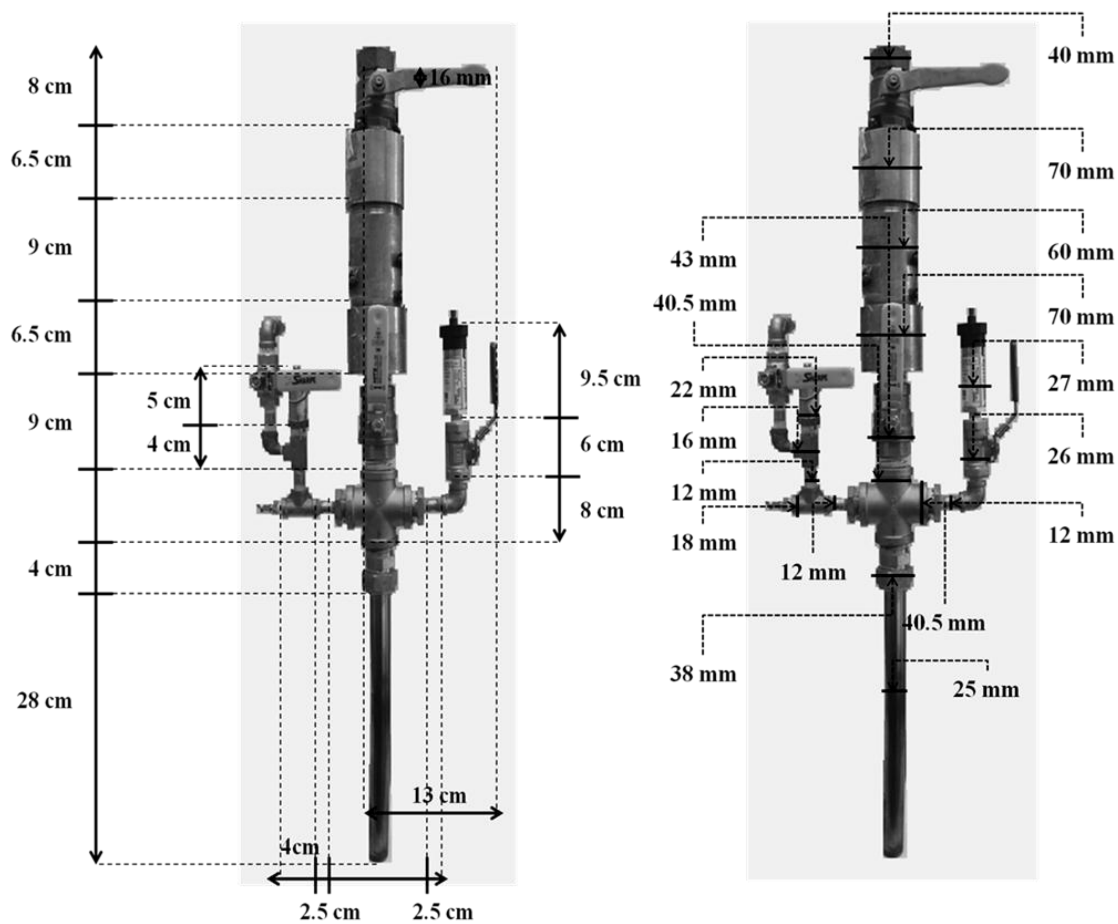



**Figure 7.17** Dimension of high pressure steaming device.

- Installation of a thermocouple that shows actual temperature inside steam chamber: the temperature inside the oven was quite different from the temperature inside the equipment due to all the air contained in the oven that acts as an isolator.
- Installation of a pressure transducer: actual pressure inside the steam chamber can be read using this pressure transducer. This accessory is protected from sudden drops in pressure by a valve that can be closed before steam decompression.
- Installation of a safety cage: for safety reason a cage around the device was built. In case of uncontrolled explosion, the cage would protect users. The cage has a top door that makes easy to remove the device from the oven.

### 7.3.1. Determination of oven temperature and sample volume

In order to find the proper temperature at which the oven must be pre-heated and the volume of the sample to be used, the following experiment was conducted. Oven was pre-heated and sample was introduced to the steam chamber (values for each experiment are shown in Table 7.9). The temperature inside the steam chamber should increase up to 210˚C, which was the highest target temperature to be part of the CCD (Table 4.2).

