Universidade de Aveiro Departamento de Engenharia Mecânica
2016

SMRITIKANA DUTTA

ANÁLISE DE VIABILIDADE DE BIODIESEL DERIVADO DE MICROALGAS

FEASIBILITY ANALYSIS OF MICROALGAE DERIVED BIODIESEL

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia de Mecânica, realizada sob a orientação científica do Professor Fernando José Neto da Silva, Professor Auxiliar do Departamento de Engenharia Mecânica da Universidade de Aveiro e Co-orientação da Doutora Margarida Isabel Cabrita Marques Coelho, Professora Auxiliar do Departamento de Engenharia Mecânica.

Este trabalho foi financiado pela Fundação para a Ciência e Tecnologia (FCT) através da bolsa de doutoramento (SFRH/BD/76592/2011), e do project EnerBioalgae (Ref. SUDOE- Ref. SOE2/P2/E374)

o júri

presidente

Prof. Doutor Amadeu Mortágua Velho da Maia Soares
Diretor do Departamento de Biologia, Universidade de Aveiro

Prof. Doutor Jesús Torres Palenzuela
Professor Titular, Faculdade de Ciências Experimentais Universidade de Vigo, Espanha

Prof. Doutor Francisco Gabriel Acién Fernández
Professor Titular, Universidade de Almería, Espanha

Prof. Doutor António Carlos Mendes de Sousa
Professor Catedrático Aposentado, Universidade de Aveiro

Prof. Doutora Maria Fernanda Cardoso Rosa
Investigadora Principal Aposentada, Instituto Nacional de Engenharia, Tecnologia e Inovação, I.P.

Prof. Doutor Fernando José Neto da Silva
Professor Auxiliar, Universidade de Aveiro

Acknowledgements

I deeply indebted to numerous persons and institutions for providing the adequate support required to complete this work. In particular, I would like to acknowledge the financial support received from the Foundation for Science and Technology (FCT) (Ref. SFRH/BD/76592/2011), project EnerBioalgae (Ref. SUDOE- Ref. SOE2/P2/E374), and Luso-American Development Foundation (FLAD).

My heartiest gratitude to my supervisors Prof. Dr. Fernando Silva and Prof. Dr. Margarida Coelho for their constant support and commitment. You have been a tremendous mentor for me. I must express my special appreciation and thanks for encouraging my research and allowing me to grow as a researcher. Thank you!

I thank to the Department of Chemistry and Department of Biology of University of Aveiro for their immense cooperation in the experimental work of the thesis.
My big thank you to late Prof. Dr. José Grácio for his constant encouragement and Prof. Dr. Antonio Sousa for the remarkable support, at TEMA.

Also, my thanks to all DEMs secretary staff: thank you!

I thank Dr. Philip Pienkos, Ryan Davis, Christopher Kinchin, Eric Tan, Abhijit Dutta, for the international training period I spent in Denver, which gave me remarkable opportunity to learn a lot in research. An additional thank you to Anna, Mike and San for all the support and strength during my stay in Denver.

Many thanks to the friends I came across in this journey. I acknowledge my peers Jorge, Paulo, Behnam, Ismael, for providing me the chance to learn from each other. Big thank you to Marija and Araliya for the infinite support every time we met.
A special thanks to Tridib for his true friendship and patience!

And to the best family a girl could ask for: Thank you for being my strongest pillar and providing me everything I needed all my life.
I dedicate this thesis to my dearest nephews and niece: Debraj Maity, Biswanath Brahma, and Shibani Brahma -their innocence brings the best of my ability today!

palavras-chave

Microalgas, extracção, biodiesel, testes de qualidade, Avaliação de Ciclo de Vida (ACV), viabilidade técnico-económica

Resumo

A obtenção de biocombustíveis a partir de microalgas pode revelar-se uma alternativa potencialmente atrativa comparativamente à produção de combustíveis fósseis a sua utilização possui vantagens significativas do ponto de vista de minimização da poluição ambiental.

O objetivo principal desta tese consiste em investigar os procedimentos necessários à eliminação das lacunas entre os testes laboratoriais e a produção industrial em grande escala. Para tal, procedeu-se à investigação e análise das tecnologias necessárias à viabilidade comercial da produção de biodiesel a partir de microalgas.

Algumas estirpes de microalgas foram cultivadas em condições distintas e os seus lípidos foram extraídos usando várias técnicas. De seguida produziu-se biodiesel através do processo de transesterificação. Testes à qualidade do biodiesel produzido foram conduzidos de acordo com os padrões Europeus de qualidade com base na normativa EN14214:2003. Adicionalmente, foi feito um estudo de impacto económico e ambiental abrangendo todo o processo de produção.

Os resultados experimentais obtidos permitiram determinar a altura ótima da colheita da biomassa e revelaram o papel do fotoperíodo e do fornecimento de CO2 à cultura. A análise da qualidade da produção de biodiesel a partir de microalgas cultivadas nas regiões de Aveiro, Almeria e Vigo confirmou a sua aptidão usando a normativa Europeia. Os resultados indicaram ainda que a utilização de águas residuais como meio de crescimento conduziu à necessidade de menos nutrientes.

A análise económica comparativa sugeriu que a valorização dos co-produtos pode representar uma condição necessária à viabilidade do processo. uma modernização do projeto de instalação (por exemplo, a valorização do coproduto, de modo a aumentar as receitas do sistema).

A Análise de Ciclo de Vida indicou que a fase de extração teve o maior impacto nas emissões (é responsável por 94% de gases por efeito de estufa – GEE e 84% de energia utilizada e obtida a partir de combustíveis fósseis – FEC) enquanto a fase de cultivo permitiu reduzir as emissões totais (-4% de GEE e -2% de FEC). O estudo de sensibilidade sugeriu um decréscimo acentuado nas emissões (8.9 e 4.5 vezes de GEE e FEC, respetivamente) usando recuperação dos solventes usados nos processo de extração a 95% face ao valor de 50%.

A conclusão deste trabalho realçou a potencialidade das microalgas como matéria-prima no que concerne à produção de biodiesel. Se os riscos de contaminação forem evitados, as grandes lagoas abertas desempenharão um papel fundamental para a viabilidade global da otimização do processo produtivo de biomassa em grande escala.

keywords

Microalgae, Extraction techniques, Biodiesel, Quality testing, Life Cycle Analysis (LCA), Techno-economic analysis (TEA)

Abstract

Microalgae derived biofuel is becoming attractive as a renewable fuel, promoting potential advantages resulting from its capability to reduce environmental pollution.

The main objective of this thesis is to investigate the requirements to bridge the gap between laboratory scale testing and its industrial production. The research was focused on the characterization and analysis of the technologies required to obtain commercially viable microalgae derived biodiesel. Several microalgae strains were cultivated in different conditions and their lipids were extracted using different techniques and chemically trans-esterified to biodiesel. Quality testing of the produced biodiesel was performed (in accordance to the European biodiesel quality standard EN14214:2003)

An economic and environmental study was developed for the whole production pathway.

This approach projects that maximum biomass is obtained at the end of the 'exponential phase' of microalgae growth and that high illumination increases the biomass concentration irrespective of the aeration rate. Quality analysis confirmed that the analyzed properties of biodiesel produced from microalgae's grown in Aveiro, Almería and Vigo region comply with the European standard. The use of wastewater as a growth medium does not compromise biodiesel quality and implies the use of less nutrients.

A comparative economic analysis showed that the facility design used in this work requires upgrading since co-product valorization can add revenue and improve feasibility.

The life cycle assessment study highlights the high contribution to emissions from the extraction step (94% greenhouse gas emission -GHG and 84% fossil energy consumption - FEC) while the cultivation step adds positive value to the total emissions (-4% GHG and -2% FEC). A sensitivity study highlighted the relevant reduction in emission (8.9 times GHG emission and 4.5 times FEC) with 95% solvent recovery when compared with 50% solvent recovery. The work concludes that microalgae have the potential to become a good feedstock for biodiesel production and if contamination risks can be avoided then large open ponds will play a vital role in large quantity biomass production hence optimizing overall feasibility.

TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

NOMENCLATURE

ALU Algal Lipid-extraction and Upgrading
ANL Argonne National Laboratory
ASTM American Society for Testing and Materials
BGY Billion Gallons per Year
CO_2 Carbon Dioxide
CN Cetane Number
CHP Combined Heat and Power
DAF Dissolved Air Flotation
DW Dry Weight
EU European Union
EC European commission
FAME Fatty Acid Methyl Esters
FA Fatty Acid
FFA Free Fatty Acid
FER Fossil Energy Ratio
FID Flame Ionization Detector
FEC Fossil Energy Consumption
GHG Greenhouse Gas
GC Gas Chromatography
GGE Gasoline Gallon Equivalent
H_2O Water
Ha Hectare
HHV Higher Heating Value
HTL Hydrothermal Liquefaction
IV Iodine Value
LCA Life Cycle Analysis
LAME Linolenic Acid Methyl Ester
MFSP Minimum Fuel Selling Price
NER Net Energy Ratio
NREL National Renewable Energy Laboratory
O_2 Oxygen
PBR Photo-bioreactor
PVC Poly Vinyl Chloride
PAR Photosynthetically Active Radiation
RPM Revolutions per Minute
RFS Renewable Fuel Standard
RDB Renewable Diesel Blend-stock
RA Resource Assessment
SCF Super Critical Fluids

SERI Solar Energy Research Institute
STP Standard Temperature and Pressure
SN Saponification Number
TAG Triacylglycerol
TEA Techno Economic Analysis
TCD Thermal Conductivity Detector
UA University of Aveiro
WTW Well To Wheel
WW Wastewater
VR Recommended maximum value (Decree Law 236/98)
VL Legal value (maximum permissible value (Decree Law 236/98)
FCS Segmented Streaming
AAS Atomic absorption spectrometry
EAM Spectrometry molecular absorption
OXY Rust
EE Emission spectrometry
Cl Ion chromatography
SPME Solid phase microextraction;
INF Combustion and Infrared
Dic. Pot . Potassium dichromate
LQ Llimit of quantification
SMEWW Standard methods for the examination of water and wastewater

NOMENCLATURE IN EQUATIONS

C_f - Final cell concentration (mg/L)

f - Time (seconds)

C_i - Initial cell concentration (mg/L)

m´ - Specific growth rate ($seconds^{-1}$)

T – Transmittance (dimensionless)

I0 - Intensity of the incident light beam (W/m^2)

I - Intensity of the light coming out of the sample (W/m^2)

A - Absorbance (dimensionless)

ε - Molar absorptivity ($L\ mol^{-1}\ cm^{-1}$)

C´ - Concentration of compound in solution ($mol\ L^{-1}$)

C - Fatty acid methyl esters (FAME)

ΣA - Total peak area of fatty acids $C_{14:0}$- $C_{24:1}$

C_{IS} - concentration of the internal standard solution (mg/mL)

V_{IS} - Volume of the internal standard solution (mL)

m - Mass of the sample (mg)

L - Linolenic acid methyl esters (LAME)

A_{IS} - Internal standard peak area

A_L - Linolenic acid methyl ester peak area

1 INTRODUCTION

The first chapter of this doctoral thesis presents the main motivation for this research. In order to justify the relevance of the research topic, recent work on microalgae as an alternative fuel for transportation sector, their quality and impact on the economy and environment is discussed. The research is interdisciplinary and includes a wide range of methodologies, such as data collection, case studies, data analysis of experimental work and modeling. This work is conducted in the framework of documenting plausible technological pathways for conversion of algal biodiesel in order to make it commercially viable.

Then the need for developing sustainable liquid fuels and microalgae's role as a potential fuel feedstock is defined in section 1.1.1 and section 1.1.2, respectively. The main objectives of this research are defined in section 1.2.
Finally, the thesis structure is highlighted in section 1.3.

One of the factors that contributed to the increasing energy crisis (the energy derived from fossil-fuel derivatives that include crude oil and its products, such as petroleum and natural gas) is that the demand placed on the resources is more that the supply rate. Worldwide oil consumption is expected to increase by around 30% between 2007 and 2035, while coal and natural gas consumption grows by 50% (Berardi, 2015), and it will be induced by economically developed nations especially China and India. In that respect, the European Union (EU) energy consumption is expected to level out in future times although world energy consumption will continue to rise due to increasing global population and economic growth (Leder & Shapiro, 2008). The EU has set a target for 10% of the energy used in road and rail transport to be derived from renewable sources by 2020 (Hamje, et al., 2014). The demand of biodiesel has increased due to the fluctuations in oil price and with the adoption of government measures (EU Directive 2003/30/EC) to promote the use of biofuels or renewable fuels for transport (Vicente , et al., 2007).

Meanwhile, the rising oil prices in the 2000s stimulated a revival of interest in algal biofuels and increased the funding with the private companies in the field. In 2013, Exxon Mobil pulled back after four years and $100 million from a joint venture of $600 million with Synthetic Genomics realizing that algae fuel is probably 25 years away from commercial viability (Carroll, 2013). On the other hand, Solazyme began commercial sales of algal biofuel in 2012, Sapphire Energy in 2013 and Algenol announces to market and distribute commercial ethanol from algae for fleets in 2017 (Voegele, 2012) (Herndon, 2013) (Energy.gov, 2015).

Biofuels are renewable fuels made from organic matter (biomass), which can replace or blend with fossil fuels. The major motivation for the use of biofuels comes from the transportation sector. Biofuels are becoming relevant, both in terms of blending with diesel (in small percentages) for application in light duty vehicles and for bus fleets. The pursuit for sustainable energy consumption has become of vital importance, from the production to transport, in order to develop more efficient and competitive industries. Transportation fuels are mostly liquid as the vehicles usually require high energy density to perform and are easiest to burn clean. Biofuels, in the form of liquid

fuels derived from plant materials, are entering the market, driven by factors such as high oil price, the need of increased energy security and concerning greenhouse gas emissions. Although, biodiesel has received considerable attention with its biodegradable, renewable and nontoxic characteristics, its production has several problems since they compete with food crops and food prices. While it is true that usually biofuels are produced and used locally in Europe but this trend is changing recently in northern Europe due to industrial use of different biofuels. European research and testing clearly indicates that biodiesel has potential to replace petroleum diesel. Many countries have adopted various policy initiatives, specifically legislations to promote and regulate the use of biodiesel in Germany, Italy, France, Austria and Sweden (Demirbas, 2008). But at the same time for technical and economic reasons biodiesel production in accordance to the required quality standards cannot be made from single oil but is feasible from mixtures or oil blends: biodiesel can be blended with diesel for application in light duty vehicles and bus fleets. To be used in the transportation sector it is imperative to obtain a suitable renewable feedstock which can be converted to fuel, with a focus on the economic and environmental feasibility. This brings to the contribution and potential posed by microalgae as a feedstock. The heating value of a fuel is the amount of heat released during the combustion and can be measured in units of energy per unit of the substance and can be determined using a bomb calorimeter. The higher heating values (HHVs) of biodiesel are comprised between 39-41 MJ/kg, lower than those for gasoline (46 MJ/kg), mineral diesel (45MJ/kg) or liquefied petroleum gas (49 MJ/kg). A biodiesel blend is a blending of biodiesel with mineral diesel and can be formulated in different concentrations. Biodiesel blends are referred to as BXX; XX representing the amount of biodiesel in the blend. Biodiesel blends present a good balance of cost, emissions, compatibility with weather conditions, materials compatibility etc. Biodiesel offers better greenhouse gas (GHG) benefits when compared with conventional diesel fuel since the emissions are roughly proportional to the blend level (Sheehan, et al., 1998). However the use of B100 could increase nitrogen oxide emissions and requires equipment modifications.

In USA, the Renewable Fuel Standard (RFS2) (Schnepf & Yacobucci, 2013) mentions the annual use of 29.3 million tons of biofuels in 2008 rising to at least 117 million tons per year of renewable fuel production and blending into the transportation fuel by 2022, of which at least 52 million tons should be from cellulosic biomass feedstock. In 2014, 24.6 million tons of fuel (out of which 11.4 million tons is oil, 3.6 million tons is renewables, 3.4 million tons is natural gas, 2.5 million tons is coal and 3.7 million tons is hydroelectricity) were consumed in Portugal which was less in 2013 by 0.3 million tons (BP stats, 2015). Global primary energy consumption increased by just 0.9% in 2014, a marked deceleration over 2013 (+2.0%) (BP stats, 2015). However, in order to replace this entire oil consumption it would require to use approximately 340,000 km^2 of cultivation lands with sunflower, 2500 km^2 of land with microalgae at 30% lipid content and 1000 km^2 of land if microalgae with 70% lipid content were used (Chisti, 2007) (Cabral, 2008). The oil consumption data represents an accumulation of crude oil, shale oils, oil sands, natural gas liquid, biogasoline, biodiesel, coal and natural gas.

The attraction of using microalgae (widely recognized as the third generation feedstock) for biofuels is their tremendous oil generating capability, as they could produce up to 58,700 L oil per hectare, which would be one or two magnitudes higher than any other energy crop (Chisti, 2007). Beyond the use of algae as a fuel source it also plays a role as a food supplement, a stabilizing agent, fertilizer, in the cosmetic industry etc. Nevertheless, its commercialization is the biggest challenge. Understanding every stage of the whole production process and their optimization is necessary for a successful scenario.

1.1 MOTIVATION

The global energy demand continues to rise due to worldwide increasing population rate and economy growth. The main motivation behind the present thesis: is to characterize the main transformation technologies required to produce microalgae-

derived biodiesel examining the cost structure and their environmental impacts to produce commercially viable high quality fuel. To perform this work, different microalgae strains, their cultivation process and various extraction methods were analyzed in order to find suitable technological pathways.

1.1.1 The need for sustainable liquid fuels

Requirement for oil is increasing at its fastest pace, depletion of conventional fossil oil reserves, and their negative environmental impact leaves the transportation sector with the most challenging question ‘ can a specific alternative fuel be used to fill our cars??’ . Energy demand in Europe seems to decrease lately yet the worldwide demand still continues to grow due to global population rate and economy (Figure 1-1).

Figure 1-1 Global oil demand from 2012 to 2015 (IEA, 2015)

Research showed that worldwide biodiesel production is increasing. Figure 1-2 shows the trend of biodiesel production from 1991 to 2010 (Energy data, 2010).

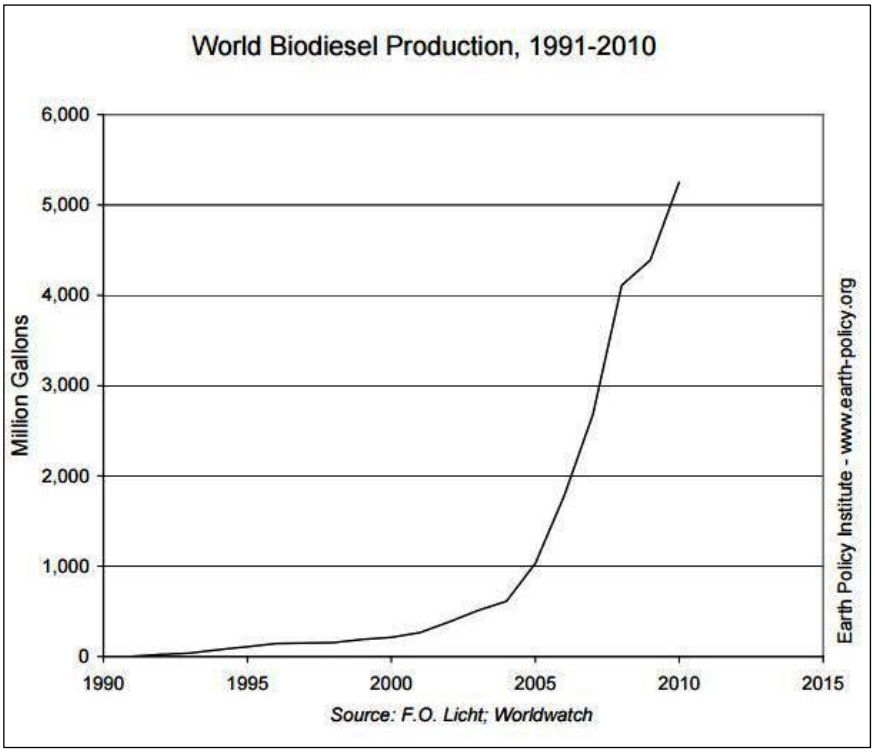


Figure 1-2 World biodiesel production from 1991 to 2010 (Energy data, 2010)

Table 1-1 reports the biodiesel production projected for the leading countries and the world in 2010 (Energy data, 2010).

Table 1-1 Projected biodiesel production in five leading countries and the world in 2010 (Energy data, 2010)

Country	Production (Million Gallons)
United States	750
Argentina	690
Germany	660
France	630
Brazil	510
World	5,253

Global biofuel production reached 120 billion liters in 2013 providing 3.5% of the world's transport fuels demand (Anselm Eisentraut, 2013). The production increased from 110 billion liters in 2012 and it is estimated to increase to 135 billion liters in 2018 (Anselm Eisentraut, 2013). The 2010 global supply of crude oil was about 72 million barrels/day which was less than the demand of about 86 million barrels/day (IEA, 2010). Globally, annual biodiesel production increased from 15,200 barrels/day in 2000 to 294,690 barrels/day in 2010 and the consumption has also increased from 8,400 thousand barrels/day to 313,770 thousand barrels/day from 2000 to 2010 (Index mundi, 2016). Annual production of pure biodiesel B100 in the USA was 1,359 million gallons in the year 2013 and 1,270 million gallons on 2014 (Petroleum & other liquids, 2015). According to available sources (Menegaki, 2011), the European Union's (EU) dependence on energy imports is already 53.1% and is expected to increase reaching 70% by 2020. Energy consumption in EU by the transport sector in 2013 turns out to be 31.6% (decreased by 1.4% from 2011 (EU transport in figures, 2013) of the total energy consumption (Figure 1-3) and biofuel consumption for transport in Portugal is 264.1 ktoe (EU transport in figures, 2015).

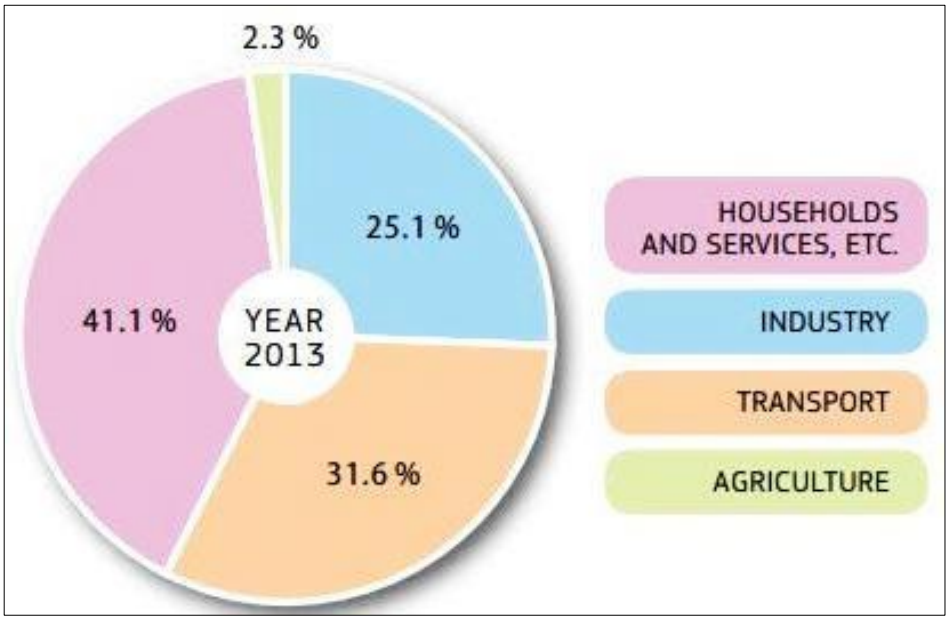


Figure 1-3 Final energy consumption in EU – by sector. Source: Eurostat June 2015 (EU transport in figures, 2015)

German Engineer Rudolf Diesel with his visionary idea in 1912 quoted *" The use of vegetable oils for engine fuels may seem insignificant today. But such oils may become in the course of time as important as the petroleum and coal tar products of the present time"* (Nitske & Wilson, 1965). The concept of using renewable energy was first demonstrated at the World Exhibition in Paris in 1900, where the first engine to run on peanut oil was shown.

1.1.2 Microalgae- an alternative fuel feedstock

In 1942 Harder and von Witsch were the first to propose microalgae as a source of lipids for food or fuel (Borowitzka, 2012). Microalgae are organisms capable of photosynthesis that are less than 0.4 mm in diameter. Microalgae are classified as Eukaryota, organisms whose cells contain complex structures enclosed within membranes although conventionally they have been regarded as simple plants within the study of botany. They have no roots, stems, or leaves but do have chlorophyll and other pigments for carrying out photosynthesis. Algae are usually found in damp places or bodies of water and thus are common in terrestrial as well as aquatic environments. Algae usage as a biofuel feedstock addresses the issues of food vs. fuel, the land occupation, the carbon footprint and the scalability.

Algae grow naturally all over the world and consume CO_2 from the environment. Their growing capabilities are massive and they reproduce in a limitless manner under optimal conditions. More than 100,000 different species (in various forms and color) of plantlike organisms belong to the algae family (Newman, 2008). Algae are easy to please since their basic needs are water, sunlight and carbon-dioxide. Nutrients such as nitrogen, phosphorous and potassium can serve as fertilizer for better algae biomass cultivation. Also, silica, iron and several trace elements can be important marine nutrients. Study by Larsdotter (2006), emphasized the growth of marine microalgae with commercial nitrate, phosphate and other micronutrients served as additional supplements.

The basic reaction in water (H_2O) that facilitates the growth of microalgae is {CO_2 + light energy + H_2O = glucose + oxygen (O_2) + H_2O}. Carbon dioxide plays an important role in the cultivation. Their abundance in the culture enhances the microalgae production. Hence, algae biodiesel manufacturers can build biodiesel plants close to the energy manufacturing plants that produce lots of carbon-dioxide. Billions of years ago the earth atmosphere was filled with CO_2 and no life. Life on earth started with cyanobacterium and algae.

These photosynthetic organisms sucked the atmospheric CO_2 and released O_2. Some namely *Neochloris oleabundans* (fresh water alga) and *Nannochloropsis sp.* (marine water alga) have higher oil contents under nitrogen shortage. *Scenedesmus obliquus* presents the most adequate fatty acid (FA) profile (linolenic, polysaturated FA's) and *Chlorella vulgaris* have high cell growth with low lipid content.

Microalgae based biofuels are an appealing choice because (Yang, et al., 2011):

- High cell growth rate (cell doubling time of 1-10 days)
- High lipid content (more than 50% by dry weight)
- Less land usage (15-300 times more oil production than conventional crops on a per-area basis) and
- High carbon dioxide (CO_2) absorption and uptake rate

Chlorella vulgaris is one of the most promising candidates for the commercial lipid production due to their faster growth and easier cultivation. Some researchers find that after carbon, nitrogen is the second most important nutrient to micro-algal growth comprising may be 10% of the biomass (Chisti, 2007).

The oil contents of various strains of microalgae are presented in table 1-2 (Chisti, 2007), and the factors that influence their growth rates are presented in table 1-3 (Larsdotter, 2006).

Table 1-2 Oil content of microalgae strains (Chisti, 2007)

Microalga	Oil Content (% dry weight)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Cryphtecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

Table 1-3 Physical, chemical, and biological factors that influence the growth rate of microalgae strains in high rate algae pond (Larsdotter, 2006)

Category	Factor
Abiotic	Light (quality, quantity)
Physical and chemical	Temperature Nutrient concentration O_2, CO_2 pH Salinity Toxic chemicals
Biotic	Pathogen (bacteria, fungi, viruses) Predation of zooplankton Competition between species
Operational	Mixing Dilution rate Depth Addition of bicarbonate Harvesting frequency

Production of biofuel from microalgae was revived during the oil embargo and oil price rises of the 1970s, leading the US Department of Energy to initiate the ‘ Aquatic Species Program’ in 1978. This Program spent $25 million over 18 years aiming to develop algae liquid transportation fuels that would be price competitive with

petroleum fuels. Over 3,000 algal strains were screened and further researched at the Solar Energy Research Institute (SERI) in Golden, Colorado. The most significant findings were that growth and lipid production were ‘ mutually exclusive’ , genetic engineering might overcome the natural limitations of algal strains and adequate species varies with place and season (Sheehan, et al., 1998). It was estimated that un-extracted algae oil would cost $59-186 per barrel (Sheehan, et al., 1998) and hence it was unable to compete with petroleum costing less than $20 per barrel in 1995 (Fishman, et al., 2010). In 1996, due to budget pressure, the program was abandoned. Despite the advantages, the main difficulty associated with feasible and environmentally acceptable industrial microalgae derived biofuel so far is scaling up the process. More investment in research applied to various fields of technology and demonstrated in pilot plants is required in order to visualize the commercialization of microalgae derived biofuel.

1.2 OBJECTIVES

The main objective of this PhD thesis is to analyze the requirements needed to bridge the gap between laboratory scale and industrial production of microalgae derived biodiesel.

The specific objectives include:

1. The characterization of the transformation technologies required to produce microalgae-derived biodiesel, from microalgae harvesting to oil processing, by analysis of the material, energy flows and examination of their environmental and economic impacts at each stage of the production cycle;

2. The investigation of possible improvements in the technological efficiency, cost structure, ability to scale up algal growth and lipid extraction facilities in order to produce commercially feasible high quality biodiesel fuel;

3. Analysis of the integration of algae oil extraction and trans-esterification methods in order to enhance the economic and energy prospective;

4. Development of a Life Cycle Assessment (LCA) for evaluation of the environmental impacts of the extracted biodiesel;

5. Conduction of an economic analysis of the microalgae-derived biodiesel production pathway;

6. Comparison between a conventional and integrated pathway of the entire chain of microalgae cultivation to biodiesel production.

This work started with the EnerBioAlgae project along with partnership including three entities in Spain (the University of Vigo as coordinator, the University of Almería, and the Energy Institute of Galicia), one in France (Université de Pau et des Pays de l'Adour), and one in Portugal (University of Aveiro) with the main goal to study the potential for the production of biodiesel from different microalgae strains and to assess its overall impact with a LCA. Biofuel development needs responsible policies, capital and transformation costs to ensure a sustainable commercialization.

1.3 THESIS STRUCTURE

The present thesis contains 8 main chapters and its structure is explained as follows. The *first chapter* provides an introduction to the subject and outlines the need for

microalgae as an alternative feedstock for sustainable liquid fuel production. The research objectives are presented as well in this chapter.

In the *second chapter* a literature review of microalgae as an alternative feedstock is conducted and the barriers associated with the feasibility of the production process are discussed.

Chapter 3 refers to the global methodology that was developed and applied within the present thesis for qualifying biodiesel production, quality analysis, and conducted economic and environmental feasibility.

Chapters 4 and 5 present the research on microalgae derived biofuel from microalgae cultivation to biomass harvesting and biodiesel production. These chapters include experimental results of algae growth in different conditions and biodiesel extraction with different techniques and the quality analysis in accordance to European standard. For each of these chapters, a specific literature review is conducted and the methodologies employed for each specific experiment are explained. Results and discussions are presented and concluding remarks summarize the obtained results.

Chapters 6 and 7 describe the techno economic analysis (TEA) and the life cycle analysis (LCA). These chapters address the two economic and environmental study of the whole production process. Case studies are analyzed and compared.

Chapter 8 summarizes the overall results, highlights the main conclusions of the research and outlines some of the issues that should be developed in future research.

2 MICROALGAE: AS ENERGY FEEDSTOCK

This chapter focuses on understanding the previous studies with microalgae as a liquid biofuel energy source.

First, the literature review is presented, followed by the barriers identified in literature associated with the success of this technology.

2.1 LITERATURE REVIEW

Among various biomass sources, a microalga has been identified as a suitable third generation biomass feedstock for the production of liquid biofuels such as biodiesel, bioethanol and bio-oil (Lee, et al., 2015) (Hu, et al., 2008) (Gouveia & Oliveira, 2009). This microorganism uses CO_2 {providing greenhouse gases (GHG) mitigation benefits (Sawayama, et al., 1995)}, water and sunlight to grow and produce oils offering several advantages over other biomass sources (Chisti, 2007). Transformation of CO_2 and nutrients into organic matter is achieved by photosynthesis, a natural process through which plants use light energy, CO_2 and H_2O to make sugar or carbohydrates (Ö sterlind, 1950) (Sheehan, et al., 1998) (Smeets, et al., 2007) (Bhutto, et al., 2011) converting them to fuels through various chemical processes (Richardson, et al., 2012) (Agarwal, 2007) (Williams, et al., 2012).

The advantages of using microalgae for biofuel production are:

- When grown using sunlight, they consume CO_2 for the cultivation and release O_2. Emissions from power plants, ethanol facilities and other sources can supply CO_2 for good biomass productivity;
- The growth rate is exceptionally high. They can be harvested every day which provides a potential to produce a volume of biomass and biofuel at-least five to ten times higher than other productive crops (Tredici, 2010);
- Nutrients like nitrogen and phosphorous enhance their growth;
- They can be cultivated within non-productive, non-arable lands such as deserts, coasts and offshore marine environments. Their ability to grow in wastewater/seawater/brackish water is an important feature.

Despite all these theoretical advantages, there are still social, economic, environmental and technical challenges associated with microalgae biofuel production process and its

use for transportation purposes. The assessment for microalgae biofuel and its feasibility for commercialization still require research (Chisti, 2008). The important components of microalgae are lipids, carbohydrates and others such as proteins. Making use of these components to improve the industrial value of biodiesel is necessary, some ideas for example are, the residual biomass gathered in the production can be used as animal feed, and produce methane by anaerobic digestion; waste glycerol can be transformed into a precious chemical (Meng, et al., 2009).

Various conversion methods as shown in figure 2-1 can result in the successful production of biodiesel, bioethanol, bio-oil and methane (Lee, et al., 2015).

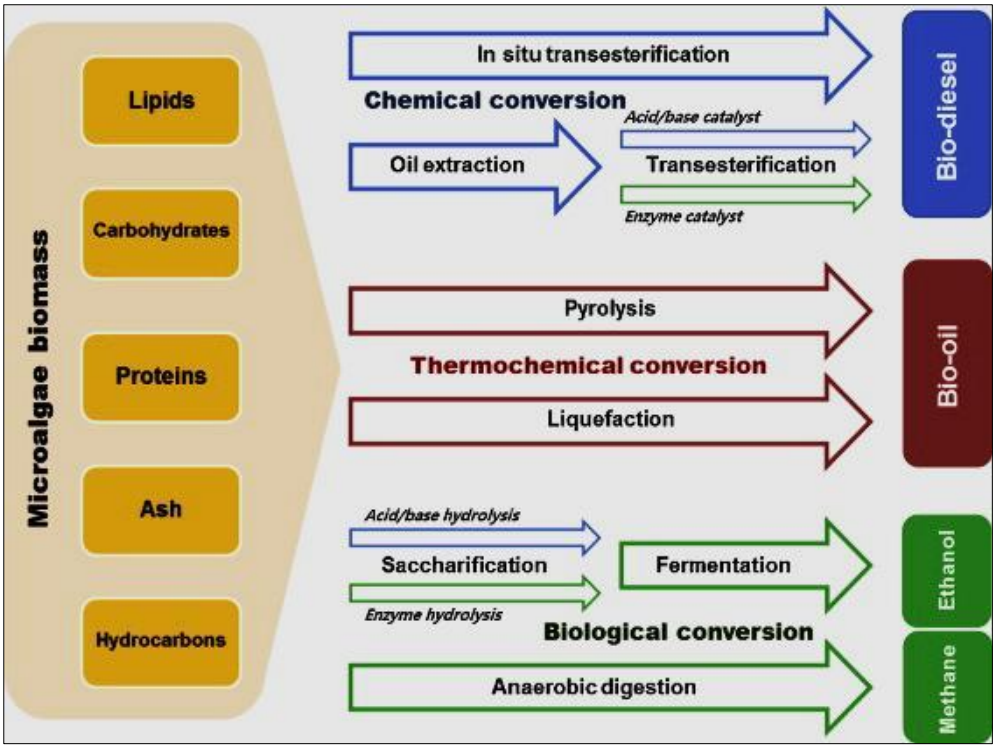


Figure 2-1 Approaches for liquid biofuel production from microalgae (Lee, et al., 2015)

Although microalgae promise to be a potential feedstock, their use for biofuel production is not economically feasible due to a high production cost. Culture cost can be expected to decrease with the development of new modified photo-bioreactor (PBR) along with the utilization of microalgae species with high lipid content (Chen,

et al., 2011). Also, genetic and metabolic modifications of microalgae can improve the cost-effectiveness of the biofuel production (Tredici, 2010). Currently, biodiesel attracted the interests of many researchers, governments and traders and gaining recognition as a renewable fuel. Biodiesel is non-toxic, biodegradable and has lower GHG emissions when used in diesel engines as compared to fossil diesel (Lam , et al., 2009). Vegetable oils from soybean, rapeseed, sunflower and palm oil were have been and are used as the main feedstock for the production of first generation biodiesel and this utilization of crops and land has received objections from the public and from non-governmental organizations. Hence, a second generation biodiesel was obtained from non-edible oils such as *Jatropha curcas L.* which did not require any major modifications on the equipment and process flow. Jatropha trees can grow easily on non-arable or wasteland but require regular irrigation and heavy fertilization for high oil yield. Also, the high concentration of free fatty acid (FFA) required an additional pretreatment step (Lam , et al., 2009).

A realistic value of microalgae biomass yield is between 15 and 25 ton/ha/year (Mujeeb, et al., 2016). With an assumption of 30% lipid content, study by Lam & Lee (2012), showed that this value totals a lipid production of 4.5-7.5 ton/ha/year which is higher when compared to oil production from soybean (0.4 ton/ha/year), rapeseed (0.68 ton/ha/year), palm oil (3.62 ton/ha/year) and jatropha (4.14 ton/ha/year). Hence, production of microalgae for biodiesel production requires less land and is very effective. Besides, microalgae are also a good feedstock for bioethanol production as they contain carbohydrates which can be used as carbon source or substrate for fermentation (Harun, et al., 2010). Crucial factors related to the success of biodiesel production process are selection of species, cultivation, and harvesting and lipid extraction (Wijffels & Barbosa, 2010). Barriers associated with scaling-up of the process indicate the need for developing new ideas (such as using sea/wastewater as culture medium to reduce the usage of nutrients (Park, et al., 2011), various geographical locations for growth etc.) to improve growth rate, lipid content and harvesting efficiencies to optimize the whole production process (Yang, et al., 2011). Figure 2-2 illustrates the steps involved in microalgae derived biodiesel production

process (Rashid, et al., 2014). The first step is the algae cultivation phase using a closed PBR or an open pond system. Appropriate species selection and culture conditions are important for growing algae with high lipid and fatty acid contents (Griffiths & Harrison, 2009). Studies on the economy and energy consumption of the cultivation systems will help in optimizing the systems which will allow commercializing the final fuel (Popp, et al., 2014). Rashid, et al., (2014) explains that the dewatered concentrated biomass slurry is then pretreated for extracting lipid through solvents. This lipid extraction can be carried out via wet or dry routes and finally trans-esterification of the extracted lipids will convert them to biodiesel. Glycerin is obtained as a co-product of the process.

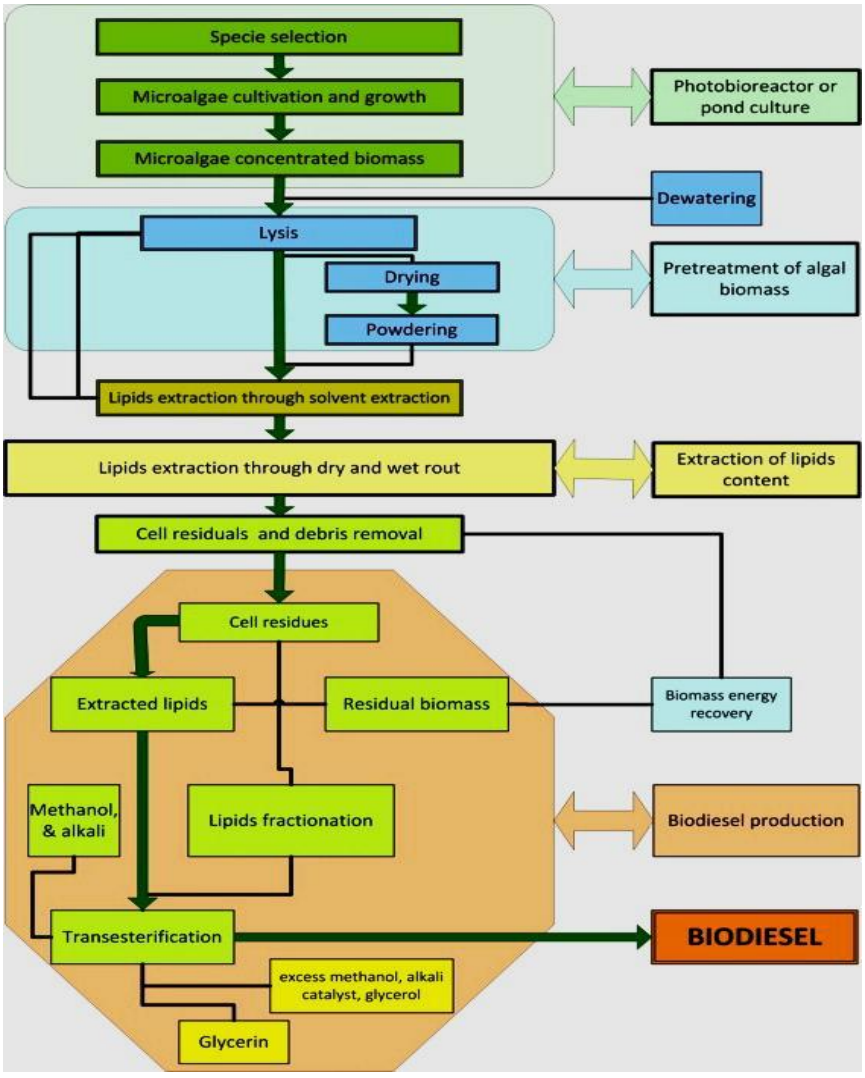


Figure 2-2 Biodiesel production process from microalgae (Rashid, et al., 2014)

There are advantages and disadvantages associated with the cultivation system. Generally, open ponds are associated with high risk of contamination, excessive land requirement and limited location due to adverse climate except for some microalgae strains which can survive in extreme environments such as *Spirulina*: high pH, *Dunaliella*: high salinity, or *Chlorella*: high growth rate (Huang, et al., 2010). On the other hand, closed PBRs are considered to be expensive (Rashid, et al., 2014). Closed systems are suggested for the production of long-chain fatty acids due to the controlled cultivation environment and are more suitable for microalgae's which are readily contaminated by other microbes (Huang, et al., 2010). Nevertheless, due to the high cost in terms of operation and capital investment and the small scale due to the complexity of bioreactor design compared to open pond system, it might not be economical to produce biodiesel on a large scale by enclosed PBRs.

