UNIVERSIDADE DO ALGARVE

PRODUCTION OF BIOFUELS FROM MICROALGAL BIOMASS

Henrique Miguel Mata Carvalho

Thesis to obtain the Master in Environmental Engineering

Work done under the guidance of:
Prof. Doutor Luísa Paula Viola Afonso Barreira
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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Henrique Carvalho

Agradecimentos

Esta secção de agradecimentos, não me permite agradecer, como devia, a todas as pessoas que, ao longo deste projeto me ajudaram, direta ou indiretamente, a cumprir os meus objetivos e a realizar mais esta etapa da minha formação académica.

Desta forma, deixo apenas algumas palavras, poucas, mas um sentido e profundo sentimento de reconhecido agradecimento.

À Prof.ª Luísa Barreira, ao Prof.º Raúl Barros por toda a orientação, estímulos, desafios, apoio e conhecimento transmitido durante a realização deste trabalho. E ao Prof.º João Varela por toda a ajuda e sugestões transmitidas no decorrer deste ano.

À empresa Necton, SA, e às Águas do Algarve, SA por me terem facultado a microalga estudada, os inóculos utilizados e o equipamento para medição da composição do biogás.

À Vera Gomes, ao Jorge Carlier, à Ana Assunção, à Ana Constantino e à Prof.ª Maribela Pestana, por toda a ajuda e colaboração nas diferentes fases deste trabalho.

A todo o grupo MarBiotech pela colaboração, ajuda e incentivo durante todo o decorrer deste trabalho.

Aos meus amigos, que de alguma maneira me ajudaram e motivaram no decorrer desta fase da minha vida.

Em especial aos meus pais, irmão e família a quem tudo devo, por todo o apoio, paciência e compreensão que me deram ao longo do tempo.

Por último quero dedicar esta dissertação ao meu avô e à minha tia, sempre foi um sonho dela ver-me completar os estudos.

Abstract

With the increase in greenhouse gases (GHG) concentrations on the atmosphere, and the problems associated with it, the interest in the development of microalgae-derived biofuels has grown significantly in recent years. Microalgal biomass presents several advantages over feedstocks commonly used for the production of biofuels. However, current production costs are still too high and uncompetitive when compared to fossil fuels. In order to reduce the production costs of microalgal-based biofuels, the development of a biorefinery combining the production of different biofuels was previously proposed.

In the present work, biodiesel was synthetized from the biomass of *Botryococcus braunii*, and the properties of the produced biodiesel were assessed according to the European and American specifications. The defatted biomass obtained as a co-product was further upgraded by anaerobic digestion into biogas, using different consortia of bacteria. The net energy and mass balance of the biofuels produced were made, to discuss the viability of microalgal-based biodiesel production coupled with the valorisation of the spent biomass under a biorefinery concept.

B. braunii biomass had an lipid content of 14.8%, after the transesterification the yield was 46.8%, and the final biodiesel yield after purification was 30.5%. The predominant fatty acids present on the biodiesel were C16:0; C18:2*n*6 and C18:1. The biodiesel produced fulfilled the international specifications, except for the parameters density, viscosity and phosphorus content.

The highest yields obtain on the anaerobic digestion were under mesophilic conditions (35°C). The digestion of the defatted biomass (without lipids, 369 mL biogas/g VS) performed similar or better results than the raw biomass (333 mL biogas/g VS). The defatted biomass reached a biogas methane content of 82% and 72% for 35°C and 25°C, respectively.

The experimental results obtained at a laboratory scale were scaled up with a process simulation software (SuperPro Designer) to produce 1000 kg of biodiesel from 16.4 t of biomass, which will lead to a production of 2687 kg of methane, making an overall energy production of 48145 kWh. However, the energy spent on the process was 94341 kWh.

Key-words: Microalgae, Biodiesel, Biogas, Lipid extraction, Anaerobic digestion, *B. braunii*.

Resumo

O setor energético e dos transportes são os mais poluentes a nível dos gases de efeito estufa na União Europeia representando 60 e 20%, respetivamente. O aumento dos gases de efeito estufa na atmosfera, e os problemas inerentes a estes (aumento da temperatura, secas mais frequentes e por períodos de tempo mais longos, maior pluviosidade e aumento do nível médio da água do mar e aumento da acidificação dos oceanos). A depleção dos combustíveis fósseis e a instabilidade política nos países produtores, podem levar à flutuação dos preços dos combustíveis. Todos estes problemas inerentes ao consumo dos combustíveis fósseis tem levado ao desenvolvimento de novas fontes de combustíveis.

Os biocombustíveis produzidos a partir de biomassa algal têm crescido significativamente nos últimos anos. Estes apresentam várias vantagens em comparação com a matéria-prima mais utilizada para a produção de biocombustíveis (menores áreas de cultivo, menores quantidades de água utilizada e não competem com os bens alimentares primários), no entanto os custos de produção atuais ainda são elevados e não conseguem competir com os combustíveis fósseis. A fim de reduzir os custos da produção de biocombustíveis a partir de microalgas, desenvolveu-se o conceito de bio refinaria, combinando a produção de diferentes biocombustíveis, tais como biodiesel, bioetanol e biogás.

No presente trabalho, o biodiesel foi sintetizado a partir da biomassa da microalga *Botryococcus braunii* e as propriedades do biodiesel produzido foram avaliadas de acordo com as especificações europeias (EN 14214) e americanas (ASTM D6751). A biomassa desengordurada, obtida como coproduto, foi digerida por digestão anaeróbica (a 25 e 35°C) e transformada em biogás, utilizando diferentes consórcios de bactérias (provenientes das Estações de Tratamento de Águas Residuais de Silves e Lagos). O biogás produzido foi analisado de forma a ser conhecida a proporção entre dióxido de carbono e metano, e a existência de sulfureto de hidrogénio (principal contaminante obtido na produção de biogás, e principal razão para não ser possível o seu armazenamento) obtido após a digestão. Para concluir foram realizados balanços de massa e energético dos biocombustíveis produzidos, de forma a discutir a viabilidade da produção do biodiesel à base de microalgas juntamente com a valorização da biomassa tendo por base um conceito de bio refinaria.

A biomassa algal apresentou um conteúdo lipídico de 14.8%, e após a extração dos lípidos, estes sofreram uma transesterificação e formaram o biodiesel. Este passo apresentou um rendimento de 46.8%, sendo que foi sujeito um passo seguinte de purificação com rendimento de 30.5% de forma a cumprir os padrões exigidos na Europa e Estados Unidos da América. Os ácidos gordos predominantes no biodiesel produzido foram os C16:0, C18:2*n*6 e C18:1, entre os quais, os ácidos gordos insaturados apresentam-se em maioria, 55%. Os ácidos gordos saturados que providenciam uma maior estabilidade oxidativa representam 43%. O biodiesel produzido cumpre a maioria das especificações exigidas pelas normas, excluindo apenas os parâmetros de densidade, viscosidade e fosforo.

Durante a digestão da biomassa original e desengordurada a 35°C foi realizada uma adição de um grama de biomassa no dia 42 e foi realizada num total de 73 dias. O rendimento da digestão da biomassa original pelo consórcio de Lagos foi de 284 mL biogás/g SV, por sua vez o rendimento da produção de biogás pelo inóculo de Silves foi de 312 mL biogás/g SV, para a digestão que durou 42 dias. O rendimento da digestão da segunda adição de biomassa (dia 42 a dia 73) foi de 308 mL biogás/g SV com o inóculo de Lagos e de 322 mL biogás/g SV com o inóculo de Silves. O rendimento total (73 dias de digestão) foi de 333 mL biogás/g SV para ambos os inóculos utilizados. O rendimento da digestão da biomassa desengordurada pelo consórcio de Lagos foi de 369 mL biogás/g SV (melhor resultado obtido nesta experiência), por sua vez o rendimento da produção de biogás pelo inóculo de Silves foi de 297 mL biogás/g SV, para a digestão que durou 42 dias. O rendimento da digestão da segunda adição de biomassa (dia 42 a dia 73) foi de 364 mL biogás/g SV com o inóculo de Lagos e de 308 mL biogás/g SV com o inóculo de Silves. O rendimento total (73 dias de digestão) foi de 359 mL biogás/g SV com o inóculo de Lagos e de 320 mL biogás/g SV com o inóculo de Silves. O inóculo de Lagos obteve um rendimento de 276 mL biogás/g SV na digestão da biomassa inicial, por sua vez o inóculo de Silves obteve um rendimento de 269 mL biogás/g SV na digestão dessa mesma biomassa, os rendimentos obtidos foram semelhantes para ambos os inóculos utilizados, esta experiencia foi realizada durante 46 dias. A biomassa desengordurada ainda apresentou uma produção mais reduzida durante os mesmos 46 dias da biomassa original. O inóculo de Lagos apresentou um rendimento de 172 mL biogás/g SV para esta mesma biomassa. O inóculo de Silves obteve um rendimento superior com 193 mL biogás/g SV. A composição do biogás foi apenas realizada na digestão anaeróbica da biomassa

desengordurada a 25 e 35°C com o inóculo de Silves. A composição do biogás a 35°C foi de 82% de metano, 18% de dióxido de carbono e não foi detetada a presença de sulfureto de hidrogénio. A composição do biogás a 25°C foi de 72% de metano, 23% dióxido de carbono e 2 ppm de sulfureto de hidrogénio.

Através de um programa de modelação (SuperPro Designer), os resultados obtidos experimentalmente foram extrapolados para uma produção de 1000 kg de biodiesel, consequentemente a biomassa desengordurada produziu 2687 kg de metano. O rendimento energético do processo foi de 48145 kWh, com um custo de produção de 94341 kWh. As principais matérias-primas para a produção de 1000 kg de biodiesel foram 16.4 t de microalga (peso seco), 200 t de metanol, 179 t de hexano e 2226 t de água, a maioria dos solventes utilizados foram reciclados e utilizados nos próximos processos de produção. Para o reaproveitamento desses solventes foi necessária a sua evaporação, este foi o processo mais dispendioso a nível energético, representando 85% do consumo energético. A exclusão do processo de evaporação desta biorrefinaria resultaria num balanço energético positivo de 33844 kWh.

Palavras-chave: Microalgas, Biodiesel, Biogás, Extração de lípidos, Digestão anaeróbica, *B. braunii*.

Abbreviations

AD	Anaerobic digestion
C/N	Carbon/nitrogen ratio
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C16:2	Hexadecadienoic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2*n*6	Linoleic acid
C18:3*n*6	Linolenic acid
CFPP	Cold filter plugging point
CN	Cetane number
CNG	Compressed natural gas
COD	Chemical oxygen demand
DB-Lagos	Defatted biomass digested with the inoculum from Lagos
DB-Silves	Defatted biomass digested with the inoculum from Silves
DW	Dried weight
EC	European Community
EGCM	External gas control module
EU	European Union
FAME	Fatty acids methyl esters
GHG	Greenhouse gases
HCl	Hydrochloridric acid
HHV	High heating value

IV	Iodine value
LCA	Life cycle assessment
LCC	Life cycle costing
LCI	Life cycle inventory analysis
LCIA	Life cycle impact assessment
LCSF	Long chain saturated factor
MP-AES	Microwave plasma-atomic emission spectrometer
MUFA	Monounsaturated fatty acids
NEB	Net energy balance
Ni/MH	Nickel-metal hydride battery
PBR	Photobioreactor
PUFA	Polyunsaturated fatty acids
RB-Lagos	Raw biomass digested with the inoculum from Lagos
RB-Silves	Raw biomass digested with the inoculum from Silves
SFA	Saturated fatty acids
SFE	Supercritical fluid extraction
STP	Standard temperature and pressure
TAG	Triacylglycerol
TLC	Thin layer chromatography
TSS	Total suspended solids
UV	Ultra violet
VS	Volatile solids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

Index

Index of Figures

Index of Tables

1. Objective

The present work aims to assess the effectiveness of a biorefinery to process the biomass of *Botryococcus braunii* into biodiesel, and further upgrade the residual biomass obtained after lipid extraction into biogas by means of anaerobic digestion.

In order to achieve this main objective a series of secondary objectives must be met, such as:

- Extraction of the lipid fraction from the biomass using low cost solvents and production of biodiesel;
- Assessment of the properties of the produced biodiesel according to the European (EN 14214) and American legislations (ASTM D6751);
- Test the effectiveness of different inocula (bacterial consortia) to effectively digest the residual microalgal biomass;
- Test the effectiveness of different temperatures for the anaerobic digestion of residual biomass into biogas;
- Assess the composition of the produced biogas;
- Characterize the mass and net energy balance of the whole process.

2. Framework

On the 20th century, the organic chemicals industry based on crude oil refineries presented a huge importance to the economy, but faced with increasing greenhouse problems, the 21st century biomass refinery will need significant development and re-structure (Clark *et al.*, 2006).

The increase in greenhouse gases can lead to the rise of the surface temperature, heat waves will occur more often and on longer periods of time, precipitation will be more intense and sea level may rise (IPCC, 2014). In the near future, depletion of fossil fuels, and political instability on the producer countries, may cause fluctuations in oil prices. Therefore, new sources of fuels must be sought and developed. One of the most promising, due to their energy density, are the biofuels produced from biomass, the main ones are biogas, biodiesel and bioethanol. Biomass feedstocks can be from many different sources (e.g. wheat, corn, rapeseed, palm oil, soy, Jatropha, sugar cane, municipal waste and starch), although the oleaginous plants are the most used (Naik *et al.*, 2010; Pereira *et al.*, 2013a).

However, the use of oleaginous plants for the production of biofuels is not sustainable on a larger scale, for three main reasons:

- Huge crop areas needed for cultivation;
- Massive quantity of freshwater for irrigation;
- Direct competition with food production, which would lead to a general increase in food prices (Chisti, 2007; Schenk *et al.*, 2008; Zhu, 2014).

Microalgae appear as a more sustainable alternative. They are suitable sources of lipids, due to the high capability to accumulate them, can be grown in non-arable lands using salt or brackish water and they do not compete with food production (Chisti, 2007; Mussgnug *et al.*, 2010; Pereira *et al.*, 2013b; Schenk *et al.*, 2008; Varela *et al.*, 2014). However, microalgal biomass production is expensive and, to date, microalgal-based biodiesel cannot compete with the low price of fossil fuels. Therefore, the biorefinery concept applied to microalgae biomass has emerged. This concept relies on the full exploitation of all biochemical constituents, through the development of different products and upgrade of residues produced throughout the process, in order to enhance the final value of a given microalgal biomass (Chisti, 2007; Pereira *et al.*, 2011).

3. State of the art review

3.1. Fossil fuels as energy sources and greenhouse gases problems

The industrial revolution escalated the global energy demand, and the prices of fossil fuels tend to increase proportionally to the depletion of the fossil fuel reserves (Mussgnug *et al.*, 2010). Human activities are perturbing the world's climate; the emissions of greenhouse gases (GHG) for the transportation and energy sectors are the most problematic, representing more than 20 or 60%, respectively in the European Community (Mata *et al.*, 2010). Moreover, atmospheric methane (CH_4), nitrous oxide (N_2O) and carbon dioxide (CO_2) levels have raised in the past years (Cherubini, 2010; IPCC, 2014). GHG contributes to global warming, and has significant effects on the environment. In addition, absorption of CO_2 by the oceans may decrease the water pH and such acidification may cause problems on the ecosystems biodiversity (IPCC, 2014; Mata *et al.*, 2010). Over the past decade the transportation sector has shown the highest growth in GHG emissions. The primary source of energy for this sector is oil, and this demand is expected to rise 60% until 2030 (Cherubini, 2010). To reduce GHG produced by fossil fuels, renewable energy sources must be implemented. In this respect, there are many different technologies already being employed, such as solar energy, hydroelectric, thermal, photovoltaic, wind, biofuels, among others (Mata *et al.*, 2010).

Concerning the replacement of fossil fuels for transportation, although there are different alternatives currently established (e.g. electricity and hydrogen driven vehicles) they are only viable for light-duty vehicles. Heavy transportation (e.g. lorries, ships, planes) and industrial machinery will probably require liquid biofuels, because the energy density available on batteries is considerably lower than that of liquid-based fuels (Guzzella & Sciarretta, 2005; Pereira *et al.*, 2013a).

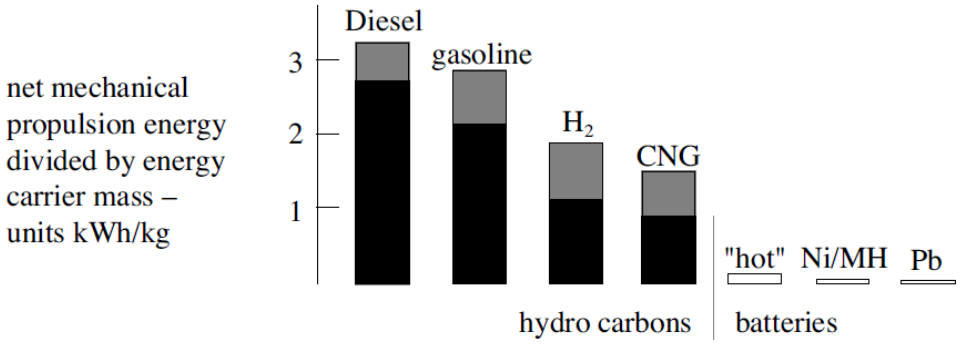

Figure 3.1 - Estimates of energy density of several on-board energy carriers (Guzzella & Sciarretta, 2005).

In this context, the EU wants to reach the target of 10% of renewable biofuels in the transport sector until 2020 (Pacini *et al.*, 2013).

3.2. Biofuels

Biofuels are divided into two main categories: 1st and 2nd generation biofuels. First generation biofuels are produced from raw materials that compete with food and feed industries. Therefore, some ethical, political and environmental concerns were raised (Cherubini, 2010; Mata *et al.*, 2010; Naik *et al.*, 2010; Schenk *et al.*, 2008).