**Table 7.9** Experiments for oven temperature and sample volume.

| **Experiment** | **Oven temperature (˚C)** | **Sample volume (ml)** |
|---|---|---|
| E1 | 400 | 20 |
| E2 | 600 | 20 |
| E3 | 800 | 20 |
| E4 | 400 | 40 |
| E5 | 600 | 40 |
| E6 | 800 | 40 |

Figure 7.18 and Figure 7.19 show the results for temperature and pressure inside the steam chamber. From Figure 7.18 it can be seen that only when the oven was pre-heated at 800˚C the system was able to reach the target temperature of 210˚C. Therefore, 800˚C was selected as the temperature at which the oven was pre-heated for the experiments presented in Chapter 4. There was a significant effect of sample volume on the time required to reach 210˚C when oven was pre-heated at 800˚C. When the sample was 20 ml

of distilled water, it took about 24 minutes, but when the sample volume was 40 ml the time required to reach the same temperature was about 40 minutes. In order to reduce the amount of energy consumed during the process the sample volume selected was 20 ml.

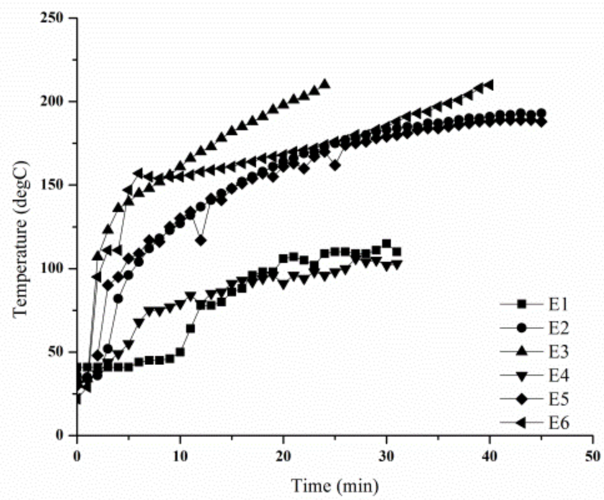



**Figure 7.18** Profile of temperature in HPS conditions test.

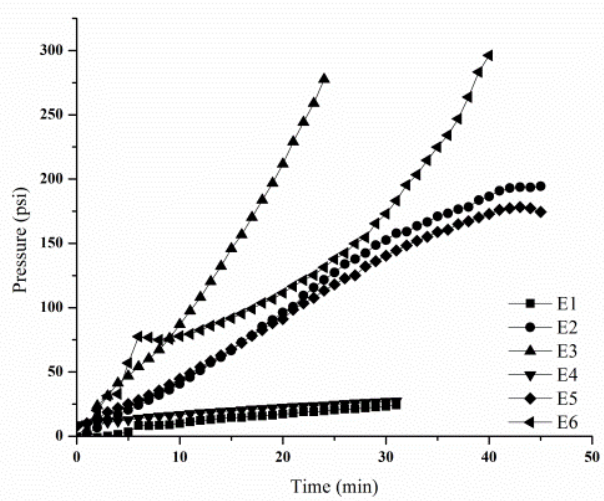



**Figure 7.19 Profile of pressure in HPS conditions test.**

### 7.3.2. Experiments reproducibility for temperature and pressure

In order to check the reproducibility of the experiments done in the HPS device, a test was conducted. The objective was to confirm that the device follows the same behaviour every time an experiment is done under the same conditions. The oven was pre-heated at 800˚C and sample volume was 20 ml. The test was done with pure distilled water and algae sample at 1 $gL^{-1}$. Figure 7.20 and Figure 7.21 show the profiles of temperatures and pressure for each replicate ef the test.