Significant drawbacks are included in the selection of a convenient harvesting technology as there is no single best method yet. The choice of the preferable approach depends on various factors like algae species, ionic strength of growth medium, etc. which in the end largely impact the cost and quality of products. Energy intensive methods can be a challenging barricade in the commercialization considering the energy contribution to the total cost. The high cost involved with harvesting can be due to the dewatering processes in order to increase the solid content from < 1.0% to a consistency of up to 20% solids (Singh, et al., 2013). Researchers have been extensively reviewing the harvesting and dewatering methods to produce advancements in the reduction of the operating cost (Harun, et al., 2010) (Golueke & Oswald, 1965) (Mohn, 1980) (Shelef, et al., 1984) (Mohn & Contreras, 1991) (Lee, et al., 1998) (Grima, et al., 1999) (Grima, et al., 2003) (Spolaore, et al., 2006) (Henderson, et al., 2009) (Khan, et al., 2009) (Uduman, et al., 2010) (Gultom & Hu, 2013).

Pretreatment of the algal slurry plays a role in the extraction process; lipid extraction process through the wet route consumes more energy than when the dry route is used by almost about 2.8 times (Xu, et al., 2011). The wet route has a slightly lower fossil

energy ratio (FER) when compared to the dry route. Figure 2-3 shows examples of the dry and wet routes extraction processes.

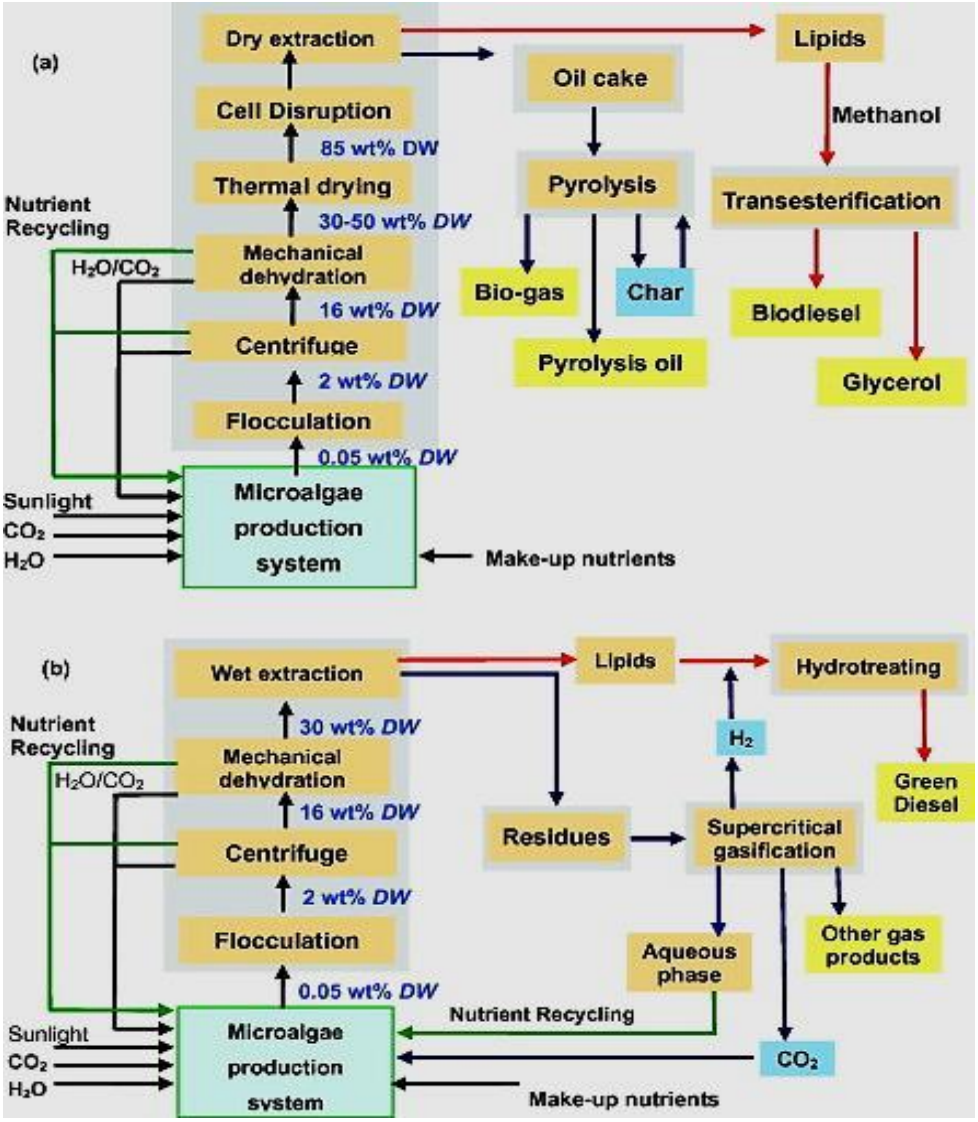


Figure 2-3 Microalgae to biofuel route a) the dry route, b) the wet route (Xu, et al., 2011)

The dry route can increase the shelf life of the final product but requires efficient and energy intensive technologies which are not economically feasible (Prakash, et al., 1997) (Beatriz & Voltolina, 1997) (Leach, et al., 1998) (Desmorieux & Decaen, 2005) (Nindo & Tang, 2007) (Mata, et al., 2010) (Mata, et al., 2011). More studies on drying efficiency and cost-effectiveness are hence required to maximize the net energy output of microalgae biofuels. Major constraints to algal biofuel production are the methods

to extract lipids from microalgae. Due to the micro-sized complex cells and rigid cell wall, oil expellers are inadequate (Chi, et al., 2006) (Cho, et al., 2012). Non-traditional costly methodologies such as organic solvents, electroporation, ultra-sonication, soxhlet, enzymatic and supercritical CO_2 can be adopted. The drying temperature during lipid extraction affects both lipid content and composition (Widjaja, et al., 2009). Freeze drying retains the composition although it is a very expensive method while high temperature drying decreases the content of triacylglycerol (TAG). Commercial production of lipid in huge quantities from algal strains of 10% lipid content with an accurate extraction method can be feasible (Kumar, et al., 2014).

A combination of enzymatic and mechanical/solvent extraction can be a more promising lipid extraction method (Kumar, et al., 2014). Solvent extraction requires extracting oil from the microalgae cells by repeated washing or percolation with an organic solvent. Possibly 98% FA's can be extracted with various chemical solvents like hexane, ethanol, mixture of hexane-ethanol, benzene, cyclohexane etc. (Geciova, et al., 2002) (Richmond, 2003) (Miao & Wu, 2006) (Macías-Sánchez, et al., 2007) (Mercer & Armenta, 2011). According to authors (Xu , et al., 2006), a potential industrial production of liquid fuel from microalgae was observed when large quantity of oil was extracted from *Chlorella protothecoids* (cells grown under continuous illumination) by using *n*-hexane, and converted into biodiesel by acidic transesterification. This study characterized the obtained biodiesel by a high heating value of 41 MJ kg^{-1}, a density of 0.864 kg L^{-1}, and a viscosity of 5.2×10^{-4} Pa s (at 40 °C). Also, new techniques using solvent extraction assisted by microwave technology present promising results. The main benefits are the reduction of the extraction time and of the energy and solvent used (Kingston & Jassie, 1989) (Kingston & Haswell, 1999) (Zlotorzynski, 1995). In fact, Virot, et al. (2008), showed that combining microwave heating and soxhlet method can reduce the extraction time from 8 hours to 32 minutes. By far, the disadvantage of these processes is the high costs associated with the required equipment and operation in order to maximize the net energy output of the fuels. Optimizing a successful lipid extraction method and converting it to biodiesel is vital. All the extraction methods appear promising at the laboratorial scale but require attention for large-scale commercialization. The main

methods of chemically converting oil to diesel are trans-esterification, pyrolysis and emulsification and among these, trans-esterification is, by far, the most used and important method to produce cleaner and environmentally safe fuel (Afonso, 2009). New techniques can be established integrating algae oil extraction and trans-esterification processes.

2.2 IDENTIFIED BARRIERS

There are several challenges and prospects that's comes across with the commercialization of microalgae derived biodiesel (Scott, et al., 2010). Critical technical challenges occur with cultivation systems, species selection, culture stability and contamination issues, harvest, lipid extraction and separation and quality control. Economic challenges are dominated by total cost, water usage, land, nutrient source and cost.

- Biodiesel generally is more expensive than petroleum diesel largely due to the high cost of the feedstock. Selecting the right algae species and building a cost effective cultivation unit, no matter the size of the facility or its geographical location, is critical.
- Low biomass concentration in the culture and low oil content is a main limitation.
- Challenges with the genetic and metabolic engineering effects the overall performance of the microalgae fuel production.
- Chemical and physical stress factors changes the microalgae biomass lipid content and composition. The optimization of strain specific culture conditions is of large complexity with many interrelated factors that can be limiting.
- Extraction methods can significantly affect the lipid yield.

- Lipid extraction methods usually consist of two steps: cell disruption (which greatly depends on size, shape and wall structure) and solvent extraction (which depends on lipid composition). These processes can be energy intensive becoming a hurdle towards the feasibility.
- The major conversion technologies (microalgae to biodiesel) are comprised by many complex processes which economically maximize the entire production cost.
- Biomass harvesting technology is energy dependent and the small cell size makes it quite costly.
- Pond design for microalgae cultivation is also a challenge to ensure good cultivation of microalgae. Temperature, nutrients and light should be adequately controlled to optimize light intensity, photo oxidation and inhibition for better growth. Microalgae farming are costly and complex.
- Energy required by the light sources used for the microalgae cultivation should be economically efficient.
- Cost of carbon source and production of CO_2 can be of concern.
- Drawbacks associated with the predation by protozoa and contamination by other algae species which causes collapse of the microalgae cultures can compromise large scale production units.
- Important operating disadvantages of biodiesel in comparison with petroleum diesel are cold start problems, low energy content and fuel pumping problems due to its viscosity. Ideally the purpose of trans-esterification process is to lower the oil viscosity but chemical solvents are an additional cost.

Some technological strategies to develop cost effective microalgae biofuel production system are:

- Co-product strategy: producing high value co-products (e.g. nutraceuticals) beside the main-product (biodiesel) and by-product (glycerin) can potentially add revenue to the system. However, new technological steps to

obtain such co-products must be experimented in order to address the process complexity (Li, et al., 2008) (Brennan & Owende, 2010).

- Using solar panels and wind power generators to produce energy for the system to work will be advantageous.
- Burning unused algal residues to produce electricity for the system.
- Using genetically modified technology for maximization of microalgae biomass and lipid production (Radakovits, et al., 2010).
- Developing cost-effective technologies for biomass harvesting and drying.

3 METHODOLOGY

A microalgae biodiesel pathway model was developed incorporating different technological strategies. This begins with a literature review assessing the interest of the research and fields (biomass conversion, feedstock processing and biochemical conversion methodologies) where improvement is required. The approach resulted in better understanding of the whole-chain process and identification of the factors which can influence the final outcome for feasibility.

In fact, to implement the knowledge it is important to follow a plan with appropriate design and culture conditions for proper microalgae cultivation. In that respect, suitable techniques are also required for better lipid extraction from the cultivated biomass and that demands the understanding of their methodologies. Acid trans-esterification was favored for FAME conversion in order to avoid saponification (obtained with base trans-esterification). The quality measurements of produced biodiesel via gas chromatography (GC) are operated to see the viability of its usage in the European transportation sector. Finally, assessing the economics and emission rates associated with the microalgae to biodiesel process is vital and, by doing so, new strategies will be established to reach for the rising energy demand. Figure 3-1 presents a synopsis of the work presented in the following chapters of this doctoral thesis.

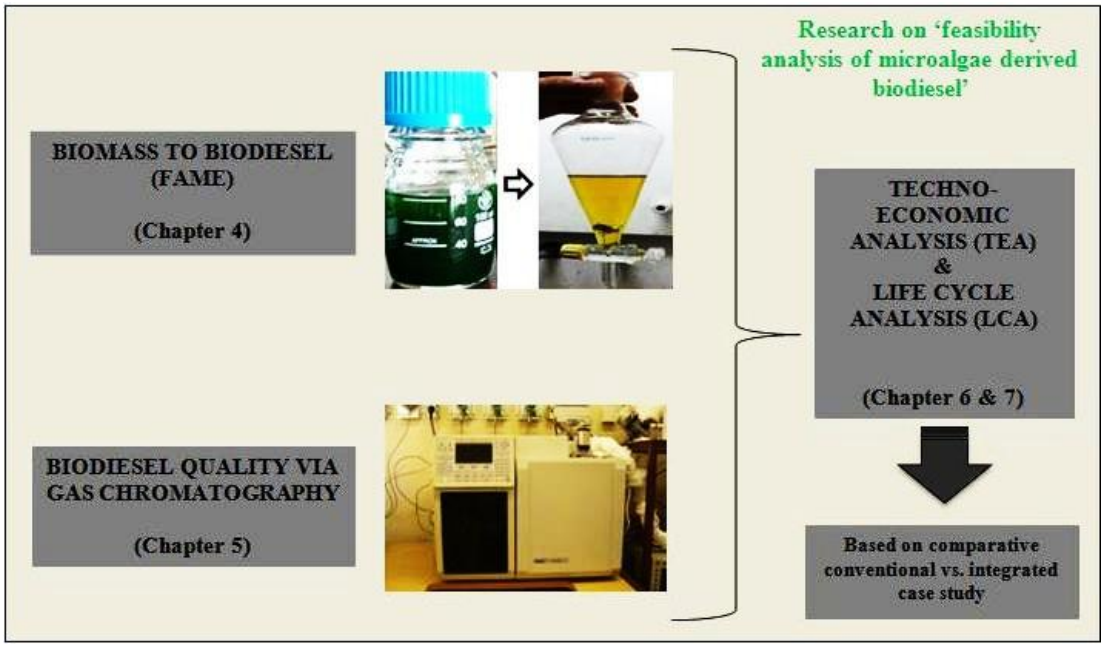


Figure 3-1 Overview of the chapter corresponding to the thesis 'Feasibility analysis of microalgae derived biodiesel'

This research started with the analysis of different microalgae strain growths and identifying proper culture conditions for their better growth rate. Literature review shows that a critical step is related to the lipid extraction technique. Both wet and dry biomass was used for the experimental part of this research and acid trans-esterification was considered to convert lipids to FAME (or biodiesel) since the method is less complicated (excludes the formation of soap during the conversion unlikely the base catalyzed trans-esterification). After converting the extracted crude oil to biodiesel the quality analysis of the final product in accordance to EN14214 shows its viability in the European market. The considered system is incomplete without an analysis focused on a techno-economic assessment (TEA) and life cycle analysis (LCA). In order to perform that, comparative case study based on two different technological design models (conventional and integrated case studies) for microalgae biodiesel production was conducted.

The different variables analyzed in every stage of the research are shown in table 3-1, elaborating the analysis conducted within the experimental part of this doctoral thesis.

Table 3-1 Tests examined in the thesis work

Strains → / **Performed analysis** ↓	***Chlorella vulgaris***	***Scenedesmus***	***Chlorophyta***	***Nannochloropsis gaditana***
Growth statistics and strain productivity ***(Chapter 4, section 4.3.1, 4.3.2)***	x			
Photoperiod and aeration rates on strain productivity and biodiesel quality ***(Chapter 4, section 4.3.2, 4.3.3; Chapter 5, section 5.3.2)***	x			
Influence of wet and dry biomass on biodiesel quality ***(Chapter 5, section 5.3.1)***	x	x	x	x
Impact of culture medium (freshwater and wastewater) and extraction technique (3-stage cross current continuous and soxhlet) on biodiesel quality ***(Chapter 5, section 5.3.3)***	x			
Determination of biodiesel quantity and their physical property ***(Chapter 5, section 5.3.4)***				x

Figure 3-2 describes the global methodology of the research. Chapter 4 to 7 will present the specific methodologies, as well as will explain the specific research objectives, review of technical literature, results and conclusions.

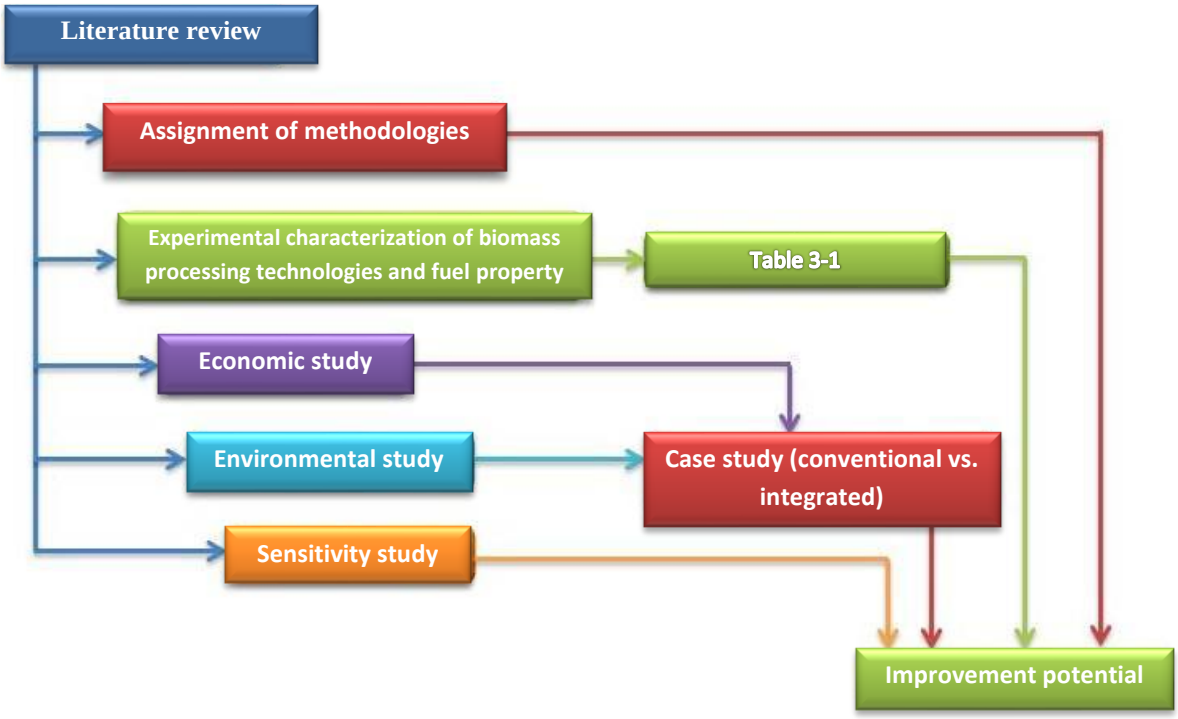


Figure 3-2 Description of the steps in the research for final improvement of the fuel

Engineering, literature knowledge and understanding biofuel needs, using innovative empirical and analytical techniques, is therefore the methodologies used in this study. This work provides an in-depth analysis, understanding and comparison of the technological pathways developed by University of Aveiro (Portugal) and National Renewable Energy Laboratory (NREL), Golden, Colorado, USA. A 3 months collaborative internship at NREL allowed developing for comparison between a conventional and an integrated approach.

4 BIOMASS PRODUCTION

This chapter will begin by presenting a background analysis on the importance of selecting appropriate microalgae strain, the influence of their yield and lipid content, on different cultivation systems and conditions contributing for high biomass production. The following section will describe the cultivation methods used for culturing different microalgae strains. The growth of *Chlorella vulgaris* was studied and the impact of varying photoperiod and aeration rate on biomass cultivation were examined. Also, the harvesting methodology was characterized.

Finally, the obtained experimental results for different case studies is presented and discussed.

4.1 LITERATURE REVIEW

- **Strain:**

Despite of the major drawbacks associated with microalgae production process described in the literature, a right strain selection can be seen as the initial step towards feasibility of the process of producing biofuel from microalgae. The important question arises *'which is the best microalgae strain viable for renewable biofuel production?* A strain is a genetic variant of a particular species of organism. Algae's fast growth rate definitely makes them one of the potential feedstock sources for biofuel, which comprises of three components: proteins, carbohydrates and lipid (Suganya, et al., 2016). The need for selecting a sustainable biomass feedstock is the driver behind the evaluation conducted in this section.

Lipids are a group of naturally occurring molecules that include monoglycerides, diglycerides, triacylglycerol's (TAG's), phospholipids and others. These are molecules that contain hydrocarbons making the building block of living cells (Donot , et al., 2013). Suganya, et al. (2016), expressed that chemical composition percentages of microalgae varies with their type (Table 4-1), containing about 40-50% (Demirbas & Demirbas, 2011) of the total mass of fatty acids (which can be extracted and converted to biodiesel).

Table 4-1 Chemical composition of different microalgae strains (% dry weight) (Suganya, et al., 2016)

Microalgae	Protein	Carbohydrates	Lipid
Scenedesmus obliquus	50-56	10-17	12-14
Scenedesmus quadricauda	47	-	1.9
Scenedesmus dimorphus	8-18	21-52	16-40
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Chlorella pyrenoidosa	57	26	2
Spirogyra sp.	6-20	33-64	11-21
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Prymnesium parvum	28-45	25-33	22-39
Tetraselmis maculata	52	15	3
Porphyridium cruentum	28-39	40-57	9-14
Spirulina platensis	46-63	8-14	4-9
Spirulina maxima	60-71	13-16	6-7
Synechoccus sp.	63	15	11
Anabaena cylindrica	43-56	25-30	4-7

Microalgae strains such as, *Chlorella vulgaris, Spirulina maxima, Chlamydomonas reinhardtii, Nannochloropsis sp., Neochloris oleoabundans, Scenedesmus obliquus, Nitzchia sp., Schizochytrium sp., Chlorella protothecoides, Dunaliella tertiolecta* have been investigated in order to select the best species in terms of lipid content both for quantity and quality (Chisti, 2007) (Xu , et al., 2006) (Rodolfi, et al., 2009) (Miao & Wu, 2006) (Lopes, et al., 2009) (Gouveia & Oliveira, 2009) (Gouveia, et al., 2009) (Morowvat, et al., 2010). The understanding of algae growth physiology, morphology and cell composition are required to improve the selection methods of microalgae strains. In a recent study by Nwokoagbara, et al. (2015), *Scenedesmus sp.* was selected as the ideal strain when compared to other strains like *Heynigia sp., Niracticinium sp., Chlorella vulgaris, Chlorella sorokiniana and Auxenochlorella protothecoides* due to its high lipid content and rapid growth rate.

The total oil quantity is a combination of biomass productivity and lipid content, whereas the quality is mostly related to the composition and distribution of the fatty acids (FA) which they contain. A mixture of unsaturated (palmitoleic 16:1, oleic 18:1, linoleic 18:2, linolenic 18:3) and saturated FA's (palmitic 16:0, stearic 18:0) constitute the oil extracted from microalgae. The quality and quantity of total obtained biofuel is directly influenced by the category and amount of lipids produced by a microalgae strain. As reported by Barupal, et al.(2010), the lipid content of microalgae ranges from 4.5% to 80% of its dry weight and strains offering more than a quantity of oil which exceeds 50% dry weight of the extracted oils can be a feasible option for industrial production of biodiesel. Microalgae lipid content and yield affects the overall economic sustainability of biofuel production. Traditional approaches for lipid characterization of algal species are gravimetric and spectrophotometric techniques which evaluate total hydrocarbon accumulation in the cells (Barupal, et al., 2010).

Additional factors play key roles when screening a certain species for viable production of oil. Contamination is one of them. It is an issue faced during bulk cultivation of microalgae and definitely needs special attention. In the work of Perrier, et al. (2015), it is showed that *Nannochloropsis occulata* is a widely used marine algae with a typically lipid content higher than 30% of dry matter. Its small in size makes it difficult to harvest, however this strain is very resistant to contaminants and climatic variations as compared to many other strains

Cell wall controls the biological and biomechanical stabilities of the cell, and thus its understanding might become essential to choose appropriate lipid extraction techniques for the production process (Kim, et al., 2016). Strong cell walls in microalgae make them less desirable for the production process: the mean extractability of species without cell walls was 2.64 times more that with strong cell walls (Mendoza, et al., 2015). At the same time, carbohydrate-rich microalgae cell wall is a determining factor in the strain selection because microalgae grow faster fixing CO_2 at a higher rate than terrestrial plants. Microalgae based carbohydrates are mainly in the form of starch and cellulose and are much easier to convert to monosaccharides when compared with lignocellulosic materials (Ho, et al., 2013).

- **Cultivation systems:**

These micro-sized organisms utilize light energy and CO_2 to multiply and generate biomass using photosynthesis. Another factor which potentially affects the lipid content and yield is the culture environment. These environments can be artificial or man-controlled ponds, natural ponds and bioreactors. Lipid productivity depends on the cultivation system i.e., whether an open system (exposed to environmental conditions) or a closed system (controlled conditions). The cultivation system design can directly influence process economics and environmental sustainability.

A PBR is a complex closed system (controlled environment built with a transparent array of tubes or plates and the microalgae broth is circulated inside) which requires a column degassing to remove O_2 produced during the photosynthesis process. The light energy can be obtained from direct sunlight or artificial illumination. Such system, despite being expensive maximizes the algae growth rate through its design: efficient distribution of light, mechanism of CO_2-O_2 exchange and pH and temperature control to protect the culture from contamination. This system reduces the culture evaporation rate, maintains an equilibrium temperature during the cultivation period, reduces the risk associated with biomass fouling and can be mounted indoors or outdoors at any angle saving space. Figure 4-1 shows these two types of cultivation systems (left is an example of open system and right is a closed system).

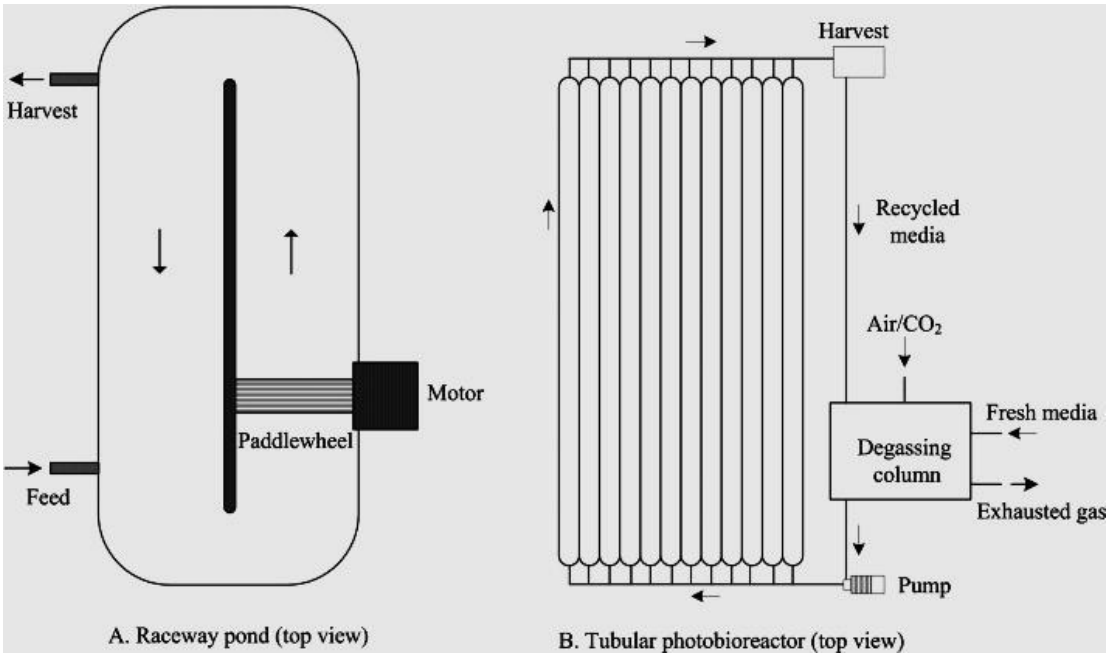


Figure 4-1 Example of (A) raceway pond and (B) tubular photo-bioreactor (PBR) (Suali & Sarbatly, 2012)

Closed systems typically are more complex than open ponds or tanks. There are three types of PBRs: vertical column, tubular and flat panels (Wang, et al., 2012). Again tubular PBRs can be divided into vertical, flat, annular, fermenter or internally illuminated types (Suali & Sarbatly, 2012).

Tubular PBRs (oriented horizontally or vertically) made from glass or plastic show promising potential at production scale with centrally installed utilities (like pump, sensors, lights and aeration valves) considering their high surface/volume ratio and since they can support high biomass productivity. PBR design requires attention concerning the maximization of light considering the type of material used for construction and their surface/volume ratio. Commonly used materials include polyethylene, glass, plexiglass, poly vinyl chloride (PVC), and acrylic-PVC due to their optical transparency. These reactors have been used in producing antioxidant *Carotenoid astaxanthine* from freshwater algae *Haematococcus pluvialis* or producing food supplements from algae *Chlorella vulgaris* (Bellou, et al., 2014). Culture parameters including illumination rates, aeration rates, nutrient input, and dilution rates etc. affect the cellular growth, lipid and fatty acid composition. Previous studies successfully involved cultivation in tubular PBRs of different microalgae species such as *Phaeodactylum* and *Haematococcus* (García-Malea, et al., 2008) (Benavides, et al.,

2013), *Nannochloropsis* (Cheng-Wu, et al., 2001), *Scenedesmus almeriensis* (Pawlowski, et al., 2014), *Tetraselmis suecica* (Michels, et al., 2014) etc. but the system design needs tailoring accordance to particular strain physiology and growth characteristics (Sforza, et al., 2012). Tubular PBR gained popularity in scaling up and high volumetric biomass production since light enters the reactor walls at various angles (Slegers, et al., 2013).

Despite the high initial capital investments associated with PBRs, the advantages they offer for microalgae culture include:

- high photosynthetic efficiency resulting in increased biomass concentration and production
- encouragement of the algae cell growth by providing a controlled integrated environment (efficient CO_2 supply, light capturing and distribution, capability to provide lightness/darkness alternating periods, effective mixing mechanisms, uniform temperature and pH control)
- prevention of contamination by predators during the cultivation period (a built-in cleaning mechanism can reduce contamination without stopping the cultivation)
- provision of a decreased culture medium loss via evaporation when compared to open pond systems
- independence upon land location, since PBRs can be mounted indoors or outdoors, vertically or horizontally or even at an angle
- no effects on biomass culture due to seasonal changes.

An important caveat to this section is that the development of PBRs for industrial or massive oil production from microalgae requires an understanding of the aspects strongly involved with the system sustainability, both economically and environmentally.

A typical open pond is normally 0.25-0.4 m deep, operates in a closed loop, oval shaped and exposed to natural environment. A paddlewheel is used to circulate the culture and prevent biomass sedimentation. Open ponds are the most widely used cultivation

systems due to their low cost of construction, maintenance and operation and for these reasons are very promising for large scale production. All necessary nutrients and CO_2 are pumped in the culture and illuminated directly from sunlight. Although, the construction methods are simple, these systems suffer from high water losses due to evaporation and are prone to contaminations from predators. Using non-toxic supple plastic liners might minimize water losses and land deterioration. Paddlewheels operated by electric motors maintain a constant mix of algae biomass in open raceway ponds promoting the cell growth but also adds to the capital costs and maintenance.

Another design developed for commercial scale production is the plastic bag PBRS due to their low cost and great sterility (Mata, et al., 2010) (Martínez-Jerónimo & Espinosa-Chávez, 1994) (Borowitzka, 1999).

Optimizing the outdoor conditions (namely, location, rainfall, sunlight intensity, operation time, culture temperature, pond depth, CO_2 delivery system, mixing technologies, power consumption etc.) can improve the overall performance. Utilization of wastewater streams can reduce the consumption of treated water and can provide good quantity of nutrients (Cai, et al., 2013). The logistic factors affecting the feasibility of the whole production process in considering the location of the system are rainfall, solar radiation, temperature, seasons, operating days, land slope, potential nutrient sources, water cost, land cost and others. Desert locations with high availability of sunlight are not suitable due to water scarcity and evaporation rates. Bennette, et al. (2014), presents that a location having a rainfall rate of less than 1 m per year is actually desirable since high rainfall can destroy the culture. Constructing ponds on the seashore with marine microalgae and plenty availability of saline water is favorable. In many countries, open ponds cannot operate 365 days since in winter the drop in the temperature can become unacceptable for microalgae growth. Pond depth vastly influences other production parameters like power consumption, mixing, light efficiency, culture temperature, areal productivity etc. An evaluation by Sutherland, et al.(2014), presented 200% increased areal productivity in an algal pond at a depth of 40 cm compared to 20 cm. A natural open environment brings many advantages for large

quantity biomass production but at the same time it exposes the culture to predators which can severely diminish biomass yields.

- **Operational parameters:**

Optimization of microalgae biomass production in PBR is achieved by manipulation of important operational parameters like light (intensity, capture, distribution, utilization and photoperiod cycle), aeration rates, temperature, pH and degree of culture mixing.

Depending on the microalgae species, cultures may be the phototropic or heterotrophs. Phototrophs require light, CO_2 to reproduce and cultivating them in an indoor environment implies an additional cost but also is excellent to keep contaminations away. The provision of larger amounts of CO_2 helps increasing the lipid yield, however incurs costs. The use of industrially emitted CO_2 can possibly be a solution. Light plays an important role in microalgae cultivation via photosynthesis which converts CO_2 and water into glucose and O_2. This glucose is used to fuel the micro cell sized organisms. The effect of light supply is directly related with cell growth rate and strongly influences biomass productivity. Low light intensity level limits the algae cell growth rate (photo-limitation) while excessive light intensity destroys the culture (photo-inhibition). A strategy to deal with such a delicate aspect would be to supply sufficient light intensity for the first few days of culture to increase the initial microalgae inoculum growth; later, as cells grow stronger and healthier, the light intensity can be increased. The production of *Spirulina* biomass with increasing light supply intensity from 8 to 30W/m^2, resulted with a linear increase in specific growth rate. In the range of 30-50W/m^2, the growth rate reaches a plateau and the culture suffered photo-inhibition with a higher light intensity (>50W/m^2) (Watanabe & Hall, 1995) (Vonshak, 1997) (Carvalho, et al., 2011). As cell growth rate is light dependent, the light spectrum available for microalgae photosynthesis is in the wavelength of 390 to 720 nm range which corresponds to photosynthetically active radiation (PAR), which actually accounts for about 50% sunlight (Suh & Lee, 2003). Furthermore, radiance outside this wavelength (non- PAR) primarily causes temperature rise in the culture damaging the

cells. Studies were conducted using spectral shifting creating an artificial increase of the photosynthetic efficiency in the PAR range using florescent dye solution absorbing non-PAR but this is a costly modification (Prokop, et al., 1984). Another important consideration related to high quantity biomass production is the photoperiod; this is the rhythm of light/dark cycles. Microalgae culture performance in PBR depends on the duration of light periods, which means that a decrease in biomass production is evident with a decrease in the duration of light period (Eduardo, et al., 2009). An adequate light distribution within the culture is attained by rapid culture mixing, equalizing the time spent by the cells near the surface, where they are exposed to a very high light intensity (Scott, et al., 2010) (Liao, et al., 2014) (Khoeyi, et al., 2012). Also, CO_2 concentration, nitrogen deprivation, phosphate limitation, silicon deficiency and iron supplementation play a significant purpose in the biomass culture and in the lipid content of microalgae (Rodolfi, et al., 2009) (Hu & Gao, 2003) (Hsueh, et al., 2009) (Griffiths & Harrison, 2009) (Liu, et al., 2008). Marine microalgae *Nannochloropsis sp.* studied at different light intensities (50, 100 and 200 μmol photons m^{-2} s^{-1}) and photoperiod cycles (24:0, 18:6 and 12:12 h light:dark) resulted in a maximum concentration of 6.5×10^7 cells mL^{-1}, with lipid content of 31.3 % at a 100 μmol photons m^{-2} s^{-1} intensity and a 18:6 h period (Suzana, et al., 2013). A slow growth pattern of *Chlorella vulgaris* was obtained under a low light intensity of 37.5 μmol photons m^{-2} s^{-1} but increased with intensity from 37.5 to 62.5 μmol photons m^{-2} s^{-1}; further increase in intensity to 100 μmol photons m^{-2} s^{-1} decreased the total biomass (Khoeyi, et al., 2012). Microalgae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* cultured under various light irradiances (36, 72, 96 and 126 μmol photons m^{-2} s^{-1}) resulted in high lipid concentration with higher irradiance values; however, for 96 and 126 μmol photons m^{-2} s^{-1}, the lipid production was no higher than for 72 μmol photons m^{-2} s^{-1} due to micro-algal light saturation (Gonçalves , et al., 2013). Hence, light regime has the potential to impact cell growth and lipid productivity, making it one of the major controlling factors for microalgae biomass production.

Improving the culture mixing by circulating the growing medium should be one of the most commonly adopted strategies for proper light distribution. Culturing *C. sorokiniana* with four static mixers reached 15-70% higher productivity than without

mixers (Ugwu, et al., 2002). Nevertheless, higher velocity adversely damages the cells and is not often practical (Contreras, et al., 1998). In conclusion, adequate mixing implies uniform dispersion of algae cells in the culture with uniform distribution of nutrients and better exposure to light avoiding deposition of algae on the bottom.

Within this context, the cleanability of the PBR preventing the walls from biofilm formation is critically important since it reduces the contamination chances and provides proper light transmission.

The aeration of external gases in PBR is an important aspect of the design to maximize microalgae production.

Previous research confirms that seawater containing wastewater is a good medium for *Nannochloropsis sp*. and productivity is improved with a mixer of 15% of CO_2 and 85% of air aeration rate (Jiang, et al., 2011). Research results reports the elevated growth rate of *Nannochloropsis sp.* by 39% with a rise in CO_2 concentration from 350 to 2800 $\mu l\ l^{-1}$ in photoautotrophic culture and by 21% in mixotrophic culture (Hu & Gao, 2003). The maximum growth rate of *Thermosynechococcus sp.* was 2.7 d^{-1} at 10% CO_2 concentration further decreasing with increased concentration from 10% to 40%, while for *Nannochloropsis sp. Oculta* the maximum growth rate was 1.6 d^{-1} obtained at 5% and 8% of CO_2 concentration (Hsueh, et al., 2009). A study by Christenson & Ronald, (2011), used medium strength domestic wastewater values (with enough nutrients such as nitrogen and phosphorus in each liter) to produce 0.6 g of algae with a total of 77.6 million kg day^{-1}. Thus, it appears the total biomass production with high lipid content could be enhanced by increasing the duration of light period and higher inflow rates. Also, wastewater can be a proper medium for biomass production.

A significant challenge in PBR design is devising a cost effective temperature control mechanism. Temperature is an environmental variable: seasonal alterations as well as daily fluctuations affect the growth of microalgae. Study by Renaud, et al. (1995) resulted that tropical marine microalgae *Isochrysis* sp. and *T. Isochrysis* sp. grew slow at 35°C, while *N. closterium* only grew in the 20°C – 30°C range and *N. paleacea* is low-

temperature tolerant with cells slowly growing at 10°C. The authors also showed that *N. paleacea* produced the highest lipid content at 10°C, while the others produced the maximum lipid content at 20°C (Renaud, et al., 1995). Without any control, the culture temperature in a PBR can increase to values which are 10°C – 30°C higher than the ambient temperature. Additional cooling mechanisms can be employed to maintain the culture temperature (submersion of the entire culture in a water pool, spraying with water, shading and incorporation of a cooler).

Another conditioning factor is the acidity of the medium. The ideal pH range for most microalgae is 7.0-9.0. However, some strains can have more acidic or basic environments. For example, the optimal pH of cyanobacterium *Spirulina platensis* is in the range of 8.0 – 10.0 (Costa, et al., 2004). It is pivotal to maintain the optimum pH range in the culture since extreme pH can cause cell breakage leading to total culture damage. But a change in pH is possible due to the dissolution of CO_2 in the culture medium. A relevant study reported an initial decrease of pH in the culture from 8 to 7.2 but after four days of growth the pH rises up to 7.5 as with higher cell concentration the CO_2 consumption by the cells is also higher (Sforza, et al., 2012). Injection of pure CO_2 in tubular PBRs can control the pH but this constitutes almost 30% of the overall biomass production cost (Martínez-Jerónimo & Espinosa-Chávez, 1994).