The majority of the 1st generation biofuels are produced from animal fats, starch, vegetable oils and sugar. The main feedstocks used are wheat, corn and rapeseed, and the primary biofuels produced are biodiesel, bioethanol, starch-derived biogas and, in smaller quantities vegetable oils, biomethanol, and bioethers. The advantages of first generation are the easier conversion of high sugar or oil content into biofuel; some studies showed a reduction in GHG emissions and fossil energy consumption compared with the conventional diesel and gasoline on a Life Cycle Assessment (LCA; Cherubini, 2010). To overcome the limitations of first generation biofuels, the second generation biofuels gained increasing attention (Cherubini, 2010; Naik *et al.*, 2010; Schenk *et al.*, 2008).

Second generation biofuels, do not compete with food crops, are carbon neutral or have a carbon negative impact on the CO_2 cycle. The main feedstocks used for its production, are residues from agriculture, forestry, industry, microalgae and lignocellulosic crops (Naik *et al.*, 2010; Schenk *et al.*, 2008), and the main goal is to enhance biofuel production

sustainability (Antizar-Ladislao & Turrion-Gomez, 2008). This generation of biofuels shows improvements on land use efficiency, net energy balance (NEB), water efficiency and environmental performance over the first biofuel generation (Cherubini, 2010; Mata *et al.*, 2010; Schenk *et al.*, 2008), and has the potential to be more price competitive with fossil fuels (Naik *et al.*, 2010).

The three main biofuels produced worldwide are biodiesel, biogas and bioethanol, they substitute diesel, natural gas and gasoline, respectively, with minor engine modifications (Naik *et al.*, 2010).

Biodiesel is a mixture of fatty acids methyl esters (FAME), normally produced from rapeseed, sunflower, soybean, palm oil or waste edible oils. The major producers are Germany, USA, France, Italy and Austria (Cherubini, 2010; Razon & Tan, 2011). Biodiesel presents several advantages over fossil fuels: it is renewable, biodegradable, has reduced particulate emissions, it improves engine lubricity and some are non-toxic. One of the major problems of biodiesel when compared to the regular fuels are the production costs, and normally they need to be heavily subsidized by governments (Razon & Tan, 2011).

The principal feedstocks used on the production of bioethanol are corn-starch, sugarcane, sugar beet and wheat. The biggest producers are USA and Brazil; who alone represent 62% of the global production. Bioethanol production consists on the fermentation of the sugars and a distillation (Cherubini, 2010; Kim & Dale, 2004).

Anaerobic digestion can be done using different biomass feedstocks. In that process anaerobic bacteria can break either organic matter or cell walls and a mixture of carbon dioxide, methane and other gases in smaller quantities are released. The main contaminant is hydrogen sulphide. Contaminants and toxic gases should be separated from the biogas, which can be a substitute to natural gas (Lantz *et al.*, 2007; Naik *et al.*, 2010; Schenk *et al.*, 2008). Due to this, the biogas usually is burn in combined heat and power plants to produce electric energy, avoiding storage (Schenk *et al.*, 2008).

Organic materials used on anaerobic digestion are mainly from corn, manure, organic waste and grasses. If waste residues are the principal constituent of the feedstock used, the biogas can be categorized as 2nd generation biofuel (Cherubini, 2010). The main gases produced are methane (50-80%), carbon dioxide (20-50%) and traces of hydrogen sulphide (0-0.4%) due to the metabolic action of methanogenic bacteria, while the solid

remainder can be used as organic fertilizer (Lantz *et al.*, 2007; Naik *et al.*, 2010). The digestate incorporate all the non-degradable substances present in the original feedstock. The degradation increase the nitrogen availability. Consequently, the fertilization efficiency will increase (Lantz *et al.*, 2007).

The anaerobic digestion is divided in four stages: stage 1 – hydrolysis of the lipids, protein and polysaccharides to form monomers and oligomers (e. g. fatty acids, sugars, peptides and amino acids); step 2 – acidogenesis due to the release of propionate, alcohols and butyrate (volatile fatty acids); step 3 – acetogenesis converting the volatile fatty acids into acetate; step 4 – methanogenesis, formation of the methane and carbon dioxide, through the consumption of the acetate (Deppenmeier *et al.*, 1996). The main groups of bacteria who conduct the anaerobic biodegradation are: hydrolytic and fermentative (step 1), acidogenic bacteria (step 2), homoacetogenic and acetogenic bacteria (step 3), and methanogenic bacteria (step 4; Leschine, 1995; Vergara-Fernández *et al.*, 2008). Anaerobic digestion can be operated in two different conditions, mesophilic (35°C) or thermophilic (55°C) (Gunaseelan, 1997; Harun *et al.*, 2010). Anaerobic digestion can occur in aqueous environments, consequently is able to use feedstock sources with high water content without a drying step (Ward *et al.*, 2008).

Microalgae biomass has a great potential for the production of the three major biofuels. Their cells can accumulate a large amount of lipids, usually in the form of triacylglycerols (TAG), carbohydrates, proteins and fats. Figure 3.2 shows the steps for biofuels production (Pereira *et al.*, 2013a; Zhu, 2014).

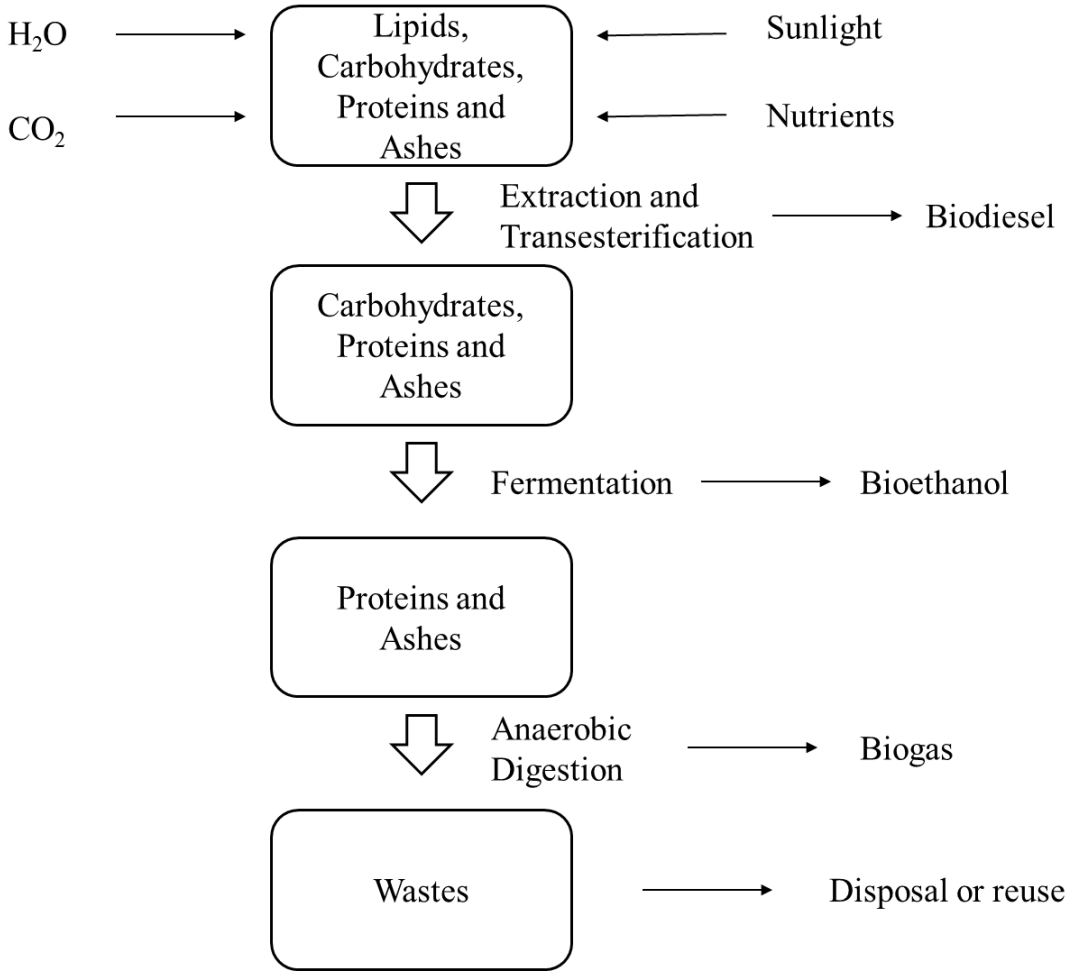

Figure 3.2 - Microalgae potential for the production of biofuels (Adapted from Zhu, 2014).

3.3. Microalgal-based biofuels

Microalgae display several advantages compared to oleaginous plants for the production of biodiesel and other biofuels, namely:

- Higher photosynthetic efficiency;
- High oil content, which can reach 70% of dried biomass (see table 1 for a comparison between different biomass feedstocks);
- Fast growth rates (e. g. microalgae can double their biomass weight within 24 h, and during exponential growth it can be as low as 3.5 h);
- Can be cultivated on sea water or brackish water;
- Nutrients required for their growth can be obtained from wastewater (e.g. nitrogen and phosphorus);

- Possibility of cultivation on non-arable land, and consequently not competing with the food and feed markets (Chisti, 2007; Mussgnug *et al.*, 2010; Pereira *et al.*, 2013b; Rodolfi *et al.*, 2009; Zhu *et al.*, 2014).

Table 3.1 - Oil productivity on the different biomass sources, and land area required (Adapted from Chisti, 2007).

Comparison of some sources of biodiesel			
Crop	**Oil yield (L/ha)**	**Land area needed (M ha) [a]**	**Percent of existing US cropping area [a]**
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[b]	136900	2	1.1
Microalgae[c]	58700	4.5	2.5

[a] For meeting 50 % of all transport fuel needs of the United States.
[b] 70 % oil (by wt) in biomass.
[c] 30 % oil (by wt) in biomass.

Nevertheless, the economics of the whole process are still not able to compete with fossil fuels (Razon & Tan, 2011; Zhu *et al.*, 2014). One of the key disadvantages of microalgae production is the cost of harvest and water removal (Razon & Tan, 2011). Nevertheless, in a near future, microalgae-based biofuels seem to be the only renewable biofuel able to compete with oil-based fuels (Chisti, 2008; Rodolfi *et al.*, 2009).

The typical composition of microalgae biomass is proteins (40-60%), lipids (5-60%), carbohydrates (8-30%), nucleic acids (5-10%) and other valuable minor compounds (pigments, anti-oxidants, fatty acids and vitamins). These components present a wide range of applications: carbohydrates are appropriate feedstock to microbial growth or production of bioethanol; lipids can be used to produce biodiesel (Uggetti *et al.*, 2014); and the long chain fatty acids, pigments, and proteins can be used as nutraceutical and pharmaceutical products (Gangadhar *et al.*, 2016; Pereira *et al.*, 2011; Uggetti *et al.*, 2014).

Generally, the oil content of microalgae compared to terrestrial plants is higher; some species can produce 50-60% of lipids (dry weight). The principal methods to extract the lipid content of the microalgae are solvent extraction, oil press, ultrasound and supercritical fluid extraction. Research has shown that *Chlorella vulgaris*, *Nannochloropsis* sp., *B. braunii*, *Nitzschia laevis*, *Parietochloris incise* and *Schizochytrium sp*. are suitable for biodiesel production (Uggetti *et al.*, 2014). With the right stress conditions it is possible to induce the lipid content of many microalgae strains (e. g. nitrogen and phosphorus deficiency, salinity; Mata *et al.*, 2010) . Microalgal oil is converted to biodiesel after transesterification. Biodiesel produced has to meet the EU and USA normatives to be sold on those countries (table 3.2). The biodiesel is composed of different types of FAME, saturated (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Biodiesel must present a low degree of unsaturation of the FAME and a high quantity of SFA and MUFA to abide to the properties regulated by the American and European specifications (Pereira *et al.*, 2013b). The density and viscosity of the biofuel produced can develop problems in the engines, forming deposits. Higher density and viscosity decreases the flow of fuel to the engine, decreasing his power (Knothe, 2005). The Cetane number (CN) represents the ignition quality of the biofuel, and can be correlated to the octane number of the gasoline. High CN can lead to low ignition delays, and vice versa. Higher CN can be derived by the high quantity of saturated fatty acids. Consequently, the higher the CN, the higher the quality of the biofuel and lower emissions of nitrogen oxides to the atmosphere (Knothe, 2005). The Iodine Value (IV) quantifies the unsaturated fatty acids and the iodine content of the biodiesel sample. Iodine value increases with the unsaturation of the biodiesel (Francisco *et al.*, 2010; Ramos *et al.*, 2009). High heating value (HHV) is the heat released during the combustion of the fuel, commonly determined by a bomb calorimeter or estimated using a group contribution method (Levine *et al.*, 2014). Calcium, magnesium, potassium, sodium and phosphorus are some of the pollutants controlled by the environmental agencies. Consequently the biodiesel sample should not exceed the maximum allowed in the legislations. Cold filter plugging point (CFPP) measures the capability of the biodiesel to pass through 0.45 µm filter at the lowest temperature. High CFPP can lead to plugging filters, tubbing line and wax settling, which will create problems on the engine. The higher the saturation of the biodiesel, the higher the melting point (Ramos *et al.*, 2009).

Table 3.2 - Comparison between the EN14214 and ASTM D6751 legislations.

Properties	**Unit**	**EN 14214**	**ASTM D6751**
Viscosity (40°C)	mm^2s^{-1}	3.5-5.0	1.9-6.0
Density (15°C)	Kg/L	0.86-0.90	n.a.
Cold filter plugging point (CFPP)	°C	n.a.	n.a.
Iodine Value	g I/100g	≤120	n.a.
Cetane number	-	≥51	≥47
PUFA (≥ 4 double bonds)	% mass	≤1	n.a.
Linolenic acid	% mass	≤12	n.a.
HHV	MJ/kg	n.a.	n.a.
Ca	mg/kg	≤5.0	≤5.0
Mg	mg/kg	≤5.0	≤5.0
K	mg/kg	≤5.0	≤5.0
Na	mg/kg	≤5.0	≤5.0
P	mg/kg	≤4.0	≤10

One promising approach to substitute the natural gas resources for anaerobic fermentation seems to be fast-growing algae with high lipid, protein and starch content (Mussgnug *et al.*, 2010). Studies with the anaerobic digestion and conversation rates of *Macrosystis pyrifera*, *Tetraselmis*, *Gracilaria tikvahiae*, *Hypnea* and *Ulva* have concluded that these species are promising (Vergara-Fernández *et al.*, 2008). Microalgae can be easily fermented when compared with land plants due to the absence of lignin in their composition (Harun *et al.*, 2011; Schenk *et al.*, 2008; Wiley *et al.*, 2011; Zhu, 2014).

Microalgae biomass can achieve similar or higher ratio of methane to carbon dioxide production to those of organic matter, 55-75% and 25-45% respectively (Harun *et al.*, 2010; Naik *et al.*, 2010; Schenk *et al.*, 2008). The higher biogas production efficiency is related to the microalgae cell wall properties: the easier the wall breaking, the higher biogas production (Uggetti *et al.*, 2014). Depending on the biomass, the typical yield varies from 0.15 to 0.65 m^3 of biogas per kg of biomass; assuming the average values of biogas energy content, microalgae could provide 9360 MJ of energy per metric ton of biomass (Chisti, 2008).

The carbon/nitrogen (C/N) ratio is also important to the yield of the anaerobic digestion: lower C/N ratios can be inhibitory, while high C/N ratios can lead to nitrogen deficiency, taking to a slower decomposition of the residue by the bacteria consortium. Microalgae biomass has a typical C/N ratio of 6. Microalgae with high protein content can be

inhibitory to the development of the methanogenic bacteria, due to the production of ammonia. Some studies have shown that a C/N ratio of 25 to 32 has a positive effect on the methane yield and, to improve the process, a secondary substrate can be added to fulfil the lack of nutrients of the initial substrate (Debowski *et al.*, 2013; Uggetti *et al.*, 2014; Wiley *et al.*, 2011). Sialve *et al.* (2009) compared the production of biogas from microalgae biomass before and after lipid extraction, and it seems to be more profitable to perform the anaerobic digestion of the biomass before lipid extraction.

If the composition of the substrates is known, it is possible to calculate the biogas yields (equation 1 and 2), although the variability in composition could be a problem (Wiley *et al.*, 2011). For microalgae, the biogas yield will probably be less than the theoretical predictions mainly because of the resistance of the cell walls (Wiley *et al.*, 2011). Angelidaki & Sanders (2004) provided a stoichiometrical balance to estimate the theoretical methane yield,

$$(1)\ C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right)H_2O \rightarrow \left(\frac{n}{2} - \frac{a}{8} - \frac{b}{4}\right)CH_4 + \left(\frac{n}{2} - \frac{a}{8} - \frac{b}{4}\right)CO_2$$

$$(2)\ B_0 = \frac{\left(\frac{n}{2} - \frac{a}{8} - \frac{b}{4}\right) \times 22.4}{12n + a + 16b}$$

Where B_0 is the theoretical methane yield (L CH_4/g VS), and 224 the molar volume of methane (STP conditions).

Table 3.3 - Theoretical methane yields for the three major substrates of microalgae (Adapted from Wiley *et al.*, 2011).

Substrate	**Composition**	**CH_4 yield (L/g VS)**
Lipids	$C_{57}H_{104}O_6$	1.014
Proteins	$C_6H_{13.1}O_1N_{0.6}$	0.496
Carbohydrates	$(C_6H_{10}O_5)_n$	0.415

3.4. Microalgal cultivation and harvesting

As previously mentioned, the microalgal biomass production is expensive compared to crops; even on the microalgal cultivation there are different costs for the different large-scale production setups (Chisti, 2007; Debowski *et al.*, 2013; Wiley *et al.*, 2011). Most important factors to the production are sun light, nitrogen, phosphorus (acts as limiting micronutrient, due to the complexation of metal ions), iron, silicon and carbon dioxide (Chisti, 2007; Mata *et al.*, 2010).