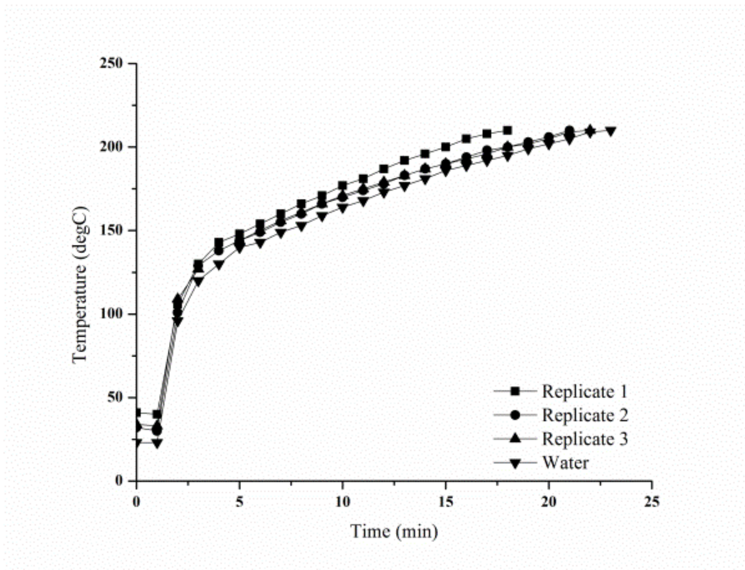



**Figure 7.20** Profile of temperature in reproducibility test.

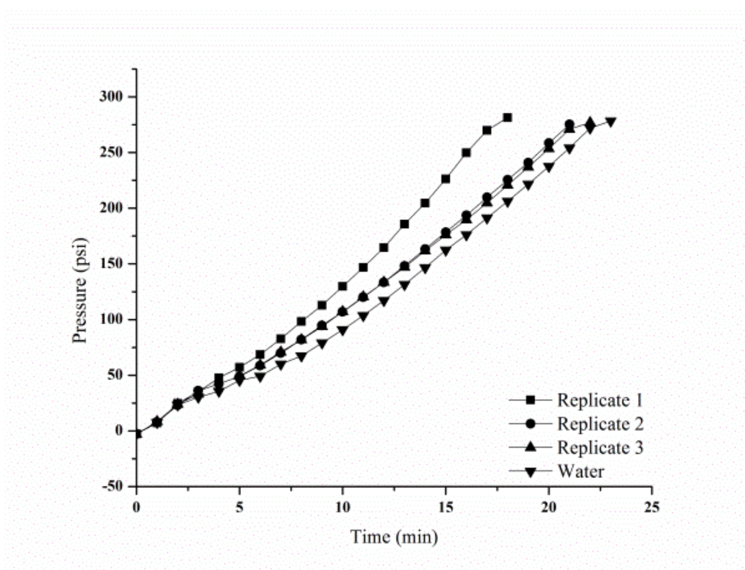



**Figure 7.21** Profile of pressure in reproducibility test.

From Figure 7.20 and Figure 7.21, it can be seen that all the replicates followed a very similar profile of temperature and pressure; this means that the HPS device is reliable since it has the same behavior in different runs with the same conditions.

### 7.3.3. Experiments reproducibility for lipid extraction from microalgae

Also reproducibility of lipid recovery was tested after HPS of microalgae samples. For this test 1 gram of freeze dried algae was added to 20 ml sample in water. The protocol for HPS was previously described (Chapter 4). The objective was to prove that the amount of bio-crude obtained after every HPS treatment was the same for microalgae under the same conditions. Table 7.10 shows the total oil recovered and the extraction efficiency for each replicate. It was concluded that the bio-crude recovery process using HPS had a good reproducibility.

**Table 7.10** Reproducibility of bio-crude recovery applying HPS.

| Replicate | Algae in 20 ml (g) | Oil recovered (g) | Extraction efficiency (%) |
|---|---|---|---|
| Replicate 1 | 0.9885 | 0.1221 | 55. 46 |
| Replicate 2 | 1.0523 | 0.1254 | 53.53 |
| Replicate 3 | 1.0168 | 0.1248 | 55.11 |

## Curriculum Vitae

### Ana-Maria Aguirre

## EDUCATION

### Graduate programs

2010-2014 Doctorate in Chemical and Biochemical Engineering.
The University of Western Ontario. Faculty of Engineering. Department of Chemical and Biochemical engineering.
Courses average grade: 94.25/100

2008-2010 M.Sc. Biotechnology. **Honorific distinction for Master thesis.**
National University of Colombia. Faculty of Science. School of Biosciences.
Master thesis title: Study of the production of peruvoside and other secondary metabolites in free and immobilized cell cultures of *Thevetia peruviana*.
Courses average grade: 92/100

### Bachelor program:

2003-2008 Biological Engineering
National University of Colombia. Faculty of Science. School of Biosciences.
Emphasis: Industrial Biotechnology
Courses average grade: 84/100

## RESEARCH EXPERIENCE

### Research assistant:

2010-present Research assistant at Department of Chemical and Biochemical engineering.
University of Western Ontario. Faculty of Engineering.

2008-2010 Researcher at Industrial Biotechnology Group. Category A according to Colciencias (Colombian Administrative Department of Science, Technology and Innovation).
National University of Colombia. Faculty of Sciences.

2008 Coordination of the Bioconversions Laboratory (support the implementation of projects).

National University of Colombia. Faculty of Sciences.

| | |
|---|---|
| 2006-2007 | Research assistant at Bioconversions Laboratory.<br>National University of Colombia. |

## TEACHING EXPERIENCE

| | |
|---|---|
| Winter 2011-2012-2013 | Teaching assistant for the course "Bioprocess Engineering".<br>The University of Western Ontario. Faculty of Engineering.<br>Duties: Preparing material for laboratory session, assisting students in laboratory and marking reports. |
| Winter 2014<br>Fall 2012 | Teaching assistant for the course "Biochemical Engineering".<br>The University of Western Ontario. Faculty of Engineering.<br>Duties: Preparing material for laboratory session, assisting students in laboratory and marking reports. |
| 2009 | Instructor for the courses "Industrial Fermentations" and "Bioprocess Modeling and Simulation" for the Biological Engineering undergraduate program.<br>National University of Colombia. Faculty of Sciences.<br>Duties: Teaching the courses. |
| 2006-2008 | Teaching assistant for the courses "Biochemical Engineering", "Bioreactors Design" and "Bioprocess modeling and simulation".<br>Duties: Tutorials and marking assignments and exams.<br>National University of Colombia. Faculty of Sciences. |