- **Harvesting techniques:**

The literature overview regarding PBRs leads to the segment which presents the elaborate cultivation conditions used in culturing different strains in a tubular PBR as described in section 4.2.2.

A suitable harvesting method performs the solid-liquid algae separation by physical, chemical or biological methods. Solid-water separation an energy dependent process which contributes to almost 20-30% of the total biodiesel production cost (Grima, et al., 2003). It seems that the convenient harvesting method selection depends on microalgae

size and culture density. The algae harvesting cost can be high because of the low biomass fractions in the culture broth. Several harvesting methods namely sedimentation, centrifugation, filtration and ultra-filtration and flocculation are severely conditioned by the type of microalgae (Zmora & Richmond, 2003) and strongly affects the final product quality, the oil yield and the processing costs (Uduman, et al., 2010). To provide a reliable analysis, a previous study by Brennan & Owende (2010), reported that harvesting can be better understood as a two-step process:

- Bulk harvesting: The primary purpose of this technique is to separate biomass from the bulk suspension, with the solid matter reaching up 2-7% using flocculation, flotation, or gravity sedimentation (Uduman, et al., 2010).
- Thickening: This step focuses in the concentration of the slurry, mostly with filtration and centrifugation and is an energy driven step (Brennan & Owende, 2010).

Bulk harvesting:

Gravity sedimentation is the common method practiced for separating microalgae in wastewater treatments. The success with gravity settling depends on microalgae density. According to Edzwald (1993), for low density microalgae, particles cannot be separated by settling. Despite its simplicity, sedimentation is a very slow method (0.1-2.6 m/h) (Choi, et al., 2006), however Uduman, et al.(2010) suggested that the technique can be improved (by making it time efficient) using lamella separators (designed to separate particulates from liquids) and sedimentation tanks. Gravity sedimentation is suitable for harvesting large size microalgae, such as *Spirulina* (300-500 μm). Quite often flocculation is used to enhance the efficiency of gravity sedimentation.

As microalgae carries a negative electrical charge that prevents them from self-aggregation in the algae culture, addition of chemicals known as flocculants can reverse the process. Flocculation is a process where dispersed particles aggregate together form to large particles for faster sedimentation. The chemicals used for flocculation are known as flocculants. The most effective flocculants for microalgae recovery are cationic flocculants (Schenk, et al., 2008). These chemicals coagulate microalgae

without affecting their composition and toxicity. Examples include aluminum sulphate Al_2 $(SO_4)_3$, ferric chloride $FeCl_3$, ferric sulphate $Fe_2(SO_4)_3$, etc. In another study, authors Divakaran & Pillai (2002), reported that a biodegradable organic flocculant (e.g. chitosan) does not contaminate the microalgal biomass since it is produced from natural sources. On the other hand, successful harvesting can be obtained using inorganic flocculants at sufficiently low pH (Uduman, et al., 2010). Despite this advantage, coagulation with inorganic flocculants suffers from drawbacks namely the flocculant concentration (which causes the separation) leading to large quantity of sludge; the process if sensitive to pH level and, the final product is often contaminated by added aluminum or iron salts. There are certain species which can induce natural flocculation or auto-flocculation. Bilanovic, et al. (1988), mentioned that auto-flocculation is a result of precipitation of carbonate salts with algae cells in elevated pH, a consequence of photosynthetic CO_2 consumption with algae. Adding flocculants to the system can enhance the separation of microalgae and the sedimentation rate. Flotation is a gravity separation process in which air or gas bubbles are attached to solid particles and carrying to the liquid surface.

Chen, et al. (2011), states that according to the bubble sizes used in the process, this application can be divided into dissolved air flotation (DAF), induced air flotation (IAF) and electrolytic flotation. Chen, et al., (1998) indicated that flotation is more valuable than sedimentation in what regards microalgae separation.

DAF is an effluent treatment process based on pressure reduction of water stream that is pre-saturated with air. It clarifies water or wastewater by the removal of suspended matter such as oil or solids by dissolving air creating tiny bubbles (10-100µm) which adhere to the suspended matter causing the suspended matter to float to the surface of the water where it can be removed by a device. This process works well for large volumes (making it commercially viable) greater than 10,000 m^3/day (Uduman, et al., 2010). Key factors with DAF are the pressure of the tank, the recycle rate, the hydraulic retention time and the floating rate of particle. Chemical flocculation along with DAF can be combined with good results to separate microalgae (Uduman, et al., 2010).

According to Grima, et al. (2003), the disadvantage of this approach is the contamination caused by flocculants which significantly deteriorates the final product.

IAF is based on bubble formation by a high speed mechanical agitator with an air injection system (Chen, et al., 2011). It entails 700-1500µm bubbles formed with the help of a high speed mechanical agitator with an air injection system (Sukenik & Shelef, 1984) (Rubio, et al., 2002).

The electrolytic method is another potential path without requiring any chemicals; in this technique, a negative electric charge drives the algae out of the solution. Included benefits comprise environmental compatibility, energy efficiency, safety, cost effectiveness etc. (Petruševski, et al., 1995).

Thickening:

Filtration is the method mostly applied both at laboratorial scale and in large-scale applications. Filtration has some drawbacks such as membrane clogging, formation of compressible filter cakes and high cost. Microstrainer and vibrating screen filters are two devices having several advantages in microalgae harvesting. Grima, et al. (2003), found that filters operating under pressure or vacuum recover large microalgae, although they fail with bacterial sized organisms. Tangential flow filtration is not only a high rate method for harvesting with a recovery rate of 70-89% of freshwater algae but also helps in retaining the structure, properties and motility of the recovered algae (Chen, et al., 1998). Grima, et al. (2003), showed that within 2-5 minutes about 80-90% microalgae can be recovered with laboratory centrifugation conducted on a pond effluent with a mass of 500-1,000g. Centrifugation is a very useful method to possibly concentrate initial slurry from a concentration of about 10-20 g/L to a thick algal paste with a concentration about 100-200 g/L (Rubio, et al., 2002). The disadvantage that comes along with centrifuge is the exposure of cells to high gravitational and shear forces which can damage the cell structure (Mollah, et al., 2004). Moreover, such devices are easily cleanable or sterilized to avoid contamination.

Among all the available harvesting techniques, filtration and centrifugation were used in this thesis study for harvesting the microalgae biomass (section 4.2.3).

Highlights of the research:

An extensive study on PBR utilization was required since the experimental part of this thesis involved the microalgae growth in a tubular PBR.

The factors for selecting proper strain are biomass productivity, growth rate, oil yield, resistance to contamination, harvesting requirements, culture environment, and physiology. Some of the major questions raised from this literature review are:

1) Is it possible to use untreated water or wastewater to grow good microalgae biomass?
2) How are the growth rates for the tested strains?
3) How do photoperiod and aeration rates affect the growth rate?

The objective for the experimental work described in the present chapter was to address these questions. The following section acknowledges the microalgae strains examined for the experimental part of this study.

4.2 METHODS

4.2.1 Strains tested

The purpose of the present research emerged from the need to find an alternative fuel source using water sources of lagoon Ria of Aveiro. In total, three strains of green microalgae {*Chlorella vulgaris, Chlorophyta* (unspecified mixture of several microalgae strains), and *Scenedesmus}* were inoculated and cultured. The extracted lipids were characterized in qualitative terms. In addition, lyophilized biomass of *Nannochloropsis gaditana* and *Scenedesmus* were received from Almería University and *Chlorella vulgaris* was obtained from Vigo region for oil extraction and further

characterization. All the biodiesel obtained from the strains was tested and the obtained results were compared to those prescribed by the European biodiesel quality standard EN 14214:2003.

4.2.2 Cultivation conditions

In this section the design of the tubular PBR used in culturing microalgae at Aveiro and the conditions used for culturing the various microalgae strains will be described.
A vertical tubular PBR with a total 30L volume (Aqualgae No. 6C-FBR-005-2011) was used to culture the microalgae strains. The PBR is a stainless steel structure consisting of six acrylic columns (each with a 110 mm outer diameter and an approximate volume of 5 L) and 10 fluorescent tube lights (TLD 36W/865) (Figure 4-2).

Figure 4-2 Left picture shows the 6 columns, lights; Right picture shows the frontal view of the cabinet (Photo credits: Smritikana Dutta, University of Aveiro)

Each column forms 2 groups: Group 1 is composed by columns numbered 1, 2 and 3 from left to right and Group 2 is composed by remaining columns. Two pH and temperature sensors were inserted in columns 2 and 4. Water from Ria de Aveiro was collected and transported to the laboratory. To minimize contamination, the lagoon

water was disinfected with ozone before using for the culture. Figure 4-3 shows the collection of lagoon water and figures 4-4 to 4-10 show the PBR used for algae cultivation. 120 liters of fresh water were collected on 11th of May 2012. The collected water was analyzed. The data is shown in the annexes later. The applied aeration rate in the culture was estimated from the readings provided by a CO_2 analyzer, model Testo 435.

Figure 4-3 Collection of water from the Lagoon Ria in Aveiro
(Photo credits: Margarida Coelho)

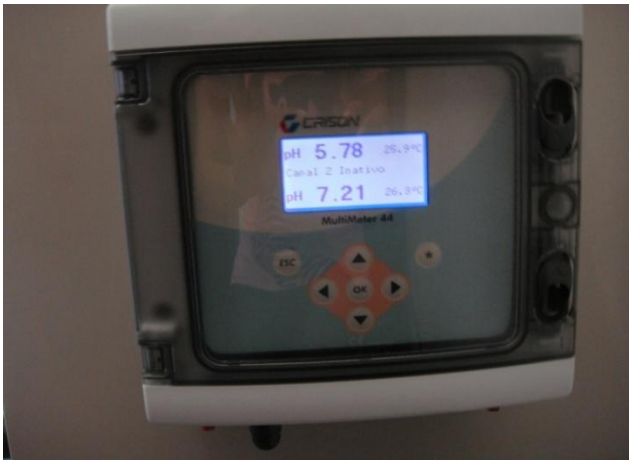


Figure 4-4 Multi-meter connected to the PBR to maintain the pH level of the culture
(Photo credits: Smritikana Dutta)

Figure 4-5 Left: CO_2 bottle, Right: CO_2 and air mixed and aerated to the culture. Green tubes: CO_2, Blue tubes: Air (Photo credits: Smritikana Dutta)

Figure 4-6 Microalgae culture in the PBR (Photo credits: Smritikana Dutta)

Figure 4-7 Addition of regular nutrients (Photo credits: Smritikana Dutta)

Figure 4-8 Collection of sample to measure daily cell growth (Photo credits: Smritikana Dutta)

Figure 4-9 Collection of microalgae samples from every column to compare their growth rates (Photo credits: Smritikana Dutta)

Figure 4-10 Daily collected 80 ml of microalgae culture was collected and replaced with water and nutrients (Photo credits: Smritikana Dutta)

Figure 4-6 to 4-10 shows the cultivation of green microalgae in the PBR, daily addition of nutrients and collection of samples for measuring the daily growth rate.

Assessing the growth rate of Chlorella vulgaris:

Three different microalgae strains were cultured: *Chlorella vulgaris, Chlorophyta,* and *Scenedesmus.* The daily growth rate was measured for *Chlorella vulgaris* in order to study the influence of microalgae's growth parameters.

An initial inoculum of 0.5L/column (3L in total) with an initial cell concentration of $8.56X10^6$/L supplied from Biology Department of the University of Aveiro was used for inoculation. All six columns were cultured under the same conditions and the total cultivation period lasted for 10 days. Before starting the cultivation the following operations were conducted to reduce culture contaminations.

- *Calibration of sensors:* Calibration of pH probes with standard buffer solutions was carried.

- *Cleaning:* Columns were filled with water collected for the experiments from, and bleach (concentration: 1,5g chlorine/L of water) for 12 hours approximately. After, the columns were rinsed with disinfected ozonized water a (5 minutes delay for the ozone to evaporate from 5L of water is necessary). Additionally, the column lid covers and pH probes were cleaned with a damp cloth to prevent contamination.

- *Inoculation:* The inoculum was placed in each column with disinfected ozonized water. At the start, the recommended amount of nutrients was doubled and added to provide extra growing capabilities[1]. Table 4-2 shows the amount of nutrients and their composition which were added to each column.

[1] Aqualgae. Personal communication.

Table 4-2 Nutrients for microalgae culture

NUTRIENT	COMPOSITION	AMOUNT (ml/5L/day)
Solution A	$NaNO_3$, $NaH_2PO_4.2H_2O$	10
Solution B	$MgSO_4.7H_2O$	5
Solution C	Ferric citrate, Na_2-EDTA, $Na_2MoO_4.2H_2O$, $MnCl_2.4H_2O$, $ZnCl_2$, $CuSO_4.5H_2O$, $CuCl_2.6H_2O$, Boro, SeO_2; Vitamin: biotin, cyanocobalamin (B12), thiamin	2

Only four tube lights were turned on for the first initial four days of culture to avoid photo-inhibition and the CO_2+Air gas mixture was aerated at a lower velocity through the columns avoiding cell destruction. On the fifth day, all 10 fluorescent tube lights were turned on. Microalgae culture samples were collected from each column every day at the same time to determine their daily cell growth rate and the same amount and concentration of medium was restored by adding water and nutrients. The culture pH was kept between 7.2 - 7.5; the ambient temperature was maintained at about 22°C and a 12 hours photo-period of light/darkness was used.

Effects of photoperiod and aeration rates:

A different batch of *Chlorella vulgaris* was cultured to investigate the effect of different illumination periods and various aeration rates in the tubular PBR. Microalgae were cultivated with varying illumination periods of 12:12 h and 24:00 h. The first set of microalgae was cultivated with 12/12h: light/ darkness and aerated with a 4 l min^{-1} of CO_2 and air (v/v) mixture inflow rate. A second set of cultures was conducted at continuous illumination (24 hours of light and zero darkness) with different aeration rates of 1.5 l min^{-1}, 3 l min^{-1} and 4 l min^{-1} respectively. The total CO_2 concentration in the gaseous mixture was kept at 21% representing 0.32 l min^{-1}, 0.63 l min^{-1} and 0.84 l min^{-1}of CO_2 flow rate. The initial four days of culture occurred with a constant photon flux density of 242.2 μmol

photons m^{-2} s^{-1} (four tube lights) avoiding photo-inhibition. Later the light intensity was increased to 401.4 μmol photons m^{-2} s^{-1} (ten tube lights). For the first set of cultures, the daily cell growth was measured, while for the second set a cell counting was done on the 2^{nd}, 7^{th} and 9^{th} day of culture. A mass flow-meter was used to set the aeration rates in columns. A pH of 7.2 -7.5 and 22°C temperature was maintained for this experiment.

4.2.3 Harvesting

All the cultured samples were harvested by resorting to vacuum filtration and centrifugation. The advantage with vacuum filtration is that it leaves a smaller degree of impurity in between the solid and liquid phases. The mixture of solid and liquid is poured through a filter paper in a Buchner funnel and solid is trapped by the filter while the liquid is drawn through the funnel into the flask below, by a vacuum. However, this process was time consuming since 5L can require approximately 24 hours to separate the biomass slurry from the culture medium (Figure 4-11). This technique separates a solid product from a solvent or liquid reaction mixture.

Figure 4-11 Vacuum filtration, 5L of microalgae culture
(Photo credits: Smritikana Dutta)

Centrifugation was hence used to separate algae. Centrifugation uses the sedimentation principle, where the centripetal acceleration causes denser particles to move outward in the radial direction and at the same time, objects that are less dense are moved to the center. In a laboratory centrifuge using sample tubes, the acceleration causes denser particles to settle to the bottom of the tube, while low-density substances rise to the top.

For a 5L volume medium, a 50 minutes period at 3000 rpm speed was used to retrieve the same amount of biomass obtained with vacuum filtration. Thus, microalgae biomass was gathered, centrifuged, and eventually transformed into a wet paste like substance or algae slurry.

4.3 EXPERIMENTAL RESULTS AND DISCUSSION

4.3.1 Microalgae growth

A typical micro-sized algae organism passes through five stages of life classified as: lag or induction phase, exponential phase, declining growth rate phase, stationary phase and death phase.

In the ‘induction phase’ little increase in cell density occurs. During this period the culture requires adaptation to the new environment before cells start reproducing. This period is attributed to physiological adaptation of cell metabolism to growth, as is characterized by the increase of the levels of enzymes and metabolites involved in cell division and carbon fixation (Barsanti & Gualtieri, 2010).

The ‘exponential phase’ is the key to success of algae massive production because in this period the cell density increases as a function of time according to an exponential function (eq. 4-1):

$$C_f = C_i . e^{m´f} \quad \text{(eq. 4-1)}$$

In this equation:

C_f is the final cell concentration; f is time (unit: seconds)

C_i is initial cell concentration (time =0) and m´ is the specific growth rate (unit: 1/seconds).

As mentioned before, the specific growth rate is dependent on parameters like species type, light intensity, temperature, availability of nutrients, etc.

During the period designated by ‘declining phase’ cell division slows down as physical and chemical factors limit culture growth.

The fourth stage is designated by ‘stationary phase’ and during this period cell concentration reaches its maximum value and stagnates. During this phase dilution of the culture with more medium can renovate the growth rate.

The ‘death phase’ occurs when the culture starts deteriorating and nutrients depletion makes the culture incapable to sustain itself. At this stage, cell density decreases quickly and the total culture collapses. Figure 4-12 depicts the growth dynamics of *Chlorella vulgaris* in each column displaying the results obtained from the daily sample collection.

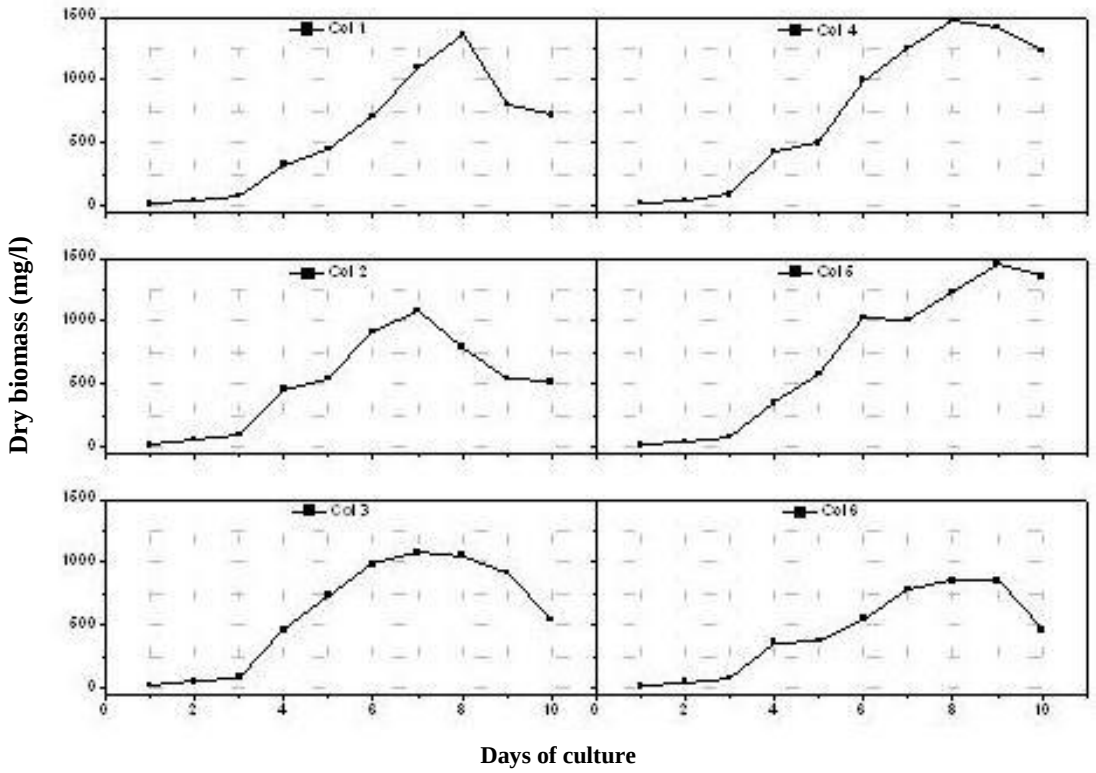


Figure 4-12 Chlorella vulgaris growth dynamics in different columns
Data credits: Dr. Paula Gonçalves (Department of Biology, University of Aveiro)

The instrument used to measure the cell growth rate was a viability analyzer, Vi-CELLTM XR, Beckman Coulter. Overall, the average maximum growth occurred between the seventh and the ninth day of culture.

The highest dry biomass concentration (with ~20% moisture) was obtained on the eighth day with **1476.7 milligrams/l**. The increase in the cell concentration estimated was from 16.7 mg/l (day 1) to 1476.7 mg/l (9^{th} day-stationary phase), then the cell multiplication slows down (1353.3 mg/l) (10^{th} day-beginning of the death phase).

4.3.2 Effect of photoperiod and aeration rates on algae growth

The obtained experimental results were used to highlight the influence of the photoperiod duration and the aeration rate upon growth rate and total biomass production.

The work was performed in two set of experiments in order to compare the influence of each parameter on growth rates. The first set involved the cultivation of *Chlorella vulgaris* at 12:12 h photoperiod with 4 l min^{-1} air aeration rate (0.84 l min^{-1} CO_2) measuring daily cell growth and their absorbance of light energy at 750 nm. The second set involved culturing *Chlorella vulgaris* at 24:00 h photoperiod with 1.5 l min^{-1}, 3 l min^{-1} and 4 l min^{-1} air aeration rates.

Growth statistics of *Chlorella vulgaris* at 12:12h with 4 l min^{-1} air aeration rate are shown in figure 4-13 (A, B). Specific growth rate is the increase in cells per unit time and generation time is the time taken for a cell population to double in number.

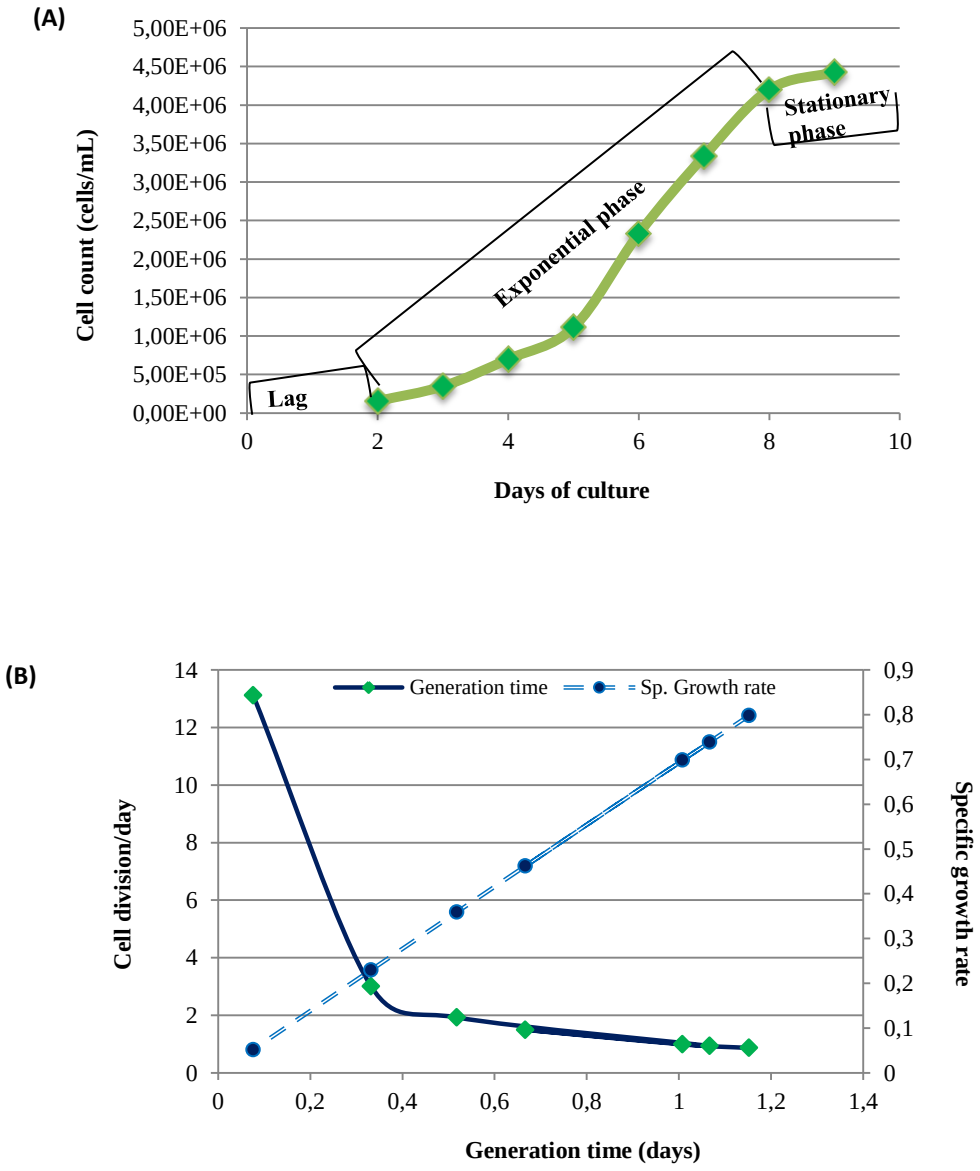


Figure 4-13 (A, B) Growth statistics of microalgae Chlorella vulgaris with 12:12h photoperiod and 4 l min-1 air aeration rate (0.84 l min-1 CO2)

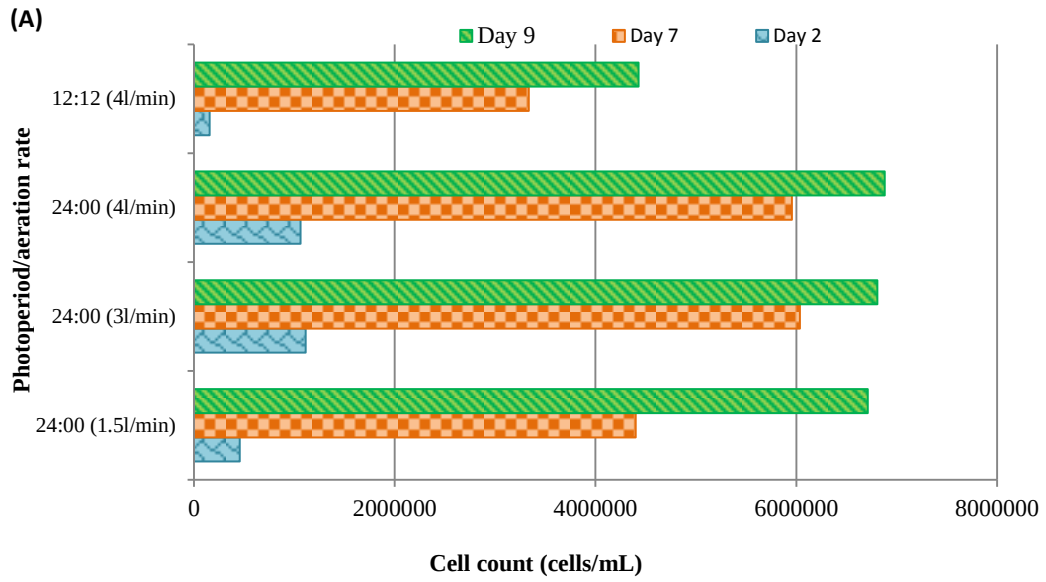
Figure 4-13A presents an exponential cell evolution phase from the 2nd day of culture until 8th day when it enters the stationary phase.

The figure demonstrates the four different life stages of microalgae: lag phase, exponential or log phase, stationary phase which leads to the final stage (death phase). The exponential phase shows a balanced growth pattern - with regular cell division by binary fission and

growth by geometric progression. The growth rate resembles the growth reported with other microalgae species which gradually increases from second day of culture (Padmanabhan & Shaleesha, 2012).

Growth rate shows a high rise in cell population of 42×10^5 cells/mL by the 8^{th} day. Figure 4-13A confirms the success of *Chlorella vulgaris* in adapting to its imposed environment and its robustness measured through a high cell count or total biomass. Balanced growth imitates a first order chemical reaction as explained in figure 4-13B. The specific growth rate of the culture is linearly related to number of cell divisions per day and generation time is the time interval required for cell population to double.

Figure 4-14 (A, B) demonstrates the influence of recurring light and dark periods on *Chlorella vulgaris* growth with varying air influx rate.



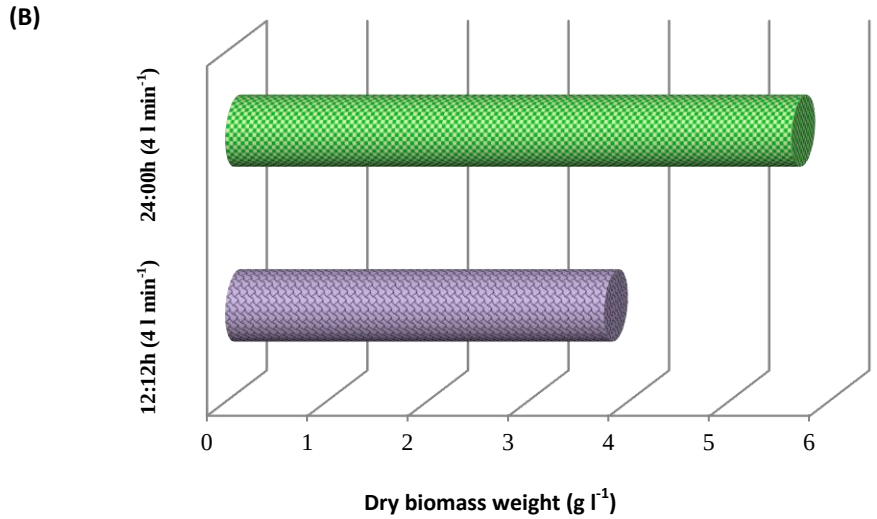


Figure 4-14 (A, B) Chlorella vulgaris growth and biomass productivity under different illumination period and aeration rates

A comparison with total cell growth concentration after 2nd, 7th and 9th day of production is plotted in figure 4-14A. Culture concentration is higher with continuous illumination (24:00h) as compared to 12:12h irrespective of the aeration rates. This result confirms previous research where continuous illumination is investigated as the best condition with respect to lipid production (Wu, et al., 2012) (Khoeyi, et al., 2012). However, total cell growth count at aeration rates 3 l min^{-1} and 1.5 l min^{-1} with 24 hours lighting period also

shows good prospect for biomass production. It can be concluded that the duration of lighting periods on algae growth can has impact on total biomass productivity. Different aeration rates have less remarkable effect on total growth. In this research, continuous illumination with 4 l min^{-1} influx resulted in maximum cell population and is the most favorable condition for attaining high biomass and biodiesel quantity. Whereas, *Chlorella vulgaris* growth on 7^{th} day with 1.5 l min^{-1} aeration rate and 24:00h photoperiod showed less interesting results compared to other aeration rates which points out the importance of higher aeration rates for better growth. Figure 4-14B compares the dry biomass weight obtained after harvesting the culture with 4 l min^{-1} inflow rate and photoperiods (12:12h, 24:00h). A total biomass concentration of 3.769 g l^{-1} was obtained with the 12:12h photoperiod and 5.637 g l^{-1} with the 24:00h photoperiod.

4.3.3 Light absorbance, transmittance and cell concentration

Absorbance is dimensionless and is the process when cells absorb light to multiply or for their growth. It is directly proportional to cell growth count or rate. In figure 4-15 the absorbance versus cell concentration per day per column is plotted. The figure displays the contrasting growth progress till the samples reached their highest growth rate.

The experimental results were obtained by culturing the same microalgae strain (*Chlorella vulgaris*) under similar conditions (same PBR but different columns) (refer section 4.3.1).

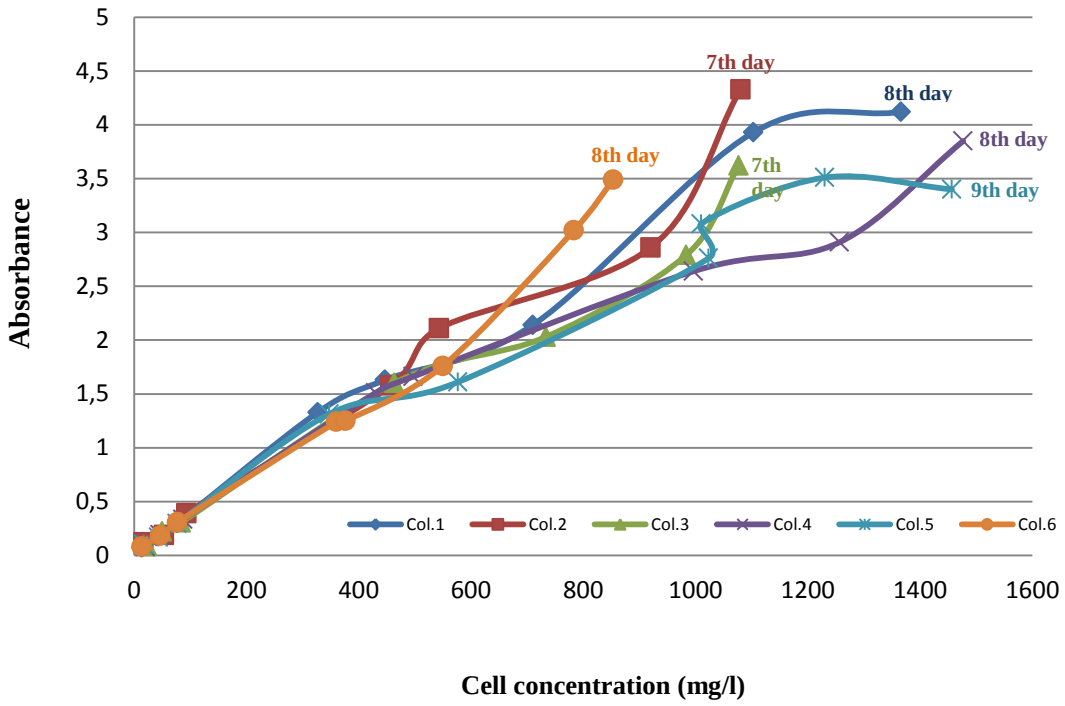


Figure 4-15 Absorbance vs. Cell concentration for different column of same culture
Data credit: Dr. Paula Gonçalves (Department of Biology, University of Aveiro)

Table 4-3 shows the highest biomass productivity obtained in every column of the PBR with the corresponding light energy absorbed by the *Chlorella vulgaris* cells.

Table 4-3 Highest biomass productivity obtained per column
Data credit: Dr. Paula Gonçalves (Department of Biology, University of Aveiro)

Column Number	ABS750	Dry biomass (mg/l)
1	4,12	1366
2	4,33	1080
3	3,62	1076,7
4	3,85	1476,7
5	3,4	1456,7
6	3,49	853,3

As discussed earlier that the proliferation of algae growth is a result of a combination of many environmental factors which includes nutrients, temperature, sunlight, mixing conditions etc. however, the combination of factors that trigger an algae cultivation is not well understood. A parameter that is optimal for one set of culture is not necessarily optimal for another. Figure 4-15 and table 4-3 clearly defines that the same strain under the same PBR can result with different biomass quantity.

On the other hand, an interesting graph illustrates the importance of light energy for better algae growth. The results used are from the experiment using *Chlorella vulgaris* cultured with 12:12 h photoperiod and 4 l/min aeration rates which brings to the concept of transmittance. It is a relationship between the amount of light that is transmitted to the detector once it has passed through the sample (I) and the original amount of light (I0) expressed (eq. 4-2)as:

$$T = I / I0 \qquad \text{(eq. 4-2)}$$

Where:

I0 = intensity of the incident light beam (W/m^2)

I = intensity of the light coming out of the sample (W/m^2)

Transmittance decreases exponentially with cell concentration while absorbance increases linearly with cell concentration obeying Beer's law which defines a relationship between the concentration of a solution and the amount of light absorbed by the solution (eq. 4-3):

$$A = \varepsilon dC' \qquad \text{(eq. 4-3)}$$

In this equation:

A = absorbance (dimensionless);

ε = molar absorptivity ($L\ mol^{-1}\ cm^{-1}$);

d = path length of the cuvette containing the sample (cm);

C´ = concentration of the compound in the solution ($mol\ L^{-1}$)

The relationship between transmittance (T) and absorbance (A) can be expressed by the following (eq. 4-4):

$$A = \log_{10}(1/T) \qquad \text{(eq. 4-4)}$$

Figure 4-16 plots a relationship of light transmittance and absorbance with respect to the growth concentration.

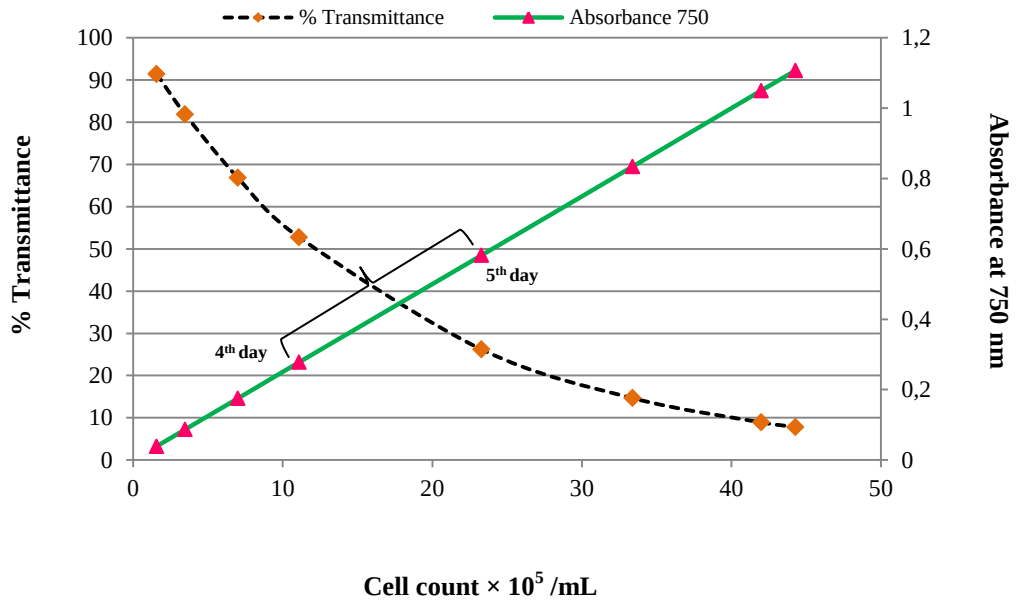


Figure 4-16 Growth statistics of *Chlorella vulgaris* with 12:12 h photoperiod and 4 l/min aeration rate

Cell growth rate shows a noticeable difference before and after 4th day of culture due to the increase in light intensity from 242.2 µmol photons m^{-2} s^{-1} to 401.4 µmol photons m^{-2} s^{-1}. The light intensity for the first 4 days was 242.2 µmol photons m^{-2} s^{-1} in order to avoid photo-inhibition but after that was raised to 401.4 µmol photons m^{-2} s^{-1} until harvest, allowing the cells to absorb more light radiation which resulted in immediate increased biomass population. As illustrated in the figure, the cells are noticed to multiply drastically between 4th to 5th days and continued to do so until the stationary phase.

These results validate the assumption that the absorptive capacity of micro-sized cells is directly proportional to its concentration which depends on light energy to convert into chemical energy for growth.

4.4 CONCLUDING REMARKS

The success in massive scale algae cultivation and its application as a feedstock for biofuel, source of nutritious food and supplements have been one of the recent debate areas. Their success will mark a milestone on the way for the algae based biofuel as industry. Managing the right species selection and their cultivation conditions can result in suitable outcome. This is the critical issue for algal producers. Algae have chlorophyll, but do not possess stem, roots, and leaves – structures that characterize land plants and can manufacture their own food through the process of photosynthesis removing huge amount of CO_2. However, they are considered to be more productive than traditional crops. Light duration and intensity directly affects the growth rate. High aeration rates do not play a decisive role on biomass production although; proper mixing of the culture is important which enables every cell to expose to light equally.

The exponential growth stages of microalgae were displayed in the results and their cultivation requires attention to achieve desired biomass production. High oil production count on two aspect: high oil content algal cells and also fast multiplying cells. Boosting growth of lower oil content cells with additional nutrients might result in high oil production but can be detrimental step to the overall economy due to the high cost of nutrients. Such a situation can be tackled by opting wastewater treatment facilities as a medium of algae culture, reducing the need for necessary nutrients, and for controlling pollution (less CO_2 emissions). Other problems encountered are contamination and low productivity. Commercial PBRs with industrial controlled growing systems can eliminate or reduce the contamination problem generating large culture volumes.

Efficient harvesting technique is required for microalgae biofuel industry. There is no universal adequate method still exists and it is an active research area. Centrifugation is considered to be expensive approach but works efficiently for small scale production. In case for large scale production designing multi-stage harvesting techniques (variety of techniques is organized in some sequence) might be profitable for maximum and efficient biomass retrieval. Algae cell characteristics are essential to determine a suitable cultivation design and convenient harvesting process.