Microalgae can present different metabolisms and can shift between those responding to stress conditions. The main are:

- Photoautotrophically – light is the only source of energy used for the photosynthesis;
- Heterotrophically – organic compounds are the only source of energy, sun light is not required;
- Mixotrophically – the main energy source is photosynthesis, but carbon dioxide and organic compounds have an important roll on growth;
- Photoheterotrophycally – organic compound, are used as carbon source, and solar light is used to convert them (Mata *et al.*, 2010).

In figure 3.3 it is possible to see the growth phases and the nutrient depletion over time (Mata *et al.*, 2010).

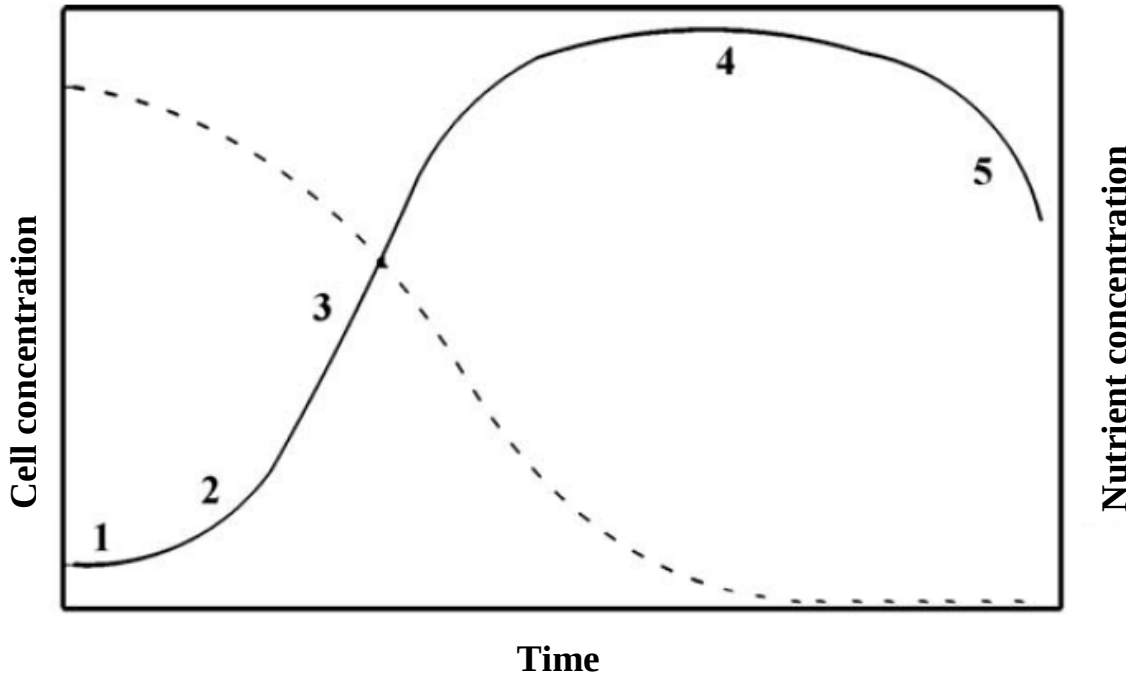

Figure 3.3 - Representation of microalgae growth rate (solid line) and nutrients concentration (dashed line) over time; (1) lag phase; (2) exponential phase; (3) linear phase; (4) stationary phase; (5) decline phase (Adapted from Mata *et al.*, 2010).

The two most studied methods for microalgal growth in large scale are raceway ponds and tubular photobioreactors (PBR; Chisti, 2007; Mata *et al.*, 2010).

Raceway ponds (figure 3.4) can be built in concrete or compacted soil, and have a recirculation channel with a small depth (0.15-0.3 m). During daylight the culture is fed, and a paddlewheel promotes the mixing and the water is circulated continuously around the circuit to prevent microalgae sedimentation. The corners are round with baffles, and the harvesting step is at the end of the channel (Chisti, 2007; Schenk *et al.*, 2008). Open systems have lower building, operation and maintenance costs. The disadvantages are water evaporation, water temperature control due the environmental conditions, and contamination of the culture by other species. With that, the productivity yields can be affected. To overcome the proliferation of undesired species the cultivation of extremophiles is encouraged (Borowitzka, 1999; Chisti, 2007; Mata *et al.*, 2010; Schenk *et al.*, 2008; Wiley *et al.*, 2011).

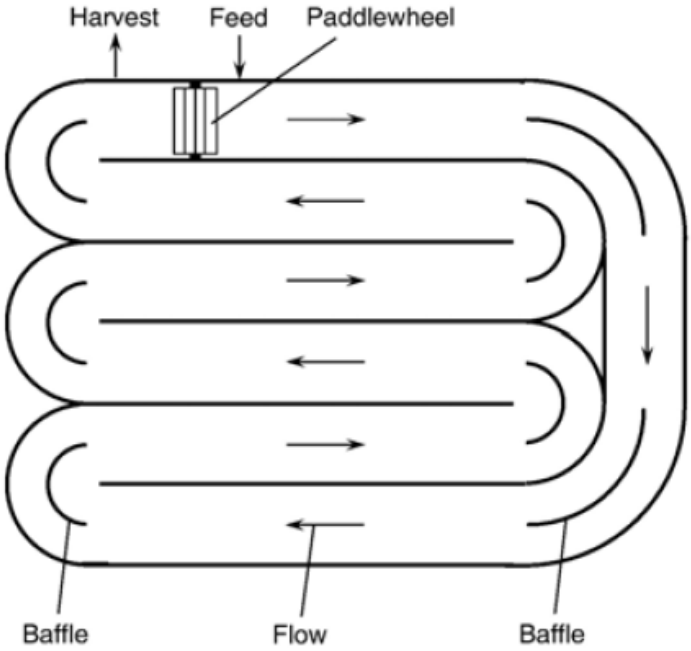

Figure 3.4 - Aerial view of a raceway pond (Chisti, 2007).

There are many different types of photobioreactors, in which design and operation methods are the main distinguishing factors. The most popular photobioreactors types are flat panels, cylindrical, tubular and disposable (Richmond, 2004). Due to the high diversity of microorganisms, and the differences on the optimal conditions for their culture (nutritional, light requirements and resistance to stress), it is difficult to design a PBR that fits all microalgae species (Richmond, 2004). The major criteria for the construction of PBR are, temperature regulation, durability of the construction material, orientation, inclination, mixing and surface to volume ratio (Mata *et al.*, 2010; Richmond, 2004).

Photobioreactors (figure 3.5) allow the production of a single microalgae species, preventing contaminations with other species or microorganisms, and the optimal conditions for this culture can be achieved. Light, mixing, pH, CO_2 losses can be monitored to ensure a higher volumetric production and higher cell concentration (Borowitzka, 1999; Chisti, 2007; Mata *et al.*, 2010; Richmond, 2004; Schenk *et al.*, 2008). The strongest limitations of the PBR are the high building and operation costs, difficulty of scaling up, bio-fouling, oxygen accumulation on the system, overheating and cell damage by shear stress (Mata *et al.*, 2010; Richmond, 2004).

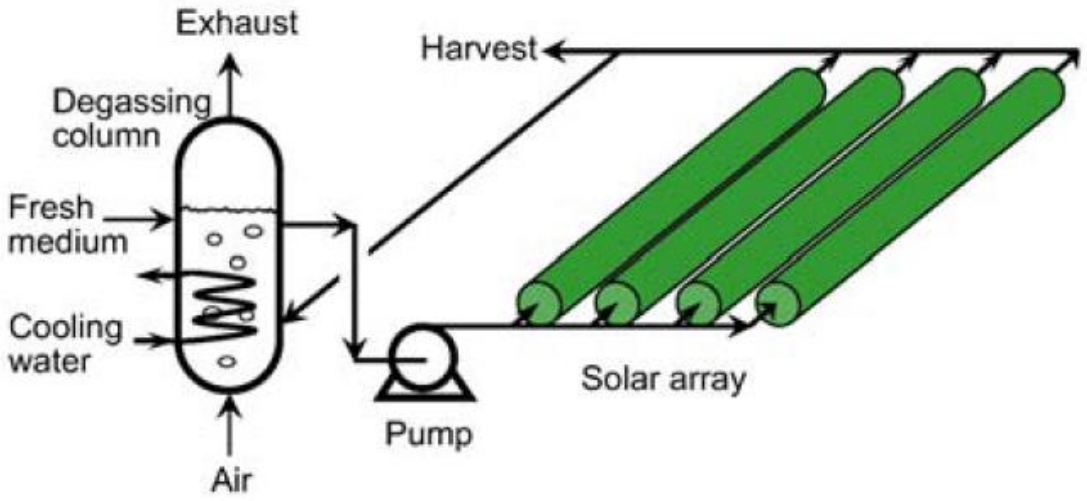

Figure 3.5 - Photobioreactor with horizontal tubes on a parallel distribution (Chisti, 2007).

Comparing raceway ponds and PBR, the price of operation costs of the raceway ponds are cheaper (one order of magnitude lower than the closed systems), but the biomass productivity and the oil yield per hectare is lower (Borowitzka & Moheimani, 2013; Chisti, 2007; Richmond, 2004; Wiley *et al.*, 2011). Due to the differences on the two productions facilities they should not be viewed as competing technologies (Mata *et al.*, 2010; Richmond, 2004).

After the cultivation of the microalgae comes one of the most expensive parts of the production: the harvesting of the culture. Harvesting can contribute 20 to 30% of the production costs. The usual processes are sedimentation, centrifugation, filtration and ultra-filtration. In some cases a flocculation step is needed to aggregate the microalgae to enhance the sedimentation if the cell size of the microalgae is small. For large sized microalgae, in a small-scale production, the best method is filtration and for microalgae with small size and fragile cells the most indicated processes are micro or ultra-filtration. Depending on the desired product quality, the harvesting method may change. For low value products sedimentation is the most used, while for high value products the recovery has to be more effective, and so the best harvesting process is centrifugation (Harun *et al.*, 2010; Mata *et al.*, 2010; Richmond, 2004).

3.5. Oil extraction

Oil extraction demands cell wall disruption. The most used processes to achieve this are expeller/oil press, liquid-liquid extraction (solvent extraction), supercritical fluid extraction (SFE) and disruptive ultrasound techniques (Harun *et al.*, 2010; Mata *et al.*, 2010; Pereira *et al.*, 2013a).

Oil presses and expellers are appropriate to nuts and seeds, or dried microalgal biomass. This method has a yield of 75%, being the less effective method (Harun *et al.*, 2010).

Solvent extraction by organic solvents is one of the most effective methods, the solvent breaks the cell walls and because of the high solubility of the lipids on organic solvents they are extracted. There are many different organic solvents that can be used on this process (e.g. benzene, acetone, chloroform, cyclohexane), but the most used due to its high extraction capability and low cost price is hexane (Harun *et al.*, 2010).

Supercritical fluid extraction uses high temperatures and pressures, is the most efficient method to extract the oils (up to 100%) and other desired products from the cells. This method can achieve high yields, high product concentration and is extremely time efficient (Harun *et al.*, 2010; Pereira *et al.*, 2013a).

Ultrasound methods are still in an initial stage of development but have already showed potential achieving a yield of 90% extraction of fatty acids and pigments. The mechanism involves the application of ultrasonic waves that create cavitation bubbles around the cells, whose collapse releases the oils (Harun *et al.*, 2010).

3.6. Transesterification

Transesterification is the conversion of triglycerides into three fatty acid ester molecules and one of glycerol (figure 3.6). Usually methanol is the solvent used to conduct this process. Transesterification occurs in three steps, first the triglycerides are converted to diglycerides, afterwards to monoglycerides and to conclude the reaction to glycerol. This reaction is an equilibrium, for each mole of triglyceride three mol of methanol are required, and the result are three mol of methyl esters and one of glycerol. To ensure the reaction in the way of the fatty acid production, methanol is used in excess (6:1). Typically, yields exceed 98%. To speed up the transesterification, catalysts are used; the most common are acids (e.g. sulfuric, phosphoric, hydrochloric or organic sulfonic),

bases (e.g. sodium hydroxide, sodium ethoxide, potassium hydroxide and potassium ethoxide) and enzymes (e.g. lipases), although the latter cannot compete with the price of the first (Chisti, 2007; Mata *et al.*, 2010; Naik *et al.*, 2010; Pereira *et al.*, 2013a).

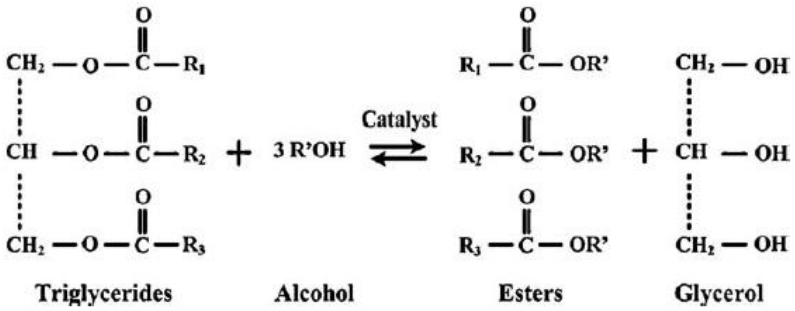

Figure 3.6 - Transesterification of triglycerides (Mata *et al.*, 2010).

3.7. Biorefinery

A biorefinery has the same principle of the oil-based refineries, in which crude oil can produce many different petrochemicals. The same concept can be applied to biomass, from which different bio-products and biofuels are produced, maximising the value of the intermediates and final products, and consequently decreasing the overall costs. The biorefinery concept centralizes many hybrid technologies from different fields, such as polymer chemistry, bioengineering, agriculture and food science (Chisti, 2007; Ohara, 2003).

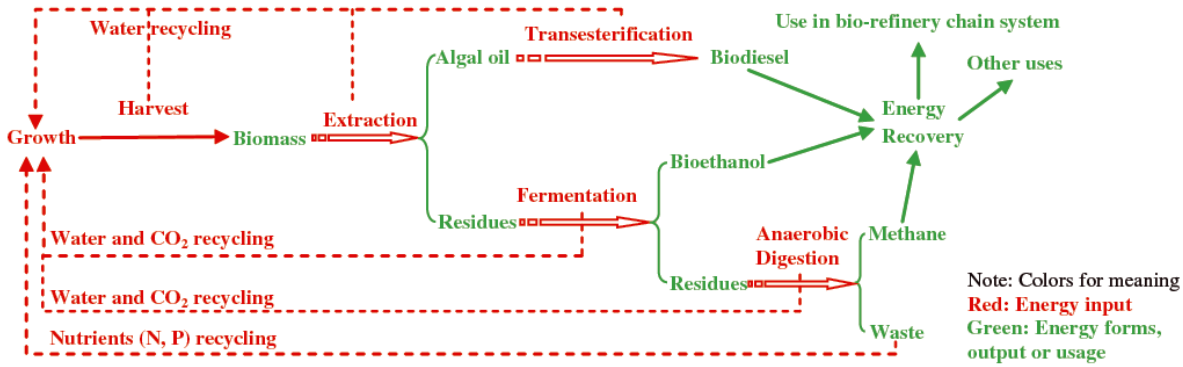

Figure 3.7 – Energy and mass incomes and outcomes on a microalgae biorefinery (Zhu, 2014).

This concept can accept different biological feedstocks and convert them to many different products including chemicals, energy, different generation fuels and materials (Clark *et al.*, 2006; Fitzpatrick *et al.*, 2010). Figure 3.7 shows the energy demands and outputs of a biorefinery.

One of the challenges for the implementation of a biorefinery concept is to deal with the wastewater effluents from bioethanol and biohydrogen processes; these streams are loaded with organic matter that can be converted to biogas and the residual solids can be used as fertilizers for agricultural soil (Kaparaju *et al.*, 2009).

On a biorefinery concept the use of non-renewable energy resources and environmental impacts has to be minimized. Considering an environmental perspective it is required to analyse the carbon, nitrogen and water cycles, their interdependences and the environmental impacts carried by a life cycle assessment (LCA; Cherubini, 2010).

3.8. Life cycle assessment

LCA is a tool to quantify the environmental impacts and resources consumption during the entire product life cycle, from the raw material acquisition or production, to the waste management (disposal) or the recycling program (Finnveden *et al.*, 2009; Rebitzer *et al.*, 2004; ISO 14040). The first scientific reports regarding this topic emerged in the 1990's, but were often criticized by the scientific community. Since then, a huge development has happened, there are guidelines and the process was homogenised (Finnveden *et al.*, 2009). In the past years, LCA has become a powerful tool for companies, governments or non-government organizations for evaluating, improving, quantifying and comparing goods and services on their potential and environmental impacts (Guinée *et al.*, 2011; Rebitzer *et al.*, 2004; ISO 14040).

A LCA is divided in four parts (figure 3.8): goal and scope definition, life cycle inventory analysis (LCI), life cycle impact assessment (LCIA) and interpretation (Finnveden *et al.*, 2009; Rebitzer *et al.*, 2004; ISO 14040). The LCA does not contemplate the costs of the product life cycle. For that purpose, a Life Cycle Costing (LCC) is normally done. However, it is strongly recommended that both are done simultaneously (Guinée *et al.*, 2011; Rebitzer & Hunkeler, 2003; ISO 14040).