## PUBLICATIONS

| | |
|---|---|
| 2014:Paper | Aguirre and Bassi. Investigation of High Pressure Steaming as a thermal treatment for lipid extraction from *Chlorella vulgaris*". Bioresource technology, Vol. 164, pages 136-142. |
| 2013: Paper | Aguirre and Bassi . (2013). Investigation of Biomass Concentration, Lipid Production, and Cellulose Content in *Chlorella vulgaris* Cultures Using Response Surface Methodology. Biotechnology and Bioengineering, Vol. 110, Issue 8, pages 2114-2122. |
| 2012: Book Chapter | Bassi, A.; Saxena, *P*; Aguirre, A.M. Mixotrophic algae cultivation for energy production and other applications (Accepted for publication). |
| 2012: Paper | Aguirre, A.M.; Bassi, A.; Saxena, *P*. Engineering challenges in biodiesel production from microalgae. Critical Reviews in Biotechnology. 2012; Early Online: 1–16. ISSN 0738-8551 print/ISSN 1549-7801 online. DOI: |

10.3109/07388551.2012.695333.

2011: Book Arias, M.; Angarita, M.J.; Aguirre, A.M. Plant cell *In vitro* culture. Editorial Académica Española (Spanish academic editorial). ISBN-10: 3846561509, ISBN-13: 978-3846561508. 180 pages.

2009: Paper Arias, M.; Angarita, M.J.; Aguirre, A.M.; Restrepo, J.M.; Montoya, C. Strategies for the improvement of secondary metabolites production in plant cell cultures. Revista Facultad Nacional de Agronomía (Journal of the National faculty of agronomy). 62(1): 4881-4895.

2009: Paper Arias, M.; Aguirre, A.M.; Angarita, M.J.; Montoya, C.; Restrepo, J.M. Engineering aspects of the *In vitro* plant cell culture for the production of secondary metabolites. Dyna, 157:109-121. (2009).

**CONFERENCES, SEMINARS AND WORKSHOPS**

2014 Aguirre, A.; Bassi, A. Study of the Effect of High Pressure Steaming on Lipid Recovery from Microalgae. Research Bridges. Sarnia. June 6th, 2014. Oral presentation. (First prize for oral presentation).

2014 Aguirre, A.; Bassi, A. Study of the effect of high pressure steaming on biofuels recovery from microalgae. Ontario-Quebec Biotechnology Meeting. Toronto. May 15-16, 2014. Oral presentation. (First prize for oral presentation).

2013 1.01x: Energy 101. A course of study offered by UTAustinX an online learning initiative of University of Texas Systems through edX. https://www.edx.org/course/utaustinx/utaustinx-ut-1-01x-energy-101-667#.U4TvIvldV1o

2012 Aguirre, A.; Bassi, A. Response surface modeling of the effect of Carbon Dioxide and Nitrate Concentration on *Chlorella vulgaris* growth, lipid production and cellulose content. The 2nd International Conference on Algal Biomass Biofuels and Bioproducts. San Diego-USA. June 10-13, 2012. Oral presentation.

2012 Arias, M; Angarita, M; Restrepo, J; Aguirre, A; Arias, J. *X* international symposium on plant biotechnology. Cayo Santa Maria-Cuba. June 19, 2012.

2011 Aguirre, A.; Bassi, A. Effect of carbon dioxide and nitrate concentration on *Chlorella vulgaris* cultures. 61st Canadian Chemical Engineering conference. London-Canada. October 23-26, 2011. Oral presentation.

2011 The Teaching Assistant Training Program. Teaching support center, The University of Western Ontario.

| | |
|---|---|
| 2010 | Communication in the Canadian classroom. Teaching support center, The University of Western Ontario. |
| 2008 | First International Biotechnology meeting and exhibition Hall, and First Biotechnological Fair. Medellin-Colombia. March 10-14. |
| 2008 | Fundamentals in liquid chromatography (Theory and Training). Lab-Instruments Ltd. November 14. |
| 2007 | Aguirre, A.; Montoya, C.; Bedoya, A. PID controller design for the *Saccharomyces cerevisiae* growth in a continuous bioreactor for biomass production. XXIV Colombian Congress of Chemical Engineering. |
| 2005 | II Symposium of bio-factories: Advances of Biotechnology in Colombia. Medellin-Colombia. March 9-11. |