5 BIODIESEL PRODUCTION

Harvested biomass is used for lipid extraction using different treatments and techniques. The objective of the present chapter is analysis of the quality of microalgae derived biodiesel. To start with, a literature review based on previous research on cell disruption, effective extraction approaches, chemical reaction 'trans-esterification' and the general regulations for high-quality fuel in accordance to the European standard for biodiesel (EN 14214:2003) is conducted.

The influence of input parameters, such as different microalgae strains, varying illumination periods and aeration rates, cultivation mediums (freshwater vs. wastewater), wet vs. dry biomass, different biomass to lipid extraction hypothesis, upon quality and quantity will be discussed.

Outputs parameters such as fatty acid methyl esters, linolenic acid methyl esters, iodine value, density, viscosity, were characterized to assess the trans-esterified lipids or biodiesel.

Lastly, the results of all extracted and converted biodiesel samples are presented and discussed followed by some conclusions.

5.1 LITERATURE REVIEW

After harvesting, the obtained algae slurry (biomass) needs to be treated so that the oils or lipids can be extracted fully for further conversion to final product (biodiesel). One of the drawbacks revealed is the resistance of strong algae cell wall against extraction techniques and some pretreatment is appropriate.

Sialve, et al. (2009), mentions that microalgae are a good source for biomass with high amount of lipid, protein and carbohydrate (besides many conventional energy crops) but their tough cell wall is a limiting factor which requires appropriate cell disintegration technique. Algae cell disruption is a complex and energy intensive stage in the production which consists in rupturing the cell wall allowing for the release of intracellular products such as oil, starch, ethanol and also added value compounds. Cell disruption of algal biomass can be performed by different physical methods. Three industrially relevant principal disruption methods are bead mill homogenizers, ultrasonic disintegrators and lyophilization or freeze fracturing. Proper cell breakage enhances the obtained lipid quantity from biomass, contributing to higher quantity oil production.

The basic mechanism of a 'bead mill homogenizer' is the rapid crushing action of glass or ceramic beads colliding with the cells. This method has been used for years to disrupt microorganisms and has been considered a good choice to disrupt spores, yeast, fungi, cells of cyanobacteria, microalgae etc. The bead size is important and 0.5 mm is preferable for microalgae. According to Lee, et al. (2010), bead beating using bead mill homogenizers was the most effective extraction method.
'Ultrasonic disintegrators' generates sonic pressure waves in a liquid medium forming microbubbles which collapse violently resulting in shock waves with enough energy to break the cell membranes. Although it is a widely used technique for cell disruption yet such instruments (power output usually ranges from 10 to 375 watts) generates considerable heat in the process. It has been suggested that combining ultrasound method with solvents like chloroform/methanol can result in a complete extraction of microalgae fatty compounds (Converti, et al., 2009). A study by Halim, et al. (2012), determined the

disruption efficiency of several methods on microalgae *Chlorococcum sp.*, such as high pressure homogenization (73.8% disruption), sulphuric acid treatment (33.2%), bead beating (33.2%) and ultrasonics (4.5%). A review assessed different mechanical disruption technologies inducing liquid shear, ultrasonics and freeze press suggesting that the disruption method choice is microorganism related (Chisti & Moo-Young, 1986). Another work with different cell disruption techniques (microwave heating; heating for 8 hours at 100°C; freezing overnight at -15°C; French press and ultrasonic) on algal biomass of *Nannochloropis salina* indicated that the cell disruption by heating, microwave and French press increased biogas production and the cell wall degradation rate. When compared to the untreated sample the biogas production was increased by 58% with the heating approach, by 40% when microwave was used and by 33% while using French pressing (Schwede, et al., 2011).

'Lyophilization' is a method of drying which works by freezing the material and reducing the surrounding pressure to allow the frozen water in the material to sublimate directly to the gas phase from the solid phase. But they are the most expensive drying methods and hence not recommended for large-scale production.

An ideal extraction technique needs to be lipid specific in order to minimize the non-lipid contaminants and also selective towards desirable lipid fractions such as neutral lipids containing mono, di and trienoic fatty acid chains (Halim, et al., 2011). An energetically efficient lipid extraction route is critical for successful upgrading of the process. Despite of all laboratory lipid extraction protocols available, determinant variables for microalgae lipid extraction are often misinterpreted leading to difficulties at industrial scale-up (Ronald, et al., 2012). Several extraction procedures can be found in books and articles aiming at the improvement of lipid recovery from any kind of organisms, tissues or cell types but the most popular extraction procedure for lipids is the one developed by Folch, et al. (1957). This procedure remains one of the best described and the most commonly used by lipidologists around the world. Some other procedures were proposed by Bligh & Dyer (1959), which used solvent mixtures made of chloroform/methanol and ethanol/diethyl ether.

Algae can be extracted using several procedures can be classified as: *physical extraction and chemical extraction* (Demazel, 2008). Combining these methods might result in

obtaining maximum oil yields while consumed time, energy and material for the process is minimized.

Physical extraction:

The simplest method is *mechanical crushing*. Microalgae retain their oil content in their cell, which can be 'pressed' out with an oil press. Oil presses are methods capable of extracting up to 80% of algae oil (Origin Oil, 2010). Also, the combination of mechanical pressing and chemical solvents results in a high percentage of extracted oil. Algae vary widely in their physical attributes and different oil press configurations work distinctively for different algae strains and the limitations are their high maintenance costs and energy intensity (Rodriguez, et al., 2015).

Osmotic shock is a sudden reduction in osmotic pressure for the cells to rupture in a solution and release of algae cellular components.

Ultrasonic extraction generates ultrasonic waves creating cavitation bubbles in a solvent material. Bubbles collapse resulting in shock waves that break down the cell walls. This method enhances basic enzymatic extraction without any caustic chemicals. The treatment accelerates extraction and increases the oil yield (Hielscher- Ultrasound Technology, 1999). Disadvantages include the energy intensity and the difficulties of implementing the technology at an industrial scale. Ultra-sonication on untreated wet microalgae structures increases the cell disruption rate with high power and reaction time. Power at 500 watts for 30 mins resulted in cell sizes decreasing from 2.78 μm to 1.68 μm and cell wall thickness decreasing from 0.08 μm to 0.05 μm. The increase in ultra-sonication time from 5 to 30 mins with 150 watts resulted in cell sizes decreasing from 2.72 μm to 2.38 μm and cell wall thickness first increased to a peak of 0.22 μm and then decreased (Cheng, et al., 2014). A study reported that the maximum intracellular substance released was obtained with ultrasonic energy intensity of 0.4 kWh L^{-1} (Keris-Sen, et al., 2014).

Chemical extraction:

Solvent assisted extraction included chemicals like benzene, ether and hexane for rupturing cell walls. Extraction methods with hexane, despite being reported to be less efficient than with chloroform are less toxic, have minimal affinity towards non-lipid contaminants and

have higher selectivity towards neutral lipid fractions (Geciova, et al., 2002) (Macías-Sánchez, et al., 2007). Solvents are effective in releasing up to 95% of oil but the use of caustic chemicals causes several problems.

Soxhlet extraction has some attractive advantages which might add value to the whole process scalability, requiring little training. The method involves repeated circulation of the sample bringing it into contact with fresh portions of solvent at high temperature, promoting equilibrium. Algae oil is extracted through repeated washing, or percolation, with an organic solvent such as hexane or petroleum ether. However, long extraction time and large usage of solvent hinders the system making it expensive and prone to environmental constraints. Lipid yields were improved when lipids were extracted from microalgae *Chlorococcum sp.* using soxhlet and hexane (lipid yield of batch extraction was 0.015 g lipid/g dried biomass and that of soxhlet extraction was 0.057 g lipid/g dried biomass) (Ronald, et al., 2012). A recent study has shown that the use of ultrasonic waves improves microalgae oil extraction by using methods such as soxhlet, maceration, and ultra-sonication in sequence; the combination of these processes resulted in lipid yields of 1.58%, 1.03%, and 1.77% for 18 hours, 8 hours, and 2.33 hours (Suarsini & Subandi, 2011). Despite improved lipid recovery, crude lipids from soxhlet had less polyunsaturated fatty acids due to the elevated thermal degradation in the process (Guckert, et al., 1988).

In e*nzymatic extraction*, the enzymes use water as a solvent material to degrade the microalgae cell walls. This makes the separation process easier since the use of any caustic chemicals is prevented and with low environmental impact. However, enzymatic extraction is expensive when compared to hexane extraction.

Supercritical fluids (SCF), such as carbon dioxide, tend to act as very effective solvents and are vastly used by industry, for example, for extracting caffeine from coffee beans and pharmaceutical compounds from plants. It can be called as a fluid state of CO_2 (held at or above its critical temperature and pressure) behaving like a gas in air at standard temperature and pressure (STP), or as a solid (dry ice) when frozen. If the temperature and pressure are increased higher from STP it reaches at or above the critical point, where it adopts properties midway between a gas and a liquid. At this stage CO_2 penetrates algae cells causing them to rupture and enabling the release of oil (Amaro, et al., 2011) (Mendes,

et al., 2006) (Pourmortazavi & Hajimirsadeghi, 2007) (Taylor, 1996) (Thana, et al., 2008) (Marchetti & Errazu, 2008). Supercritical CO_2 extraction is a promising green technology which may override the use of traditional organic solvents in lipid extraction methods. The economic assessment shows that biodiesel produced from the SCF technology is comparable to conventional catalytic transesterification, and is competitive with petroleum-derived fuels if waster oil is used as a feedstock (Wen, et al., 2009). Marchetti & Errazu (2008), studied different biodiesel production process using vegetable oils (with high content of FFA's) and reported that the supercritical technology is attractive alternative from a technical point of view. Lipid extraction of *Chlorococcum sp.* was performed using supercritical CO_2 resulting in lipids that decreased with temperature and increased with pressure, but no significant differences were found in the lipid profile with the varying processing conditions (Halim, et al., 2011).

Also, few new techniques using solvent assisted extraction with microwave technology have given promising results. The main benefits of these methods are the reduction of extraction time, energy and solvent used (Zlotorzynski, 1995) (Kingston & Jassie, 1989) (Kingston & Haswell, 1999). Combining methods like microwave heating and soxhlet also reduced the extraction time from 8 hours to 32 mins (Virot, et al., 2008). By far the main disadvantage of these processes seems to be the high costs associated with both the required equipment and the operation.

Some chemical alteration (such as trans-esterification, pyrolysis and emulsification) should be done to the lipid structure before they are ready to use as a fuel and, the trans-esterification reaction is, by far, the most used and important method to produce cleaner and environmentally safe fuel. *Trans-esterification* is a chemical alteration of extracted lipids to fatty acids using an alcohol and a catalyst. Biodiesel is briefly defined as a long chain of alkyl (methyl, ethyl, and propyl) esters of vegetable or animal fat. Extracted fatty acid esters cannot be classified as biodiesel until they meet the EN 14214 (European Standard EN 14214:2008+A1, 2009) quality specifications in Europe, and the ASTM D6751 in the USA.

An ester group reacting with an alcohol in presence of a catalyst is the basic chemistry of a trans-esterification reaction. In the following figure, the organic group R is an alcohol and

the R′, R″, R‴ are esters. For the purpose of biodiesel production from microalgae, lipid extraction is followed by a reversible reaction named trans-esterification or alcoholysis which results in biodiesel and glycerol (Sharma & Singh, 2009). Figure 5-1 depicts the most common reaction to obtain biodiesel from TAG through trans-esterification reaction. Trans-esterification reaction is quite sensitive to various parameters, namely the type of catalyst, reaction temperature, etc.

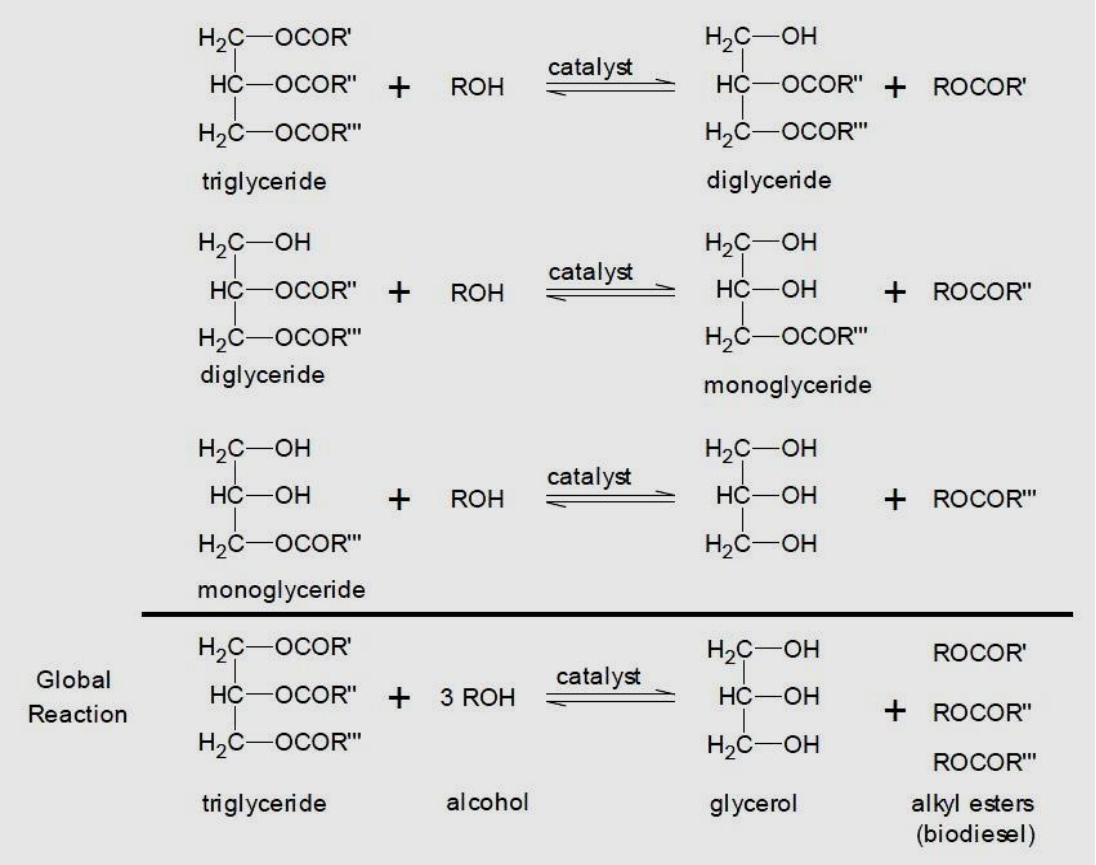


Figure 5-1 Trans-esterification reaction for biodiesel production (Afonso, 2009)

Biodiesel production contains FFA's, phospholipids, sterols, water, odorants and other impurities. For this reason, it is not recommended to use lipids directly as a fuel.

Variables such as FA composition and FFA content of the oil are dependent on the trans-esterification reaction. Previous research emphasizes that mechanical stirring results in better yield compared to magnetic stirring (Sharma & Singh, 2008). Parameters like Saponification Number (SN), Iodine value (IV), Cetane Number (CN), among others, determine the acceptability of the oil for the trans-esterification reaction.

Some important variables influencing the trans-esterification reaction are:

Catalysts:

Sulphuric acid (H_2SO_4) is the most commonly used acid catalyst whereas sodium hydroxide (NaOH) and potassium hydroxide (KOH) are used as alkaline catalysts. Acid catalysts are slow reaction but are insensitive to FFA and water content of the extracted lipid and they are easily separated from the final product. On the other hand, alkaline catalysts are relatively cheaper and easily available and they are faster than acid catalyzed transesterification, but involve sensitivity to FFA content of the extracted lipid and also prone to saponification (Lam, et al., 2010). Among KOH and NaOH, research reported higher yield with KOH (91.67%) as compared to that obtained with NaOH (85.9%) (Vicente , et al., 2004). Previous study by Karmee & Chadha (2005), states that with KOH (homogenous catalyst), 92% yield was obtained under optimum conditions in 1.5 hours reaction time; whereas, yield of 83% was achieved with ZnO (heterogeneous catalyst) in 24 hours reaction time. Homogenous catalysts (which are in the same phase as the reactants) have few disadvantages in their application while heterogeneous catalysts (which are in a different phase from the reactants) have been tried to overcome the drawbacks of homogenous catalysts (like magnesium oxide, calcium oxide etc.). FFA is saturated or unsaturated monocarboxylic acids naturally occurring in oils. A high FFA value leads to high acid value. To use alkaline catalyst for trans-esterification, FFA should be within a desired limit (ranging from 0.5% to < 3%). Previous research confirms this level of FFA (Zhang, et al., 2003) (Fangrui & Milford, 1999) (Freedman, et al., 1984) (Tiwari, et al., 2007) (Ramadhas, et al., 2005) (Sahoo, et al., 2007) (Canakci & Gerpen, 1999). Trans-esterification reaction is a one-step process for oils with FFA within the range and a two-step procedure for oils with FFA higher than the range, since it requires pretreatment.

Previous research confirms that a water content lower than 0.1% decreases the conversion of ester to a significant extent (Canakci, 2007). According to Meher, et al. (2006), the catalysts and unused solvents were in the lower glycerol layer whereas some of the catalysts, solvents and glycerol were in the upper biodiesel layer. After separating the layers and purifying (via several hot water washes) the moisture from washed biodiesel can be removed using anhydrous sodium sulphate (Na_2SO_4).

*Molar ratio: T*he commonly employed molar ratio for a 2-step reaction is 6:1 for acid trans-esterification and 9:1 for alkali catalyzed trans-esterification. For a single step trans-esterification reaction, the 10:1 molar ratio has been used frequently, although an optimum molar ratio may vary from 6:1 to 13:1 (Sharma, et al., 2008).

After 'trans-esterification', the resultant FAME undergoes a purification process in order to respect the minimum required contents for free glycerol, soap, metals, alcohol, free fatty acids, catalyst, water and glyceride established by the European and American standards. A high presence of these compounds in the biodiesel strongly affects the fuel properties, engine life and performance. The FAME quality testing of the extracted microalgae lipids ensures their potential for biodiesel production. An analysis based on the FAME, saturated and unsaturated methyl ester composition, LAME and IV can be used to predict the critical parameters and the overall potential of the biodiesel. Technologies used to extract, transform and purify the feedstock into oil not only determine whether they meet the fuel standard but also the condition of the produced quantity of free and bonded glycerin, which in return defines the purity and quality of the biodiesel. One of most commonly used purification method of the FA esters is vigorous washing with warm water. This technique is efficient in removing methanol, glycerol, sodium compounds, free fatty acid esters and soaps (Berrios, et al., 2011).

The regulations for the quality of the biodiesel produced in accordance with the European biodiesel quality specification EN 14214:2003 for European markets are explained below (eq. 5-1, 5-2, 5-3). Within standard EN 14214, the test-method EN 14103 defines FAME and LAME contents required to assess the quality criteria of biodiesel production.

FAME is calculated using the formula:

$$C = \frac{\sum A - A_{IS}}{A_{IS}} \times \frac{C_{IS} \times V_{IS}}{m} \times 100\% \quad \text{..... (eq.5-1)}$$

Where:

C = Fatty acid methyl esters (FAME)

$\sum A =$ total peak area of fatty acids $C_{14:0}$–$C_{24:1}$;

A_{IS} = internal standard (methyl heptadecanoate) peak area;

C_{IS} = concentration of the internal standard solution, in mg/mL;

V_{IS} = volume of the internal standard solution used, in mL;

m = mass of the sample, in mg.

To calculate the retention times of the fatty acid methyl esters, a FAME standard needs to be run. The method of analysis uses the sample mixture to determine the response and retention time of each component experimentally. The results are then based on an area percentile and the final value is calculated using equation 5-1.

According to test-method EN 14103, the result for the total FAME content should be greater than 90% (Ruppel & Huybrighs, 2008). Also, the oil extraction method can directly influence the fatty acid profile depending on the efficiency of polar and neutral lipids extraction (Wang & Wang, 2012). Charge distribution of neutral or non-polar lipids is evenly distributed. Non-polar molecules do not dissolve well in water; in fact, polar and non-polar molecules tend to repel each other as oil and water and will separate from each other even if they are shaken vigorously in an attempt to mix them.

LAME is calculated by:

$$L = \frac{A_L}{\sum A - A_{IS}} \times 100\% \quad \text{..... (eq. 5-2)}$$

Where:

L = Linolenic acid methyl esters (LAME)

$\sum A$ = total peak area $C_{14:0} - C_{24:1}$;

A_{IS} = internal standard (methyl heptadecanoate) peak area;

A_L = linolenic acid methyl ester peak area.

Total linolenic acid ($C_{18:3}$) content should be higher than 1% and lower than 15% (Wang & Wang, 2012) (Munari, et al., 2007).

IV is the mass of iodine in grams absorbed by 100 grams of a chemical substance and is a measure of total unsaturation compounds in the fatty acids. The EN14214 specification allows a maximum of 120 for the iodine number (Schober & Mittelbach, 2007).

The iodine value is obtained from:

Iodine value = X g iodine / 100 g sample	……..…… (eq. 5-3)

The most common topic related to the iodine value of biodiesel is the oxidation stability, because the oxidation process affects fuel quality (Sahoo, et al., 2007).

5.2 METHODS

This section describes the methodologies used for lipid to FAME conversion. The extracted oil was subjected to acid trans-esterification and its quality was analyzed.

5.2.1 Harvested biomass to FAME

To account for the impact of wet biomass (algae gathered, centrifuged and eventually transformed into a wet pastelike substance) and dry biomass (the pastelike algae slurry is dried) on the quality of the final product, this thesis includes an analysis of this extractions conducted in parallel for microalgae *Chlorophyta* and *Scenedesmus* biomass. Table 5-1 presents the strains and the biomass type used for this analysis.

Table 5-1 Wet/dry biomass for lipid extraction

Strain	Wet biomass	Dry biomass
Chlorella vulgaris	x	x
Scenedesmus	x	x
Chlorophyta	x	x
Nannochloropsis gaditana		x

The wet extraction process adopted experimentally was adapted from the method used by Bligh & Dyer, (1959). Such method reports benefits simultaneous lipid extraction and phase separation during the process (Bligh & Dyer, 1959). In previous research, reports mentioned the use of methanol to improve the lipid content by a better separation of inorganic and organic phases; also, chloroform–methanol were chosen as the lipid extractant albeit its negative environmental and health impacts (Widjaja, et al., 2009) (Converti, et al., 2009) (Smedes & Thomasen, 1996). Many reports are available concerning the production of lipid from wet microalgae (Chisti, 2007) (Widjaja, et al., 2009) (Sharma & Singh, 2009) (Hu, et al., 2008) (Petkov & Garcia, 2007).

For the dry extraction process the microalgae biomass were lyophilized or freeze-dried. Freeze-drying is a dehydration process used by freezing the samples by reducing surrounding pressure to sublimate directly from the solid phase to the gas phase.

Algae cells were ruptured with an ultrasound and the released lipids were mixed with solvents which were later evaporated using a rotary evaporator or a water bath at around 70ºC to obtain the crude lipid. Figure 5-2 shows the water bath used for solvent evaporation during the experimental part phase.

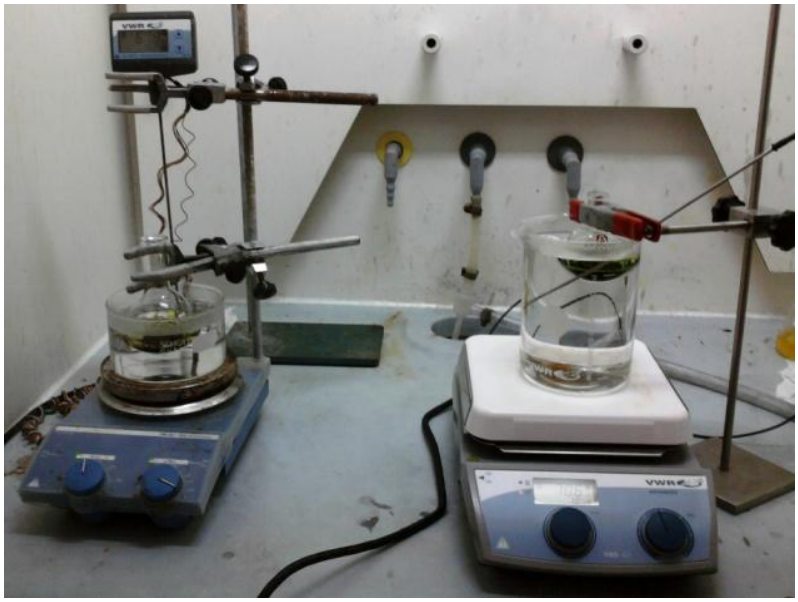


Figure 5-2 Evaporation of the liquid phase in a water bath
(Photo credits: Smritikana Dutta, University of Aveiro)

The extraction process included *magnetic stirring*, *vacuum filtration*, *ultrasound* and *evaporation* and after trans-esterification, the biodiesel was characterized by gas chromatography.

Microalgae tissues were homogenized with chloroform/dichloromethane: methanol (2:1). The lipid samples were reacted with methanol containing 6% m/m anhydrous HCl at 60°C for 6 hours. Solvent to lipid ratio used was 30:1 (v/m) (Dutta, et al., 2011). Hexane was used as a catalyst to recover the FAME separating the organic and inorganic phases.

Figure 5-3 shows the separation of the phases. The density of the organic phase is lower than water density so the organic phase stays up. The organic phase was collected for further calculations and the inorganic phase was discarded. Warm distilled water was used to purify the mixture by washing vigorously until the pH of the organic phase becomes neutral. The organic phase was further analyzed for FAME characterization.

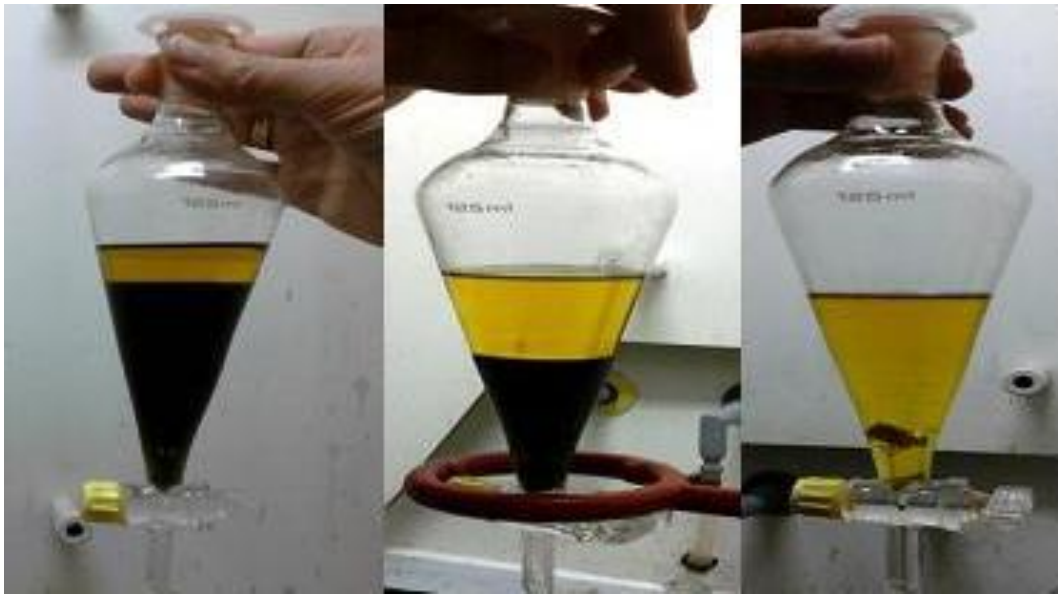


Figure 5-3 Separation of phases
(Photo credits: Smritikana Dutta, University of Aveiro)

Purification of the obtained FAME (with several hot water washes) followed by the evaporation of solvent hexane (at ~55°C) resulted with the final outcome. Figure 5-4 displays the obtained pure biodiesel.

Figure 5-4 Pure biodiesel extracted from microalgae in Aveiro
(Photo credits: Smritikana Dutta, University of Aveiro)

Finally, after trans-esterification, all biodiesel samples were ready for the qualitative analysis (Petkov & Garcia, 2007). FAME analysis was performed in a gas chromatograph

with a split injection into an analytical column with a polar stationary phase and a flame ionization detector. A gas chromatograph (Varian 3800 GC) equipped with flame ionization detector (FID) and thermal conductivity detector (TCD) in series, and a capillary 1079e VICE pneumatic valve (with oven) gas injector was used for quality assessment. Figure 5-5 shows the GC used for the characterization of the produced biodiesel and table 5-2 presents its operating conditions.

Figure 5-5 Gas Chromatography Varian 3800 (Photo credits: Smritikana Dutta, University of Aveiro)

Table 5-2 Parameters of Gas Chromatography

Gas Chromatograph (Varian 3800)	**Conditions**
Inlet temperature	220^{o} C
Column flow	2 mL/min
Injection volume	0.5 µL
Oven program initial temperature	50^{o} C
Ramp	6^{o} C/min
Oven program final temperature	200^{o} C
Hold time	5 min
Column	DB1-ht, 15 m, 0.32 mm x 0.1 µl

The samples were subjected to a silylation procedure {using 30µL oil, 100µL pyridine, 100µL bistrimethylsilyltrifluoroacetamide (BSTFA) and 50µL trimethylchlorosilane (TMCS), 70ºC, and 30 minutes} before injecting in the GC. Silylation is one of the most practiced derivatization technique (where a chemical compound is transformed into a product). Functional groups like hydroxyl, carboxylic acid, amine, thiol, phosph that present a problem with GC detection can be derivatized using silylation reagents. This involves the replacement of acidic hydrogen with an alkylsilyl group, e.g,-$SiMe_3$. Such derivatives results in products which are less polar, more volatile and thermally more stable. The silylating reagent is usually selected based on its reactivity and selectivity towards the compound, the intended application, the stability of the derivative, and its abundance/nature of reaction byproducts.

Methyl heptadecanoate ($C_{18}H_{36}O_2$) was used as an internal standard ($C_{17:0}$). The fatty acids were identified comparing the retention time of standards from the GC peaks.

5.2.2 Extraction techniques on wastewater and freshwater cultures

The main purpose of this section is to describe the methodology used for extracting lipid from microalgae cultured in freshwater and wastewater sources and the quality analysis of the lipid transformed to biodiesel.

Chlorella vulgaris was cultivated at Vigo, Spain in medium with:
(a) Freshwater and
(b) Wastewater (WW)

Lipids were extracted from the dried biomass separately at University of Aveiro with two different extraction methods:
(a) 3-stage cross current/ultrasonic extraction and
(b) Soxhlet extraction

The water used for the microalgae growth from the wastewater treatment plant of Guillarei (Spain) was analyzed and the results are shown in table 5-3. The final composition of the culture medium used in order to maintain the growth of *Chlorella vulgaris* is illustrated in table 5-4.

Table 5-3 Analysis of wastewater samples [Prof. Jesus Torres, data provided from experimental results obtained at Vigo University (personal communication)]

Analyzed Parameters	Methods of analysis	Units	Guillarei samples
pH	PE-04 Electrometer	pH unit	6.2
Temperature (in situ)	PE-04 Electrometer	ºC	20.4
Total solid	PE-58 Gravimeter	mg/L	312
Dissolved solids	PE-37 Gravimeter	mg/L	312
Volatile dissolved solids	PE-37b Gravimeter	%	27
Suspended solids	PE-06 Gravimeter	mg/L	< 2
Settleable solids	PE-55 Imhoff Cone	mL/L	<0.1
BOD_5	PE-02 Oxymeter	mg O_2/L	18.2
Total organic carbon	PE-77 C Analyzer	mg C/L	6.4
COD	PE-01 Volumeter	mg O_2/L	34
Total nitrogen	PE-79 N Analyzer	mg N/L	14.9
Organic nitrogen	PE-78 Kjeldhal	mg N/L	0.6
Ammonia nitrogen	PE-05 Selective Electrode	mg N/L	0.8
Nitrite	PE-29 VIS Spectrophotometer	mg N/L	0.02
Nitrate	PE-28 VIS Spectrophotometer	mg N/L	13.5
Organic phosphate	PE07b VIS Spectrophotometer	mg P/L	0.2
Inorganic phosphate	PE-07 VIS Spectrophotometer	mg P/L	2.6
Alkalinity	PE-11 Volumeter	mmol/L	1.2
Conductivity	PE-03 Electrometer	µS/cm	411
CDOM	PE-90 VIS Spectrophotometer	m^{-1}	16.4
Sulphates	PE-38 Nephelometer	mg/L	31.6
Chlorides	PE-17 Volumeter	mg/L	69.3
Oils and fats	PE-43 Gravimeter	mg/L	< 2
Total coliform	PE-49 Membrane filtration	UFC/100mL	440
VOCs	PE-68 P&T CG/MS	µg/L	< 0.01

Table 5-4 Composition of the culture medium used for maintaining the strain [Prof. Jesus Torres, data provided from experimental results obtained at Vigo University (personal communication)]

Stock	g/L stock	Final concentration in the medium (µM)
$NaNO_3$	27.2	320
KH_2PO_4	2.72	20
$CaCl_2$	2.5	170
$MgSO_4$	7.5	304
NaCl	2.5	428
H_3BO_3	11.42	185
$NaHCO_3$	15	208
$Na_2EDTA\ 2H_2O$	4.36	11.7
$FeCl_3\ 6H_2O$	3.15	11.7
$ZnSO_4\ 7H_2O$	8.82	30.7
$MnCl_2\ 4H_2O$	1.44	7.28
Na_2MoO_4	0.71	0.9
$CuSO_4\ 5H_2O$	1.57	6.29
$CoCl_2\ 5H_2O$	11.9	0.05
K_2CrO_4	1.94	0.01

Lipid extraction treatment of the dried biomass via 3-stage cross current continuous and soxhlet extraction techniques was carried out as described below:

a) <u>3-stage cross current solvent extraction:</u>

This extraction scheme was conducted by adding fresh solvent to every stage of the reaction and the corresponding extract is obtained and used in order to maximize the total lipid extraction from same quantity of dry biomass.

The method is illustrated in figure 5-6. 5 gram of dry and pulverized *Chlorella vulgaris* tissues were homogenized at every stage with 25 ml of dichloromethane: methanol (2:1, v/v) solvent.

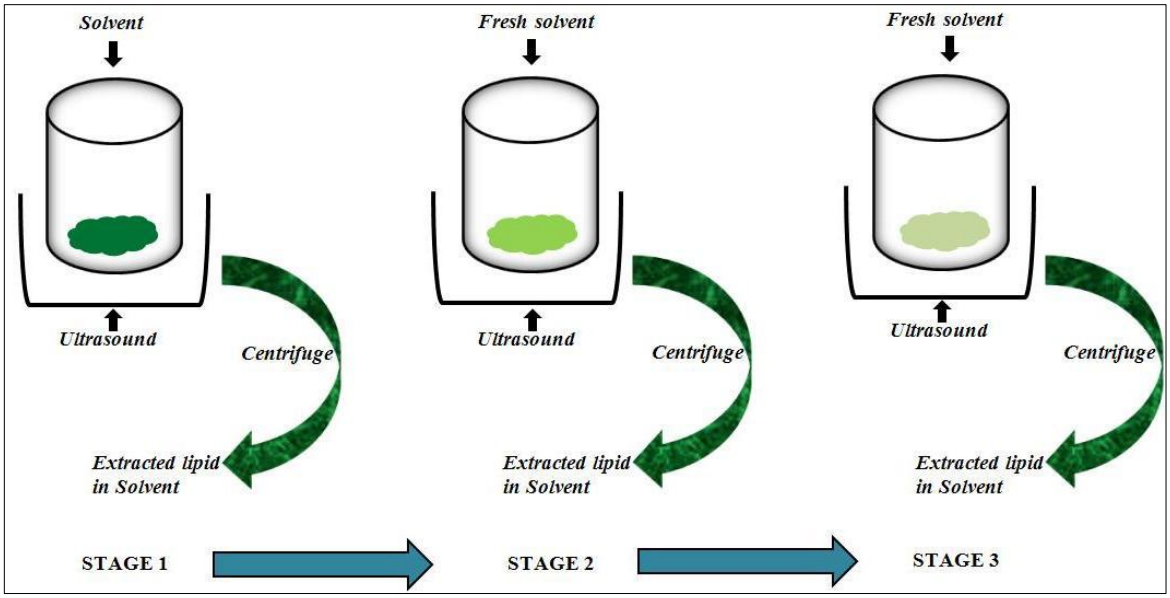


Figure 5-6 3-stage cross current continuous extraction procedure flowchart diagram

Each stage was followed by 60 minutes of ultra-sonication using a Branson 5510E-DTH system, with an ultrasonic processor of 42 kHz, 135W. The extract was centrifuged at 3000 rpm for 10 minutes. At the end of each stage, the total extracted lipids were separated and the biomass was fed with fresh solvent and the process was repeated all over again. The experiment was replicated and the final result was averaged.

b) <u>Soxhlet extraction:</u>

This method involves lipid extraction from solid material by repeated washing or percolation using organic solvents (Figure 5-7).

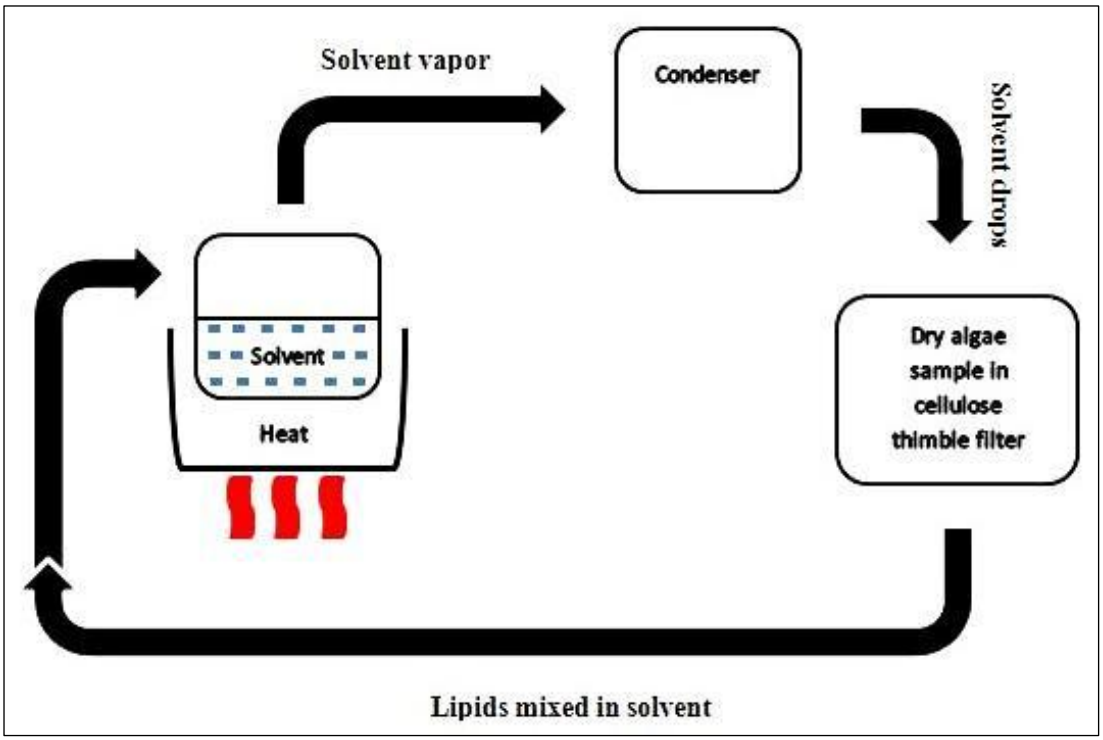


Figure 5-7 Soxhlet extraction procedure flowchart diagram

A continuous flow soxhlet extraction method was used with 5 gram of dry pulverized *Chlorella vulgaris* samples with 250 ml of dichloromethane: methanol (2:1, v/v) solvent. The extraction process lasted for 270 minutes. A previous study with continuous soxhlet method confirmed that this method is considered more effective than other processes (Dutta, et al., 2011). The experiment was performed simultaneously with two soxhlet extractors placed side by side (Figure 5-8).

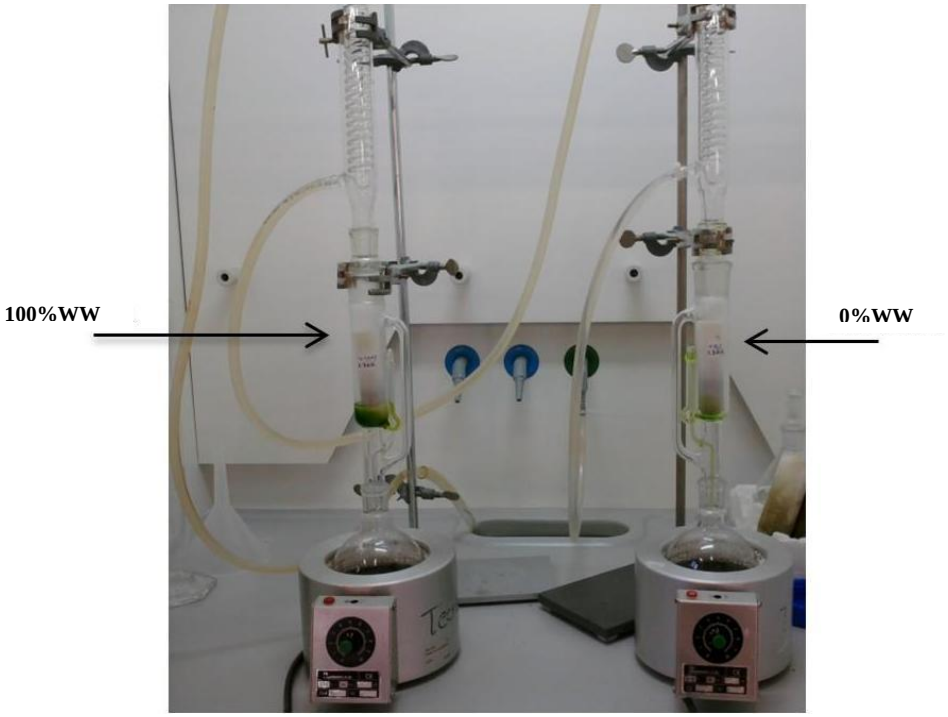


Figure 5-8 Soxhlet extraction for biomass samples (Photocredit: Smritikana Dutta, University of Aveiro)

A rotary evaporator at 90 rpm and 50°C was used to separate the lipids from solvents. Samples were left in the oven at 50°C to make them completely solvent free. Figure 5-9 shows a picture of the hexane recovery using a reflux condenser column after lipid extraction.