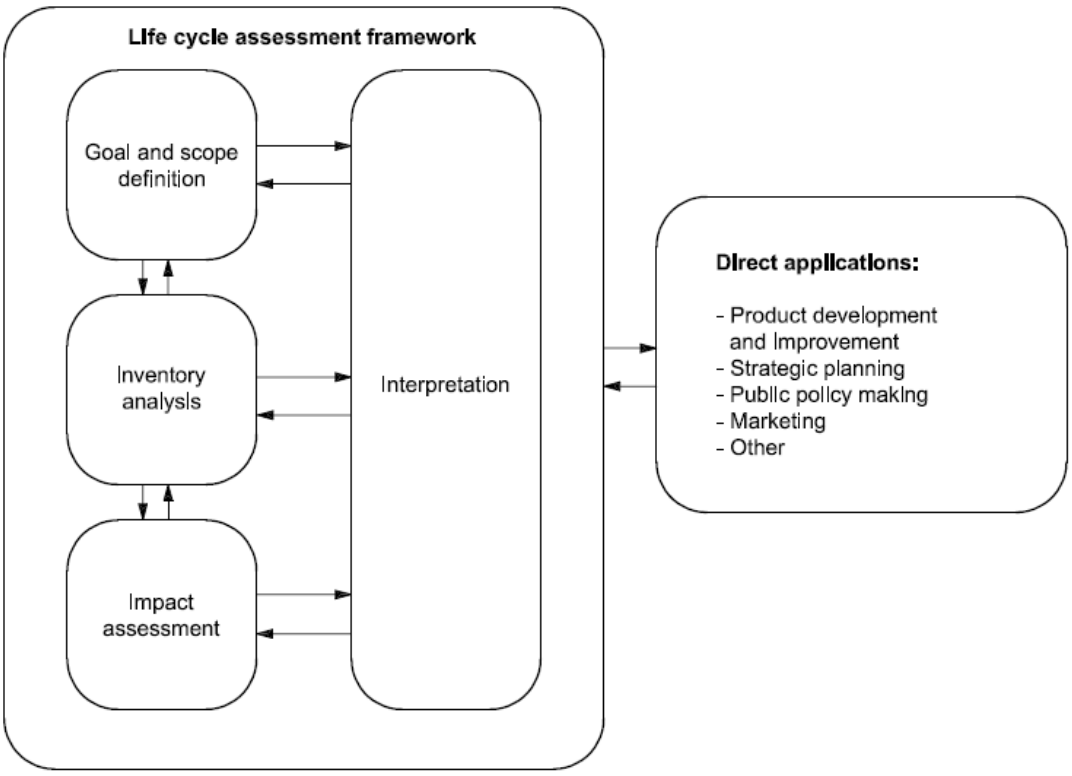

Figure 3.8 - Life cycle assessment framework (ISO 14040).

3.9. Microalgae (*Botryococcus braunii*)

B. braunii was the species researched on this thesis, due to the high quantity of lipid fraction reported on previous studies (75%), and the capability to accumulate high quantities of hydrocarbons (Mata *et al.*, 2010; Metzger & Largeau, 2005).

B. braunii is a unicellular photosynthetic green microalgae, belonging to the group of chlorophyceae (chlorophyta) that can be found in brackish and freshwater, and in all types of climates and environmental conditions. This strain was already reported in distinct locations, namely: USA, Portugal, Bolivia, France, Ivory Coast, Morocco, Philippines, Thailand and West Indies (Banerjee *et al.*, 2002). Contrarily to most microalgal species, this strain has the capability to accumulate significant amounts of hydrocarbons (e.g. alkadienes, trienes, triterpenes and tetraterpenes). Normally, this microalgae forms colonies (botryoid organisation of individual pyriform-shaped cells), as shown in figure 3.9 (Borowitzka & Moheimani, 2013; Metzger & Largeau, 2005).

Most studies show that significant differences exist among strains of this microalgae species, mainly the type of hydrocarbons synthesized, due to the growth environment (e. g. laboratory or wild growth; Metzger & Largeau, 2005).

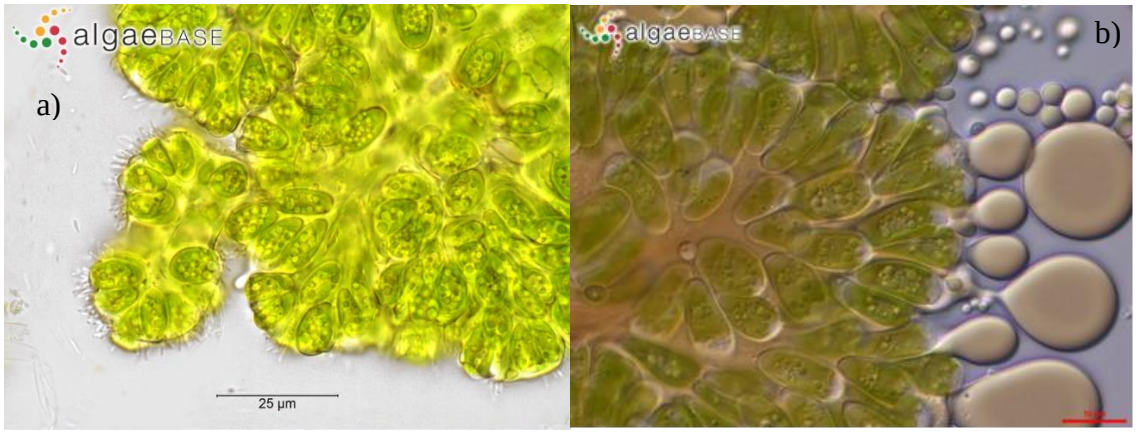

Figure 3.9 – a) Microscopy visualization of B. braunii colonies (scale bar = 25 µm, Algaebase, 2015); b) Microscopy visualization of B. braunii excreting oil from the colonies (scale bar =10 µm Algaebase, 2015).

4. Materials and methods

4.1. Growth of microalgal biomass

The microalgal biomass of *B. braunii* was supplied in the form of a dried powder by NECTON S.A., Portugal. Cultures were grown outdoors on green wall airlift photobioreactors (figure 4.1). The culture was harvested by centrifugation, and stored at -20ºC. The frozen paste was later freeze-dried in an industrial equipment and placed in airtight bags until use.

Figure 4.1 - Green wall airlift photobioreactor at NECTON S.A. facilities (Pereira, 2009).

4.2. Determination of total lipids

The amount of total lipids was determined according to the Bligh and Dyer method (Bligh & Dyer, 1959), with some adaptations. Dried microalgae (20 mg per replicate) was homogenised in 10 mL tubes with 0.8 mL of distilled water for 20 min, at room temperature. Afterwards, 2 mL of methanol and 1 mL of chloroform was added and the mixture was homogenized in ice for 60 seconds with a disperser (IKA Ultrathurrax T25). Later, 1 mL of chloroform was added and the samples were homogenized for 30 s, followed by the addition of 1 mL of distilled water and further homogenization for 30 s. After homogenizing the samples were centrifuged at 5000 *g* for 10 minutes, the

chloroform was extracted to new tubes, and 1 mL was pipetted to a previously weighted tube. Finally the tubes were dried at 60ºC, until the chloroform was completely evaporated and further weighted.

4.3. Biodiesel production

4.3.1. Lipid extraction

Lipids were extracted following the protocol described in Balasubramanian (2013) with few modifications (figure 4.2). Dried microalgae (50 g per replicate) was placed in 250 mL of solvent mixture of hexane, methanol and ethyl acetate (3:2:0.05; HPLC grade), and stirred at reflux temperature for 2 hours. After refluxing, the mixture was separated in falcon tubes and centrifuged for 5 min at 5000 *g*. After the first extraction the algae cake was further extracted with 250 mL of solvent mixture, under reflux conditions for 30 min (2nd extraction) and later for 15 min (3rd extraction). The three lipid fractions were pooled and filtered through a Whatman nº 4 filter paper. The solvent mixture was removed from the microalgal oil using a rotatory evaporator under reduced pressure.

Figure 4.2 - Extraction of the lipids from the B. braunii biomass.

4.3.2. Lipid transesterification and biodiesel purification

Biodiesel was synthesised (figure 4.3) according to the protocol described by Gangadhar (2016) with minor modifications. The extracted oil was mixed with a methanolic solution (2% sulphuric acid) and stirred at reflux temperature for 5 hours. To confirm the conversion efficacy, the mixture was allowed to reach room temperature, and thin-layer chromatography (TLC) was performed using 98:2 hexane and ethyl acetate as eluent mixture. After reaction completion the solvent was removed up to ¼ of the initial volume using a rotatory evaporator. Afterwards, hexane was added to extract the FAME and the mixture was centrifuged at 5000 *g* for 5 min. The hexane fraction was separated from the residues (e.g. methanol and glycerol) on a separating funnel with distilled water until the pH reached the value of 6. Afterwards a saturated NaCl solution was used to help break the emulsions generated in the process. To completely eliminate the water, anhydrous sodium sulphate was added, which was afterwards removed by filtration. The hexane phase was evaporated using a rotatory evaporator.

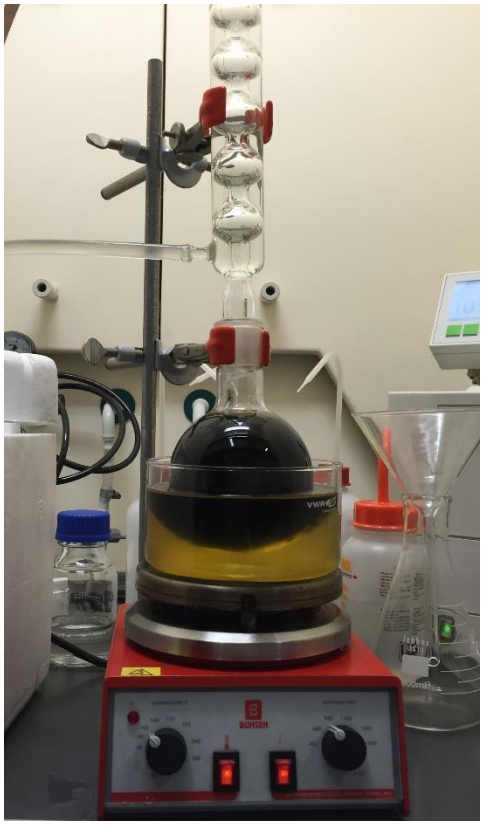

Figure 4.3 - Transesterification of the lipids to produce the biodiesel.

To remove the contaminants from the biodiesel mixture (e.g. pigments), bentonite was used according to the procedure described in Peña (2015). Briefly, for each gram of biodiesel, 10 mL of a solvent mixture (99:1 hexane and diethyl ether) and 2 g of bentonite were used. The mixture was stirred over 24 h at 40ºC, and centrifuged at 10000 *g* for 10 min. The hexane fraction was collected, filtered using a 0.45 µm filter, and evaporated using a rotatory evaporator to obtain the final biodiesel.

4.3.3. Determination of fatty acid methyl ester profile of the biodiesel

The fatty acid methyl ester (FAME) profiles of the biodiesel samples were determined using a Bruker GC-MS (Bruker Scion 456-GC - TQ) equipped with a ZB5-MS capillary column (25 m × 0.25 mm internal diameter, 0.25 µm film thickness, Agilent). The carrier gas was helium at 1 mL/min, while the injector and detector were maintained at 300°C in S/SL mode. The oven temperature was programmed for 60°C (1 min), 30°C min^{-1} to 120°C, 4 °C min^{-1} to 250°C, 20°C min^{-1} to 300°C, and hold for 4 min at this temperature. Identification and quantification of the FAME was performed by comparing the retention times of the FAME in the biodiesel samples with those of an external standard (Supelco 37 Component FAME Mix, Sigma-Aldrich) and further confirmed by comparison of the MS spectra. For quantification purposes, a separate calibration curve was generated for each of the FAME in the standard.

4.3.4. Assessment of biodiesel properties

Density was measured on a 5 mL pycnometer (Gay-Lussac, adjusted 5 cm^3), and kinematic viscosity was measured using a viscometer (Pobel, Micro Ubbelohde Ic, according to ISO 3105) at 40ºC. The cetane number (CN) was determined using the cetane number of each fatty acid methyl ester (CN_c) and relative amount of each fatty acid methyl ester in the mixture (A_c), the equation 3 was used (Knothe, 2014):

(3) $$CN_{mix} = \sum A_c \times CN_c$$

The estimation of the cold filter plugging point (CFPP) and long chain saturated factor (LCSF) was calculated with the following equations 4 and 5 (Ramos *et al*., 2009):

(4) $$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24)$$

(5) $$CFPP = 3.1417 \times LCSF - 16.477$$

C(n) is the relative amount of individual fatty acid methyl ester, present on the mixture where (n) represents the number of carbons on the fatty acid chain.

The iodine value was calculated according to the factors and equations established in the EN14214 (2008).

The High heating value (HHV) was estimated according equation 6 (Fassinou, 2012):

(6) $$HHV = \frac{(\sum x_i A_i)}{\sum x_i}$$

Where x_i is the mass fraction of the FAME i and A_i is the HHV of the pure FAME i estimated according to a group contribution method (Levine *et al.*, 2014).

4.4. Determination of Ca, Mg, K, Na and P

The determination of these elements in biodiesel was performed in an Agilent 4100 Microwave Plasma – Atomic Emission Spectrometer (MP-AES), with an External Gas Control Module (EGCM), allowing air injection into the plasma to prevent carbon deposition in the torch. The plasma was stabilized using nitrogen. An inert OneNeb nebulizer was used to increase nebulization efficiency. Calibration standards were prepared at concentrations of 0.5, 1, 5 and 10 ppm by diluting a 500 ppm S21+K solution (Conostan) with Shellsol (Shell). All standards were matrix matched with Blank Oil 75 (Conostan). The samples were spiked with S21+K 0.5 ppm and the spikes measured to validate the method. The wavelengths for the quantification of each element are presented in table 3.

Table 4.1 – Wavelengths of the elements.

Element	**Wavelength (nm)**	**Nebulizer pressure (kPa)**
Mg	285.213	240
Ca	422.673	240
Na	588.995	240
K	766.491	240
P	213.618	240

4.5. Anaerobic digestion of raw and spent biomass

For the production of biogas two inocula were tested, from two different anaerobic digestion reactors, both from Águas do Algarve, SA wastewater treatment plants (WWTP Lagos and Silves). Moreover, two different temperatures (25ºC and 35ºC) and two different biomasses (raw and defatted) were tested.

Dried biomass (1 g per replicate) was placed with 40 mL of inoculum, 10 mL of distilled water and 0.5 g of sodium bicarbonate ($NaHCO_3$) in clamp-top 100 mL vials. Capped with a rubber septum and clamped with an aluminium crimp seal at the experimental temperature. To assure the anaerobic conditions and start the digestion the head space of each reactor was purged with nitrogen before clamped. Procedural blanks were prepared without the biomass sample. The experiment (figure 4.4) was done in triplicate.

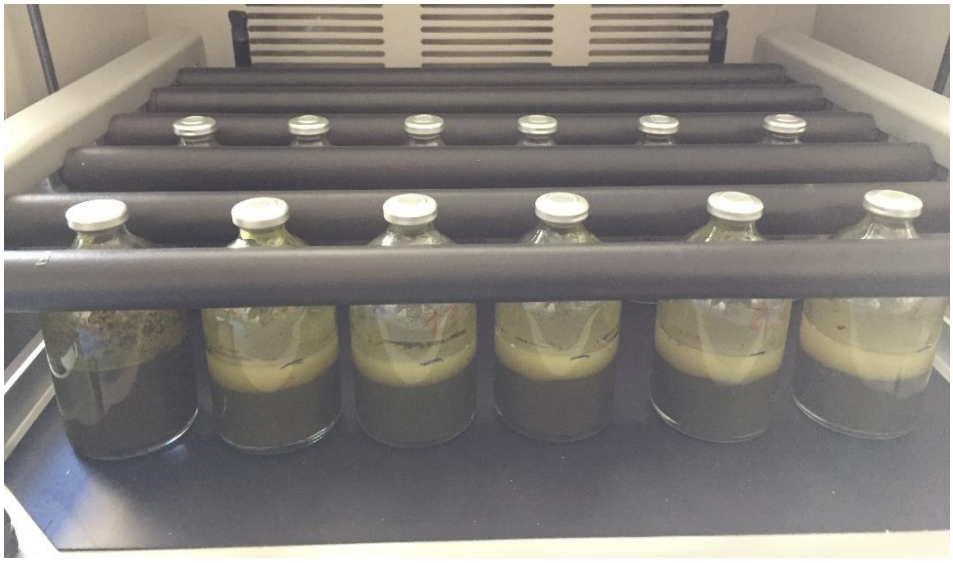

Figure 4.4 - Biogas production experiment.

Due to the low amount of biogas produced with the initial experimental design tested, a new experimental setup was designed using 10 g of defatted biomass, 400 mL of Silves inoculum, 100 mL of distilled water and 5 g of $NaHCO_3$. Two replicates for each temperature were tested and an additional biomass feed of 10g was performed at day 14 of the experiment. The produced biogas was stored on tedlar bags for gases linked to the incubation vials via tubing (figure 4.5).

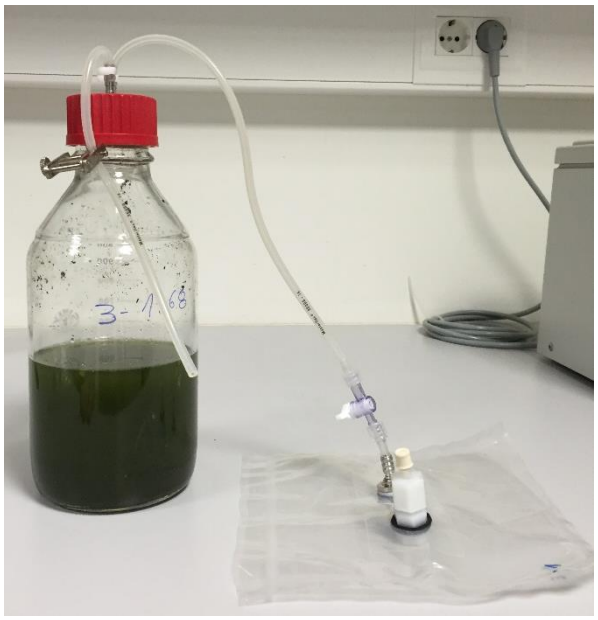

Figure 4.5 - Anaerobic digester linked to the tedlar bag.