Figure 5-9 Chemical solvent hexane recovery after microalgae lipid extraction (Photocredit: Smritikana Dutta, University of Aveiro)

5.2.3 Determination of fuel quantity and property

This experiment was executed mainly to determine the quantity of pure biodiesel from the dry microalgae biomass. Microalgae *Nanochloropsis gaditana* grown in PBRs at Almería University was used for this test and the lipid extraction was performed by the soxhlet technique. The acid trans-esterified FAME was characterized by GC. Water content of the biodiesel was detected and the density and viscosity of the extracted biodiesel was measured with a Stabinger Viscometer SVM 3000.

5.3 EXPERIMENTAL RESULTS AND DISCUSSION

The qualities of the obtained biodiesel are shown here.

5.3.1 Biodiesel quality from wet and dry microalgae biomass

- ***Chlorella vulgaris*:**

  Results presented in figure 5-10 and table 5-5 depicts the total FAME profile of wet-route biodiesel extraction from *Chlorella vulgaris*, and the LAME and iodine values according to the European standard.

  Column 1 & 2 are representing the quality of algae culture in the same PBR (with same culture environment) to ensure biomass quality equilibrium.

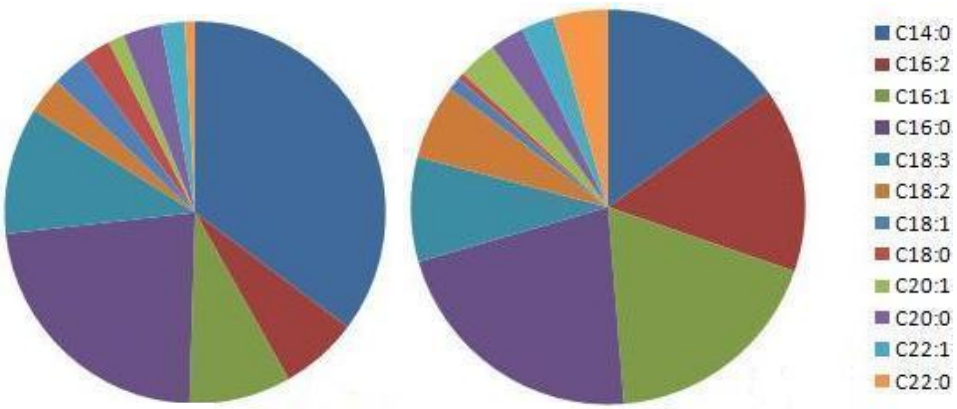


Figure 5-10 FAME profile of wet biodiesel extraction from *Chlorella vulgaris*
a) Column 1 & b) Column 2 of the PBR used

The total lipid content of micro-algal oil is a mixture of saturated and unsaturated fatty acids (Meng, et al., 2009). With such unique profiles of fatty acid esters, the results showed higher quantity of saturated long chain such as palmitic (C16:0), (C18:0) acids and also higher percentage of monounsaturated compounds especially oleate acid (C18:1) and palmitoleic acid (C16:1). Total FAME content is approximately 95%.

Table 5-5 LAME and Iodine value percentages of Column 1 and Column 2

Sample	LAME (%)	IODINE VALUE
Column 1	13	88.3
Column 2	9.4	59.3

LAME and IV percentages are in accordance with the European biodiesel standard. Results confirm that *Chlorella vulgaris* grown with fresh-water with an appropriate growing method and correct extraction procedure has the potential to produce biodiesel in accordance with the requirements for the European market (Coelho, et al., 2013).

- ***Chlorophyta and Scenedesmus*:**

  Results reported in figures 5-11, 5-12 and 5-13 compares the FAME profiles for *Chlorophyta* and *Scenedesmus* extracted with the wet and dry process routes.

  The two different strains were cultured parallely (3 columns cultured with *Chlorophyta* and the other 3 columns cultured with *Scenedesmus*) using same culture condition. Analysis of FAME confirms the feasibility of lipid extraction from wet algae and their conversion to biodiesel. Both the microalgae strains have high and almost equal potential for biodiesel production.

  The results also show that, the dry extraction techniques are more effective in producing high-quality biodiesel, irrespective of the type of microalgae as shown on a previous publication (Coelho, et al., 2013).

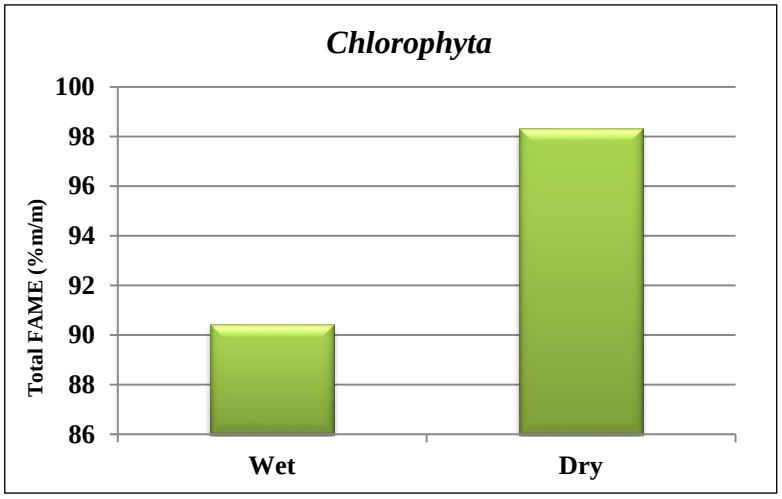


Figure 5-11 : FAME profile comparison for microalgae strain *Chlorophyta* by wet and dry biomass

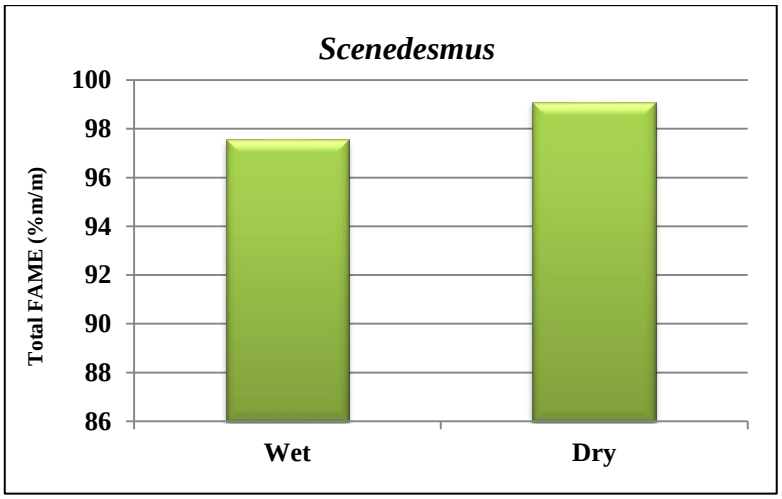


Figure 5-12 FAME profile comparison for microalgae strain *Scenedesmus* by wet and dry biomass

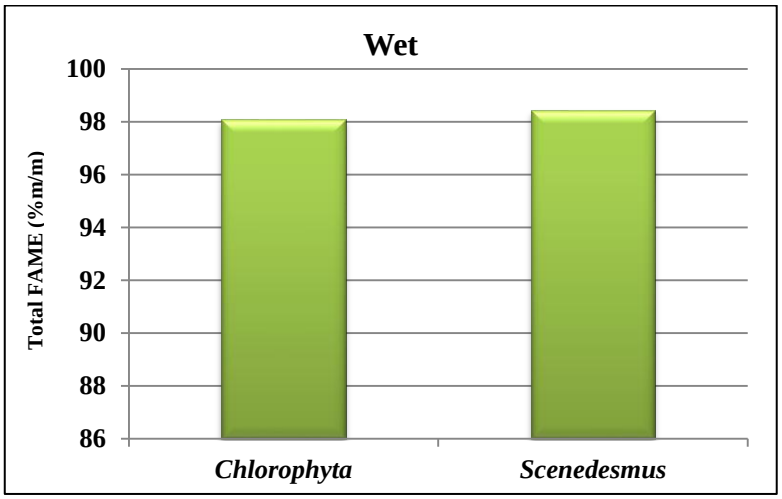


Figure 5-13 FAME profile comparison for microalgae strains *Chlorophyta* and *Scenedesmus* by wet biomass

Results on LAME content and IV also confirm that both strains have high potential according to the European standard (Table 5-6).

Table 5-6 LAME and Iodine value percentages for *Chlorophyta* and *Scenedesmus*

Sample	LAME (%)		IODINE VALUE	
	Wet	*Dry*	*Wet*	*Dry*
Chlorophyta	5.6	5.3	48.4	48.8
Scenedesmus	2.4	2.0	34.1	51.6

The compositions of each extracted lipid samples were plotted in an integrated tri-plot graph (Figure 5-14) representing 100% of monounsaturated, polyunsaturated and saturated methyl ester composition respectively plots. According to the biodiesel quality criteria specified in the European standard EN14214: the yellow zone (right) represents good cetane number and iodine value. On the region on the left, yellow: a good cold filter plugging point is obtained; the green one represents the set of properties which allow for biodiesel that meets the standard; the orange region contain the set of properties which are not appropriate for biodiesel production (Ramos, et al., 2009).

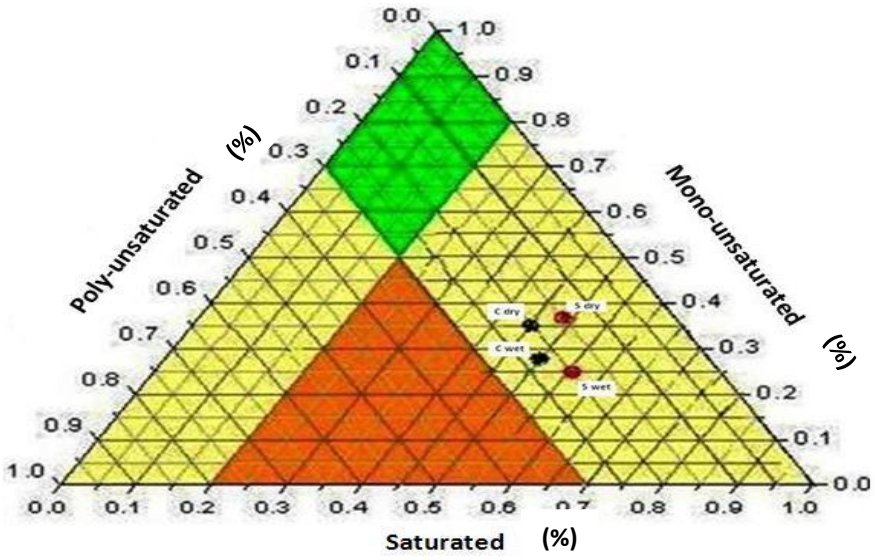


Figure 5-14 Distribution of monounsaturated, polyunsaturated and saturated methyl esters – *Chlorophyta* and *Scenedesmus*

The results obtained are contained in the area that meets the limit of the cetane number and iodine value (yellow, right). These results show a high content of saturated methyl esters, according to EN 14214 (Coelho, et al., 2013). It can be concluded that both *Chlorophyta* and *Scenedesmus* microalgae offer high potential for biodiesel production if an appropriate extraction method and proper execution of the procedure is followed. The dry process produces better results than the wet extraction process.

- ***Nannochloropsis and Scenedesmus*:**

  The total FAME and LAME values of dry microalgae cultivated and lipid extracted and trans-esterified are presented below. These samples were cultured in Almería region and were extracted in Aveiro. The distribution of monounsaturated, polyunsaturated and saturated methyl esters are plotted in a tri-graph (Figure 5-15).

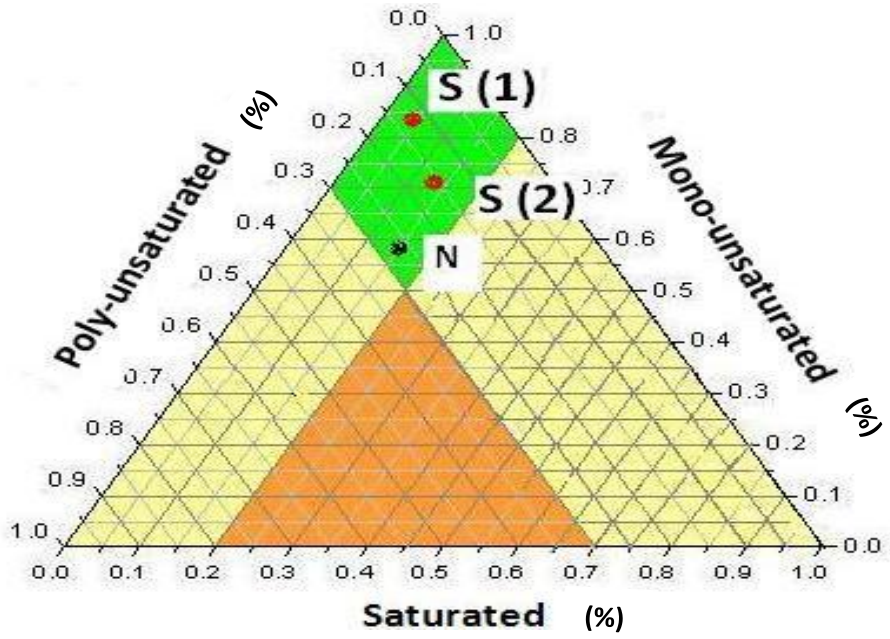


Figure 5-15 Distribution of monounsaturated, polyunsaturated and saturated methyl esters – *Nannochloropsis* (N) and *Scenedesmus* (S1, S2: two different samples of same algae *Scenedesmus*)

Figure 5-15 shows good capability for biodiesel production of all the strains, where N, S(1), and S(2) are named to identify the microalgae strains (*Nannochloropsis gaditana* and *Scenedesmus*).

The quality potential is achieved by comparing the obtained results with the standards limits present in the European norms. Total FAME and LAME percentages are presented below in Table 5-7.

Table 5-7 LAME and FAME values of microalgae strains

SAMPLE	Total FAME (%)	Total LAME (%)
Nannochloropsis gaditana [N]	99.6	10.2
Scenedesmus [S(1)]	100	4.5
Scenedesmus [S(2)]	99.9	7.5

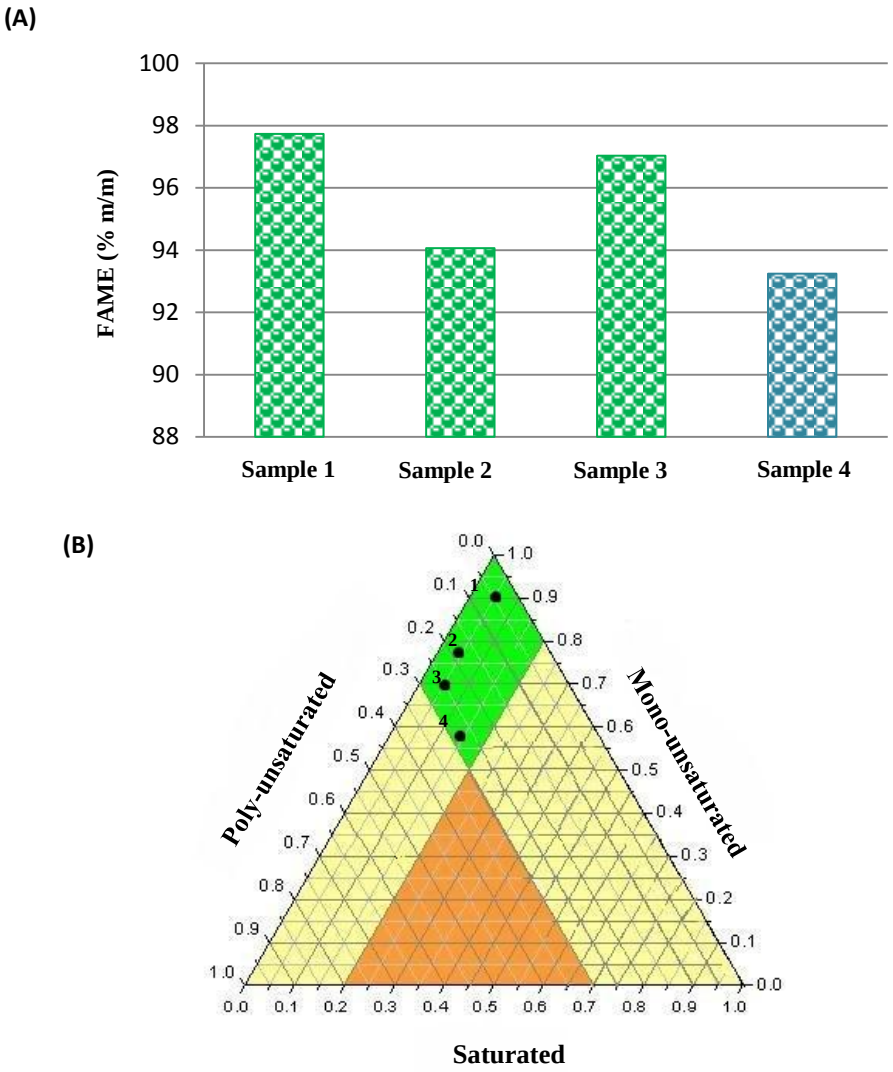
5.3.2 Effect of photoperiod and aeration rates on biodiesel quality

Table 5-8 explains the conditions of the extracted biodiesel samples from *Chlorella vulgaris*. Figure 5-16 (A, B) illustrates the quality of extracted biodiesel from *Chlorella vulgaris* according to the varying illumination and aeration rates (refer section 4.3.2, Chapter 4).

Table 5-8 Photoperiod and aeration rate conditions

	Sample 1	Sample 2	Sample 3	Sample 4
Photoperiod (24:00h)	x	x	x	
Photoperiod (12:12h)				x
Aeration rate (1.5 l min^{-1})	x			
Aeration rate (3 l min^{-1})		x		
Aeration rate (4 l min^{-1})			x	x

Total FAME, saturated and unsaturated acids under different photoperiods and aeration rates are plotted. Figure 5-16A shows that the total FAME values comply with the standard, confirming their potential as biodiesel as feedstock. The cultures with continuous illumination period (24:00h) show better results than with 12:12h illumination period. Sample 1 and sample 3 have FAME content higher than 97%, sample 2 FAME content is 94% while sample 4 has the minimum FAME percentage of 93%. Figure 5-16B represents the distribution of methyl ester composition of each extracted lipid sample. All samples contained more than 50% monounsaturated methyl ester and a low content of polyunsaturated methyl ester.



Triangular graph: Sample **1**, Sample **2**, Sample **3**, Sample **4**

Figure 5-16 (A, B) Quality analysis of microalgae *Chlorella vulgaris* grown under different photoperiod and aeration rates

LAME and iodine values are showed in table 5-9.

Table 5-9 LAME content and iodine value calculated for biodiesel from *Chlorella vulgaris* cultured with different photoperiod and aeration rates

	Sample 1	Sample 2	Sample 3	Sample 4
LAME (%)	1.3	4.6	3.9	4.6
IODINE VALUE	76.8	105.3	105.8	116.4

All the samples are in accordance to the EN14214 standard (Gouveia, et al., 2009). Sample 4 (high IV) shows the oil to be highly unsaturated. Hence it is expected to have a low cloud point which is a positive feature for utilization under winter conditions while diminishing its oxidation stability. Sample 1 (low IV) has a high cloud point and better oxidation stability.

5.3.3 Influence of using wastewater and freshwater algae on final quality

The ensuing results compare the influence on the lipid quality of green microalgae *Chlorella vulgaris* with fresh water (0% WW) and wastewater (100% WW) used in the culture (refer section 5.2.2). Lipids were extracted and trans-esterified in Aveiro. Two different extraction methods were used to conduct this experiment and the results obtained are illustrated below.

a) <u>3-stage cross current continuous extraction method</u>

This method was obtained by adding fresh solvent to every stage of the reaction to obtain high lipid quantity from the same quantity of biomass. FAME yield by microalgae weight was obtained by plotting the FAME content obtained after trans-esterification according to the type of water used during biomass production. Figure

5-17 presents a comparison of the FAME yield percentages by weight for 0%WW and 100%WW samples at every stage of extraction.

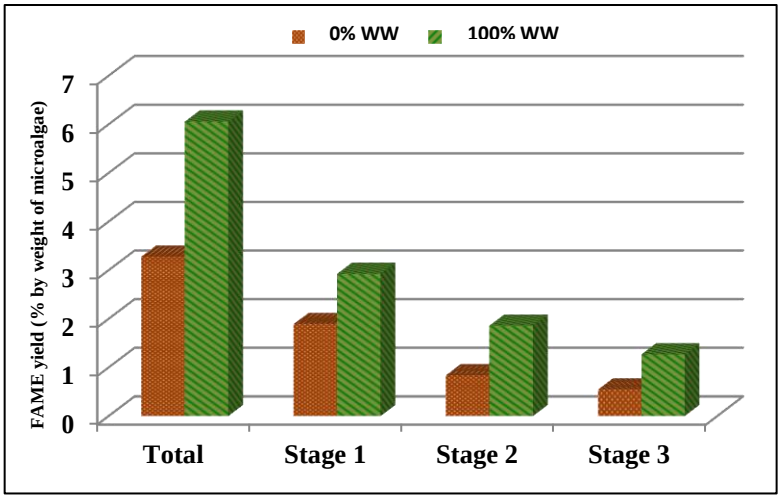


Figure 5-17 FAME yield by weight of algae with 0%WW and 100%WW *Chlorella vulgaris*

A similar pattern in the FAME yield was observed for both samples. A decrease in FAME yield explains the weight loss of biomass occurred at each stage of the extraction method. The results from figure 5-17 follow an inverse exponential curve. Total lipid is directly proportional to the amount of biomass used for extraction although the differences in extracted FAME percentages are big. 100%WW samples offer higher FAME yield which suggests that they constitute a better raw material than 0%WW samples grown with fresh water.

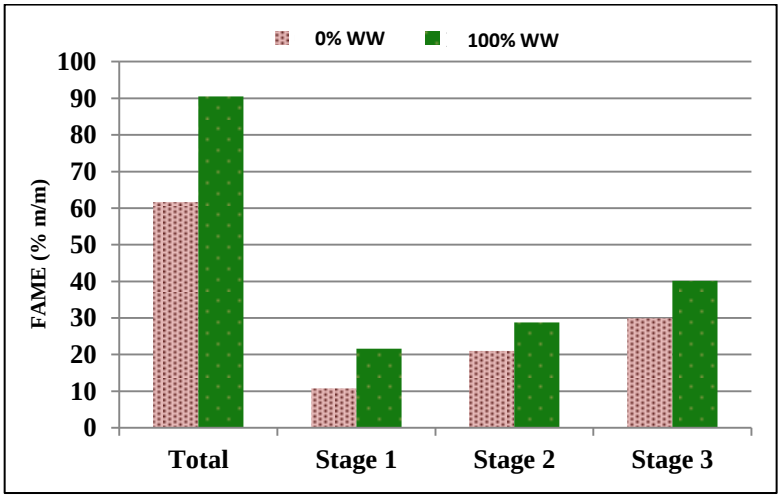


Figure 5-18 FAME percentage via GC with 0%WW and 100%WW *Chlorella vulgaris*

Figure 5-18 presents the results achieved by calculating the methyl ester peak patterns via GC. There is a gradual rise in the profile since the amount of extracted lipid increases with every stage despite of the biomass loss suffered due to the continuous extraction method. An increased time of extraction and the addition of fresh solvent ruptured the cells better at each stage. Total FAME yields are aligned with those shown in figure 5-17 highlighting the potential advantages of wastewater usage. According to the method highlighted in the standard EN 14103, total FAME content should be higher than 90% (Ruppel & Huybrighs, 2008).

Thus, it can be confirmed that wastewater grown samples provide a good resource for biodiesel production with a total 91% FAME content.

b) Soxhlet extraction method

Soxhlet extraction was conducted on fresh water and wastewater grown *Chlorella vulgaris* samples. Figure 5-19 compares the FAME yield percentages by algae weight, crude lipid weight and gas chromatography.

FAME by crude lipid is obtained by calculating the weight after trans-esterification per Soxhlet extracted crude lipid.

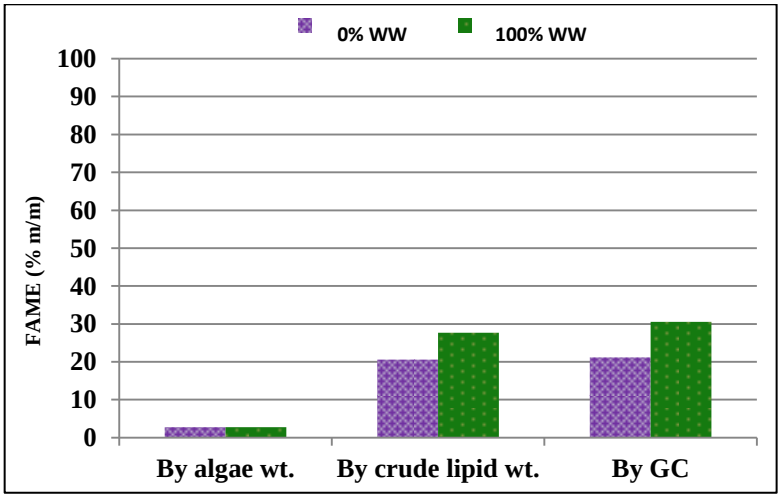


Figure 5-19 Soxhlet extracted FAME results with 0%WW and 100%WW *Chlorella vulgaris*

100%WW samples offer better results when compared with 0% WW but the overall quality of extracted biodiesel or FAME percentages is not satisfactory.

5.3.4 Determination of fuel quantity and property

This section describes the results obtained concerning the quantity and physical properties of the obtained biodiesel from microalgae *Nanochloropsis gaditana* cultivated in the PBRs at University of Almería (refer section 5.2.3).

50 grams of dry *Nanochloropsis gaditana* biomass were extracted using a soxhlet with 850 ml of chemical solvent {a mixture of dichloromethane and methanol (2:1 v/v)} which resulted in **20 ml of biodiesel**.

This biodiesel was further characterized by GC and the total FAME percentage was 90.2. The water content obtained in the pure biodiesel was 245 mg/kg which is also within the range of the standard (the upper limit of biodiesel water content is 500 mg/kg (Postigo, et al., 1995). Viscosity is one of the most important properties of biodiesel and it expresses the magnitude of internal friction. Density is a key fuel property that directly affects the engine performance characteristics. Cetane number and the heating value are related to the density. These parameters influence fuel atomization and combustion processes that take place in diesel engines (Nita & Geacai, 2011). High viscosity means poor atomization and inaccurate fuel injection while a low viscosity makes it easier to pump and atomize into finer droplets (Islam & Beg, 2004). Experimental results for viscosity and density measurements for the investigated fuel in the temperature range of 10ºC to 80ºC are shown in figure 5-20 and figure 5-21.

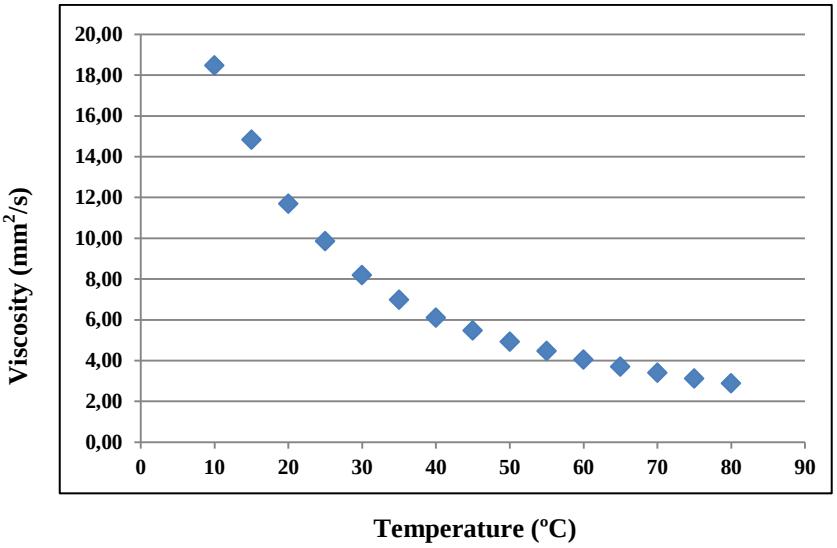


Figure 5-20 Viscosity versus temperature for extracted biodiesel from *Nannochloropsis gaditana*

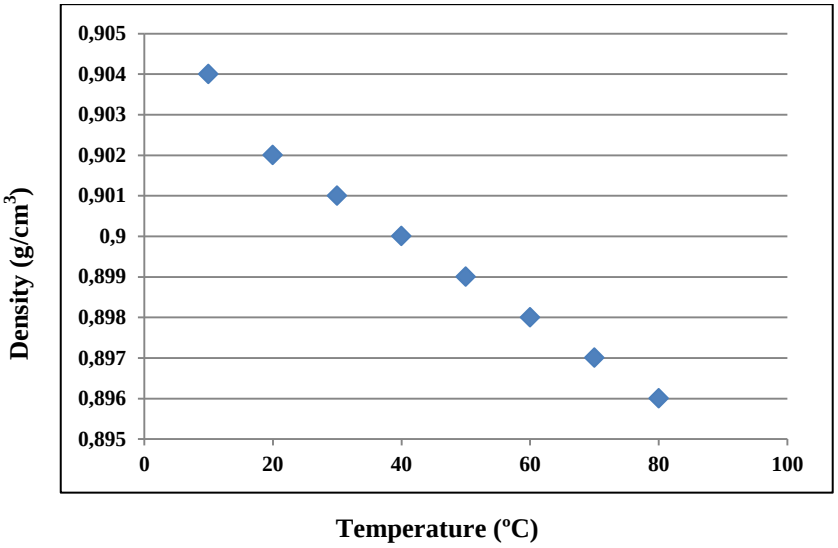


Figure 5-21 Density versus temperature for extracted biodiesel from *Nannochloropsis gaditana*

In this study the viscosity of the extracted pure biodiesel at 40ºC is 6mm^2/s which is almost close the upper limit of biodiesel viscosity at 40ºC (5mm^2/s) (Postigo, et al., 1995). Density varied between 0.896 and 0.904 g/cm^3. The density of biodiesel obtained at 15ºC is 0.903 g/cm^3, which is slightly above the upper limit of density (Postigo, et al., 1995).

5.4 CONCLUDING REMARKS

For the success of quality biodiesel production from microalgae strains, the extraction techniques are the most severe threat. Viable techniques (in terms energy and cost) can make the whole biodiesel production process feasible. Choosing a cheap extraction methodology and reusing the solvents will increase the feasibility of the production process. More study on this topic is essential to design an optimal extraction technology. Several methods, mainly classified under: physical extraction methodologies and chemical extraction methodologies can be used for algae lipid extraction.

Wet extraction of biodiesel samples resulted in good FAME percentages, confirming its suitability according to European standard; however, dry extraction offered better results but the dry extraction is energy and economic intensive process (Aziz, et al., 2013).

3-stage cross current or 3-step ultra-sonication is a more powerful method to rupture the micro-sized algae cells and for lipid extraction than a Soxhlet extraction technique.

The results significantly indicate that wastewater grown microalgae can offer advantages for biodiesel production. FAME percentages of 100%WW samples were consistently higher than for 0%WW samples. A combination of wastewater culture medium for algae growth and extraction using ultra-sonication resulted with good quality lipid production. An approach to economically achieve large quantity of biodiesel would require reduced use of solvent for lipid extraction.

Tri-plot graph shows that the distribution of the fatty acid compositions. FAME, LAME contents and IV percentages confirm that microalgae lipids can be successfully used for biodiesel production. *Chlorella vulgaris* with continuous illumination period, a high aeration rate and a suitable extraction technique results in the most favorable condition for increased total biomass productivity, biodiesel quantity and quality. *Chlorella vulgaris*, *Nanochloropsis gaditana* and *Scenedesmus* can be acknowledged as suitable microalgae strains for biodiesel production.

6 TECHNO-ECONOMIC ANALYSIS (TEA)

The main purpose associated with the work conducted and presented in the previous chapters of this thesis has highlighted the suitability of various microalgae's towards the production of high quality biodiesel. Currently, microalgae are cultivated in relatively small-scale systems but large-scale systems are essential to make an affirmative impact on the biodiesel market. An alternative fuel must be technically feasible, economically competitive, environmentally acceptable and easily available.

An economic feasibility analysis associated with the process used for obtaining algae biodiesel aimed at large-scale application is studied in this chapter and a life cycle analysis (LCA) for the environmental feasibility with the process is discussed on chapter seven. An understanding of the entire algal biodiesel production model was established, through the development of separate data inventories analyzing the conversion pathways designed at the University of Aveiro (UA) (conventional case study) and at the National Renewable Energy Laboratory (NREL), Golden, Colorado, USA (integrated case study).

Chapter six begins with a literature review on techno economic analysis (TEA) of microalgae biodiesel, highlighting the recent research developments. Information explaining the goal and technological methodologies for the comparative conventional vs. integrated case study on TEA will be given afterwards.

Finally, the results obtained with this study are shown, described and some concluding remarks are emphasized.

6.1 LITERATURE REVIEW

The economics of microalgae production is dependent among other things, on photosynthetic productivity, and the recent research has examined different strategies to increase the overall productivity. The strategies includes investigation of various strains, improving cultivation conditions towards high biomass and lipid yield and selection of appropriate techniques for increased lipid extraction. It is important to identify the main costs and energy requirements since the technology can account for high energy consumption by extraction and drying steps. Optimization of these steps can reduce the amount of energy consumption by 46% and emissions by 48% whereas an algae strain with high lipid content (43% instead of 27%) can decrease the energy usage and emissions by 48% and 38%, respectively (Coelho, et al., 2013). The extraction step for producing biodiesel requires significant amounts of energy and solvents use and hence affect global economy of the process. Additionally, biodiesel percentage, biomass production, amount of CO_2 inflow for proper growth and lipid content of the strain creates profound differences in energy use and emissions. The cost analysis results obtained by Slade & Bauen (2013), favors a raceway pond system over tubular PBR systems with a base case algae biomass production cost of ~1.6 €/kg to 1.8 €/kg and the optimized case cost is ~ 0.3 €/kg to 0.4 €/kg of algae biomass. For tubular PBR the base case production cost is ~ 9 €/kg to 10 €/kg and the optimized case cost is ~ 3.8€/kg (Slade & Bauen, 2013). Slade & Bauen (2013), summarized the main limitations to the study of microalgae production cost is due to the data constraints and dependency on parameters which are extrapolated from laboratorial studies. For example, one of the most cited cost modeling communication published by Benemann & Oswald (1996), have used assumptions from mid-1970.

It has been discussed earlier in Chapter 4 that each approach has its own advantages and disadvantages. There is a conflicting view where the raceway ponds can add more expenses to the whole production chain than PBRs due to a significant higher volume of water usage, which is circulated and also suffers water loss due to evaporation (Jonker & Faaij, 2013). Based on 152 scenarios a model identified the biomass production cost to sequester CO_2 from industrial processes between $ 102 per ton - $ 500 per ton including the capital expenses of setting up the facilities (Bilanovic, et al., 2012). In comparison,

another study by Rezvani, et al. (2016), separates the operating costs from capital expenses in the cash flow analysis estimating the values above $440 per ton at a photosynthetic efficiency of 4%. Davis, et al. (2011), suggested a specific operating cost of $208 per ton of algae for biofuel production in the raceway with a value for PBR technology at $123 per ton. Upon completing the base case scenarios, the lipid cost to achieve a 10% return was determined to be $8.52 per gal (open ponds) and $18.10 per gal (PBR) while hydro-treating to produce a diesel blend stock brought the totals to $9.84 per gal and $20.53 per gal, for the two cases (Davis, et al., 2011). This study also concluded that significant cost improvement is possible with better microalgae strains (with high growth rates and high lipid content) (Davis, et al., 2011).

A major driver behind the microalgae derived fuel cost is the feedstock price. An economic study by hydrothermal liquefaction of defatted microalgae to bio-crude by hydro-processing showed that the feedstock cost of $33-132 per dry ton influenced the minimum fuel selling price (MFSP) by $584-869 per m^3 (Ou, et al., 2015). Published microalgae biomass costs vary from $350 per ton to $7320 per ton, depending on the strain, cultivation and extraction methods and facility locations (Alabi, et al., 2009).

Nagarajan, et al. 2013, made a comprehensive TEA of microalgae biodiesel production process resulting the production cost in the range of $0.42-0.97 per liter which requires further cost reduction for commercialization. There is also research related to the biodiesel production facilities from cooking oil and algae using simultaneous optimization and heat integration approach which highlights the alkali-catalyzed process as best for algae oil, with a cost of $0.42 per gallon biodiesel, energy consumption of 1.94 MJ per gallon biodiesel and water consumption of 0.60 gallon water per gallon biodiesel (Martín & Grossmann, 2012; Martín & Grossmann, 2014; Cruz, et al., 2014).

A study by Song, et al. (2015), aiming at the optimization of microalgae based biodiesel production by waste heat recovery and process integration estimated a reduced unit production cost of $0.592 per liter biodiesel, and approximately $0.172 per liter biodiesel was avoided by heat integration. It was also indicated that the production process contains several energy-intensive sections, mainly esterification, trans-esterification and purification (biodiesel and glycerol); the total material and energy balance of this work was simulated in PRO/II software (Song, et al., 2015). In addition, research on TEA of microalgae

remnant pyrolysis biofuels estimated fuel prices at between $1.49-1.80 per liter (Thilakaratne, et al., 2014).

The requirement for a low cost, sustainable fuel drives to address the optimization of biodiesel production process. Primary goal should include the development of long term strategic decisions of the process. Final fuel cost is still a severe limitation; however, studies are attempting to commercialize microalgae biodiesel. Lundquist, et al. (2010), stated that microalgae derived biofuel costs would range from $0.17-2.09 per liter. On the other hand, Alabi, et al. (2009), suggested a cost range of $0.88-24.60 per liter.

The final resulting cost metric is the combined cost of every single stage and economic parameters (operation and equipment costs) involved in the whole microalgae-to-biodiesel fuel production. Production of high-value co-products is an additional strategy to add revenue to the total chain production process.

6.2 GOAL

After the studies on microalgae growth which were followed by quality characterization of the extracted biodiesel and in view of the above, it may be stated that challenges are still limiting the development of microalgae as a source of biodiesel. To address these challenges, technological advancement is a must.

Figure 6-1 represents the full chain pathway of the conventional production system.

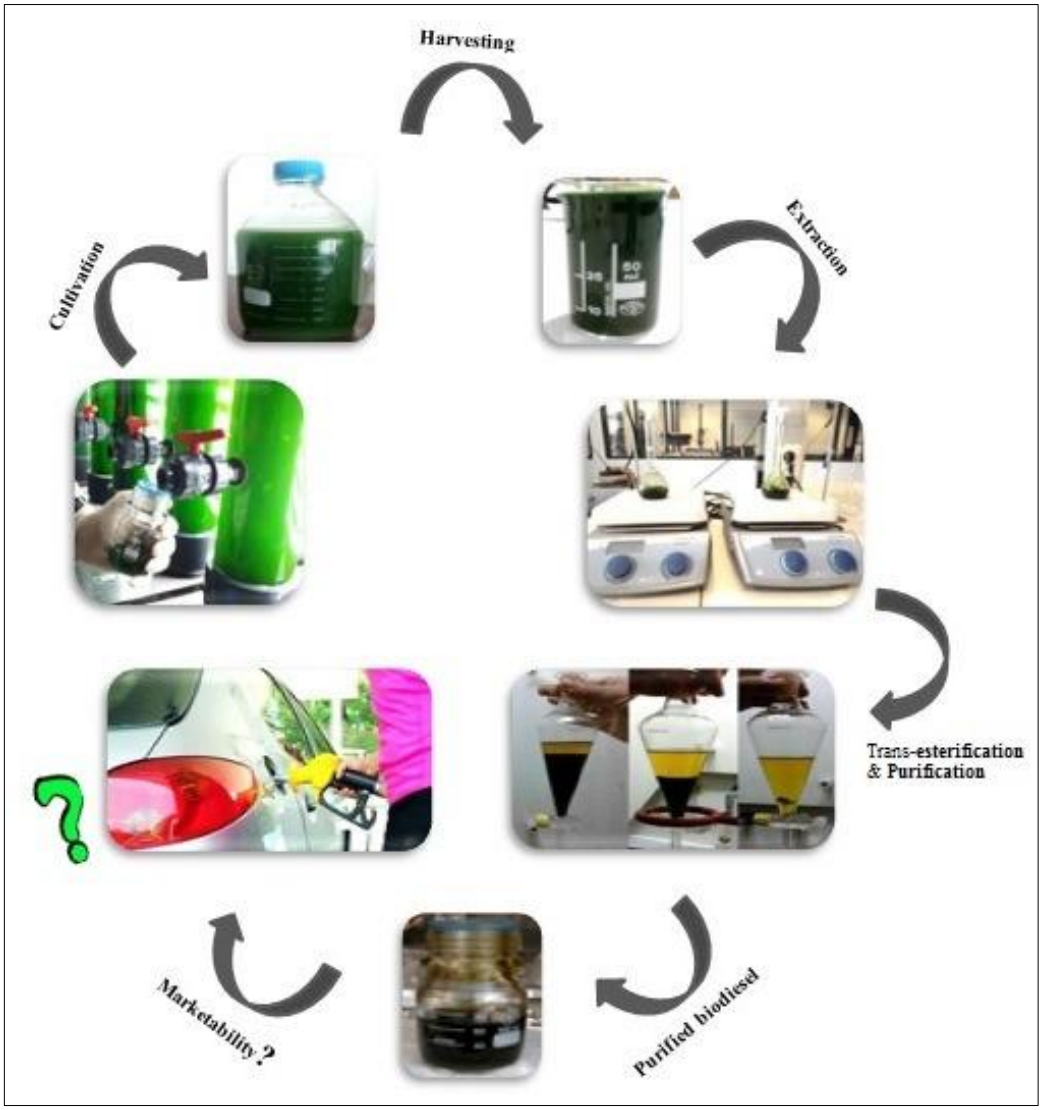


Figure 6-1 Full chain methodology from microalgae cultivation to pure biodiesel production

This chapter focuses on the economics of the process aiming at larger scale feasibility. Both strengths and challenges come along with any technological pathway. The feasibility analysis of the technological processes requires the quantification of mass and energy balances as well as of CO_2 emissions throughout the process from microalgae growth to biodiesel production. A TEA is used for assessing economic viability which in turn, determines the near and future term commercial success of a process or service since it provides costs and allows setting guidelines for the required performance.

The objective is to study the entire production process with technical and economic feasibility. The options for optimization of the microalgae biofuel production chain to maximize the oil yield and estimate the principal energy, material inputs and economic

aspects related to the process were explored within a partnership between University of Aveiro (UA) and the microalgal research group of the National Renewable Energy Laboratory (NREL). The focus was a comparative study of a large-scale algae-to-biofuel pathway in terms of environmental and economic impacts and the analyzed pathways were defined. This section provides a clear understanding of the goal and methodologies used.

Data inventories along with different technical pathways characterized the two scenarios (UA & NREL) which were chosen in-order to compare the different realities. The main processing technologies evaluated are: solvent extraction, trans-esterification and product purification (conventional pathway); fermentation, distillation, solvent extraction, product purification and hydro-treating (integrated pathway). These case studies are referred to respectively as Case A (conventional pathway) and Case B (integrated pathway). A comparison of the conversion procedures intended at the improvement of the environmental aspects was performed. Finally, the overall cost model associated with the well-to-wheel algal biofuel production process was determined using a particular set of common assumptions.

A 'pathway' in algal biofuel production is a set of processes beginning with microalgae cultivation followed by dewatering extraction, and upgrading of intermediates to finished product. This section describes some contrasting pathways in algae cultivation to provide a framework for a discussion of the sustainability impacts of the different approaches. The primary challenge in developing the techno-economic analysis (TEA) of this work was to gather the set of assumptions needed to run separate mathematical models and compare the pathways.

The present work required the following:

- Identification of the technical pathways for both Case A and Case B
- Allocation of mass and energy inputs
- Definitions for the assumptions required for performing a TEA, including:
    - microalgae productivity: 30 g/m^2/day (as average over the year)
    - facility operation: 330 days/year
    - lipid content: 41%
    - pond area: 4,050 hectares

6.3 METHODS

6.3.1 Conventional versus Integrated pathway study

The process design designated by Case A is based on the conventional biofuel production methods and includes only microalgae cultivation, biomass harvesting, lipid extraction, and final product (biodiesel) upgrading and purification steps.

The *facility design of Case A* is described as follows:

The most conventionally practiced pathway starts with algae biomass cultivation followed by dewatering (by centrifuge and filtration), homogenization and lipid extraction by the Bligh and Dyer method (1959), and a reversible trans-esterification reaction (or alcoholysis) which leads to the conversion of the extracted lipid (TAG's) to mono-esters or biodiesel (Widjaja, et al., 2009) (Dutta, et al., 2011) (Ramos, et al., 2009).
The technological steps relevant with the Case A model are represented in the form of flowcharts indicated as figures 6-2, 6-3 and 6-4.
This design incorporates the preparation of the culture medium, the sterilization process, algae growth, sedimentation, dissolved air flotation (DAF), centrifugation, lipid extraction and solvent recovery, lipid upgrading, product purification and finally the storage. Figures 6-3 and 6-4 describe the detailed design comprised within 'lipid extraction and solvent recovery' and 'lipid upgrading and product purification' processes respectively.

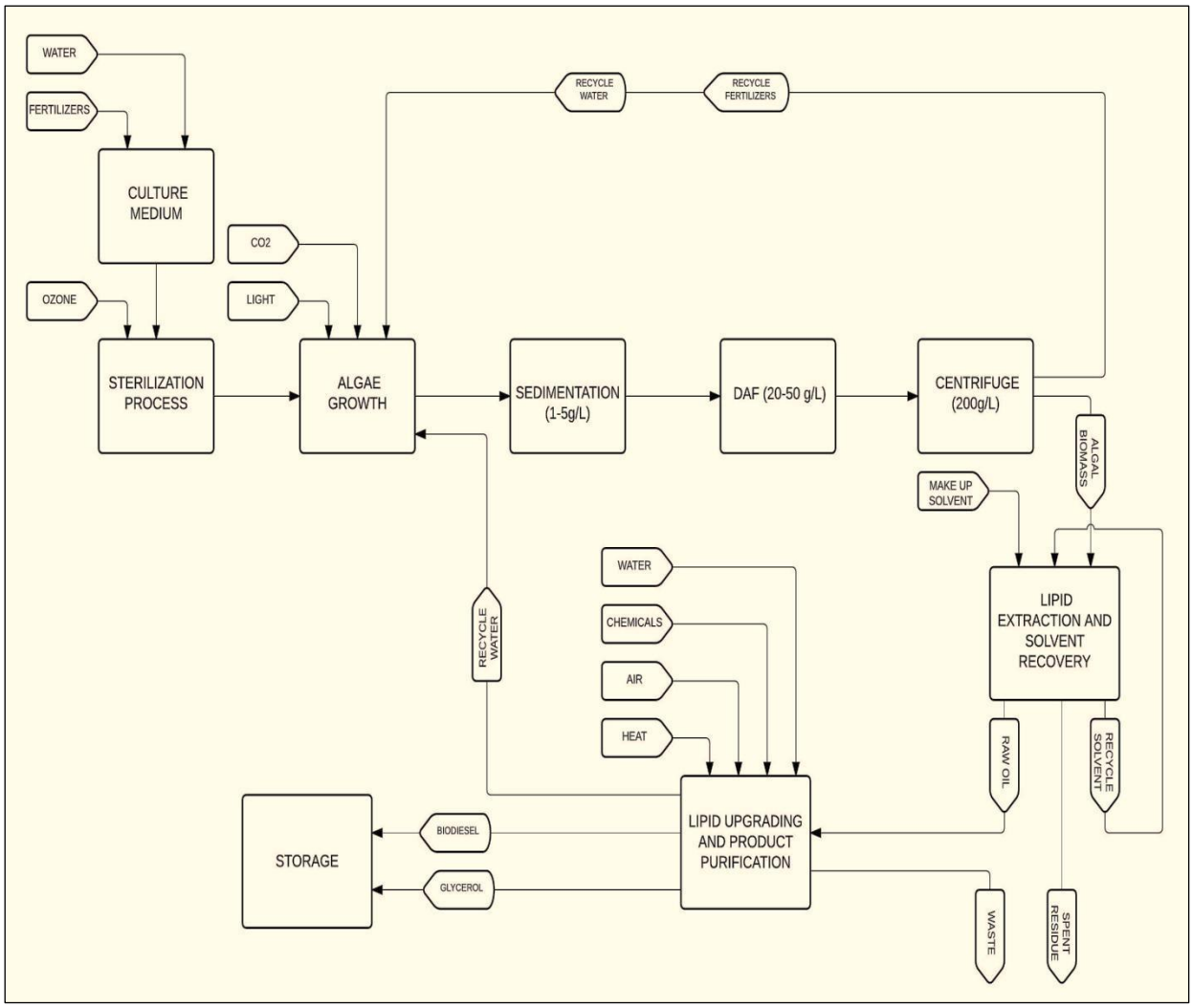


Figure 6-2 Case A: Microalgae biofuel production flowchart (Conventional pathway)

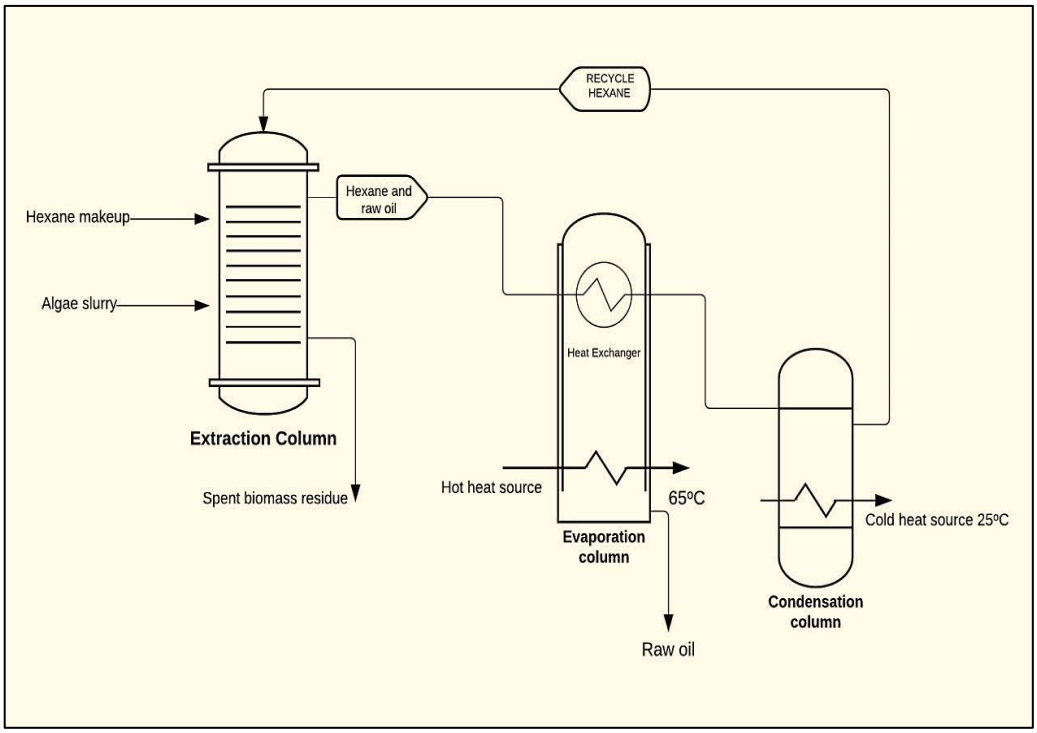


Figure 6-3 Case A: Detailed design for 'lipid extraction and solvent recovery' (Conventional pathway)

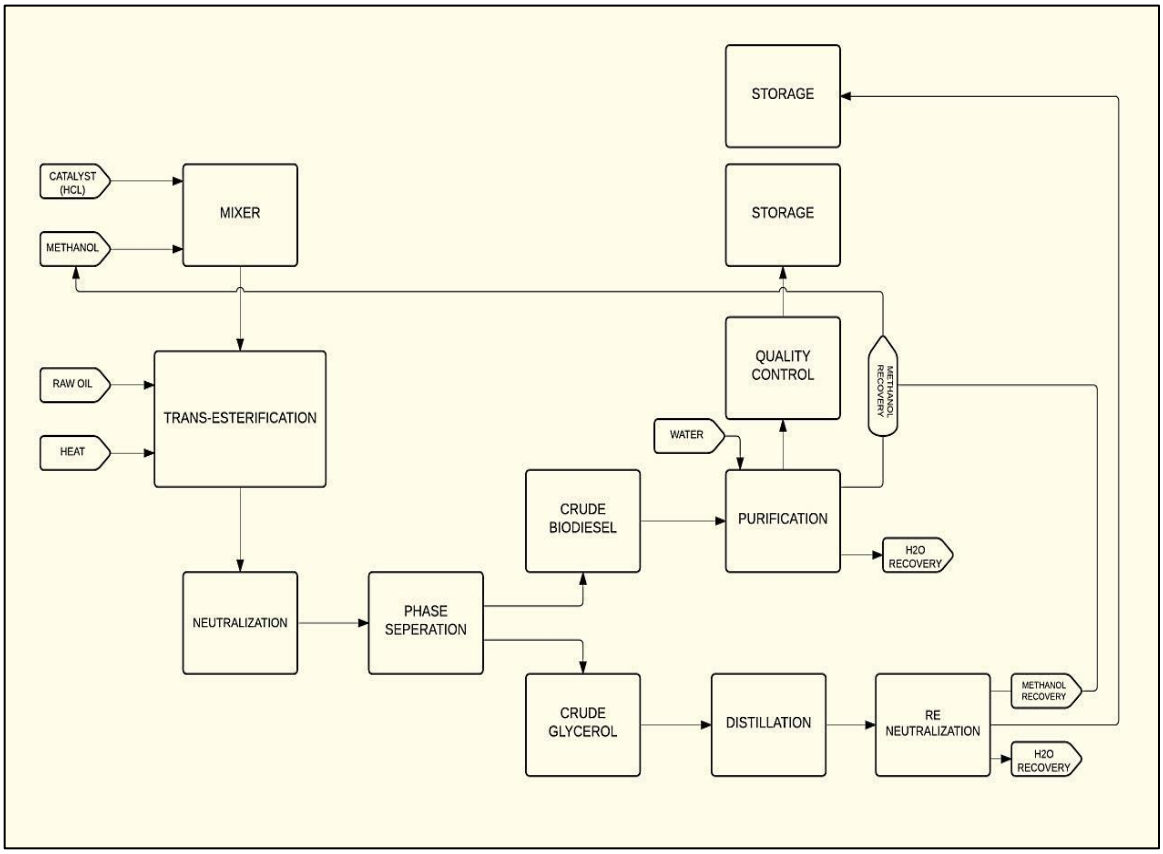


Figure 6-4 Case A: Detailed design for 'lipid upgrading and product purification' (Conventional pathway)

The ‘lipid extraction and solvent recovery’ (Figure 6-3) step consists of extraction (with hexane as the main chemical solvent) along with evaporation (at ~65ºC) and condensation (at ~25ºC) to recover and reuse solvent. The ‘lipid upgrading and product purification’ (Figure 6-4) step designates the conversion of extracted triaclyglycerol into crude biodiesel as the primary product via acid trans-esterification and glycerol is obtained as a by-product. The raw product is then purified via multiple hot water washes and taken to the storage tank. The crude glycerol from the water wash is purified via distillation. A block diagram for the purification system is shown in figure 6-5.

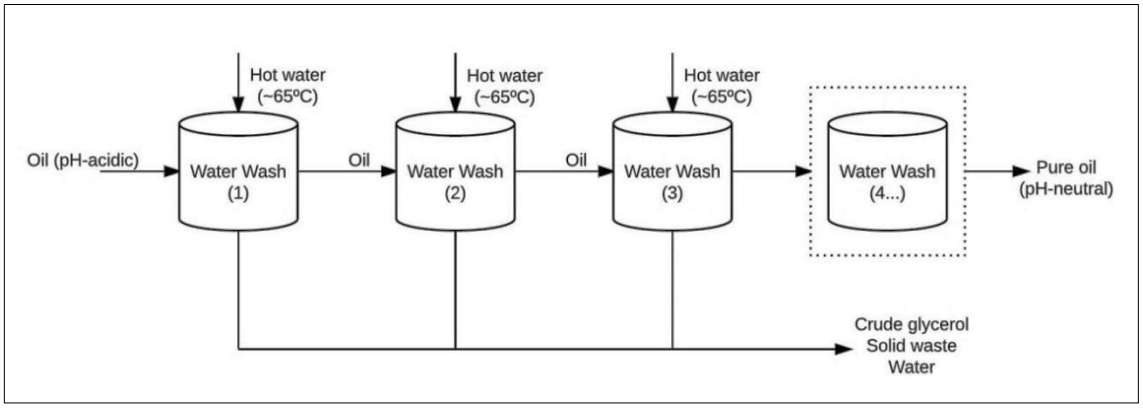


Figure 6-5 Block diagram schematic for the lipid purification to pure biodiesel (Conventional pathway)

Having established the basis for the algae-to-biodiesel pathway, a previous mathematical model developed was used to address mass and energy balance and assess process costs. This model was developed from the previous work (Acién, et al., 2012), conducted at Almería University and complemented by original work at UA, which included extraction and trans-esterification (Carmo, 2012) (Silva, 2014).

Next, *facility design of Case B* is described:

NREL has been developing design case models and process and cost target reports focused on cellulosic biofuel pathways for over 10 years. These developments were initially targeted at ethanol production but efforts have more recently been expanded to include hydrocarbon biofuel production, based on increasing interest in algae. Recently, the USA Department of Energy – Bioenergy Technologies Office (DOE-BETO) has expanded its focus to include algal biofuel pathways (Fishman, et al., 2010). The first activity was the

'Harmonization Initiative' which brought together three modeling partners such as NREL, the Argonne National Laboratory (ANL) and the Pacific Northwest National Laboratory (PNNL) who worked simultaneously to harmonize conceptual models around TEA, LCA, and resource assessment (RA) respectively. A harmonization report was published for the production of 5 billion gallons/year (BGY) of renewable diesel and was based on an 'algal lipid extraction and upgrading' (ALU) process focused on microalgae lipid extraction with all remaining residual material fed to anaerobic digestion (Davis, et al., 2012). This effort resulted in an initial baseline, a projecting out-year process and in cost improvement goals. Later in 2013 this effort was complemented repeating the process while targeting year 2022 with algae hydrothermal liquefaction (HTL) conversion pathway to achieve a minimum fuel selling price (MFSP) of $4.49/GGE (Gasoline gallon equivalent) (Davis , et al., 2014) (Jones, et al., 2014). The economics of this 2022 projected n^{th}-plant is computed to be $4.35/GGE (2011-year dollars) (Davis , et al., 2014). This was the result at a final upgraded product yield of 141.1 GGE/dry ton of biomass in the base case. This reflects a $3.05/GGE contribution from feedstock and a $1.30/GGE contribution from the conversion process (Davis , et al., 2014).

The work included in this thesis examined a case study that compared the final cost and environmental impacts associated with the two different lipid extraction and upgrading pathways using same technological assumptions. The model used reflected minimal adjustments that had been made to the front-end portion (algae growth to harvest and dewatering) of the ALU design model process (Davis, et al., 2012) to the fractionation back end (pretreatment to anaerobic digestion and CHP (combined heat and power) of the 2014 model (Davis , et al., 2014). The resulting process flowchart is shown below in figure 6-6.

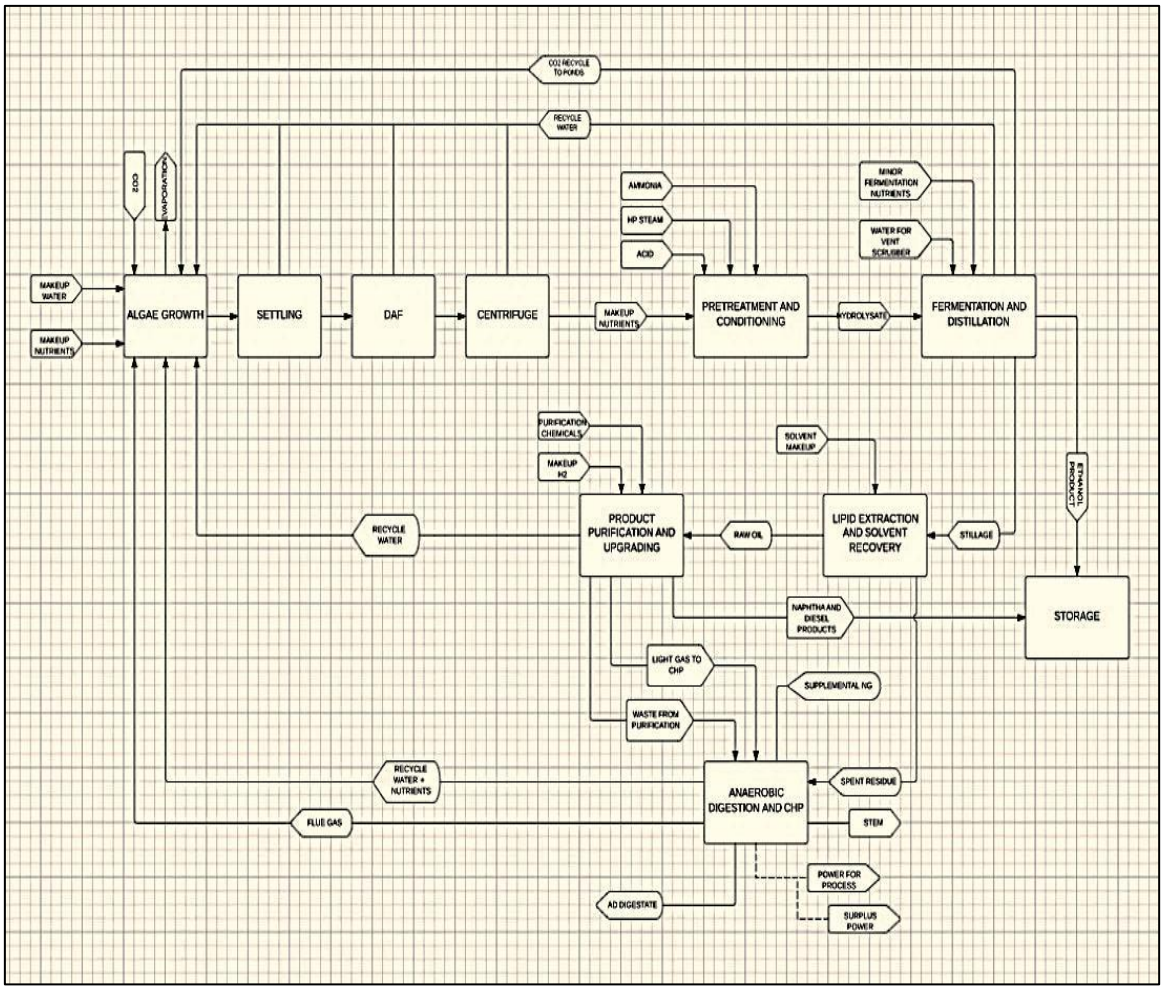


Figure 6-6 Case B: Full chain process for 'biofuel production' (Integrated pathway) (Davis , et al., 2014)

Case A and Case B adopted the same biomass cultivation technology (cultivation and growth in open pond systems). In Case B the biomass obtained is pretreated by combining it with steam and H_2SO_4 hydrolyzing the carbohydrates to sugars and allowing for efficient extraction of the wet slurry for the recovery of lipids. NH_3 is added in order to increase the pH of pretreated slurry to ~5 and then the algal sugars are valorized by fermentation to ethanol which adds both coproduct credit as well as additional biofuel yield. In the ensuing process designated as 'fermentation and distillation', most of the sugars are converted to ethanol which is distilled and purified to 99.5% using vapor-phase molecular sieve adsorption. At this stage the stillage is fed to a liquid-liquid extraction column with hexane solvent at a 5:1 mass ratio, resulting in the recovery of > 99.5% oil. This procedure is followed by purification to remove polar lipids and impurities which are presented in figure 6-7 (Davis , et al., 2014). Purification consisted of three steps: degumming,

demetallization and bleaching, for removing phospholipids, polar lipids, metals, salts and impurities.

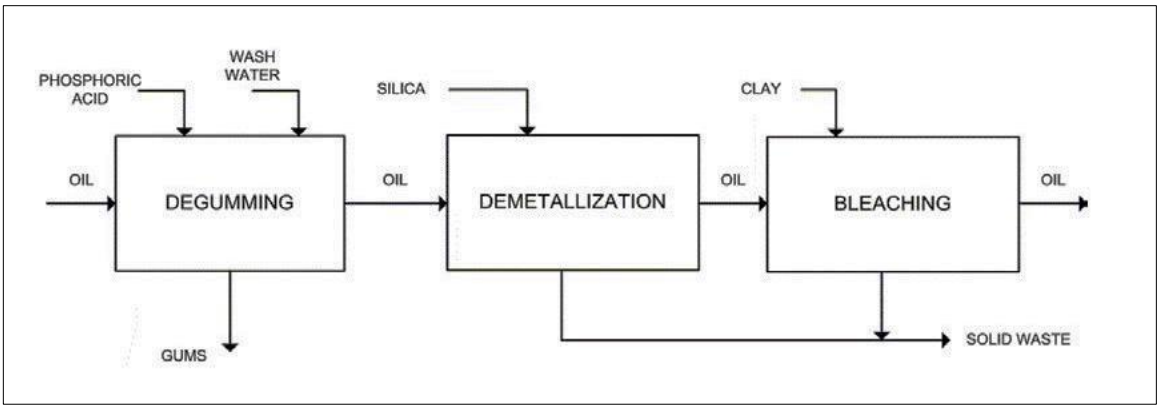


Figure 6-7 Block diagram schematic for lipid purification system (Integrated pathway) (Davis , et al., 2014)

The objective is achieved by adding phosphoric acid, wash water, silica and clay. The purified oil is then upgraded in an on-site hydro-treating facility with diesel as the primary product and naphtha as the co-product. Finally, the extracted stillage is combined with the oil cleanup and sent to anaerobic digestion, yielding biogas. This biogas was combusted in a gas turbine to generate electricity, which can be sold to the grid, as well as flue gas heat, which can be recovered and used for steam generation. A small amount of natural gas was co-fed with the biogas to the turbines in order to facilitate and improve the energy efficiency. The CO_2 from the turbine flue gas can also be captured and used for algal cultivation. Digester effluent water (which contains nutrients like nitrogen and phosphorus) and solid digestate cake (containing high nitrogen content and sold as a fertilizer) are additional co-product revenue sources.

6.4 RESULTS AND DISCUSSION

The computed TEA results obtained with the two different conversion pathways is presented in table 6-1. The biomass cost associated with the Case A is 2.9 times that of the

Case B. The total biomass cost and minimum final fuel selling price (MFSP) of the two processes are compared below.

For this study the biomass cost for Case B was considered to be ***$430/ton-biomass***. This value is obtained from the previous study at NREL targeting a 2022 algal biomass cost (Jones, et al., 2014). While, the biomass cost used for the UA-Portugal technology (Case A) was calculated to be is ***$1279/ton-biomass*** and assumes to be the present biomass cost.

Table 6-1 Techno-economic results associated with the two different algae to biofuel conversion pathways, Case A vs. Case B

	Case A	**Case B**
Biomass Cost ($/ton-biomass)	1279	430
MFSP $/GGE (in 2011-year dollars)	10.6	4.4
MFSP $/gal (in 2011-year dollars)	11.0	4.6

The computed MFSP (in 2011-year dollars) for Case B was ***$4.4/GGE***. In stark contrast, the MFSP for Case A was ***$10.6/GGE***. The economic analysis shows a vast difference between the two pathways which is mainly associated to the design. These results were obtained using the assumptions (mentioned in section 6.2.) and the data inventory created for the pathways (presented in the annexes later) in the model developed by Silva, (2014).
A techno-economic analysis is used for assessing economic viability which, in turn, determines the near and future-term commercial success of a process or service since it provides costs and sets guidelines for required performance. No credits are added to co-products in this system. The results show that the feasibility of a large scale production requires further improvements by understanding to bridge those gaps with improved strategies. Excessive usage of chemicals (hexane) is one of the reasons for additional cost; these chemicals must be recycled in the process in a higher percentage. Capturing CO_2 from single localized emitters such as power plants or cement kilns can potentially provide several possible cost and energy saving advantages. Facilities integrating power plant and

algal ponds would reduce the costs of CO_2 emissions, produce waste heat for warming ponds in the winter and provide carbon credits to the utility. As CO_2 capture by microalgae to biomass production is not feasible in the darkness thus, methods to capture, concentrate, store, and transport CO_2 from source to cultivation system (pond) will need to be developed as part of the integrated solution for capturing as much of the CO_2 emission as possible during the daytime.

'Lipid upgrading and product purification' stage is usually a cost involved technique since a lot of fresh water is required during the purification process to neutralize the biodiesel pH (Figure 6-4). The comparative results suggest the high importance of finding better value-added utilizations for co- and by-products essentially for the Case A technology. Moreover, figure 6-6 highlights how better economic figures can be obtained: despite the added complexity of the process, each of the steps adds revenue which, in turn, contributes towards the decrease of the overall final fuel cost. In order to improve Case A it is important to implement new technologies, approaches and strategies to add as much revenue as possible throughout the process pathway.

Despite the technical feasibility associated with the widely proposed pathway represented by Case A, the process shows poor economic figures at larger scale. A better knowledge and understanding is essential to increase the efficiency of each of its stages in order to decrease the overall production costs. A different approach, which relies on valorization of co-products and residues (Case B), seems to be more efficient at all levels. Hence, correct planning to increase the revenue associated with co- and by-products is expected to play an optimistic role and change the economic scenario of Case A towards successful production. With the same set of assumptions, the biodiesel cost of Case A with four varying microalgae productivities ($20g/m^2$/day, $25g/m^2$/day, $30g/m^2$/day and $35g/m^2$/day) using an open pond and PBR is shown in figure 6-8.

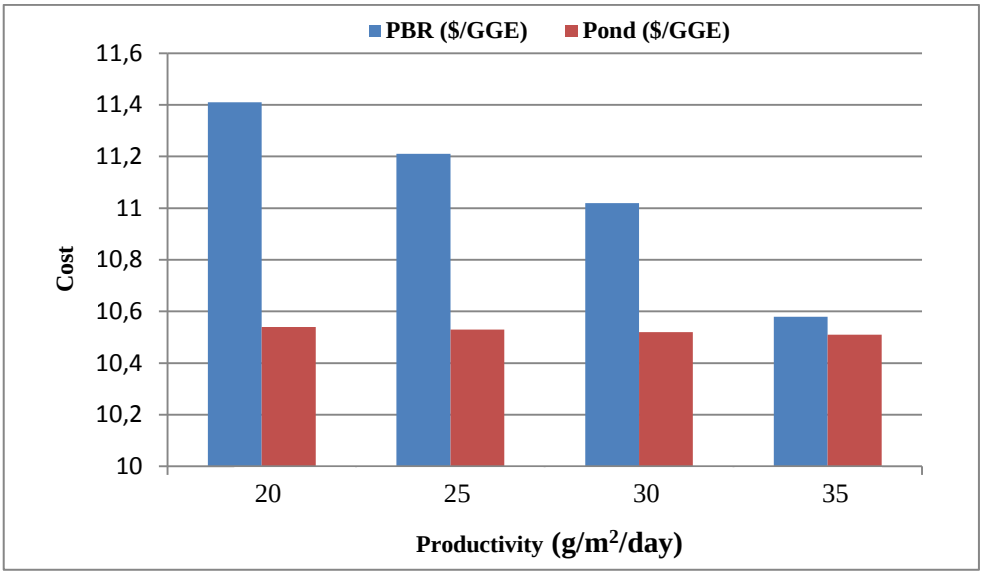


Figure 6-8 Biodiesel cost in PBR and Pond systems with varying productivities; (20g/m^2/day, 25g/ m^2/day, 30g/ m^2/day and 35g/ m^2/day)

Clearly, fuel cost associated with PBRs is expensive compared to open pond cultivation as stated previously in the literature of chapter 2, section 2.1. This resembles results from previous studies where raceway pond systems are idealized over tubular PBR systems (Popp, et al., 2014) (Slade & Bauen, 2013) (Jonker & Faaij, 2013) (Benemann & Oswald, 1996) (Bilanovic, et al., 2012) (Rezvani, et al., 2016) (Davis, et al., 2011). High cost with PBRs is due to the resources in terms of energy and economic associated to provide a controlled growth environment to the algae cells while this is not the case in ponds. Microalgae with higher productivities are better for producing final fuel at lower cost. This graph analyses the economic difference offered by the two different cultivation systems on microalgae derived biofuel cost.

6.5 CONCLUDING REMARKS

Techno-economic analysis models are used to set benchmarks and to quantify costs which helps to highlight the economic hurdles associated with a given process technology. One of

the main hurdles to the marketability of a product is the final selling price. This chapter of the thesis attempts to study the costs associated with different technical pathways of microalgae derived biodiesel production. The facility design of an integrated system clearly favors lower production costs since every co-product adds revenue to the whole process.

The conventional pathway needs upgrading which can be achieved by the co-product valorization. Instead of throwing away the algae residue after 'lipid upgrading and product purification' (refer Figure 6-4) they should be used to produce co-products like animal feel or flue gas etc. This is an important step for the economic feasibility of this process.

The biomass cost with a conventional approach is 2.9 times higher than with the integrated system. Also, the MFSP is 2.4 times higher than with integrated system. Closed photo-bioreactors increased the fuel cost when compared with ponds. Open ponds are cheaper but they present considerable contamination risks since the culture is exposed to other bacteria.

7 LIFE CYCLE ANALYSIS (LCA)

A Life Cycle Analysis (LCA) is focused on the environmental impacts of the product life cycle. This chapter primarily includes a range of analysis of microalgae derived biodiesel methodologies starting with a literature overview, a case study, discussing their corresponding results and finally a sensitivity analysis. The focus is to understand the environmental aspects related with pollution or emissions which result from a given LCA including fundamental aspects like the greenhouse gas (GHG) emissions and fossil energy consumption (FEC) to develop a sustainable scenario. The fundamental objective is to conduct a LCA on microalgae derived biodiesel contrasting different technological pathways. A whole-of-life algal biofuel production model was established, through the development of two separate data inventories supporting different scenarios (Europe, USA) and estimating the total environment benefits and drawbacks for the different stages in the production chain.

The work integrates the results obtained in the TEA for conventional and integrated biofuel production. A sensitivity analysis is performed with the conventional pathway to upgrade the decision making process for feasible fuel production.

7.1 LITERATURE REVIEW

Life cycle assessment is an environmental management tool for quantifying the input–output inventory of a system throughout its life cycle stages, and projecting the performance of the product. The technical framework for the LCA methodology, according to the ISO 14000 series (ISO 14041-43), consists of four phases: goal and scope definition; inventory analysis; impact assessment; and interpretation (Korres, et al., 2011).

LCA can be used to:

- minimize the magnitude of environmental pollutions
- conserve non-renewable resources and ecological systems
- establish smart technologies
- maximizing waste and material recycling
- develop most appropriate emission prevention techniques

In spite of its huge potential to meet global demand for transport fuels, microalgae biofuel technology has many challenges limiting their development associated with the production pathway. Therefore, under these circumstances a critical question is: *"what is the key to make microalgae derived biodiesel production sustainable by reducing the overall GHG emission?"*

The focus was on the technological breakthroughs, especially with the production stage and lipid extraction (due to their energy intensiveness) which will in return benefit for commercial production. Research by Clarens et al. (2010), featured that the energy generated from microalgae has lower environmental effects than conventional crops (due to their potential with land use and eutrophication). Some research studies concluded that certain modifications in the technology lead algal biofuel to sustainability by lowering the environmental impact than fossil fuels (Lardon, et al., 2009) (Batan, et al., 2010) (Colin, et al., 2012) (Sander & Murthy, 2010) (Brentner, et al., 2011). Findings reviewed that the selection of algae strains with high lipid content and appropriate cultivation condition to achieve high yield is important for the future of microalgae derived biodiesel production (Stephenson, et al., 2010) (Khoo, et al., 2011) (Campbell, et al., 2011) (Benemann, et al.,

2012) (Cheng & Timilsina, 2011). In order to reduce production costs, the utilization of wastewater can be used as the cultivation medium. Wastewater contains important macronutrients that are vital for microalgae growth (Chisti, 2007) (Yang, et al., 2011) (Rawat, et al., 2011) (Hosikian, et al., 2010). Recycling harvest water in the process and using wastewater as source instead of fresh water can enhance the competitiveness of microalgae based fuels (Yang, et al., 2011) (Singh, et al., 2011). According to Yang et al. (2011), using wastewater reduces 90% of total water requirement and also the need of nutrients except phosphate. Seawater contains nutrients in trace amount; additional nutrients should be added to get a desired yield; also careful attention to minimize water evaporation loss and contamination problems is required.

Stephenson, et al. (2010), suggests that there is a need of pilot scale trials of algal biodiesel production allowing LCA to operate. A plant constructed in Spain of water management of wastewater projects (including water treatment, urban sewage treatment, industrial wastewater treatment and water recycling and desalination facilities) uses treated municipal water to grow microalgae (in open pond system) and recycle back the water after harvesting to the city (Aqualia, 2016). Such a plant could produce biofuels being economically viable.

In some studies, the primary focus of a LCA investigation is on total energy demands and also on the CO_2 emissions of the production chain (Jorquera, et al., 2010) (Stephenson, et al., 2010). LCA research by Jorquera, et al. (2010), examined the microalgae growth using open and closed ponds concluding better net energy ratio (NER) results for open ponds than closed ponds. These results match the findings reported by Campbell, et al., (2009). Despite of low greenhouse potential, depletion of natural resources and high eutrophication potential a previous study quantified the environmental benefits of microalgae cultivation with flue gas input and coal-fired generated electricity (Kadam, 2002). Lardon et al. (2009), studied a scenario of microalgae derived biodiesel production comparing low-nitrogen culture condition to oilseed crops and confirms the potential of microalgae as an energy source but also reports the need of decreased energy and fertilizer consumption.

LCA demands addressing impact categories within the early stage of the process aiming for the product development (Weidema, 2000). Using LCA methodology, suitable technical alternatives for a process can be developed by cross comparing life cycle impacts based on different sensitivity criteria (Mathew, et al., 2013). LCA is a powerful tool which

can be used to analyze and optimize different production stages such as growth conditions, extraction steps and reuse of the co-products in order to contribute towards the sustainability of the process. Given the lack of existing large scale facilities, it is difficult to provide adequate data which will support the modeling efforts associated with the production on an industrial level.

7.2 GOAL AND METHODS

In order to ensure the feasibility and competitiveness of the production of products from microalgae, an environmental assessment is made for open pond cultivation systems. In this thesis a comparison of the microalgae biodiesel production pathways (Case study: Case A vs. Case B and a sensitivity study for Case A) is presented to quantify environmental impacts.

The functional unit was 1 gasoline gallon equivalent (GGE) of microalgae biodiesel using existing technology. The LCA study was conducted for an open pond system with *Chlorella vulgaris* microalgae.

The LCA of the back end model (named for the harvested biomass to end product) of Case B technology was performed and the emission values were published in previous studies (Jones, et al., 2014). The front end LCA (cultivation and dewatering) inventory list is presented below (Tables 7-1 and 7-2) (Davis, et al., 2012).

Table 7-1 Inputs for the front end LCA model (Integrated pathway) (Davis, et al., 2012)

Inputs	Values
Ammonia (NH3) (kg/h)	890
Diammonium phosphate (DAP) (kg/h)	448
Carbon dioxide (CO_2) (kg/h)	129227
Water (H_2O) (kg/h)	1762800
Flocculent (kg/h)	201
Power demand (kwh)	29057

Table 7-2 Outputs for the front end LCA model (Integrated pathway) (Davis, et al., 2012)

Outputs	Values
Water evaporation (kg/h)	943182
CO_2 lost from ponds (kg/h)	22398
Water purge (kg/h)	600750
Algal biomass harvested (kg/h)	50611
Water in harvested biomass stream(kg/h)	202444

The goal of the present task is to appropriately carry an extensive comparison of the two technological pathways targeted at their environmental improvement. LCA was used to compare the two pathways, in particular their contribution to the environmental impacts.

The fossil energy and GHG emission drivers at all stages of the production system (including algae cultivation, biomass harvesting, oil extraction, and its conversion to biodiesel via trans-esterification) is considered for Case A pathway. The objective is to develop suitable alternatives, by cross-comparing the impacts based on the criteria: solvent (hexane) recycling, energy sources and residue usage. A system boundary of "well-to-wheel" (WTW) defined the sub-process models of the microalgae biomass production, lipid extraction and solvent recovery.

The lipid upgrading for Case A is shown in figure 7-1. For this task, LCA was developed using SimaPro v.8.0.2 software since it contains the latest databases and features to quantify life cycle impacts (SimaPro, 2016).