4.5.1. Inoculum characteristics

The inocula were characterised measuring the following parameters: volatile suspended solids (VSS), total suspended solids (TSS), phosphorus (P), nitrogen (N), organic nitrogen, carbon (C), hydrogen (H), phosphate (PO_4^{3-}), nitrate (NO_3^-), ammonium (NH_4^+) and chemical oxygen demand (COD). These parameters were measured in the large scale replicates before and after anaerobic digestion.

Phosphates and ammonium were measured by spectrophotometry using a multi plate reader (Synergy 4, Biotek), at the absorbance 880 and 630 nm, respectively, using the method described in APHA (2005). For the phosphate determination the sample was filtered through a 1.6 µm filter under vacuum (Whatman GF/A), then the ascorbic acid, ammonium molybdate and potassium antimonyl tartrate trihydrate reacted and formed a blue colouration. For the ammonium determination the sample was filtered on the same way, and reacted with dichloroisocyanuric acid, phenol, trisodium citrate and sodium hydroxide formed the blue indophenol.

Nitrate was determined using a nitrate kit (HACK LANGE LCK 340). Briefly, the sample (10 mL) was placed on the cell and NitraVer 5 Nitrate Reagent Powder Pillow was added to the sample and shaked vigorously for 1 min. After that time, the tube was placed on a

stand and after the reaction time (5 min) the sample was measured in an UV spectrophotometer (HACH LANGE DR 2800).

The Chemical Oxygen Demand (COD) was determined in the mixture suspended on distilled water at a dilution of 1/20. Briefly, 2 mL of the sample was pipetted to a COD kit tube (HACH LANGE LCK 514), homogenised on a vortex, and placed on the heating block at 148ºC for 120 min. The tubes were allowed to cool for 10 min, homogenised again in the vortex, and after cooling down to room temperature the absorbance of the samples was measured in the UV spectrophotometer (HACH LANGE DR 2800).

The amount of C, H and N was performed in triplicate in samples of 1-2 mg. Samples were sealed in tin boats and weighed on a microbalance (Sartorius M5P). Prepared samples were either stored in a desiccator or immediately transferred to the automatic sampler Elemental Analyser model Vario ELIII. Nicotinamide served as reference.

For total phosphorous determination, samples from the anaerobic digestion were ignited at 550 ± 50 ºC for 14 h in the muffle furnace. After they were humidified with water and 2 mL of hydrochloridric acid (HCl), afterward the samples were filtered with bidistilled water. Then, 5 mL of solution were added to a volumetric flask with 12.5 mL of 5% ammonium molybdate and ammonium vanadate (0.25%) for 15 min. The volume of the mixture was then make up to 50 mL and the absorbance (375 nm) of the mixture measured on a colorimeter (CADAS-100).

The amount of potassium in the samples after the anaerobic digestion was performed with the same filtered sample used on the determination of the total phosphorus, on a MP-AES at 766.491 nm.

Organic nitrogen was determined using the Kjeldahl method (APHA *et al.*, 2005). The sample was boiled in H_2SO_4 afterwards the ammonium sulfate solution was mixed with sodium hydroxide (NaOH) to convert the NH_4^+ to NH_3. This was followed by the distillation of NH_3. To conclude the quantification of ammonia a titration was performed with a standard mineral acid.

Total suspended solids (TSS) and volatile suspended solids (VSS) were done under ESS Method 340.2, the solutions were filtered through a 1.6 µm filter under vacuum (Whatman GF/A). Afterwards, the filter was washed 3 times with Milli-Q water, and

dried on an oven for 1 hour at 103-110ºC. When at room temperature the filter was weighted and the amount of TSS calculated using formula (7).

(7) $$TSS\left(\frac{mg}{L}\right) = \frac{filter\ weight\ after\ dried\ (mg) - filter\ tare\ (mg)}{volume\ of\ the\ sample\ (L)}$$

For the determination of SSV the filter used on the TSS was placed in the muffle furnace and ignited at 550 ± 50 ºC for 30 min.

(8) $$VSS\left(\frac{mg}{L}\right) = \frac{TSSfilter\ weight\ after\ dried\ (mg) - filter\ weight\ after\ crucible\ (mg)}{volume\ of\ the\ sample\ (L)}$$

4.6. Biogas production

Biogas production was measured daily. The pressure of the head space was measured using a manometer (Fisher Scientific FB57057; figure 4.6). After pressure measurement the biogas was released to the environment until the pressure on the headspace reached atmospheric pressure. The pressure difference measured was converted into biogas volume using equation (9).

(9) $$V_{biogas} = \frac{P \times V_{Head} \times C}{R \times T}$$

where V_{biogas} is the biogas produced during that day (L), P the difference of absolute pressure between the day before and the present day (mbar), V_{head} the volume of head space of the digester (L), C the molar volume (22.41 L/mol), R the universal gas constant (83.14 L mbar/ k mol) and T the absolute temperature.

Figure 4.6 - Measurement of the pressure on the head space to estimate the biogas production.

Carbon dioxide (CO_2, %), methane (CH_4, %), oxygen (O_2, %) and hydrogen sulphide (H_2S, ppm) concentrations in the biogas were analysed using a Geotech Biogas 5000 Gas analyser (figure 4.7).

Figure 4.7 - Measurement of the biogas composition with a Geotech Biogas 5000.

4.7. Statistical analyses

Significant differences between the biogas production in the different combinations of treatments (Inocula – Lagos and Silves; Temperature – 25°C and 35°C; Biomasses – Raw and Defatted) were assessed using the Wilcoxon non-parametric test since the data was not normally distributed. Differences were considered significant when p-values ≤ 0.05.

5. Results and discussion

5.1. Proximate composition

The contents of proteins, lipids, carbohydrates, ashes and C/N ratio of the raw and defatted biomass are presented on table 5.1. The lipid content before the extraction process was 14.8%, and after only 1%. The protein content was 48.3 and 50.8% in the raw and defatted biomass samples, respectively, higher than the results reported by Neumann *et al.* (2015) and Sydney *et al.* (2010), which were 39.6 and 46.0% respectively. *B. braunii* had 31.1 and 42.8% of carbohydrates available on the raw and defatted biomass respectively, higher than the 22.3% obtain by Neumann *et al.* (2015) in the defatted biomass sample. The total ash content of the *B. braunii* raw biomass was 5.6% and the defatted biomass 5.7%, lower than the 7.5% reported by Sydney *et al.* (2010). The C/N ratio for the biomass samples were 6.4 and 5.8 for raw and defatted biomass, respectively.

Table 5.1 - Proximate composition of B. braunii biomass.

Composition	**Raw biomass (%)**	**Defatted biomass (%)**
Proteins	48.3 ± 0.5	50.8 ± 0.8
Lipids	14.8 ± 0.4	1.00 ± 0.10
Carbohydrates	31.1 ± 0.7	42.8 ± 0.5
Ashes	5.60 ± 0.10	5.70 ± 0.40
C/N ratio	6.40 ± 0.10	5.80 ± 0.02

5.2. Biodiesel

Lipids of *B. braunii* were extracted with a mixture of hexane, methanol and ethyl acetate (3:2:0.05; HPLC grade). The yield of the extraction was 13.8 ± 0.2% of dry weight (DW), which was confirmed by the Bligh and Dyer method (14.8 ± 0.4%; table 5.1). Higher lipid contents have been reported for *B. braunii*; Ashokkumar & Rengasamy (2012) observed a maximum lipid content of 42% in this microalgae at the exponential phase of growth, which decreased afterwards. Stirring the biomass at reflux temperature for 2h and 45min, on intercalated intervals of 120, 30 and 15min, showed to be the most efficient method

for the lipid extraction (93%; figure 5.1) suggesting that a pre-treatment to disrupt the cell wall was not necessary.

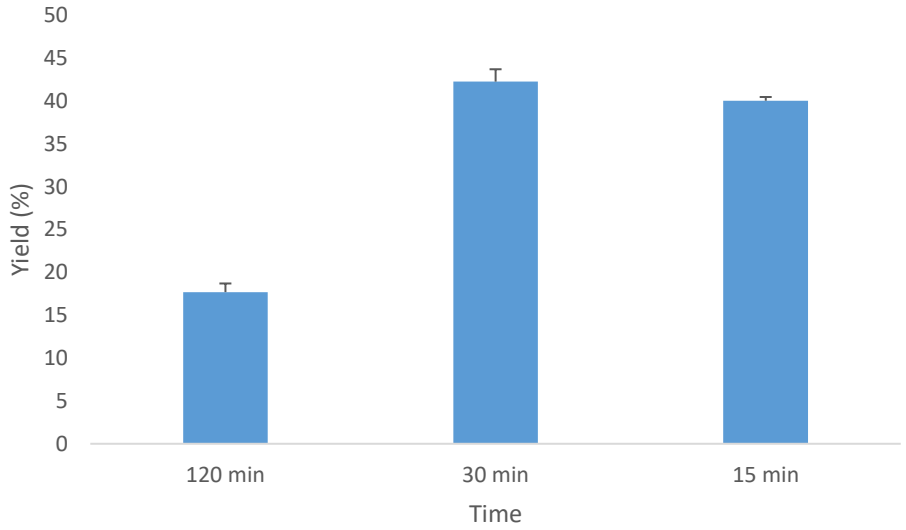

Figure 5.1 - Efficiency of the lipid extraction.

Transesterification was carried out using methanol in the presence of sulphuric acid (2% H_2SO_4 in methanol). The reaction was followed by thin layer chromatography (figure 5.2). In figure 5.2 the first spot corresponds to the TAG standard, while the second spot is the FAME standard of sunflower biodiesel. The last spot represents the transesterified lipids of *B. braunii*. This TLC confirms that the TAG from the parent oil of *B. braunii* was completely converted to FAME.

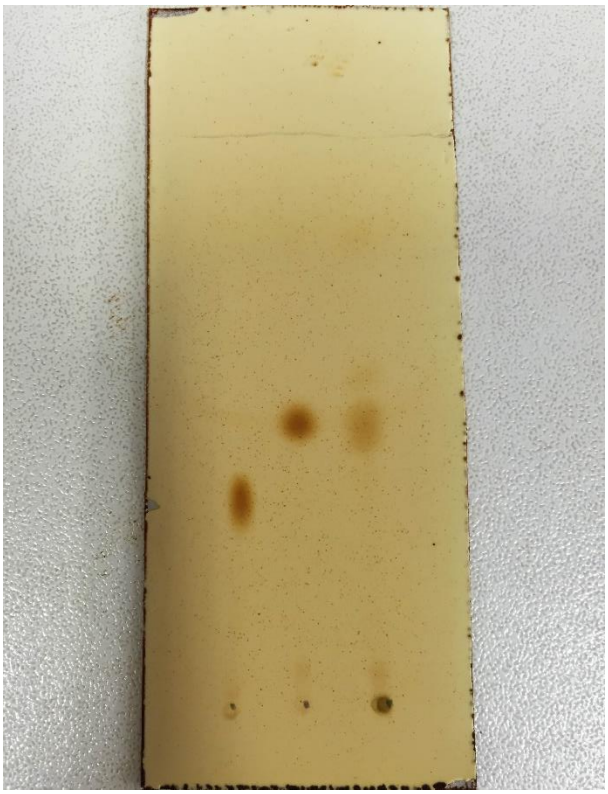

Figure 5.2 - TLC to confirm the conversion of TAG to FAME.

The crude biodiesel yield was 46.8 ± 7.5% and after the purification with bentonite 30.5 ± 8.8% (figure 5.3). Lipids can be divided in two categories, neutral lipids (mainly triglycerides and cholesterol) and polar lipids (phospholipids and glycolipids). Triglycerides are the main feedstock for biodiesel production (Huang *et al.*, 2010). However, due to the different conversion rates of the different lipid groups and losses occurring during the process, the conversion of lipids to biodiesel was not 100% effective. This is probably related to the high content of phospholipids and glycolipids commonly observed in microalgal biomass and losses occurring during the water washes and centrifugations.

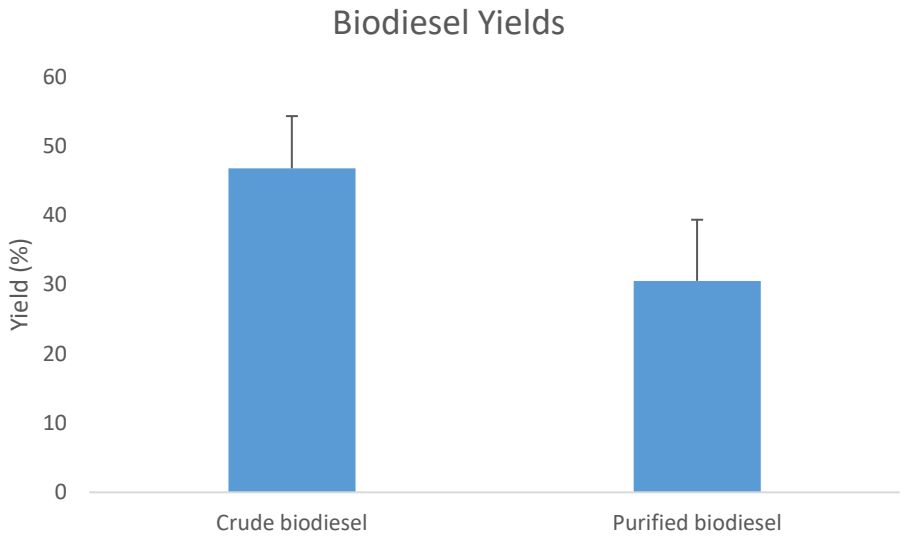

Figure 5.3 - Yields of the biodiesel conversion rates

The fatty acid composition of the biodiesel produced from *B. braunii* is presented in table 5.2. The fatty acid profile of the produced biodiesel mainly ranges from C16 to C18 fatty acids. According to Chisti, (2007), long chain carbon FAME from C:16 to C:18 are the most suitable for biodiesel production. The predominant fatty acids of *B. braunii* biodiesel were palmitic (C16:0; 42%), oleic (C18:1; 31%) and linoleic (C18:2*n*6; 16%) acids. Hexadecadienoic (C16:2), palmitoleic (C16:1), linolenic (C18:3*n*6) and stearic (C18:0) acids were also present on the biodiesel produced, but in smaller quantities. The fatty acid profile obtained in the present work matches those in previous reports (Fang *et al.*, 2004; Lee *et al.*, 2010; Yamaguchi *et al.*, 1987; Yeesang & Cheirsilp, 2011; Yoo *et al.*, 2010). Unsaturated fatty acids were the major FAME present on this biodiesel sample, representing over 55% of the total FAME. Yeesang & Cheirsilp (2011) reported that a more saturated microalgae biodiesel can improve the cetane number and oxidative stability. Saturated fatty acids represented 43% of the total FAME. Metzger & Largeau (2005) reported that the capability of this microalgae to produce long chain hydrocarbons coupled to the fatty acid composition can produce biodiesel with improved quality.

Table 5.2 - FAME composition of the biodiesel sample from B. braunii.

Carbon number	Fatty acid	%
C16:0	Palmitic acid	42.08
C16:1	Palmitoleic acid	3.71
C16:2	Hexadecadienoic acid	2.32
C18:0	Stearic acid	1.70
C18:1	Oleic acid	31.83
C18:2n6	Linoleic acid	16.64
C18:3n6	Linolenic acid	1.71
∑SFA		43.78
∑MUFA		35.55
∑PUFA		20.68

Properties of the biodiesel produced (table 5.3) were assessed according to the European (EN 14214) and American (ASTM D6751) standards.

Table 5.3 - Comparison of the properties of the B. braunii biodiesel sample with the EN14214 and ASTM D6751 legislations.

Properties	Unit	*B. braunii*	EN 14214	ASTM D6751
Viscosity (40°C)	mm^2s^{-1}	6.32 ± 0.20	3.50-5.00	1.9-6.0
Density (15°C)	kg/L	0.84 ± 0.01	0.86-0.90	n.a.
Cold filter plugging point (CFPP)	°C	-0.59	n.a.	n.a.
Iodine Value	g I/100g	68.6	≤120	n.a.
Cetane number	-	61.8	≥51	≥47
PUFA (≥ 4 double bonds)	% mass	n.d.	≤1	n.a.
Linolenic acid	% mass	1.71	≤12	n.a.
HHV	MJ/kg	39.4	n.a.	n.a.
Ca	mg/kg	2.60 ± 0.02	≤5.0	≤5.0
Mg	mg/kg	0.01 ± 0.01	≤5.0	≤5.0
K	mg/kg	0.24 ± 0.02	≤5.0	≤5.0
Na	mg/kg	1.61 ± 0.01	≤5.0	≤5.0
P	mg/kg	7.42 ± 0.36	≤4.0	≤10

The density of *B. braunii* biodiesel sample was 0.84 ± 0.01 kg/L. However, this value does not meet the EN 14214 (0.86-0.90 kg/L) limit. The ASTM D6751 does not regulate this parameter. Lower biodiesel density increases leaks during the compression on the plunger, consequently the engine will have a power loss (Knothe *et al.*, 2005).