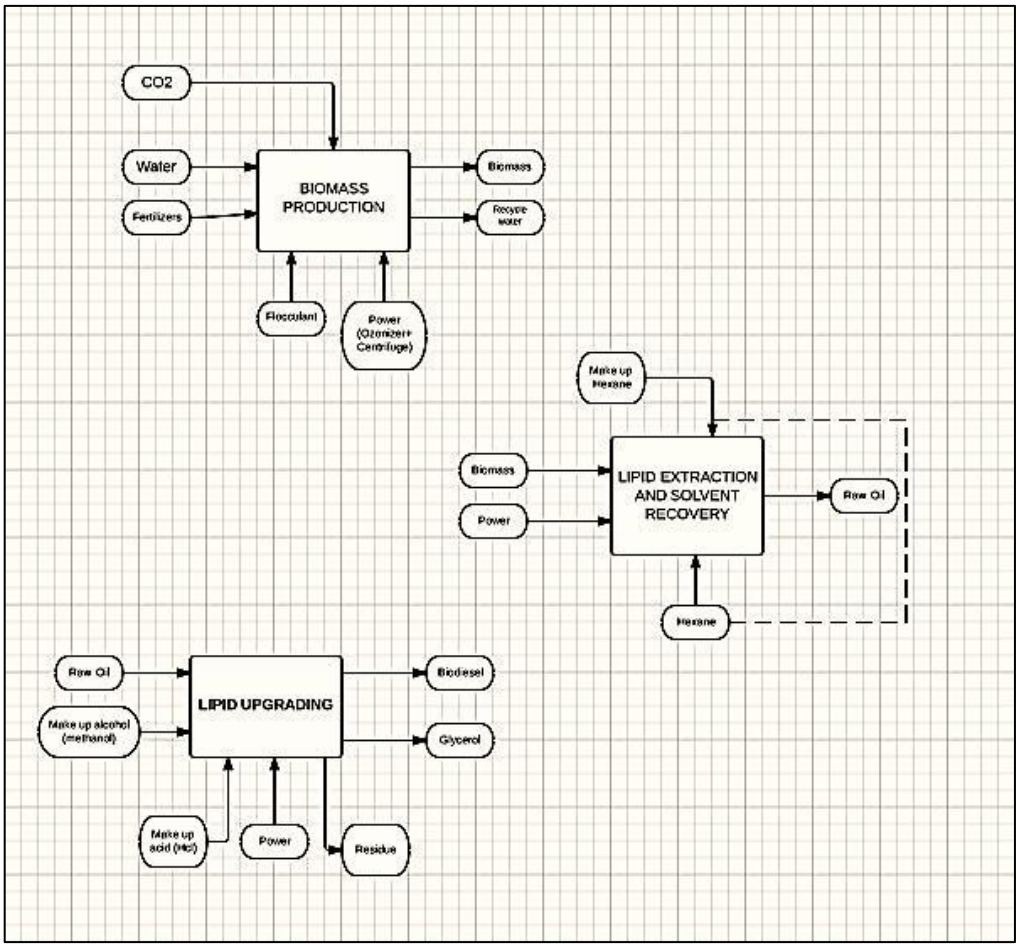


Figure 7-1 Case A: LCA scheme for the 'biofuel production' (Conventional pathway)

A whole production model was established, through the development of data inventories supporting the underlying technologies and requirements.

LCA data inventories for Case A are presented in tables 7-3, 7-4, and 7-5 (Silva, 2014).

Table 7-3 Data inventory for LCA study - biomass production stage (Conventional pathway) (Silva, 2014)

Product	**Values**
Biomass (kg/h)	151875
Resource	
Carbon dioxide (kg/h)	1389656
Water (kg/h)	25160625
Fertilizers (kg/h)	45562.5
Flocculant (kg/h)	18984
Recycled water (kg/h)	-25158677
Power	
Ozonizer (kWh)	2904
Centrifuge (kWh)	7904

Tables 7-4 and 7-5 present the data for the 'lipid extraction and solvent recovery' and 'lipid upgrading' stages respectively.

Table 7-4 Data inventory for LCA study -'lipid extraction and solvent recovery' stage (Conventional pathway) (Silva, 2014)

Product	**Values**
Raw oil (kg/h)	28580
Resource	
Biomass (kg/h)	151875
Hexane (kg/h)	2885625
Recycle hexane (kg/h)	-1442813
Power (kWh)	14561.7

Table 7-5 Data inventory for LCA study - 'lipid upgrading' stage (Conventional pathway) (Silva, 2014)

Product	**Values**
Biodiesel (kg/h)	28713
Glycerol (kg/h)	3018
Residue (MJ/h)	1933968
Resource	
Raw oil (kg/h)	28580
Alcohol (methanol) (kg/h)	32148
Recycle alcohol (kg/h)	-14499
Acid (Hcl) (kg/h)	4265
Recycle acid (kg/h)	-3625
Power (kwh)	1058

The baseline variables and assumptions used for the Case A- design are shown below,

- Energy source: Electricity mix
- Co-product residue: Lignite
- Hexane recovery: 50%
- Water (natural resource) recovery: 99%
- Alcohol (Methanol) recovery: 45%
- Acid (HCl) recovery: 85%

As already discussed in Chapter 6, 'Case A' (Figure 6-2, p. 110) presents by far a bad scenario than that of 'Case B' (Figure 6-6, p. 114). The conventional prototype is a good approach (positive on a laboratorial scale) but still needs improvement at the technological stages to make the product commercially viable. At this stage in reality only biodiesel (main product) and glycerin (by-product) is obtained and utilized in the system.

For this LCA study, it is assumed that the algae residue from the 'lipid extraction and solvent recovery' stage (Figure 6-3, Chapter 6) is converted to combustible fuel for heat production. Three different assumptions used are: lignite, natural gas and hard coal.

A sensitivity study was performed using the following variables and limits,

- Energy source: Wind, Hydro, Nuclear & Solar
- Co-product residue: Natural gas & Hard coal
- Hexane recovery: 95%

Table 7-6 represents the emission factors involved with the power and co-product variables (obtained from the SimaPro software).

LCA for the conventional pathway used the electricity -emission factor as the baseline applied to Portugal (results are presented in the sensitivity study, section 7.3.2).

Table 7-6 Emission factors used to conduct the sensitivity analysis (sources: SimaPro)

EMISSION FACTORS		
	GHG	**FEC**
POWER SOURCE	**(kg CO_2 eq/kWh)**	**(MJ eq/kWh)**
Wind power	0.0131	0.168
Hydro power	0.00581	0.0386
Nuclear power	0.015	0.177
Electricity mix	0.1415 (EDP, 2013)	20.91 (EU energy in figures, 2015)
Mix photovoltaic	0.0905	1.15
RESIDUE	**(kg CO_2 eq/MJ)**	**(MJ eq/MJ)**
Lignite	0.0384	1.52
Hard coal	0.0204	1.38
Natural gas	0.014	1.12

Results are reported in values corresponding to GHG emissions and FEC consumption rates in the following section.

7.3 RESULTS AND DISCUSSION

7.3.1 Conventional versus Integrated case study

U.S. average electricity grid mix (emission factors: GHG – 0.654 kg CO2eq/kWh, FEC – 7.46 MJ eq/kWh) was used as energy source for the baseline scenario in order to compare the emissions with the integrated pathway (Case B) (Jones, et al., 2014). The GHG emissions and the fossil energy consumption obtained for conventional and integrated processes are shown in the table 7-7 (Case A vs. Case B: whole process technology).

Results clearly favor Case B; biofuels obtained in Case A lead to 22 times higher GHG emission levels (kg CO_2 eq/MJ) and 142 times higher fossil energy consumption (MJ eq/MJ) when compared with Case B.

Table 7-7 GHG emissions and fossil energy consumption for the 'well-to-wheel' process, Case A vs. Case B

Emissions	**Case A (whole process technology)**	**Case B (whole process technology)**	**Case B (excluding algae growth)**
GHG (kg CO_2 eq/GGE)	161.63	5.34	2.87
GHG (kg CO_2 eq/MJ)	1.32	0.06	0.03
Fossil energy consumption			
Fossil Energy (MJ eq/GGE)	11350.77	58.42	29.89
Fossil Energy (MJ eq/MJ)	92.77	0.65	0.33

For the integrated pathway emissions resulting from biomass cultivation are quite relevant and in the same order of magnitude of those associated with the rest of the whole process: algae growth itself contributes 0.03 GHG (kg CO_2 eq/MJ) and 0.32 FEC (MJ eq/MJ). Algae cultivation contributes to about 50% of the total emissions in the integrated system (Case B). This is due to the process upgrading which incorporates the production of

various co-products adding revenue to the whole system (Figure 6-6). Although, the front end of the integrated technology requires modification to obtain a more feasible scenario.

Figure 7-2 (for the conventional pathway) emphasizes the high contribution to emissions and energy consumption from the extraction phase while the cultivation phase adds positive value to the balance contributing maximum in the final emission output: 94% to the GHG emissions and 84% to the total fossil energy consumption. This is due to the use of high quantity of expensive chemical solvent (hexane) by the phase.

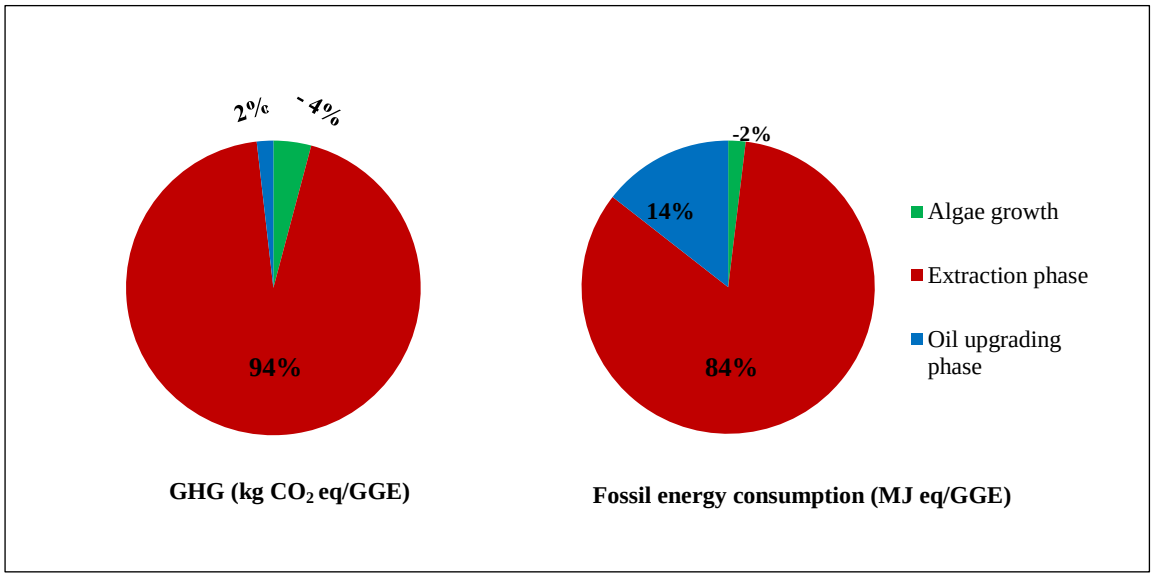


Figure 7-2 Case A: GHG emission and fossil energy consumption associated with biodiesel production stages (Conventional pathway)

Results emphasize the definite need of upgrading the extraction stage within the conventional process pathway. Excessive usage of chemicals such as hexane in this phase is definitely one of the reasons for such high emission rates.

Increasing the percentage of solvent usage recovery in the process can reduce the total emissions associated with the process and this approach has been analyzed in the sensitivity study described in section 7.3.2. Also, displacing hexane with other low emission factor solvent might contribute to better results. The microalgae biomass growth shows positive contribution when compared with rest of the process steps. Furthermore,

the emissions for microalgae biodiesel should be lower than those of conventional diesel to favor biodiesel production.

7.3.2 Sensitivity analysis (Conventional pathway)

A sensitivity analysis was performed on the results of the conventional pathway varying hexane recovery rate (95%), different residues and different electricity sources to check the feasibility in terms of emissions associated with the production chain. It is assumed that residues are replaced by lignite, natural gas and hard coal in order to bring revenue in the production system. Results displayed in tables 7-8 and 7-9 illustrate the impact of different variables on GHG emissions and fossil energy consumption rates for the conventional technology.

Table 7-8 Results of sensitivity analysis on the GHG (kg CO2 eq/GGE) emission rates

GHG (kg CO2 eq/GGE)			
Power	**Residue**	**Hexane (50% recovery)**	**Hexane (95% recovery)**
ELECTRICITY MIX (Baseline scenario)	**Natural gas**	164,2	17.1
	Lignite	158,9	11.8
	Hard coal	162,8	15.7
WIND POWER (100%)	**Natural gas**	163.8	16.7
	Lignite	158.5	11.4
	Hard coal	162.4	15.3
HYDRO POWER (100%)	**Natural gas**	163.8	16.7
	Lignite	158.5	11.4
	Hard coal	162.4	15.3
NUCLEAR POWER (100%)	**Natural gas**	163.8	16.7
	Lignite	158.5	11.4
	Hard coal	162.4	15.3
SOLAR POWER (100%)	**Natural gas**	164	16.9
	Lignite	158.7	11.6
	Hard coal	162.6	15.5

Table 7-9 Results of sensitivity analysis on the fossil energy consumption (MJ eq/GGE) emission rates

FOSSIL ENERGY CONSUMPTION (MJ eq/GGE)			
Power	**Residue**	**Hexane (50% recovery)**	**Hexane (95% recovery)**
ELECTRICITY MIX (Baseline scenario)	**Natural gas**	11470.7	2602.3
	Lignite	11384	2515.5
	Hard coal	11414.3	2545.9
WIND POWER (100%)	**Natural gas**	11409.2	2540
	Lignite	11322.5	2454
	Hard coal	11352.8	2484
HYDRO POWER (100%)	**Natural gas**	11409.2	2540
	Lignite	11322.1	2453
	Hard coal	11352.4	2484
NUCLEAR POWER (100%)	**Natural gas**	11409.2	2540
	Lignite	11322.5	2454
	Hard coal	11352.9	2540
SOLAR POWER (100%)	**Natural gas**	11412.1	2543
	Lignite	11325.4	2457
	Hard coal	11355.8	2487

It has been assumed that to environmentally improve the conventional approach that converting the algae residues obtained after the 'lipid upgrading' stage to combustible fuel can be used to generate electricity and/or heat which brings credit to the production chain. Lignite was used as the baseline assumption. Natural gas and hard coal were used as a variable in the sensitivity study to see their impact on the process. The results show that lignite consistently produces least emissions among natural gas and hard coal. Impact of different energy sources such as electricity mix, wind, hydro, nuclear and solar in the process was calculated. For the lipid extraction stage, hexane recovery at a 95% level contributes to a drastic reduction in the emission rates. Hexane recovery at 95% as compared to 50% lowers by about 9.5 times GHG emissions and by 4.5 times the FEC with natural gas and electricity mix.

Table 7-8 identifies the priority actions in the system which is predominantly based on lignite and 95% hexane recovery which results in significant GHG emission reduction when compared to natural gas and hard coal. Lignite makes the best strategy while hard coal is the second best.

The change in the electricity mix to nuclear or other power sources does not change considerably the final results, since the electricity use influence value differs by 2-3 orders of magnitude from the most relevant factors (use of raw oil, alcohol, etc.)

7.4 CONCLUDING REMARKS

Each case is unique and different in terms of species types, cultivation systems (open/closed ponds etc.), operating conditions, and the use of biomass to make different by-products. However, this does not undermine the importance of using LCA to set an overall benchmark testing feasibility of the existing production system and approach. Alternatively, technological upgradation with the life-cycle energy consumption for commercial production must be considered. In this thesis the sensitive prone area for the conventional pathway is the extraction stage (94% GHG and 84% FEC) impacting most to the total emissions while for integrated case it is the microalgae cultivation step (~50% contribution to total emissions). Recovery and re-use of solvents for the extraction step can reduce the emissions and optimize the overall cost.

Technical feasibility associated with the widely proposed pathway represented by the conventional process shows poor emission rates compared with the integrated system. A better knowledge and understanding is needed to increase the efficiency of each of its phases to decrease the overall emission rates. A different approach (integrated model: Case B), which relies on valorization of co-products and residues seems to be more efficient at all levels. Hence, correct planning to increase the revenue associated with co- and by-products is expected to play a vital role in achieving feasibility.

Closed PBRs (figure 6-8, Chapter 6), although they produce high yield microalgae biomass, they are more expensive to run than open ponds for commercial biodiesel production. However, open ponds present serious risks of culture contamination. The realistic LCA of the conventional model (without assuming the usage of ‘residue’ to add revenue in the system) used for the microalgae biodiesel production in this thesis was calculated; the results indicate an increase of 5% GHG emissions and 3% higher FEC when compared to the usage of ‘residue’.

8 CONCLUSIONS AND FUTURE WORK

The main motivation of this PhD research was to examine production of microalgae derived biodiesel in order to evaluate the constraints within the production process and the economic and environmental feasibility. The work conducted within the present thesis involved algae cultivation, biomass harvesting, lipid extraction, lipid upgrading and FAME purification. Parameters related with the main transformation technologies to produce required quality microalgae biodiesel were fully characterized.

A research gap in the field was identified as a scale up effect (experimental small scale feasibility versus the expected large scale viability). From this point different transformation technologies were analyzed. The results achieved at laboratorial scale were extrapolated by conducting TEA and LCA on a large scale process. The work conducted proved that the biodiesel obtained from microalgae is adequate and might great potential for use in vehicle engines.

Some of the results obtained were published in scientific journals or conference proceedings that resulted from this research, such as the acceptance of biodiesel from microalgae grown in lagoons in the transportation crisis, the effect of illumination period and aeration rate on the extracted fuel property, the integration of wastewater to an ensuring sustainable route towards commercialization and a comparative conventional vs. integrated technological study on TEA and LCA of the whole process.

Thus, the main objectives of this research were fully achieved and resulted in the following outcomes:

1. The main transformation technologies to produce microalgae-derived biodiesel (including harvesting to oil processing) were characterized;

2. Identification of bottlenecks towards improvements in the efficiency, cost structure, ability to scale-up algal growth and lipid extraction to produce commercially feasible biodiesel was conducted;
3. The integration of algae oil extraction and trans-esterification methods was analyzed to improve the economic prospective;
4. An economic analysis of the microalgae-derived biodiesel production was executed;
5. A LCA evaluating the environmental impacts of the microalgae produced biodiesel was developed;
6. The entire microalgae to biodiesel chain were compared carrying several case studies.

Important contribution of the research was:

- The ability to define microalgae strain potential by testing different species;
- Effect of illumination and aeration rates on biomass cultivation;
- Biodiesel with standard quality was obtained from microalgae;
- Laboratorial success of all the steps involved with the production technology;
- Identification of major constraints to large-scale microalgae derived biodiesel;
- Comparison of different transformation technologies;
- Identification and assessment of the major parameters which might influence the technical, economic and environmental outcome of biodiesel from microalgae.

General conclusions of this research:

1. Microalgae have the potential to become a renewable energy feedstock that could to some extent meet the arising demand for transportation fuels. The algae cell growth is sensitive to the cultivation parameters namely CO_2 supply for growth, light/dark regime, effective mixing mechanisms, temperature control, pH range etc. The experimental results have shown that microalgae follow an exponential growth pattern reaching a maximum cell count by the 8^{th} day of culture. The use of

continuous illumination results in higher final cell concentration compared to 12:12h photoperiods. Combination of high aeration rates and continuous illumination for microalgae growth is confirmed as a good strategy for high quantity biomass production;

2. Application of conventional technologies (microalgae to biodiesel), predicts to be in good agreement with the experimental data. Although, development of the methodology for upgrading the system towards commercial feasibility is important;

3. Wastewater usage for the microalgae cultivation seems to be an appropriate solution from a sustainable biomass production point of view. Feasibility depends largely on the lipid extraction stage which is strongly conditioned by solvent usage. Upgrading of the technological stages and proper co-product usage will directly impact on feasibility;

4. The comparative TEA found that an integrated methodology seems to be a good approach since co-products add credit to the whole production chain. In terms of future work the inclusion of co-products valorization can improve the performance of a conventional pathway;

5. LCA research results point out different behavior for different case studies. For an integrated system, algae growth was seen to be responsible for half of the total emissions. The extraction phase was dominating for the conventional system: 94% of the total emissions resulted from extraction.

Specific conclusions:

Biomass production

1. It was experimentally observed that microalgae growth dynamics passes through five stages of life as shown in previous studies. In order to obtain maximum biomass, cultures must be harvested at the fourth stage;

2. A daily comparison of *Chlorella vulgaris* growth for 10 days was performed. The average maximum growth rate occurs between the 7^{th} and the 9^{th} culture days. Microalgae cells are directly dependent on light supply for their growth. It is seen that the cells absorb maximum light energy between $8^{th} – 9^{th}$ days of culture;

3. Under continuous illumination, different aeration rates hold no dramatic effect on the total biomass production rate;

Biodiesel production

1. The trade-offs between biodiesel quality via dry and wet extraction routes were investigated for *Chlorophyta* and *Scenedesmus*. Dry techniques were more effective in producing standard quality biodiesel independently of the microalgae strain. Quality characterization of the extracted biodiesels with all strains showed potential for the European transport sector;

2. The results have shown that wastewater can be used to produce microalgae suitable for quality biodiesel production. For FAME content a total of 91% was measured with *Chlorella vulgaris* using wastewater for the culture, while 61% was obtained using potable water. FAME content showed a similar pattern independently of ultrasonication or soxhlet extraction;

3. Biodiesel was extracted from dry *Nannochloropsis gadiatana* and satisfactory values were obtained for water content, FAME, viscosity and density;

4. A realistic approach for obtaining larger quantity of biodiesel would need to control on the usage of solvent.

Techno-economic analysis

1. A comparative case study on the final fuel cost was examined using two different technological pathways: conventional and integrated. The main processing methods designed for a conventional facility includes solvent extraction, trans-esterification and product purification. For the integrated facility they include fermentation, distillation, solvent extraction, product purification and hydro-treatment;

2. The biomass cost using the designed integrated facility commonly is almost 3 times lower than that obtained in the conventional facility. Minimum fuel selling price for the integrated case is 4.35$/GGE while for the conventional facility a value of 10.55$/GGE is obtained. This result determines the requirement to develop new strategies. These strategies could include using co-products to add benefit to the system, recycling a higher percentage of the chemical solvent used, using CO_2 from power plants, using algae residue etc. for the conventional pathway in order to make it more competitive;

3. Results have shown that the PBRs are more expensive for biodiesel production than open ponds. The higher costs in PBRs are due to the required resources in terms of energy and capital/operation related to the controlled growth environment.

Life cycle analysis

1. The calculated fossil energy and GHG emission at all stages of the production system for the conventional case study showed that improvement is needed on the extraction phase for environmental feasibility. 94% of GHG emissions and 84% of fuel energy consumption is due to the lipid extraction phase which shows that the usage of solvents is definitely one of the reasons for such high emission rates. A sensitivity study showed a drastic reduction in the emission rates when the hexane recovery changes from 50% to 95%. Use of a conventional facility design results in 22 times higher GHG emissions and 142 times higher fossil energy consumption than the integrated design;

2. For the integrated design, the algae cultivation stage contributes with 50% of the total emissions.

Implementation considerations:

The adopted methodology provided the opportunity to evaluate quality enhancing parameters and the economic and environmental impact of the production process hence contributing to the decision making in areas such as:

1. Selection of right microalgae strain;
2. Selection of the optimal conditions for the microalgae cultivation;
3. Selection of the most appropriate cultivation systems to maximize high yield biomass production and minimizing the cost related with it;
4. Identification of the harvesting and extraction techniques with the highest economic impacts;
5. Analysis of lipid to biodiesel conversion methodologies and evaluation of the quality of the product;
6. Understanding which production stages have impacts on the economic and environmental feasibility of the process;
7. Comparison of the final produced fuel cost and emissions between different technological systems (such as conventional and integrated).

It must be emphasized that, since the main objective of this work was to understand and develop a technology for the entire microalgae biodiesel chain, it was not necessary to consider all possible techniques available in literature. Since, it would have been impossible in practical terms to characterize the whole production to feasibility study of all microalgae strains and technological pathways available. In terms of future work, the development of conventional pathway with new strategies would be desirable. Microalgae have the potential to be a relevant and sustainable renewable energy feedstock. In spite of the many advantages, microalgae biofuels also have some disadvantages such as costly harvesting process and low raw-biomass-to-fuel conversion rate. These limitations could be overcome by designing advanced photo-bioreactors and developing low cost

technologies for biomass harvesting, drying and oil extraction. In addition, genetic engineering technology in the can be an efficient strategy to improve biomass and biofuel production. Genetic engineering seems an important role in the production of valuable products with minimal costs. Finally, it must be pointed out that the research work in order to make it possible to upgrade it is required to optimize the impact on emissions and cost.

9 REFERENCES

Acién, F., Fernández, J., Magán, J. & Molina, E., 2012. Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnology Advances*, 30(6), pp. 1344-1353.

Afonso, M. d. C. P., 2009. Enzymatic biodiesel production from free fatty acids, Aveiro: University of Aveiro, Master's thesis.

Agarwal, A. K., 2007. Biofuels (alcohols and biodiesel) applications as fuels for internal combustion engines. *Progress in energy and combustion science,* 33(3), pp. 233-271.

Alabi, A. O., Tampier, M. & Bibeau, E., 2009. *Microalage technologies & processes for biofuels/bioenergy production in British Columbia: current technology, sustainability & barriers to implementation*: Seed Science Ltd.

Amaro, H. M., Guedes, A. C. & Malcata, F. X., 2011. Advances and perspectives in using microalgae to produce biodiesel. *Applied Energy,* 88(10), pp. 3402-3410.

Anselm Eisentraut, 2013. *Renewable energy medium-term market report. Biofuels outlook market developments and policy challenges. Market trends and projections to 2018,* s.l.: IEA.

Aqualia, 2016. *Aqualia infraestructuras,* Madrid.

Aziz, M., Oda, T. & Kashiwagi, T., 2013. Enhanced high energy efficient steam drying of algae. *Applied Energy,* 109(C), pp. 163-170.

Barsanti, L. & Gualtieri, P., 2010. *Algae: anatomy, biochemistry and biotechnology.* 2nd ed. Italy: CRC .

Barupal, D. K., Kind, T., Kothari. S. L., Lee, D.Y & Fiehn, O., 2010. Hydrocarbon phenotyping of algal species using pyrolysis-gas chromatography mass spectrometry. *BMC Biotechnology,* 10(40).

Batan, L., Quinn, J., Willson, B. & Bradley, T., 2010. Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae. *Environmental Science andTechnology,* 44(20), pp. 7975-7980.

Beatriz, C. & Voltolina, D., 1997. Viability of mass algal cultures preserved by freezing and freeze-drying. *Aquaculturural Engineering,* 16(4), pp. 205-211.

Bellou, S., Baeshen, M. N., Elazzazy, A. M., Aggeli, D., Sayegh, F. & Aggelis, G., 2014. Microalgae lipids biochemistry and biotechnological perspectives. *Biotechnology Advances,* 32(8), pp. 1476-1493.

Benavides, A. M. S., Torzillo, G., Kopecký, J. & Masojídek , J., 2013. Productivity and biochemical composition of Phaedactylum tricornutum (Bacillariophyceae) cultures grown outdoors in tubular photobioreactors and open ponds. *Biomass and Bioenergy,* Volume 54, pp. 115-122.

Benemann, J. & Oswald, W., 1996. *Systems and economic analysis of microalgae ponds for conversion of CO2 to biomass,* Berkeley.

Benemann, J., Woertz, I. & Lundquist, T., 2012. Life cycle assessment for microalgae oil production. *Disruptive Science and Technology,* 1(2), pp. 68-78.

Bennette, M. C., Turn, S. Q. & Chan, W. Y., 2014. A methodology to assess open pond, phototrophic, algae production potential: A Hawaii case study. *Biomass and Bioenergy,* Volume 66, pp. 168-175.

Berardi, U., 2015. Building energy consumption in US, EU, and BRIC countries. *Procedia Engineering,* Volume 118, pp. 128-136.

Berrios, M., Martín, M., Chica, A. & Martín, A., 2011. Purification of biodiesel from used cooking oils. *Applied Energy,* 88(11), pp. 3625-3631.

Bhutto, A. W., Bazmi, A. A. & Zahedi, G., 2011. Greener energy: Issues and challenges for Pakistan-biomass energy prospective. *Renewable and sustainable energy reviews,* 15(6), pp. 3207-3219.

Bilanovic, D., Holland, M. & Armon, R., 2012. Microalgae CO2 sequestering- modeling microalgae production costs. *Energy Conversion and Management,* Volume 58, pp. 104-109.

Bilanovic, D., Shelef, G. & Sukenik, A., 1988. Flocculation of microalgae with cationic polymers- effects of medium salinity. *Biomass,* 17(1), pp. 65-76.

Bligh, E. & Dyer, W., 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology ,* 37(8), pp. 911-917.

Bloomberg news, 2011. *Led by demand in China, energy use in projected to rise 53% by 2035,* New York.

Borowitzka, M. A., 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology,* 70(1-3), pp. 313-321.

Borowitzka, M. A., 2012. Energy from microalgae: a short history. In: *Algae for biofuels and energy*. s.l.:Springer Netherlands, pp. 1-15.

BP stats, 2015. *BP statistical review of world energy* , London.

Brennan, L. & Owende, P., 2010. Biofuels from microalgae- A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews,* 14(2), pp. 557-577.

Brentner, L. B., Eckelman, M. J. & Zimmerman, J. B., 2011. Combinatorial life cycle assessment to inform process design of industrial production of algal biodiesel. *Environmental Science Technology,* 45(16), pp. 7060-7067.

Cabral, P. H. F. G., 2008. Research on operating conditions of Photobioreactors, Aveiro: University of Aveiro, Master's thesis.

Cai, T., Park, S. Y. & Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: Status and prospects. *Renewable and Sustainable Energy Reviews,* Volume 19, pp. 360-369.

Campbell, P., Beer, T. & Batten, D., 2011. Life cycle assessment of biodiesel production from microalgae in ponds. *Bioresource Technology,* 102(1), pp. 50-56.

Campbell, P. K., Beer, T. & Batten, D., 2009. *Greenhouse gas sequestration by algae-energy and greenhouse gas life cycle studies.* Australia, Proceedings of the 6th Australian Conference on Life Cycle Assessment.

Canakci, M., 2007. The potential of restaurant waste lipids as biodiesel feedstocks. *Bioresource Technology*, 98(1), pp. 183-190.

Canakci, M. & Gerpen, J. V., 1999. Biodiesel production via acid catalysis. *American Society of Agricultural Engineers*, 42(5), pp. 1203-1210.

Carmo, M. P. S., 2012. Biodiesel de micro-algas: balanço energético e económico, Aveiro: University of Aveiro, Master's thesis.

Carroll, J., 2013. Exxon atleast 25 years away from making fuel from algae, BloombergBusiness.

Carvalho, A., Silva, S., Baptista , J. & Malcata, F., 2011. Light requirements in microalgal photobioreactors: an overview of biophotonic aspects. *Applied Microbiology and Biotechnology,* 89(5), pp. 1275-1288.

Chen, C.-Y.et al., 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology,* 102(1), pp. 71-81.

Cheng, J. J. & Timilsina, G., 2011. Status and barriers of advanced biofuel technologies: A review. *Renewable Energy,* 36(12), pp. 3541-3549.

Cheng, J. et al., 2014. Fractal microstructure characterization of wet microalgal cells disrupted with ultrasonic cavitation for lipid extraction. *Bioresource Technology,* Volume 170, pp. 138-143.

Cheng-Wu, Z., Zmora, O., Kopel, R. & Richmond, A., 2001. An industrial-size flat plate glass reactor for mass production of Nannochloropsis sp. (Eustigmatophyceae). *Aquaculture,* Volume 195, pp. 35-49.

Chen, Y., Liu, J. & Ju, Y.-H., 1998. Flotation removal of algae from water. *Colloids and Surfaces B: Biointerfaces,* 12(1), pp. 49-55.

Chi, R., Tian. J., Gao, H., Zhou, F., Liu, M., Wang, C. & Wu, Y., 2006. Kinetics of leaching flavonoids from Pueraria Lobata with Ethanol. *Chinese Journal of Chemical Engineering,* 14(3), pp. 402-406.

Chisti, Y., 2007. Biodiesel from microalgae. *Biotechnology advances,* 25(3), pp. 294-306.

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology,* 26(3), pp. 126-131.

Chisti, Y. & Moo-Young, M., 1986. Disruption of microbial cells for intracellular products. *Enzyme and Microbial Technology,* 8(4), pp. 194-204.

Choi, S., Lee, J., Kwon, D. & Cho, K., 2006. Settling characteristics of problem algae in the water treatment process. *Water Science & Technology,* 53(7), pp. 113-119.

Cho, S.-C., Choi, W.-Y., Oh, S.-H., Lee, C.-G., Seo, Y.-C., Kim, J.-S., Song, C.-H., Kim, G.-V., Lee, S.-Y., Kang, D.-H., Lee, H.-Y., 2012. Enhancement of lipid extraction from marine microalga, Scenedesmus associated with high-pressure homogenization process. *Journal of Biomedicine and Biotechnology,* pp. 1-6.

Christenson, L. & Ronald, S., 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnology Advances,* 29(6), pp. 686-702.

Clarens, A. F., Resurreccion, E. P., White, M. A. & Colosi, L. M., 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. *Environmental Science and Technology,* 44(5), pp. 1813-1819.

Coelho, M. C., Dutta, S. & Silva, F. N., 2013. Biodiesel from lagoon microalgae, acceptable alternative fuel for the transportation crunch?. *Transportation Research Record: Journal of Transportation Research Board,* Volume 2385, pp. 11-18.

Colin, B. M. & Robert, H. E., 2012. The energy return on investment for algal biocrude: results for a research production facility. *BioEnergy Research,* 5(2), pp. 341-362.

Contreras, A., Garcia, F., Molina, E. & Merchuk, J., 1998. Interaction between CO2-mass transfer, light availability, and hydrodynamic stress in the growth of phaeodactylum tricornutum in a concentric tube airlift photobioreactor. *Biotechnology and Bioengineering,* 60(3), pp. 317-325.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P. & Borghi, M. D., 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. *Chemical Engineering and Processing. Process Intensification,* 48(6), pp. 1146-1151.

Costa, J. A. V., Colla, L. M. & Filho, P. F. D., 2004. Improving Spirulina platensis biomass yield using a fed-batch process. *Bioresource Technology,* 92(3), pp. 237-241.

Cruz, V. d. l., Hernández, S., Martín, M. & Grossmann, I. E., 2014. Integrated synthesis of biodiesel, bioethanol, isobutane, and glycerol ethers from algae. *Industrial & Engineering Chemistry Research,* 53(37), pp. 14397-14407.

Davis , R., Kinchin, C., Markham, J., Tan, E. C. D. & Laurens, L. M. L., 2014. Process design and economics for the conversion of algal biomass to biofuels: algal biomass fractionation to lipid-and carbohydrate-derived fuel products, Colorado: U.S. Department of Energy.

Davis, R., Aden, A. & Pienkos, P. T., 2011. Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy,* 88(10), pp. 3524-3531.

Davis, R., Fishman, D., Frank, E. D. & Wigmosta, M. S., 2012. *Renewable diesel from algal lipids: An integrated baseline for cost, emissions, and resource potential from a harmonized model,* Colorado: U.S. Department of Energy.

Demazel, D., 2008. Use of algae as as energy source, Nantes: ECN.

Demirbas, A., 2008. Biodiesel: A realistic fuel alternative for diesel engines. 1 ed. s.l.:Springer-verlag London.

Demirbas, A. & Demirbas, F. M., 2011. Importance of algae oil as a source of biodiesel. *Energy conversion and management,* 52(1), pp. 163-170.

Desmorieux, H. & Decaen, N., 2005. Convective drying of spirulina in thin layer. *Journal of Food Engineering,* 66(4), pp. 497-503.

Divakaran, R. & Pillai, V. S., 2002. Flocculation of algae using chitosan. *Journal of Applied Phycology,* 14(5), pp. 419-422.

Donot, F., Cazals. G., Gunata, Z., Egron, D., Malinge, J., Strub, C., Fontana, A. & Schorr-Galindo, S., 2013. Analysis of neutral lipids from microalgae by HPLC-ELSD and APCI-MS/MS. *Journal of Chromatography B,* Volume 942-943, pp. 98-106.

Dutta, S., Neto, F., Coelho, M. C., Gonçalves, P. P., Silva, V. S., Kopyl, S., Soares, N., Gracio, J. & Sousa, A. C. M., 2011. Analysis of potential of high quality biodiesel production from microalgae. 19th European Biomass Conference and Enhibition, pp. 822-824.

EDP, 2013. *Origens da Eletricidade.* [Online] Available at: http://www.edpsu.pt/pt/origemdaenergia/Pages/OrigensdaEnergia.aspx [Acedido em 5 March 2016].

Eduardo, J.-L., Scoparo, C. H. G., Lacerda, L. M. C. F. & Franco, T. T., 2009. Effect of light cycles (night/day) on CO2 fixation and biomass production by microalgae in photobioreactors. *Chemical Engineering and Processing. Process Instensification* , 48(1), pp. 306-310.

Edzwald, J., 1993. Algae, bubbles, coagulants and dissolved air flotation. *Water Science & Technology,* 27(10), pp. 67-81.

Energy data, 2010. *World on the Edge- energy data- biofuels.*

Energy.gov, 2015. *Algenl announces commercial algal ethanol fuel partnership,* Washington: U.S. Department of Energy.

EU energy in figures, 2015. *Statistical pocketbook 2015,* s.l.: European Commission.

EU transport in figures, 2013. *Statistical pocketbook,* Luxembourg: European commission.

EU transport in figures, 2015. *Statistical pocketbook,* Luxembourg: European commission.

European Standard EN 14214:2008+A1, 2009. *Automotive fuels-Fatty acid methyl esters (FAME) for diesel engines- Requirements and test methods,* UK: Bristish Standard.

Fangrui, M. & Milford, H. A., 1999. Biodiesel production: a review. *Bioresource Technology,* 70(1), pp. 1-15.

Fishman, D., Majumdar, R., Morello, J., Pate, R., & Yang, J., 2010. National algal biofuels technology roadmap,U.S. Department of Energy.

Folch, J., Lees, M. & Sloane, S. G., 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry,* 226(1), pp. 497-509.

Freedman, B., Pryde, E. & Mounts, T., 1984. Variables affecting the yields of fatty esters from transesterified vegetable oils. *Journal of the American Oil Chemists Society,* 61(10), pp. 1638-1643.

García-Malea, M. C., Acién, F. G., Del, R. E., Fernández, J. M., Cerón, M. C., Guerrero, M. G. & Molina.Grima, E., 2008. Production of astaxanthin by Haematococcus pluvalis: Taking the one-step system outdoors. *Biotechnology and Bioengineering,* 102(2), pp. 651-657.

Geciova, J., Bury, D. & Jelen, P., 2002. Methods for disruption of microbial cells for potential use in the diary industry-a review. *International Dairy Journal,* 12(6), pp. 541-553.

Golueke, C. & Oswald, W., 1965. Harvesting and processing sewage-grown planktonic algae. *Journal (water pollution control federation),* 37(4), pp. 471-498.

Gonçalves , A. L., Pires, J. C. & Simões, M., 2013. Lipid production of Chlorella vulgaris and Pseudokirchneriella subcapitata. *International Journal of Energy and Environmental Engineering,* 4(14).

Gouveia, L., Marques, A., da Silva T.L & Reis, A., 2009. Neochloris oleabundans UTEX#1185: a suitable renewable lipid source for biofuel production. *Journal of Industrial Microbiology and Biotechnology,* 36(6), pp. 821-826.

Gouveia, L. & Oliveira, A., 2009. Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology and Biotechnology,* 36(2), pp. 269-274.

Griffiths, M. J. & Harrison, S. T., 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology,* 21(5), pp. 493-507.

Grima, E. M., Belarbi, E.-H., Fernández, F. G. A., Medina, A. R. & Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances,* 20(7-8), pp. 491-515.

Grima, E. M., Fernández, F. A., Camacho, F. G. & Chisti, Y., 1999. Photobioreactors: light regime, mass transfer, and scaleup. *Journal of Biotechnology,* 70(1-3), pp. 231-247.

Guckert, J. B., Cooksey, K. E. & Jackson, L. L., 1988. Lipid solvent systems are not equivalent for analysis of lipid classes in the microeukaryotic green alga, Chlorella. *Journal of Microbiological Methods,* 8(3), pp. 139-149.

Gultom, S. O. & Hu, B., 2013. Review of Microalgae Harvesting via Co-Pelletization with Filamentous Fungus. *Energies,* 6(11), pp. 5921-5939.

Halim, R., Gladman, B., Danquah, M. & Webley, P., 2011. Oil extraction from microalgae for biodiesel production. *Bioresource Technology,* 102(1), pp. 178-185.

Halim, R., Harun, R., Danquah, M. K. & Webley, P. A., 2012. Microalgae cell disruption for biofuel development. *Applied Energy,* 91(1), pp. 116-121.

Hamje, H. D., Hass, H., lonza, L., Mass, Heiko., Reid, A., Rose, K. D., & Venderbosch, T., 2014. EU renewable energy targets in 2020: revised analysis of scenarios for transport fuels, Luxembourg: European commision.

Harun, R., Singh, M., Forde, G. M. & Danquah, M. K., 2010. Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and sustainable energy reviews,* 14(3), pp. 1037-1047.

Henderson, R., Parsons, S. A. & Jefferson, B., 2009. The impact of algal properties and pre-oxidation on solid-liquid separation of algae. *Water Research,* 42(8-9), pp. 1827-1845.

Herndon, A., 2013. Tesoro is first customer for Sapphire's algae-derived crude oil, BloombergBusiness.

Hielscher- Ultrasound Technology, 1999. *Hielscher- Ultrasound Technology.* [Online] Available at: https://www.hielscher.com/algae_extraction_01.htm#Ultrasonic_Enzymatic_Extraction [Acedido em 5 March 2016].