Viscosity indicates the capability of the fuel to flow in the combustion engine, and the possibility to form deposits on the engine. Higher viscosity presents a higher tendency to develop the aforementioned problems (Knothe, 2005). The EN14214 and ASTM D6751 regulations impose limits between 3.5-5.00 mm^2s^{-1} and 1.90-6.00 mm^2s^{-1}, respectively. The biodiesel sample produced presented a viscosity of 6.32 ± 0.2 mm^2s^{-1}, which is higher than the values presented in the normatives. This value is probably related with the high degree of saturation and chain length of the fatty acids present on the biodiesel sample.

According to Knothe (2014) the CN can be estimated from the FAME profile of the microalgae biodiesel, and is related to the ignition quality of the fuel. This parameter is similar to the octane number for gasoline; a low CN leads to a high octane number and high ignition delay, and vice versa (Knothe, 2005). To meet the EN 14214 the CN has to be higher than 51 and for the ASTM D6751 higher than 47. The result obtained for *B. braunii* biodiesel was 61.8, which was in agreement with both normatives. The high CN of our biodiesel is probably related with the high quantity of saturated fatty acids present, and can lead to lower emissions of nitrogen oxides (NO_x). Some biodiesel samples require additives to increase the CN and decrease the NO_x to meet this parameter (Knothe, 2005).

The CFPP is the lowest temperature a biodiesel sample has the capability to pass through 0.45 µm filter, before that temperature the biodiesel begin to nucleate and form wax crystals suspended in the liquid phase (Knothe *et al.*, 2005). Biodiesel samples with high CFPP can lead to operating problems such as wax settling, and filter and tubing lines plugging (Ramos *et al.*, 2009). The estimated CFPP from *B. braunii* biodiesel was -0.59 °C. Usually, the higher the chain length of saturated fatty acids, the higher the melting point of the biodiesel sample. Due to the higher quantity of palmitic acid (42.08%) this sample had a high CFPP. The EN14214 and ASTM D6751 regulations does not regulate this parameter, although the CFPP should meet the minimal temperatures recorded during the year (Ramos *et al.*, 2009).

The EN 14214 regulates the PUFA and linolenic acid contents, due to the oxidation capability of these FAME, while the American standard does not regulate both. Oxidative stability is measured by the period of time passed before the FAME start aging at 110°C under a constant air stream. The oxidation stability decrease with the increase of the PUFA (Ramos *et al.*, 2009). Biodiesel with low oxidative stability could not meet the required characteristics to be commercialized (Gangadhar *et al.*, 2016). The PUFA and

linolenic fatty acid methyl ester contents of the *B. braunii* biodiesel sample were in agreement with the European standard.

The higher heating value (HHV), also known as gross calorific value or gross energy, of a fuel is defined as the amount of heat released by a specified quantity (initially at 25°C) once it is combusted and the products have returned to a temperature of 25°C, which takes into account the latent heat of vaporization of water in the combustion products. Different methods have been reported to estimate the HHV, mainly based on elemental analysis and chemical composition of the fatty acids profile (Gangadhar *et al.*, 2016). The estimation using a group contribution method (Levine *et al.*, 2014) for the HHV value of the biodiesel sample was 39.4 MJ/kg, this value meets the HHV range of other biodiesel samples (39-41 MJ/kg; Gangadhar *et al.*, 2016). While petroleum diesel had a higher value (45.8 MJ/kg; Demirbas, 2008). This estimation method underestimates systematically the value of the HHV, mostly because of the hydrocarbon content is not quantified. Due to the high capability of *B. braunii* to accumulate hydrocarbons, the experimental HHV should be higher than the estimated (Gangadhar *et al.*, 2016).

The iodine value is the quantification of total unsaturated fatty acids in the mixture of fatty acid. The result is expressed in mass of iodine (g) consumed by 100g of the biodiesel sample when adding iodine to the double bound. Iodine value indicates the propensity from the fatty acids to oxidize or polymerize and form engine deposits (Knothe *et al.*, 2005). The higher the unsaturation of the sample, the higher the iodine value (Francisco *et al.*, 2010; Ramos *et al.*, 2009). The iodine value of *B. braunii* was 68.6 g I/100g, this value was below the maximum legislated by the European standard (120 g I/100g).

The metal content of the biodiesel sample, group I (Na, K) and group II (Ca, Mg) were lower than the maximum allowed by both normatives. The EN14214 and ASTM D6751 regulations impose a maximum of phosphorus content between 4 and 10 mg/kg, respectively. The phosphorus content of this biodiesel sample meets the American standard, but does not meet the normative from the EU, probably because of the phospholipids content of the *B. braunii* biodiesel. With the separation of the different type of lipids before the transesterification, the biodiesel will meet the 4 mg/kg limit.

5.3. Biogas

The solids and elemental analysis results of both inocula and biomasses are shown in table 5.4. The inoculum obtained from Silves wastewater treatment plant (WWTP) had a higher content of TSS and VSS, compared with the one collected at Lagos WWTP. The crude and defatted biomasses had higher contents of TSS, hence a higher energy content will lead to a greater biogas production (El-Mashad & Zhang, 2010). The ratio for VSS/TSS were 78.7 and 69.6% for the Silves and Lagos inoculum, respectively.

Silves inoculum had a higher content of phosphates but a lower content of ammonium in the mixture, compared to the Lagos inoculum. Both inocula had insignificant quantities of nitrates, mainly because of the anaerobic conditions and nitrate reducers present on the digesters when the samples were taken.

Both inocula had a high content of organic N. The organic N content were 1.20 and 0.80 g/L for Silves and Lagos, respectively.

A higher content of carbon, hydrogen and nitrogen was detected in the Silves inoculum compared to the inoculum from Lagos. The higher content of C and N, led to a high C/N ratio of the inoculum, 8.4 and 7.8, respectively. The C/N ratio of the RB-Lagos and DB-Lagos mixtures were 6.7 and 6.1, respectively. The digester RB-Silves and DB-Silves had a C/N ratio of 7.0 and 6.5, respectively. These C/N ratios of the mixtures on the digesters were not ideal for the production of biogas. To increase the biogas production the C/N ratio should be 20-30 (Zhong *et al.*, 2012).

The two inocula had a high content of organic matter, which was showed by the quantity of COD. The COD content were 13.5 and 9.7 g/L for Silves and Lagos, respectively.

Table 5.4 - Properties of the 2 different inoculums and the 2 different biomasses.

	Inoculum Silves	**Inoculum Lagos**	**Raw Biomass**	**Defatted Biomass**
TSS (g/L)[a]	15.8 ± 0.5	13.7 ± 0.6	-	-
VSS (g/L) [a]	12.4 ± 0.3	9.51 ± 0.22	-	-
Organic N (g/L) [a]	1.20 ± 0.07	0.80 ± 0.05	-	-
C (%)[a]	39.0 ± 0.6	24.7 ± 2.75	49.6 ± 0.5	47.2 ± 0.5
H (%)[a]	6.03 ± 0.10	3.94 ± 0.18	7.60 ± 0.10	7.19 ± 0.07
N (%)[a]	4.67 ± 0.18	3.16 ± 0.35	7.73 ± 0.08	8.3 ± 0.06
PO_4^{3-} (mg/L) [b]	73.5 ± 0.9	49.7 ± 0.5	-	-
NH_4^+ (mg/L) [b]	58.8 ± 0.6	119.6 ± 2.4	-	-
NO_3^- (mg/L) [b]	1.33 ± 0.42	2.20 ± 0.82	-	-
COD (g/L) [a]	13.5 ± 0.2	9.67 ± 0.19	-	-
COD (g/g biomass)	-	-	1.08 ± 0.01	0.98 ± 0.02

[a] *Results obtain from the sample.*

[b] *Results obtain from the liquid phase, after filtration.*

The anaerobic digestion and cumulative biogas production of raw biomass with an incubation temperature of 35°C (mesophilic) are shown in figure 5.4, 5.5, 5.6 and 5.7.

During this experiment two additions of 1g of raw biomass were done, at days 1 and 42, before that addition the biogas production was close to 0 (2 mL). The raw biomass inoculated with the inoculum from Silves WWTP (RB-Silves) showed equal biogas productivity at day 73, when compared with the biomass inoculated with the inoculum from Lagos WWTP (RB-Lagos), namely 333 mL biogas/g VSS. In figure 5.4, two production peaks are observed: at the first day of the digestion, and at the first day after the addition of biomass had the higher production of biogas. The maximum production was 58.5 mL of biogas on day 43 (RB-Lagos), when compared with the maximum from day 1 (RB-Lagos), 36.3 mL of biogas. Probably the consortium of bacteria over time has evolved to be more capable to digest this type of biomass, the production peak of the biogas was higher after the second addition of biomass, and the lag phase was smaller. This capability of the consortium of bacteria to adapt to the biomass was reported by Bagi *et al.* (2007).

Figure 5.4 shows the trend of the biogas production of the RB-Lagos and RB-Silves over the days. Both inocula had a similar trend over the experiment time. The first day had the highest production, after the biogas production decreased until day 5. From day 5 to day 11 the production increased, after day 11 until day 42 of digestion, the biogas production

decreased almost until 0 mL. On the day 42 with the addition of the second gram of raw biomass the biogas production increased again, reaching the maximum diary productivity. From day 43 to day 46 the biogas production decreased, on day 47 the production increased again. From day 47 to day 60 the production decreased, after day 60 to the end of the anaerobic digestion (day 73) the production almost ceased (3.56-1.52 mL).

The increase from day 5 to 11 can be related to the adaptation of the inoculum to the microalgae biomass, the inoculum was optimized to digest the sludge from the wastewater treatment. On the first day of digestion, even without the adaptation of the inoculum had the second highest biogas production (only passed by day 43), probably because of the availability of highly biodegradable components on the biomass sample. On day 42 and 73 the biogas production was almost ceased. Therefore, we can assume that the microalgae biomass was already digested.

Comparing the 2 periods of digestion (figure 5.5), day 1 to 42 and 43 to 73, we can see that the digestion of the second gram of biomass added was faster than the first, the acclimation of the microorganisms to the feedstock were the main contributor, and the behaviour of the production curve was the same.

RB-Silves showed a higher biogas productivity, when compared with RB-Lagos, 312 and 284 mL biogas/g VSS, respectively (figure 5.6) during the first 42 days of anaerobic digestion. The yield of the second addition of biomass was higher than the first. RB-Silves continues to had a higher yield than the RB-Lagos, 322 and 308 mL biogas/g VSS (figure 5.7) That higher productivity of RB-Silves can be related with the C/N ratio of the inoculum (Sialve *et al.*, 2009).

A Wilcoxon test reveals no statistical difference between the experimental biogas productions of the two controls, but there were significant differences between RB-Lagos and RB-Silves after the addition of the biomass ($p \leq 0.05$).

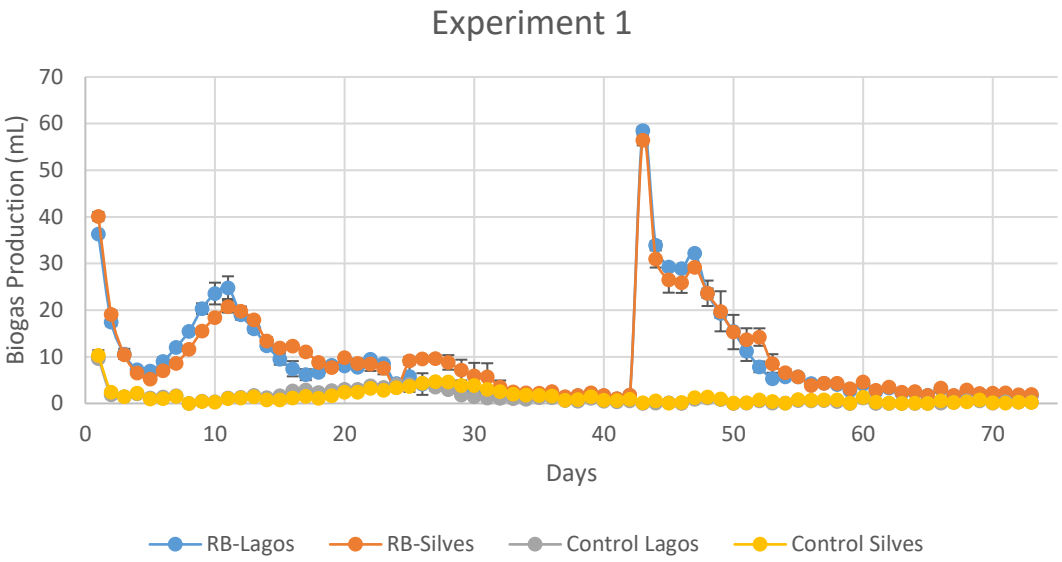

Figure 5.4 – Daily biogas production from raw biomass at 35°C inoculated with the inoculum from Lagos WWTP (RB-Lagos) and Silves WWTP (RB-Silves). At day 42, one more gram of raw biomass was added. Values are presented as means ± standard deviation.

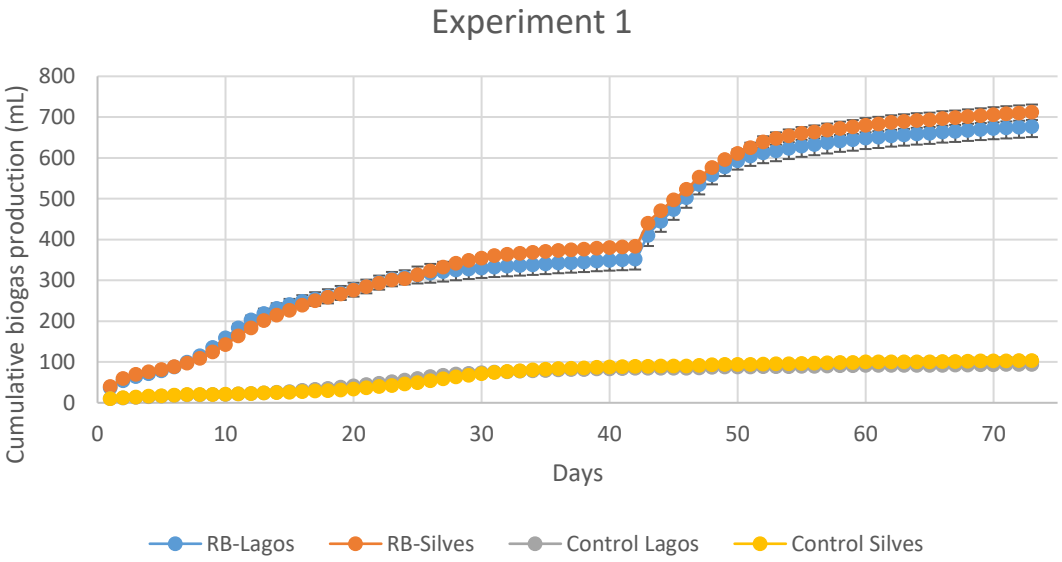

Figure 5.5 – Cumulative biogas production from raw biomass at 35°C inoculated with the inoculum from Lagos WWTP (RB-Lagos) and Silves WWTP (RB-Silves). At day 42, one more gram of raw biomass was added. Values are presented as means ± standard deviation.

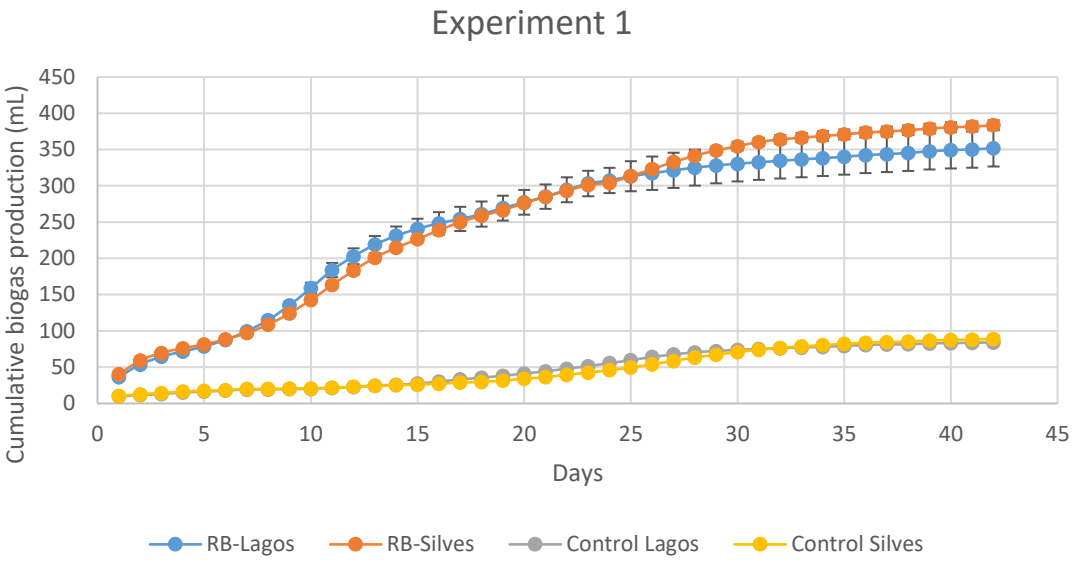

Figure 5.6 – Cumulative biogas production of the first 42 days of digestion of the raw biomass at 35°C inoculated with the inoculum from Lagos WWTP (RB-Lagos) and Silves WWTP (RB-Silves). Values are presented as means ± standard deviation.

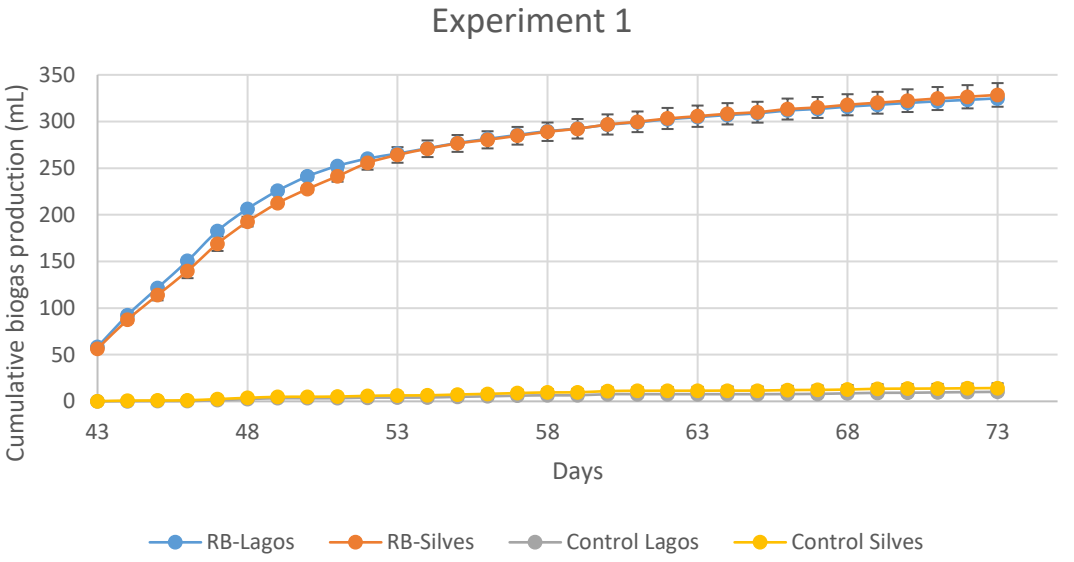

Figure 5.7 – Cumulative biogas production of the second gram of raw biomass added at 35°C inoculated with the inoculum from Lagos WWTP (RB-Lagos) and Silves WWTP (RB-Silves). Values are presented as means ± standard deviation.

Figure 5.8 shows the second experiment, i.e. anaerobic digestion of the defatted biomass inoculated with inoculum from Silves WWTP (DB-Silves) and with the inoculum from Lagos WWTP (DB-Lagos), at 35°C, for the production of biogas. This experiment was

conducted under the same conditions as the first one, with the addition of 1 more gram of biomass at day 42. Experiment 2 had the same behaviour as experiment 1, with small differences on the digestion rate. In this experiment, the bacterial consortium took more time to digest the biomass, probably due to the disruption of the cell wall, the anaerobic bacteria had more organic matter to digest, which ultimately led to a higher biogas production (figure 5.9). The biogas yield obtained with DB-Lagos and DB-Silves were 359 and 320 mL biogas/g VSS, respectively for the anaerobic digestion during the 73 days.

Figure 5.10 represents the cumulative biogas production of the first 42 days. Both inoculums showed similar performances until day 31, after that day the inoculum from Lagos appear to be more suitable for the digestion of this type of biomass, with a yield of 369 mL biogas/g VSS, while Silves had a yield of 297 mL biogas/g VSS.

On experiment 1, the second addition of biomass showed higher productivity, on this one, only the anaerobic digestion with DB-Silves presented a better biogas yield. DB-Lagos showed higher biogas productivity than the DB-Silves, 364 and 308 mL biogas/g VSS, respectively during the second period of digestion (figure 5.11). The anaerobic digestion of the defatted biomass showed higher results when compared with the raw biomass. Wiley *et al.* (2011) reported that digestion of the lipidic fraction of the microalgae were the main contributor for the biogas productivity, while Sialve *et al.* (2009) reported that the biogas yield would increase with the disruption of the cell wall of the microalgae. Overall the biomass without lipids and with the cell wall disrupted (because of the lipid extraction) presented improved biogas production.

A Wilcoxon test revealed no statistical difference between the experimental biogas productions of the two controls, and between DB-Lagos and DB-Silves after the addition of the defatted biomass ($p \leq 0.05$).

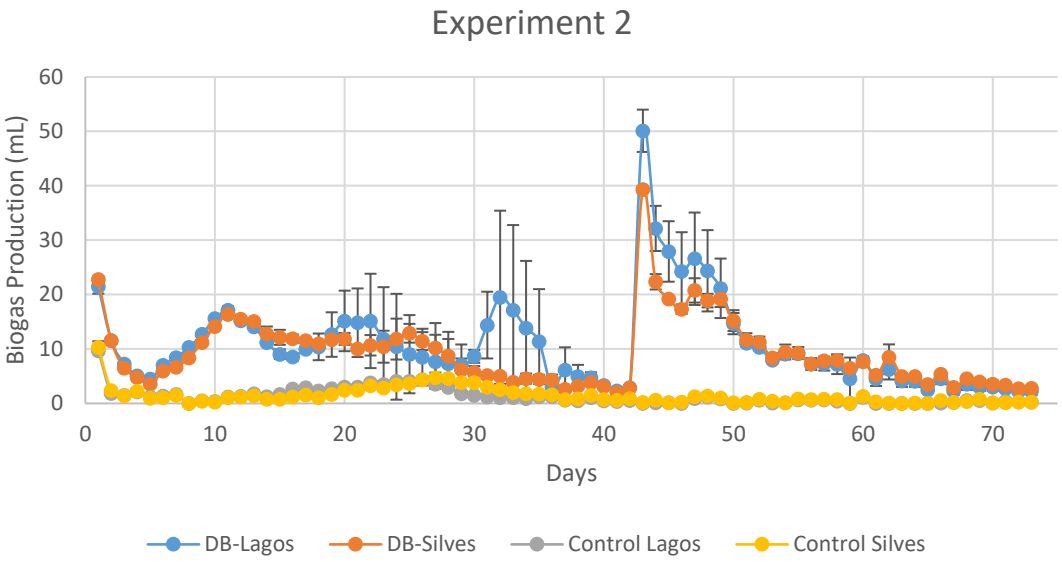

Figure 5.8 – Daily biogas production from defatted biomass at 35°C inoculated with the inoculum from Lagos WWTP (DB-Lagos) and Silves WWTP (DB-Silves). At day 42, one more gram of defatted biomass was added. Values are presented as means ± standard deviation.

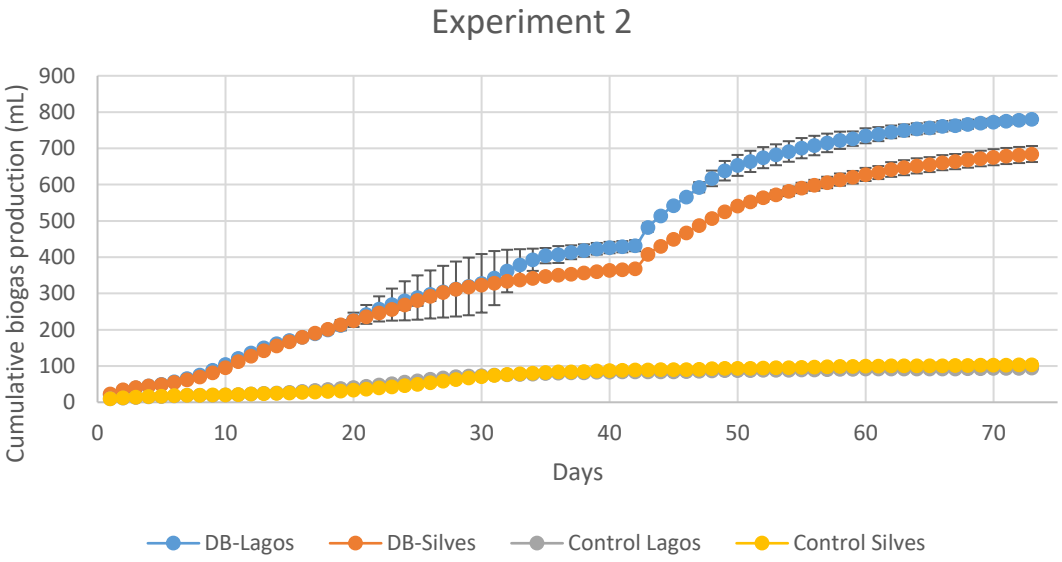

Figure 5.9 – Cumulative biogas production from defatted biomass at 35°C inoculated with the inoculum from Lagos WWTP (DB-Lagos) and Silves WWTP (DB-Silves). At day 42, one more gram of defatted biomass was added. Values are presented as means ± standard deviation.

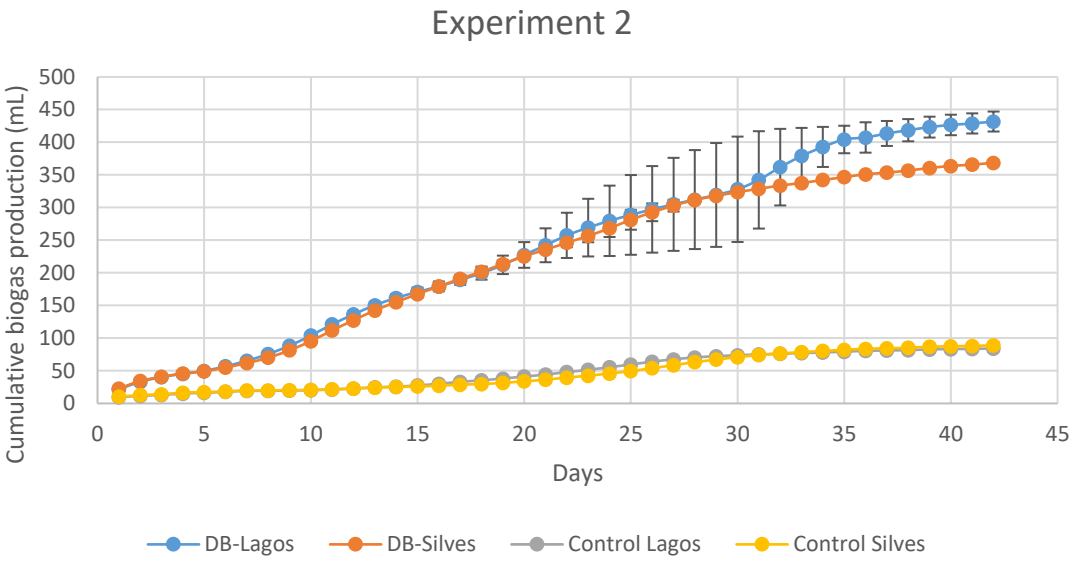

Figure 5.10 – Cumulative biogas production of the first 42 days of digestion of the defatted biomass at 35°C inoculated with the inoculum from Lagos WWTP (DB-Lagos) and Silves WWTP (DB-Silves). Values are presented as means ± standard deviation.

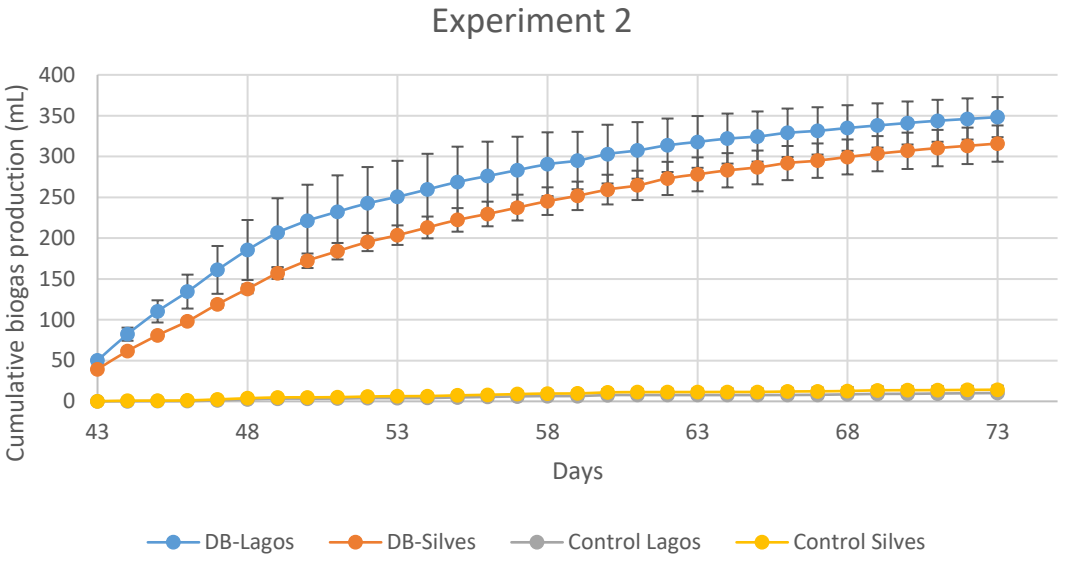

Figure 5.11 – Cumulative biogas production of the second gram of defatted biomass added at 35°C inoculated with the inoculum from Lagos WWTP (DB-Lagos) and Silves WWTP (DB-Silves). Values are presented as means ± standard deviation.

Figure 5.12 shows the third experiment, i.e. anaerobic digestion of the raw biomass inoculated with inoculum from Silves WWTP (RB-Silves) and with the inoculum from

Lagos WWTP (RB-Lagos), at 25°C, for the production of biogas. Figure 5.13 shows the cumulative biogas production of the experiment.

The experiment shows the same behaviour then the experiment at 35°C, but with a lower biogas production and a lower adaptation of the inoculum. It took 25 days to achieve the second peek of production, when it took only 11 days with the same conditions but with the temperature at 35°C.

The biogas yield obtained with RB-Lagos and RB-Silves were 276 and 269 mL biogas/g VSS, respectively for the anaerobic digestion.

A Wilcoxon test revealed no statistical differences between the experimental biogas productions of the two controls, and between RB-Lagos and RB-Silves after the addition of the biomass ($p \leq 0.05$). The same test showed no statistical difference between the biogas production of the RB-Lagos, with the variation of temperature. On other hand, RB-Silves and different temperatures showed statistical differences. Silves and Lagos inocula without the biomass addition (controls) and different temperatures showed statistical differences.

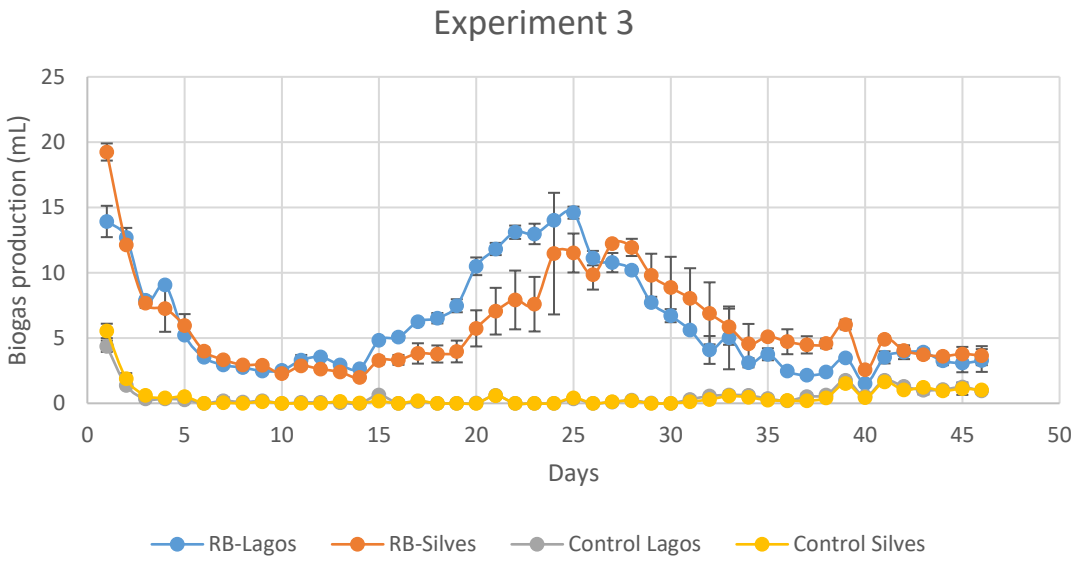

Figure 5.12 – Daily biogas production from raw biomass at 25°C inoculated with the inoculum from Lagos WWTP (RB-Lagos) and Silves WWTP (RB-Silves). Values are presented as means ± standard deviation.

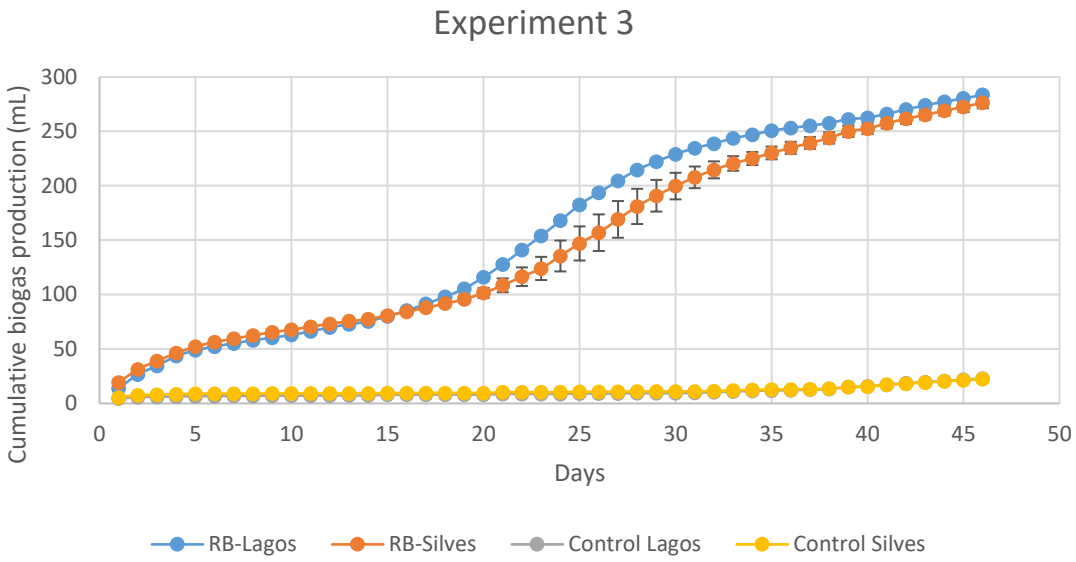

Figure 5.13 – Cumulative biogas production from raw biomass at 25°C inoculated with the inoculum from Lagos WWTP (RB-Lagos) and Silves WWTP (RB-Silves). Values are presented as means ± standard deviation.