Ho, S.-H., Huang, S. W., Chen, C. Y., Hasunuma, T., Kondo, A. & Chang, J. S., 2013. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresource Technology,* Volume 135, pp. 191-198.

Hosikian, A., Lim, S., Halim, R. & Danquah, M. K., 2010. Chlorophyll extraction from microalgae: A review on the process engineering aspects. *International Journal of Chemical Engineering,* pp. 1-12.

Hsueh, H., Li, W., Chen, H. & Chu, H., 2009. Carbon bio-fixation by photosynthesis of Thermosynechococcus sp. CL-1 and Nannochloropsis oculta. *Journal of Photochemistry and Photobiology B: Biology,* 95(1), pp. 33-39.

Huang, G., Chen, F., Dong, W., Zhang, X. & Chen, G., 2010. Biodiesel production by microalgal biotechnology. *Applied Energy,* 87(1), pp. 38-46.

Hu, H. & Gao, K., 2003. Optimization of growth and fatty acid composition of a unicellular marine picoplankton, Nannochloropsis sp., with enriched carbon sources. *Biotechnology Letters,* 25(5), pp. 421-425.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. & Darzins, A., 2008. Microalgae triaclyglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal,* 54(4), pp. 621-639.

IEA, 2010. *World energy outlook,* France: IEA.

IEA, 2015. *International energy agency- oil market report,* France.

Index mundi, 2016. *World biodiesel production and consumption by year.* [Online] Available at: http://www.indexmundi.com/energy.aspx?region=xx&product=biodiesel&graph=production+consumption
[Acedido em 2 March 2016].

Islam, M. & Beg, M., 2004. The fuel properties of pyrolysis liquid derived from urban solid wastes in Bangladesh. *Bioresource Technology,* 92(2), pp. 181-186.

Jiang, L., Luo, S., Fan, X., Yang, Z. & Guo, R., 2011. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO2. *Applied Energy,* 88(10), pp. 3336-3341.

Jones, S., Zhu, Y., Anderson, D., Hallen, R., Elliot, D., Schmidt, A., Albrecht, K., Hart, T., Butcher, M., Drennan, C. & Snowden-Swan, L., 2014. Process design and economics for the conversion of algal biomass to hydrocarbons: whole algae hydrothermal liquefaction and upgrading, Oak Ridge: U.S. Department of Energy.

Jonker, J. & Faaij, A., 2013. Techno-economic assessment of microalgae as feedstock for renewable bioenergy production. *Applied Energy,* Volume 102, pp. 461-475.

Jorquera, O., Kiperstok, A., Sales, E. A., Embiruçu, M. & Ghirardi, M. L., 2010. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. *Bioresource Technology,* 101(4), pp. 1406-1413.

Kadam, K., 2002. Environmental implications of power generation via coal-microalgae cofiring. *Energy,* 27(10), pp. 905-922.

Karmee, S. K. & Chadha, A., 2005. Preparation of biodiesel from crude oil of Pongamia pinnata. *Bioresource Technology,* 96(13), pp. 1425-1429.

Keris-Sen, U., Sen, U., Soydemir, G. & Gurol, M., 2014. An investigation of ultrasound effect on microalgal cell integrity and lipid extraction efficiency. *Bioresource Technology,* Volume 152, pp. 407-413.

Khan, S. A., Rashmi, Hussain, M. Z., Prasad, S., Banerjee & U. C., 2009. Prospects of biodiesel production from microalgae in India. *Renewable and Sustainable Energy Reviews,* 13(9), pp. 2361-2372.

Khoeyi, Z. A., Seyfabadi, J. & Ramezanpour, Z., 2012. Effect to light intensity and photoperiod on biomass and fatty acid composition of the microalgae, Chlorella vulgaris. *Aquacultural International,* 20(1), pp. 41-49.

Khoo, H., Sharratt, P. N., Das, P., Balasubramanian, R. K., Naraharisetti, P. K., & Shaik, S., 2011. Life cycle energy and CO2 analysis of microalgae-to-biodiesel: reliminary results and comparisons. *Bioresource Technology,* 102(10), pp. 5800-5807.

Kim, D.-Y., Vijayan, D., Praveenkumar, R., Han, J.-I., Lee, K., Parl, J.-Y., Chang, W.-S., Lee, J.-S. &Oh, Y.-K., 2016. Cell-wall disruption and lipid/astaxanthin extraction from microalgae:Chlorella and Haematococcus. *Bioresource Technology,* Volume 199, pp. 300-310.

Kingston, H. & Haswell, S. J., 1999. Microwave-enhanced chemistry: fundamentals, sample preparation and applications. In: Washington: American Chemical Society, p. 4729.

Kingston, H. & Jassie, L., 1989. Introduction to microwave sample preparation: theory and practice. *Analytical Chemistry,* 61(21), p. 1240A.

Knuckey, R. M., Brown, M. R., Robert, R. & Frampton, D. M., 2006. Production of microalgal concentrates by flocculation and their assessment as aquaculture feeds. *Aquacultural Engineering,* 35(3), pp. 300-313.

Korres, N. E., Thamsiriroj, T., Smyth, B. M., Nizami, A. S., Singh, A. & Murphy, J. D., 2011. Grass biomethane for agriculture and energy. In: E. Lichtfouse, ed. *Genetics, biofuels and local farming systems.* s.l.:s.n., pp. 5-49.

Kumar, R. R., Rao, P. H. & Arumugam, M., 2014. Lipid extraction methods from microalgae: a comprehensive review. *Frontiers in Energy Research,* 2(61).

Lam , M. K., Lee, K. T. & Mohamed, A. R., 2009. Life cycle assessment for the production of biodiesel: A case study in Malaysia for palm oil versus jatropha oil. *Biofpr biofuels bioproducts and biorefining,* 3(6), pp. 601-612.

Lam, M. K. & Lee, K. T., 2012. Microalgae biofuels: A critical review of issues, problems and the way forward. *Biotechnology advances,* 30(3), pp. 673-690.

Lam, M. K., Lee, K. T. & Mohamed, A. R., 2010. Homogenous, heterogenous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: A review. *Biotechnology Advances,* 28(4), pp. 500-518.

Lane, J., 2013. Algenol hits 9K gallons/acre mark for algae-to-ethanol process.

Lardon, L., Hélias, A., Sialve, B., Steyer, J.-P. & Bernard, O., 2009. Life-cycle assessment of biodiesel production from microalgae. *Environmental Science and Technology,* 43(17), pp. 6475-6481.

Larsdotter, K., 2006. Wastewater treatment with microalgae- a literarture review. *VATTEN,* Volume 62, pp. 31-38.

Leach, G., Oliveira, G. & Morais, R., 1998. Spray-drying of Dunaliella salina to produce a beta-carotene rich powder. *Journal of Industrial microbiology and biotechnology,* 20(2), pp. 82-85.

Leder , F. & Shapiro, J. N., 2008. This time it's different: an inevitable decline in world petroleum production will keep oil product prices high, causing military conflicts and shifting wealth and power from democracies to authoritarian regimes. *Energy policy,* 36(8), pp. 2850-2852.

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. *Bioresource Technology,* 101(1), pp. S75-S77.

Lee, O. K., Seong, D. H., Lee, C. G. & Lee, E. Y., 2015. Sustainable production of liquid biofuels from renewable microalgae biomass. *Journal of Industrial and Engineering Chemistry,* Volume 29, pp. 24-31.

Lee, S., Kim, S.-B., Kim, J.-E., Kwon, G.-S., Yoon, B.-D. & Oh, H.-M., 1998. Effects of harvesting method and growth stage on the flocculation of the green alga Botryococcus braunii. *Letters in Applied Microbiology,* 27(1), pp. 14-18.

Liao, Q., Lin, L., Rong, C. & Zhu, X., 2014. A novel photobioreactor generating the light/dark cycle to improve microalgae cultivation. *Bioresource Technology,* Volume 161, pp. 186-191.

Liu, Z.-Y., Wang, G.-C. & Zhou, B.-C., 2008. Effect of iron on growth and lipid accumulation in Chlorella vulgaris. *Bioresource Technology,* 99(11), pp. 4717-4722.

Li, Y., Horsman, M., Wu, N., Lan, C. Q. & Dubois-Calero, N., 2008. Biofuels from microalgae. *Biotechnology Progress,* 24(4), pp. 815-820.

Lopes, T., Reis, A., Medeiros, R., Oliveira, A. C. & Gouveia, L., 2009. Oil production towards biofuel from autotrophic microalgae semicontinuous cultivations monitorized by flow cytometry. *Applied Biochemistry and Biotechnology,* 159(2), pp. 568-578.

Lundquist, T., Woertz, I., Quinn, N. & Benemann, J., 2010. *A reslistic technology and engineering assessment of algae biofuel production,* Berkeley, California.

Macías-Sánchez, M., Mantell, C., Rodríguez, M., Ossa, E. M., Lubían, L. M. & Montero, O., 2007. Supercritical fluid extraction of carotenoids and chlorophyll a from Synechococcus sp. *The Journal of Supercritical fluids,* 39(3), pp. 323-329.

Marchetti, J. & Errazu, A., 2008. Technoeconomic study of supercritical biodiesel production plant. *Energy Conversion and Management,* 49(8), pp. 2160-2164.

Martínez-Jerónimo, F. & Espinosa-Chávez, F., 1994. A laboratory-scale system for mass culture of freshwater microalgae in polyethylene bags. *Journal of Applied Phycology,* 6(4), pp. 423-425.

Martín, M. & Grossmann, I. E., 2012. Simultaneous optimization and heat integration for biodiesel production from cooking oil and algae. *Industrial & Engineering Chemistry Research,* 51(23), pp. 7998-8014.

Martín, M. & Grossmann, I. E., 2014. Design of an optimal process for enhanced production of bioethanol and biodiesel from algae oil via glycerol fermentation. *Applied Energy,* Volume 135, pp. 108-114.

Mata, T. M., Martins , A. A., Sikdar, S. K. & Costa, C. A., 2011. Sustainability considerations of biodiesel based on supply chain analysis. *Clean Technologies and Environmental Policy,* 13(5), pp. 655-671.

Mata, T. M., Martins, A. A. & Caetano, N. S., 2010. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews,* Volume 14, pp. 217-232.

Mathew, D., Lorenzo, G. D. & Pilidis, P., 2013. *Developing a sustainable microalgae biofuel production process model through Life Cycle Assessment (LCA).* Greece, 3rdInternational Exergy, Life Cycle Assessment, and sustainability Workshop & Symposium (ELCAS3).

Meher, L., Dharmagadda, V. & Naik, S., 2006. Optimization of alkali-catalyzed transesterification of Pongamia pinnata oil for production of biodiesel. *Bioresource Technology,* 97(12), pp. 1392-1397.

Mendes, R., Reis, A. & Palavra, A., 2006. Supercritical CO2 extraction of γ-linolenic acid and other lipids from Arthrospira (Spirulina) maxima: Comparison with organic solvent extraction. *Food Chemistry,* 99(1), pp. 57-63.

Mendoza, H., Carmona, L., Assunção, P., Freijanes, K., Jara, A., Portillo, E., & Torres, A., 2015. Variation in lipid extractability by solvent in microalgae. Additional criterion for selecting species and strains for biofuel production from microalgae. *Bioresource Technology,* Volume 197, pp. 369-374.

Menegaki, A. N., 2011. Growth and renewable energy in Europe: A random effect model with evidence for neutrality hypothesis. *Energy economics*, 33(2), pp. 257-263.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q. & Xian, M., 2009. Biodiesel production from oleaginous microorganisms. *Renewable Energy*, 34(1), pp. 1-5.

Mercer, P. & Armenta, R. E., 2011. Developments in oil extraction from microalgae. *European Journal of Lipid Science and Technology*, 113(5), pp. 539-547.

Miao, X. & Wu, Q., 2006. Biodiesel production from heterotrophic microalgal oil. *Bioresource Technology*, 97(6), pp. 841-846.

Michels, M. H., Slegers, P. M., Vermue, M. H. & Wijffels, R. H., 2014. Effect of biomass concentration on the productivity of Tetraselmis suecica in a pilot-scale tubular photobioreactor using natural sunlight. *Algal Research*, Volume 4, pp. 12-18.

Mohn, F., 1980. Experiences and strategies in the recovery of biomass from mass cultures of microalgae. *Algae biomass*, pp. 547-571.

Mohn, F. & Contreras, O., 1991. *Harvesting of the alga Dunaliella-some considerations concerning its cultivation and impact on the production costs of β-carotene.*, s.l.: Berichte des Forschungszentrum Jülich.

Mollah, M. Y., Morkovsky, P., Gomes, J. A. G., Kesmez, M., Parga, J. & Cocke, D. L., 2004. Fundamentals, present and future perspectives of electrocoagulation. *Journal of Hazardous Materials*, 114(1-3), pp. 199-210.

Morowvat, M., Rasoul-Amini, S. & Ghasemi , Y., 2010. Chlamydomonas as a 'new' organism for biodiesel production. *Bioresource Technology*, 101(6), pp. 2059-2062.

Mujeeb, M., Vedamurthy, A. & Shivasharana, C., 2016. Current strategies and prospects of biodiesel production: A review. *Advances in Applied Science Research*, 7(1), pp. 120-133.

Munari, F., Cavagnino, D. & Cadoppi, A., 2007. Determination of total FAME and linolenic acid methyl esters in pure biodiesel (B100) by GC in compliance with EN 14103, Milan, Italy: Thermo Fisher Scientific.

Nagarajan, S., Chou, S. K., Cao, S., Wu, C. & Zhou, Z., 2013. An updated comprehensive techno-economic analysis of algae biodiesel. *Bioresource Technology*, Volume 145, pp. 150-156.

Newman, S., 2008. How algae biodiesel works.

Nindo, C. & Tang, J., 2007. Refractance window dehydration technology: a novel contact drying method. *Drying Technology: An International Journal*, 25(1), pp. 37-48.

Nita, I. & Geacai, S., 2011. Study of density and viscosity variation with temperature for fuels used for diesel engine. *Ovidius University Annals of Chemistry,* 22(1), pp. 57-61.

Nitske, R. W. & Wilson, C. M., 1965. *Rudolf Diesel: Pioneer of the age of power.* s.l.:University of Oklahoma Press.

Nwokoagbara, E., Olaleye, A. K. & Wang, M., 2015. Biodiesel from microalgae: the use of multi-criteria decision analysis for strain selection. *Fuel,* Volume 159, pp. 241-249.

Origin Oil, 2010. Algae harvesting, dewatering and extractio*n.* Amsterdam, Worldbiofuels markets, RAI congress centre.

Österlind, S., 1950. Inorganic cabon sources of green algae. I. growth experiments with Scenedesmus quadricauda and Chlorella pyrenoidosa. *Physiologia plantarum,* Volume 3.

Ou, L., Thilakaratne, R., Brown, R. C. & Wright, M. M., 2015. Techno-economic analysis of transportation fuels from defatted microalgae via hydrothermal liquefaction and hydroprocessing. *Biomass and Bioenergy,* Volume 72, pp. 45-54.

Padmanabhan, A. M. & Shaleesha, S. A., 2012. Microalgae as an oil producer for biofuel applications. *Research Journal of Recent Sciences,* 1(3), pp. 57-62.

Park, J., Craggs, R. & Shilton, A., 2011. Wastewater treatment high rate algal ponds for biofuel production. *Bioresource Technology,* 102(1), pp. 35-42.

Pawlowski, A., Fernández, I., Guzmán, J. L., Berenguel, M., Acién, F. G. & Normey-Rico, J. E., 2014. Event-based predictive control of pH in tubular photobioreactors. *Computers & Chemical Engineering,* Volume 65, pp. 28-39.

Perrier, B., Crampon, C., Guézet, O., Simon, C., Maire, F., Lépine O., Pruvost, J., Lozano, P., Bernard, O. & Badens, E., 2015. Production of a methyl ester from the microaalgae Nannochloropsis grown is raceways on the french west coast. *Fuel,* Volume 153, pp. 640-649.

Petkov, G. & Garcia, G., 2007. Which are fatty acids of the green alga Chlorella?. *Biochemical Systematics and Ecology,* 35(5), pp. 281-285.

Petroleum & other liquids, 2015. *Monthly biodiesel production report,* U.S. Energy Information Administration.

Petruševski, B., Bolier, G., Breemen, A. V. & Alaerts, G., 1995. Tangential flow filtration: A method to concentrate freshwater algae. *Water Research,* 29(5), pp. 1416-1424.

Popp, J., Lakner, Z., Harangi-Rákos, M. & Fári, M., 2014. The effect of bioenergy expansion: food, energy, and environment. *Renewable and sustainable energy reviews,* Volume 32, pp. 559-578.

Postigo, M. A., Garcia, P. H., Ortega, J. & Tardajos, G., 1995. Excess molar volumes of binary mixtures containing a methyl ester (ethanoate to tetradecanoate) with odd n-alkanes at 298.15 K. *Journal of Chemical & Engineering Data,* Volume 40, pp. 283-289.

Pourmortazavi, S. & Hajimirsadeghi, S., 2007. Supercritical fluid extraction in plant essential and volatile oil analysis. *Journal of Chromatography A.,* 1163(1-2), pp. 2-24.

Prakash, J., Pushparaj, B., Carlozzi, P., Torzillo, G., Montaini, E. & Materassi, R., 1997. Microalgal biomass drying by a simple solar device. *International Journal of Sustainable Energy,* 18(4), pp. 303-311.

Prokop, A., Quinn, M. F., Fekri, M., Murad, M. & Ahmad, S. A., 1984. Spectral shifting by dyes to enhance algae growth. *Biotechnology Bioengineering,* 26(11), pp. 1313-1322.

Radakovits, R., Jinkerson, R. E., Darzins, A. & Posewitz, M. C., 2010. Genetic engineering of algae for enhances biofuel production. *Eukaryotic Cell,* 9(4), pp. 486-501.

Ramadhas, A., Jayaraj, S. & Muraleedharan, C., 2005. Biodiesel production from high FFA rubber seed oil. *Fuel,* 84(4), pp. 335-340.

Ramos, M., Fernández, C. M., Casas, A., Rodríguez, L. & Pérez, A., 2009. Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology,* 100(1), pp. 261-268.

Rashid, N., Rehman, M. S. U., Sadiq, M., Mahmood, T. & Han, J.-I., 2014. Current status, issues and developments in microalgae derived biodiesel production. *Renewable and sustainable energy reviews,* Volume 40, pp. 760-778.

Rawat, I., Kumar, R. R., Mutanda, T. & Bux, F., 2011. Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Applied Energy,* 88(10), pp. 3411-3424.

Renaud, S. M., Zhou, H. C., Parry, D. L., Thinh, L.-V. & Woo, K. C., 1995. . Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae Isochrysis sp., Nitzschia closterium, Nitzschia paleacea, and commercial species Isochrysis sp. (clone T. ISO). *Journal of Applied Phycology,* 7(6), pp. 595-602.

Rezvani, S., Moheimani, N. & Bahri, P., 2016. Techno-economic assessment of CO2 bio-fixation using microalgae in connection with three different state-of-the-art power plants. *Computers & Chemical Engineering,* Volume 84, pp. 290-301.

Richardson, Y., Blin, J. & Julbe, A., 2012. A short overview on purification and conditioning of syngas produced by biomass gasification: Catalytic strategies, process intensification and new concepts. *Progress in energy and combustion science,* 38(6), pp. 765-781.

Richmond, A., 2003. Handbook of microalgal culture: biotechnology and applied phycology:Wiley-Blackwell.

Rodolfi, L., Chini, Z. G., Bassi, N., Padovani, G., Biondi, N. & Tredici, M. R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering,* 102(1), pp. 100-112.

Rodriguez, C., Alaswad, A., Mooney, J., Prescott, T. & Olabi, A. G., 2015. Pre-treatment techniques used for anaerobic disgestion of algae. *Fuel Processing Technology,* Volume 138, pp. 765-779.

Ronald, H., Danquah, M. K. & Webley, P. A., 2012. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnology Advances,* 30(3), pp. 709-732.

Rubio, J., Souza, M. & Smith, R., 2002. Overview of flotation as a wastewater treatment technique. *Minerals Engineering,* 15(3), pp. 139-155.

Ruppel, T. & Huybrighs, T., 2008. *Fatty acid methyl esters in B100 biodiesel by gas chromatography (modified EN 14103),* Shelton, CT USA: PerkinElmer, Inc..

Saeid, A. & Chojnacka, K., 2015. Toward production of microalgae in photobioreactors under temperate climate. *Chemical Engineering Research and Design* , Volume 93, pp. 377-391.

Sahoo, P., Das, L., Babu, M. & Naik, S., 2007. Biodiesel development from high acid value polanga seed oil and performance evaluation in a CI engine. *Fuel,* 86(3), pp. 448-454.

Sander, K. & Murthy, G. S., 2010. Life cycle analysis of algae biodiesel. *The International Journal of Life Cycle Assessment,* 15(7), pp. 704-714.

Sawayama, S., Inoue, S., Dote, Y. & Yokoyama, S.-Y., 1995. CO2 fixation and oil production through microalga. *Energy conversion and management,* 36(6-9), pp. 726-731.

Schenk, P. M., Skye, R., Thomas, H., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., Kruse, O., Hankamer, B., 2008. Second generation biofuels: high-efficency microalgae for biodiesel production. *BioEnergy Research,* 1(1), pp. 20-43.

Schnepf, R. & Yacobucci, B. D., 2013. *Renewable fuel standard (RFS): overview and issues,* s.l.: Congressional research service.

Schober, S. & Mittelbach, M., 2007. Iodine value and biodiesel: Is limitation still appropriate?. *Lipid Technology,* 19(12), pp. 281-284.

Schwede, S., Kowalczyk, A., Gerber, M. & Span, R., 2011. *Influence of different cell disruption techniques on mono disgestion of algal biomass.* Linköping- Sweden, Bioenergy Technology, pp. 41-47.

Scott, S. A., Davey, M. P., Dennis, J. S., Horst, I., Howe, I., Howe, C. J., Smith, D. J. L., & Smith, A. G., 2010. Biodiesel from algae: challenges and prospects. *Current Opinion in Biotechnology,* 21(3), pp. 277-286.

Sforza, E., Bertucco, A., Morosinotto, T. & Giacometti, G. M., 2012. Photobioreactors for microalgal growth and oil production with Nannochloropsis salina: from lab-scale experiments to large-scale design. *Chemical Engineering Research and Design,* 90(9), pp. 1151-1158.

Sharma, Y. C. & Singh, B., 2008. Development of biodiesel from karanja, a tree found in rural India. *Fuel,* 87(8-9), pp. 1740-1742.

Sharma, Y. & Singh, B., 2009. Development of biodiesel: current scenario. *Renewable and Sustainable Energy Reviews,* 13(6-7), pp. 1646-1651.

Sharma, Y., Singh, B. & Upadhyay, S., 2008. Advancements in development and characterization of biodiesel: A review. *Fuel,* 87(12), pp. 2355-2373.

Sheehan, J., Camobreco, V., Dufflied, J., Graboski, M. & Shapouri, H., 1998. An overview of biodiesel and petroleumdiesel life cycles, Springfield, VA: U.S. Department of Commerce.

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 alage,* Colorado: National Renewable Energy Laboratory.

Shelef, G., Sukenik, A. & Green, M., 1984. *Microalgae harvesting and processing: a literature review,* s.l.: U.S. Department of Energy.

Sialve, B., Bernet, N. & Bernard, O., 2009. Anaerobic digestion of microalgae as a necessary step to make microalgae biodiesel sustainable. *Biotechnology Advances,* 27(4), pp. 409-416.

Silva, J., 2014. *Avaliação da produção de biodiesel a partir de microalgas,* Aveiro: University of Aveiro, Master's thesis.

SimaPro, 2016. *SimaPro- World's leading LCA software.* [Online] Available at: https://www.pre-sustainability.com/simapro [Acedido em 5 March 2016].

Singh, A., Nigam, P. & Murphy, J., 2011. Renewable fuels from algae: an answer to debatable land based fuels. *Bioresource Technology,* 102(1), pp. 10-16.

Singh, M., Shukla, R. & Das, K., 2013. Harvesting of microalgal biomass. In: F. Bux, ed. *Biotechnological applications of microalgae: biodiesel and value added products.* New york: CRC press, Taylor and Francis group.

Slade, R. & Bauen, A., 2013. Microalgae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects. *Biomass and Bioenergy,* Volume 53, pp. 29-38.

Slegers, P., Beveren, P. J. M., Wijffels, R. H., Straten, G. & Boxtel, A. J. B., 2013. Scenario analysis of large scale algae production in tubular photobioreactors. *Applied Energy,* Volume 105, pp. 395-406.

Smedes, F. & Thomasen, T. K., 1996. Evaluation of the Blight & Dyer lipid determination method. *Marine Pollution Bulletin,* 32(8-9), pp. 681-688.

Smeets, E. M. W., Faaij, A. P., Lewandowski, I. M. & Turkenburg, W. C., 2007. A bottom-up assessment and review of global bio-energy potentials to 2050. *Progress in energy and combustion science,* 33(1), pp. 56-106.

Song, C., Chen, G., Ji, Na., Liu, Q., Kansha, Y. & Tsutsumi, A., 2015. Biodiesel production process from microalgae oil by waste heat recovery and process integration. *Bioresource Technology,* Volume 193, pp. 192-199.

Spolaore, P., Joannis-Cassan, C., Duran, E. & Isambert, A., 2006. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering,* 101(2), pp. 87-96.

Stephenson, A. L., Kazamia, E., Dennis, J. S., Howe, C. H, Scott, A. S., Smith, A. S., 2010. Life-cycle assessment of potential algal biodiesel production in the United Kingdom:a comparison of raceways and air-lift tubular bioreactors. *Energy Fuels,* Volume 24, pp. 4062-4077.

Suali, E. & Sarbatly, R., 2012. Conversion of microalgae to biofuel. *Renewable and Sustainable Energy reviews,* 16(6), pp. 4316-4342.

Suarsini, E. & Subandi, S., 2011. *Utilization ultrsonic to increase the efficiency of oil extraction for microalgae indigenous isolates from pond gresik, east java.*, pp. 275-279.

Suganya, T., Varman, M., Masjuki, H. & Renganathan, S., 2016. Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach. *Renewabla and Sustainable Energy Reviews,* Volume 55, pp. 909-941.

Suh, I. S. & Lee, C.-G., 2003. Photobioreactor engineering: design and performance. *Biotechnology and Bioprocess Engineering,* 8(6), pp. 313-321.

Sukenik, A. & Shelef, G., 1984. Algal autoflocculation-verification and proposed mechanism. *Biotechnology and Bioengineering,* 26(2), pp. 142-147.

Sutherland, D., Turnbull, M. & Craggs, R., 2014. Increased pond depth improves algal productivity and nutrient removal in wastewater treatment high rate algal ponds. *Water Research,* Volume 53, pp. 271-281.

Suzana, W., Ani, I. & Shaleh, S., 2013. The influence of light intensity and photoperiod on the growth and lipid content of microalgae Nannochlropsis sp.. *Bioresource Technology,* Volume 129, pp. 7-11.

Taylor, L. T., 1996. *Supercritical fluid extraction.* illustrated ed.:Wiley.

Thana, P., Machmudah, S., Goto, M., Sasaki, M., Pavasant, P. & Shotipruk, A., 2008. Response surface methodology to supercritical carbon dioxide extraction of astaxanthin from Haematococcus pluvialis. *Bioresource Technology,* 99(8), pp. 3110-3115.

Thilakaratne, R., Wright, M. M. & Brown , R. C., 2014. A techno-economic analysis of microalgae remnant catalytic pyrolysis and upgrading to fuels. *Fuel,* Volume 128, pp. 104-112.

Tiwari, A. K., Kumar, A. & Raheman, H., 2007. Biodiesel production from jatropha oil (Jatropha curcas) with high free fatty acids: An optimized process. *Biomass and Bioenergy,* 31(8), pp. 569-575.

Tredici, M. R., 2010. Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. *Biofuels,* 1(1), pp. 143-162.

Uduman, N., Qi, Y., Danquah, M. K. & M., G., 2010. Dewatering of microalgae cultures: A major bottleneck to algae-based fuels. *Journal of Renewable and Sustainable Energy,* 2(1), pp. 012701-15.

Ugwu, C., Ogbonna, J. & Tanaka, H., 2002. Improvement of mass transfer characteristics and productivities of inclined tubular photobioreactors by installation of internal static mixers. *Applied Microbiology and Biotechnology,* 58(5), pp. 600-607.

Vicente, G., Martínez, M. & Aracil, J., 2004. Integrated biodiesel production: a comparison of different homogenous catalysts systems. *Bioresource Technology,* 92(3), pp. 297-305.

Vicente, G., Martínez, M. & Aracil, J., 2007. Optimisation of integrated biodiesel production. Part I. A study of the biodiesel purity and yield. *Bioresource Technology,* 98(9), pp. 1724-1733.

Virot, M., Tomao, V., Ginies, C., Visinoni, F. & Chemat, F., 2008. Microwave- integrated extraction of total fats and oils. *Journal of Chromatography A,* Volume 1196-1197, pp. 57-64.

Voegele, E., 2012. Propel, Solazyme make algae biofuel available to the public, Grand Forks.

Vonshak, A., 1997. *Spirulina Platensis Arthrospira: physiology, cell-biology and biotechnology.* London: Taylor & Francis.

Wang, B., Lan, C. & Horsman, M., 2012. Closed photobioreactors for production of microalgal biomasses. *Biotechnology Advances,* 30(4), pp. 904-912.

Wang, G. & Wang, T., 2012. Characterization of liquid components in two microalgae for biofuel application. *Journal of the American Oil Chemists Society,* 89(1), pp. 135-143.

Wang, H.-Y., Bluck, D. & Wie, B. J. V., 2014. Conversion of microalgae to jet fuel: Process design and simulation. *Bioresource Technology,* September, Volume 167, pp. 349-357.

Watanabe, Y. & Hall, D. O., 1995. Photosynthetic CO2 fixation technologies using a helical tubular bioreactor incorporating the filamentous cynobacterium Spirulina platensis. *Energy Conversion and Management,* 36(6-9), pp. 721-724.

Weidema, B., 2000. *LCA developments for promoting sustainability.* Melbourne, 2nd National Conference on LCA.

Wen, D., Jiang, H. & Zhang, K., 2009. Supercritical fluids technology for clean biofuel production. *Progress in Natural Science,* 19(3), pp. 273-284.

Widjaja, A., Chien, C.-C. & Ju, Y.-H., 2009. Study of increasing lipid production from fresh water microalgae Chlorella vulgaris. *Journal of the Taiwan Institute of Chemical Engineers,* 40(1), pp. 13-20.

Wijffels, R. H. & Barbosa, M. J., 2010. An outlook on microalgal biofuels. *Science,* 329(5993), pp. 795-799.

Williams, A., Jones, J., Ma, L. & Pourkashanian, M., 2012. Pollutants from the combustion of solid biomass fuels. *Progress in energy and combustion science,* 38(2), pp. 113-137.

Wu, L., Chen, P., Huang, A. & Lee, C., 2012. The feasibility of biodiesel production by microalgae using industrial wastewater. *Bioresource Technology,* Volume 113, pp. 14-18.

Xu , H., Miao, X. & Wu, Q., 2006. High quality biodiesel production from a microalga Chlorella protothecoides by heterotrohic growth in fermenters. *Journal of Biotechnology,* 126(4), pp. 499-507.

Xu, L., Brilman, D. W. F., Withag, J. A. M., Brem, G. & Kersten, S., 2011. Assessment of a dry and a wet route for the production of biofuels from microalgae: Energy balance analysis. *Bioresource Technology,* 102(8), pp. 5113-5122.

Yang, J., Xu, M., Zhang, X., Qiang, H., Sommerfeld, M. & Chen, Y., 2011. Life-cycle analysis on biodiesel production from microalage: water footprint and nutrients balance. *Bioresource technology,* 102(1), pp. 159-165.

Zhang, Y., Dubé, M., McLean, D. & Kates, M., 2003. Biodiesel production from waste cooking oil:1. Process design and technological assessment. *Bioresource Technology,* 89(1), pp. 1-16.

Zlotorzynski, A., 1995. The application of microwave radiation to analytical and environmental chemistry. *Critical Reviews in Analytical Chemistry,* 25(1), pp. 43-76.

Zmora, O. & Richmond, A., 2003. Microalgae for aquaculture: microalgae production for aquaculture. In: A. Richmond, ed. *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*. Oxford, UK: Blackwell Publishing Ltd, pp. 365-379.

ANNEXES

I.

On Table I-1, results of the lagoon water are shown. The water was analyzed at the laboratory of INSA (NATIONAL HEALTH INSTITUTE DOUTOR RICARDO JORGE, IP).

Table I-1 Analysis of lagoon water collected from Ria de Aveiro.

Parameters	Analytical methods	Results	VR	VL
pH (21ºC) (E. Sorensen)	Electrometria/DSA ASQT-PE07 (2009-01-29)	7.0	-	5.0 – 9.0
Colour (mg/L PT-Co)	EAM/DSA ASQT-PE11 P (2009-01-29)	7.5	-	-
Total suspended solids (mg/L)	Gravimetria – DSA ASQT-PE24 P (2009-02-10)	7	-	-
Conductivity at 20ºC (µS/cm)	Electrometria/DSA ASQT-PE08 P (2009-01-29)	125	-	-
Smell at 25ºC (-)	Factor de diluição/DSA ASQT-PE14 P (2009-01-28)*	0	-	-
Nitrates (mg/L NO3)	CI/DSA ASQT-PE02 P (2010-07-23)	4.6	-	-
Iron (µg/L Fe)	FCS/DSA ASQT-PE03 P (2009-01-29)	$4.9e^{2}$	-	-
Total Copper (mg/L Cu)	EAA/DSA ASQT-PE09 P (2009-01-29)	<0.010 (LQ)	-	0.1
Total Zinc (mg/L Zn)	EAA/DSA ASQT-PE09 P (2009-01-29)*	<0.2 (LQ)	-	0.5
Boron (mg/L B)	EAM/DSA ASQT-PE11 P (2009-01-29)	<0.20 (LQ)	-	-
Total Nickel (mg/L Ni)	EAA/DSA ASQT-PE09 P (2009-01-29)	<0.005 (LQ)	-	0.05
Total Arsenic (mg/L As)	EAA/DSA ASQT-PE09 P (2009-01-29)	<0.0025 (LQ)	-	0.1
Total Cadmium (mg/L Cd)	EAA/DSA ASQT-PE09 P (2009-01-29)	<0.001 (LQ)	-	0.01
Total Chromium (mg/L Cr)	EAA/DSA ASQT-PE09 P (2009-01-29)	<0.01 (LQ)	-	0.05
Total Lead (mg/L Pb)	EAA/DSA ASQT-PE09 P (2009-01-29)*	<0.005 (LQ)	-	0.05
Total Cyanide (mg/L CN)	FCS/DSA ASQT-PE03 P (2009-01-29)	<0.010 (LQ)	-	0.05
Sulphate (mg/L SO4)	CI/DSA ASQT-PE02 P (2010-07-23)	14.4	-	250
Chloride(mg/L Cl)	Cl/DSA ASQT-PE02 P	13.8	-	250

	(2010-07-23)			
Biochemical Oxygen Demand (mg/L O2)	Potenciometria-Método Interno*	<3.0 (LQ)	-	5
Total Nitrogen (mg/L N)	EAM-Método Interno*	1.2	-	-
Total Organic Carbon (mg/L C)	C. Infravermelho-DSA ASQT-PE16 P (2009-01-29)	2.3	-	-
Total Phosphorus (mg/L P)	EAM/DSA ASQT-PE11 P (2009-01-29)	<0.17 (LQ)	-	1
Nitrites (mg/L NO2)	FCS/DSA ASQT-PE03 P (2009-01-29)	0.03	-	-
Total Hydrocarbons (mg/L)	FTIR/SMEWW*	<0.3 (LQ)	-	-
Total Pesticides (µg/L)	SPE-GC/MS*	0.07	-	2.5
Aluminium (µg/L AL)	FCS/DSA ASQT-PE03 P (2009-01-29)	<50 (LQ)	-	-
Calcium (mg/L Ca)	CI/DSA ASQT-PE06 P (2009-01-29)	8.8	-	-
Chemical Oxygen Demand (mg/L O2)	Dic. Pot.*	<30 (LQ)	-	-
Fluoride (mg/L F)	CI/DSA ASQT-PE02 P(2010-07-23)	0.11	-	-
Phosphate (mg/L P2O5)	EAM/DSA ASQT-PE11 P (2009-01-29)	0.051	-	-
Magnesium (mg/L Mg)	CI/DSA ASQT-PE06 P (2009-01-29)	3.2	-	-
Manganese(µg/L Mn)	EAA/DSA ASQT-PE09 P (2009-01-29)	47	-	-
Potassium(mg/L K)	CI/DSA ASQT-PE06 P (2009-01-29)	2.3	-	-
Sodium (mg/L Na)	CI/DSA ASQT-PE06 P (2009-01-29)	11.1	-	-
Polynuclear aromatic hydrocarbons (µg/L HAP)	SPE-GC/MS*	<0.06 (LQ)	-	100
Ammonium (mg/L NH4)	FCS/DSA ASQT-PE03 P (2009-01-29)	<0.05 (LQ)	-	1
Extractables Chloroform (mg/L)	Gravimetria-Método Interno*	11.9	-	-
Anionic surfasse-active substances (mg/L – sodium lauryl sulfate)	EAM-Método Interno*	<0.20 (LQ)	-	0.5

The tests marked with (*) are not included in the scope of accreditation.

II. CONVENTIONAL PATHWAY (DATA INVENTORY)

On Table II-1, II-2, II-3 and II-4 the data used for the conventional process is shown. These values are taken from the previous modeling work (Acién, et al., 2012) (Silva, 2014).

Table II-1 Technical parameters used for the production pathway

Technical Parameters	Unit	Value
CO_2 usage	kg/kg biomass	9,15
H_2O evaporation	L/m2/day	10
Mixing power consumption	W/m3	1
Labor	people/ha	0,18
Ratio V/S	m3/m2	0,25
CO_2 fixation efficiency	0	0,196721311
Dilution rate	1/day	0,02
Total culture volume	m3	1000000
Total biomass production	ton/ha/year	30
Total CO_2 consumption	ton/ha/year	274,5
Total water evaporation	ton/ha/year	30000
Water cost	€/kg	0,05
CO_2 cost	€/kg	0,1
Nutrients cost	€/kg	0,4
Fertilizers usage	kg/kg biomass	0,3
Power cost	€/kWh	0,05
Power for harvesting and others	kwh/m3 harvest	1
System cost	€/m3	10

Table II-2 Equipment, cost, size unit, and maximum capacity data

Equipment & costs	Minimum size unit	Cost (€/unit)	Max. capacity
Medium preparation	10 m^3/h	6000	2000 m^3/h
Sterilization process	10 m^3/h	20000	0 m^3/h
Air blower	200 m^3/h	2500	0 m^3/h
System	200 m^3	2000	1000000 m^3
Sedimenter	25 m^3/h	20000	2000 m^3/h
Harvest storage tank	5 m^3	600	200 m^3/h
Decanter	4 m^3/h	40000	200 m^3/h
Harvest pump	10 m^3/h	1000	200 m^3/h
CO_2 supply unit	100 kg/h	3000	36600kg/h

Table II-3 Cost of raw materials

Raw materials	€/unit
Fertilizers (kg)	0,4
H_2O (m^3)	0,05
CO_2 (kg)	0,1

Table II-4 Cost of utilities

Utilities	€/unit
H_2O loses (m^3)	0,05
Mixing power consumption (kwh)	0,05
Power for harvesting + others (kwh)	0,05
Labor	1500

III. INTEGRATED PATHWAY (DATA INVENTORY)

On Table III-1, III-2, III-3, III-4 and III-5 are the data used for the integrated process is shown. These values are taken from the previous work (Davis, et al., 2011) (Davis, et al., 2012) (Davis , et al., 2014).

Table III-1 Technical parameters and their value

Technical parameters	Unit	Value
Lipid production	MM gal/yr	10
Diesel production	MM gal/yr	9,3
Water demand	MM gal/yr	10000
H2O evaporation	gal/gal lipid	570
Water blowdown to treatment/discharge	gal/gal lipid	430
Mixing power consumption	W/m3	0
Production days	days/year	330
Land cost	$/ha	1212
Dilution rate	1/day	0,02
Total CO2 consumption	ton/year	145000
Total water evaporation	ton/ha/year	1881000
Water cost	$/kg	0,009
CO2 cost	$/kg	0,04
Nutrients cost	$/ton	5649
Fertilizers usage	ton/year	9900
Power cost (import)	$/kWh	0,08
Power cost (export)	$/kWh	0,065
System cost	$MM	195
Annual salary of workers	$/year	0
Liners	$/gal	5,43
Liners	$/acre ponds	20500

Table III-2 Cost and consumption of nutrients

Nutrients	Consumption (ton/year)	Cost ($/ton)
Ammonia	5100	407
Diammonium phosphate	4800	442

Table III-3 Harvesting cost

Auto-flocculation (1%)	$/gal	1,52
Flocculation (10%)	$/kg	10,67
Centrifuge (20%)	$/gal	0,17

Table III-4 Extraction cost

Mechanical methods (Homogenizer)	$/gal	0,51
Solvent method (butanol)	$/kg	2,08

Table III-5 System cost

Total capital cost (direct + indirect)	$MM	370
Total coproduct credits	$MM/year	6
Net operating cost	$MM/year	37