The anaerobic digestion of the defatted biomass with an incubation temperature of 25°C for biogas production is shown in figure 5.14, in figure 5.15 is shown the cumulative biogas production of this experiment.

The anaerobic digestion of the defatted biomass displays the same behaviour of the raw biomass. Figure 5.15 shows a slight difference between the yields of both inocula, 172 and 193 mL biogas/g VSS for DB-Lagos and DB-Silves, respectively. The yield of the anaerobic digestion of the defatted biomass was higher than the digestion of the raw biomass under mesophilic conditions (35°C). In contrary the yield of the anaerobic digestion of the defatted biomass at 25°C was lower than the raw biomass.

A Wilcoxon test revealed no statistical difference between the experimental biogas productions of the two controls, but there was statistical difference between DB-Lagos and DB-Silves after the addition of the defatted biomass ($p \leq 0.05$). The controls from Lagos and Silves, with a different temperature showed statistical differences. DB-Lagos and DB-Silves also showed statistical differences between the different temperatures.

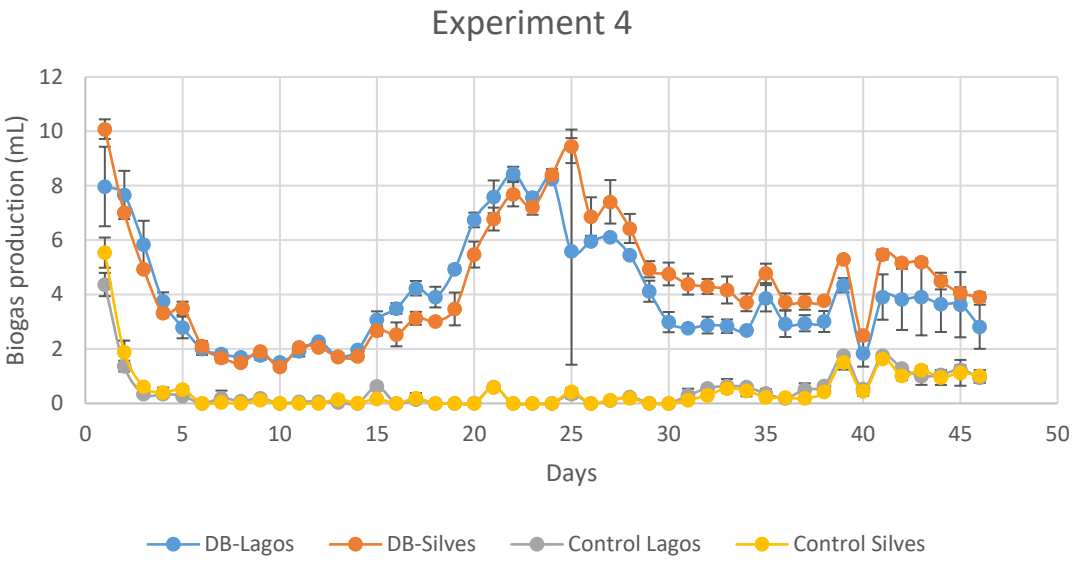

Figure 5.14 – Daily biogas production from defatted biomass at 25°C inoculated with the inoculum from Lagos WWTP (DB-Lagos) and Silves WWTP (DB-Silves). Values are presented as means ± standard deviation.

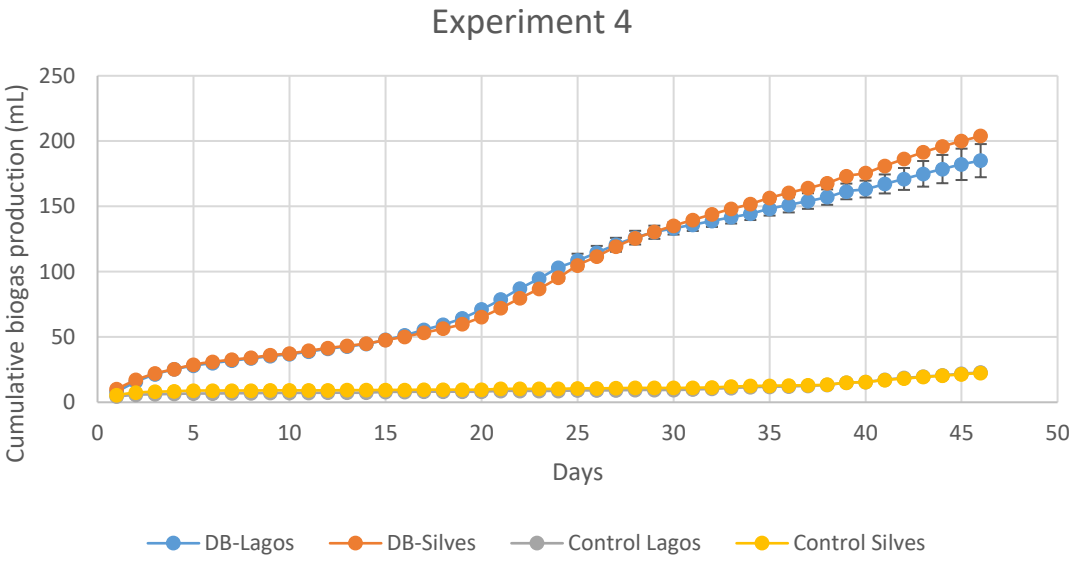

Figure 5.15 – Cumulative biogas production from defatted biomass at 25°C inoculated with the inoculum from Lagos WWTP (DB-Lagos) and Silves WWTP (DB-Silves). Values are presented as means ± standard deviation.

Table 5.5 shows the composition of the biogas samples from the DB-Silves large scale digesters (10:1).

The methane content in the biogas produced on day 15 and 16 of the digestion with the incubation temperature at 35°C was 59.4 ± 0.97%. On the following three days (17-19) was 69.2%, between the days 20 and 27 the methane content reached the highest value with 82.0 ± 0.48%. The methane content of the biogas decreased to 77.2% on the period between days 27 and 30. Overall the methane content increased continuously until day 27 and it remained almost constant thereafter. The methane content of the biogas sample from the period between days 15 and 30 of anaerobic digestion at 25°C was 72.2%.

The H_2S content on the biogas samples tested was very low (0-2 ppm). Kafle & Kim (2012) reported that the methanogens suffer an inhibition with values beyond 3000 ppm. With the low quantity of H_2S produced from the anaerobic digestion the biogas can be stored and burned, not promoting damages on the equipment (Lantz *et al.*, 2007; Mussgnug *et al.*, 2010; Weiland, 2003). Biogas produced from microalgae biomass should have low concentration of H_2S due to the low amount of sulphureted amino acids (Mussgnug *et al.*, 2010; Sialve *et al.*, 2009).

Table 5.5 - Biogas composition DB-Silves large scale digesters (CH_4, CO_2 and H_2S content).

Temperature (°C)	**Day**	**CH_4 (%)**	**CO_2 (%)**	**H_2S (ppm)**
25	15 to 30	72.2	27.8	2
35	15 to 16	59.4 ±0.97	40.6 ±0.97	0
	17 to 19	69.2	30.8	0
	20 to 27	82.0 ±0.48	18.0 ±0.48	0
	27 to 30	77.2	22.8	1

Table 5.6 shows the theoretical methane production for both biomass samples, the results of the biogas production for the experiments and the methane production of the defatted biomass.

Table 5.6 - Biogas production results, methane production and theoretical methane production.

				Lagos inoculum			Silves inoculum		
Incubation temperature (°C)	**Biomass type**	**Theoretical methane production (mL/gVS)**		**Biogas production (mL/gVS)**	**Methane production (mL/gVS)**	**Methane yield (%)**	**Biogas production (mL/gVS)**	**Methane production (mL/gVS)**	**Methane yield (%)**
35	Raw	519	1st digestion	284	234*	45	312	257*	50
			2nd digestion	308	254*	49	322	266*	51
			Total	333	275*	53	333	274*	53
	Defatted	435	1st digestion	369	304*	70	297	244*	56
			2nd digestion	364	300*	69	308	254*	58
			Total	359	296*	68	320	264*	61
25	Raw	519	Total	276	228*	44	269	222*	43
	Defatted	435	Total	172	142*	33	193	159*	36

*Value estimated with the result of the methane yield obtain in the anaerobic digestion of the defatted biomass.

All the results obtained from the anaerobic digestion were lower than the theoretical methane production, meaning that the biomass was not digested up to 100%. The percentage of digestion of the raw biomass was between 45-53%, with the best result obtained for sample RB-Lagos with an incubation temperature of 35°C. The digestion of the defatted biomass showed better results, between 56-70%, the higher result was the one obtained for the sample DB-Lagos with an incubation temperature of 35°C. Under 25°C the degradation of the biomass showed lower productivity, the raw and defatted biomass had a yield between 42-43 and 32-36%, respectively.

It was difficult to reach 100% degradation of the biomass because of the composition of the cell wall of the microalgae. Microalgal cell walls contain compounds such as cellulose and hemicellulose that are hardly biodegradable by anaerobic microorganisms (Neumann *et al.*, 2015; Passos *et al.*, 2014).

The biogas production for all the tested conditions varied between 172 and 369 mL biogas/g VS. Mussgnug *et al.* (2010) reported for different species higher, lower and identical results, *Chlorella kessleri* had a similar biogas production (335 mL biogas/g VS), when compared with this *B. braunii* strain. The best result reported by Mussgnug *et al.* (2010) was 587 mL biogas/g VS with *Chlamydomonas reinhardtii*. Zhong *et al.* (2012) with a Taihu blue algae and a C/N ratio of 6 (similar to *B. braunii*) obtained a result of 391 mL biogas/g VS, slightly higher than the results obtained in the present study. Nevertheless, the methane yield was lower, achieving 201 mL CH_4/g VS instead of 233-274 mL CH_4/g VS.

Frigon *et al.* (2013) tested two biomass samples of *B. braunii*, while Neumann *et al.* (2015) a defatted biomass of *B. braunii* in the same conditions of the ones used on this experiment, with a different inoculum. The results obtained were 343, 370 and 404 mL CH_4/g VS, respectively, these results were better than those obtained on this experiment, although the productivity increased with the defatted biomass reported by Neumann *et al.* (2015).

Dogan-Subasi & Demirer (2016) tested the anaerobic digestion of *Chlorella vulgaris* with three feedings, the results obtained were lower than those reported by Frigon *et al.* (2013), Neumann *et al.* (2015) and the present study. However, the behaviour of the production curve was the same.

The results of the anaerobic digestion of this experiment and the results of other experiments showed a high variability on the biogas and methane yield among the different microalgae species. Mainly because of the characteristics of the cell wall, although a microalgae without a cell wall could not be ideal for the anaerobic degradation, some substrates from the degradation can be inhibitors to the methanogenic bacteria, the characteristics of the inoculum used for the degradation can be other key factor (Mussgnug *et al.*, 2010; Passos *et al.*, 2014; Sialve *et al.*, 2009)

Table 5.7 shows the analytical composition of both digesters after the anaerobic digestion.

Table 5.7 – Analytical composition of the reaction mixture after digestion (DB-Silves).

	Temperature (°C)	
	25	**35**
TSS (g/L)[a]	38.3 ± 0.9	31.9 ± 1.6
VSS (g/L) [a]	34.6 ± 0.7	28.1 ± 1.6
C (%)[b]	44.3 ± 0.7	43.3 ± 0.8
H (%)[b]	6.76 ± 0.30	6.50 ± 0.13
N (%)[b]	6.09 ± 0.13	5.79 ± 0.17
C/N [b]	7.26 ± 0.16	7.47 ± 0.15
K (%)[b]	0.42 ± 0.08	0.44 ± 0.08
P (%)[b]	0.61 ± 0.06	0.45 ± 0.11
PO_4^{3-} (g/L)[c]	0.68 ± 0.04	0.59 ± 0.04
NH_4^+ (g/L) [c]	0.23 ± 0.02	0.28 ± 0.02
Organic N (g/L) [a]	3.53 ± 0.05	3.59 ± 0.04
NO_3^- (mg/L) [c]	n.d.	n.d.
COD (g/L) [a]	45.5 ± 0.9	38.8 ± 4.0

[a] *Results obtain from the sample.*

[b] *Results obtain from the solid phase.*

[c] *Results obtain from the liquid phase, after filtration.*

Degradation of biomass feedstocks with high quantity of protein, like those used on this study, can increase the inorganic ammonium ($N\text{-}NH_4^+$) concentration up to concentrations that are toxic to methanogenic bacteria (the range of the toxicity values are between 4000-6000 mg $N\text{-}NH_4^+/L$). The values obtained after the digestion were 215-

300 mg N-NH_4^+/L, which were below the toxicity values. The biogas production was not affected by the ammonium concentration (Passos *et al.*, 2015, 2014).

The concentration of phosphates and ammonium increased with the digestion. The concentration of phosphates was higher with the incubation temperature of 25°C, on the other way the ammonium concentration was higher with the incubation of 35°C. The produced NH_4^+ and PO_4^{3-} on the liquid digestate can be used as nutrient supplements (N and P source) for the production of the microalgae (Sialve *et al.*, 2009).

Due to the methanogenic bacteria and the nitrate reduction conditions nitrate was not detected (Kafle & Kim, 2013).

The biogas produced is generated from the conversion of organic compounds in substrates; the higher the VS removal, the higher the biogas production (Zhong *et al.*, 2012). Both digesters had a TSS and VSS load before digestion of 52.6 and 47.6 g/L, respectively. After digestion, the TSS composition of the anaerobic digester at 25°C had a load of 38.3 g/L and the one at 35°C, 31.9 g/L, which corresponds to a TSS reduction of 27.2% and 39.6%, respectively. The VSS quantity of the 25°C digester and the 35°C after the anaerobic digestion were 34.5 and 28.1 g/L respectively, which corresponds to 27.5 and 41% of VSS reduction. Dogan-Subasi & Demirer (2016) achieved 36% of VS reduction on the degradation of *C. vulgaris* on mesophilic conditions, and Zhong *et al.* (2012) achieved 41.26% of VS reduction. Both results were similar to the ones obtain in this study.

The COD load after the digestion of the microalgae was 45.5 g/L under 25°C of temperature which corresponds to an COD removal of 8.7%, the load under 35°C was 38.8 g/L which corresponds to a COD reduction of 22.1%. The results obtained were similar to the ones obtained by Vergara-Fernández *et al.* (2008; 17 %). Dogan-Subasi & Demirer (2016) reported a COD removal of 59, 11 and 46% at the end of the 1st, 2nd and 3rd digestion steps, respectively. The low removal on the second feed was attributed to high substrate load, which could be an explanation to the low COD removal of this experiment. The C/N ratios after the digestion were 7.26 and 7.47 for the experiments at 25°C and 35°C, respectively.

The organic N increased with the anaerobic digestion, probably due to the degradation of the proteins of the microalgae (Sialve *et al.*, 2009). The organic nitrogen content after the anaerobic digestion was 3.53 and 3.59 g/L for the for the incubation temperature of 25

and 35°C, respectively. The potassium content after the anaerobic digestion was 0.42 and 0.44% for the incubation temperature of 25 and 35°C, respectively. The phosphorus content of the digestate decreased with the incubation temperature. The phosphorus content was 0.61 and 0.45% for the incubation temperature of 25 and 35°C, respectively. The digestate can be reused as fertilizer, but before some parameters of the digestate have to be analysed (e.g. presence of heavy metals and pathogens; Dogan-Subasi & Demirer, 2016).

5.4. Energy and mass balance

The energy and mass balance of the biorefinery (figure 5.16) was quantified for the production of 1000 kg of biodiesel. This quantity of biodiesel corresponds to an energy production of 10965 kWh. The methane produced on the anaerobic digestion of the defatted biomass was 2687 kg, which corresponds to 3761255 L. The energy generated by the heat of the methane was 37195 kWh, making an overall energy production of 48145 kWh. The energy required to the production of these two biofuels was 94341 kWh.

Table 5.8 - Overall biorefinery energy outputs and inputs.

Overall biorefinery process	
Biodiesel production (kg)	1000
Biodiesel energy produced (kWh)[a]	10965
Methane production (kg)	2687
Methane production (L)	3761255
Methane energy produced (kWh)[b]	37195
Total energy produced (kWh)	48145
Total energy required to the process (kWh)	94341

[a] Computed with a high heating value 39.42 MJ/kg.
[b] Computed with a methane calorific value of 35.6 kJ/L (Sialve *et al.*, 2009).

Table 5.9 shows the raw materials used during the production of both biofuels. During the first production batch the quantity of materials was higher than the next ones, due to the recycling of the solvents. The main material used was the water (2225.5 t), due to the water wash of the biodiesel after the transesterification. The main solvents used on the first batch (methanol and hexane) decreased 85 and 56%, respectively for the next. Even though the low quantity of ethyl acetate used on the process (1.8 t), all of it was recycled

to the next batch. The microalgae used to the production of the biodiesel was 16.4 t. After the biogas production the digestate was purged, and utilized on the next batch.

Table 5.9 – Mass balance of the raw materials of the biorefinery.

Raw Material (t)	**1st batch**	**Next batches**
Dried microalgae	16.40	16.40
Methanol	200.00	30.00
Hexane	179.20	79.50
Ethyl Acetate	1.80	0.00
Sulfuric Acid	4.90	4.90
Water	2225.50	2225.50
Diethyl Ether	0.10	0.01
Bentonite	2.30	2.30
$NaHCO_3$	7.00	7.00
Inoculum	7.70	0.00

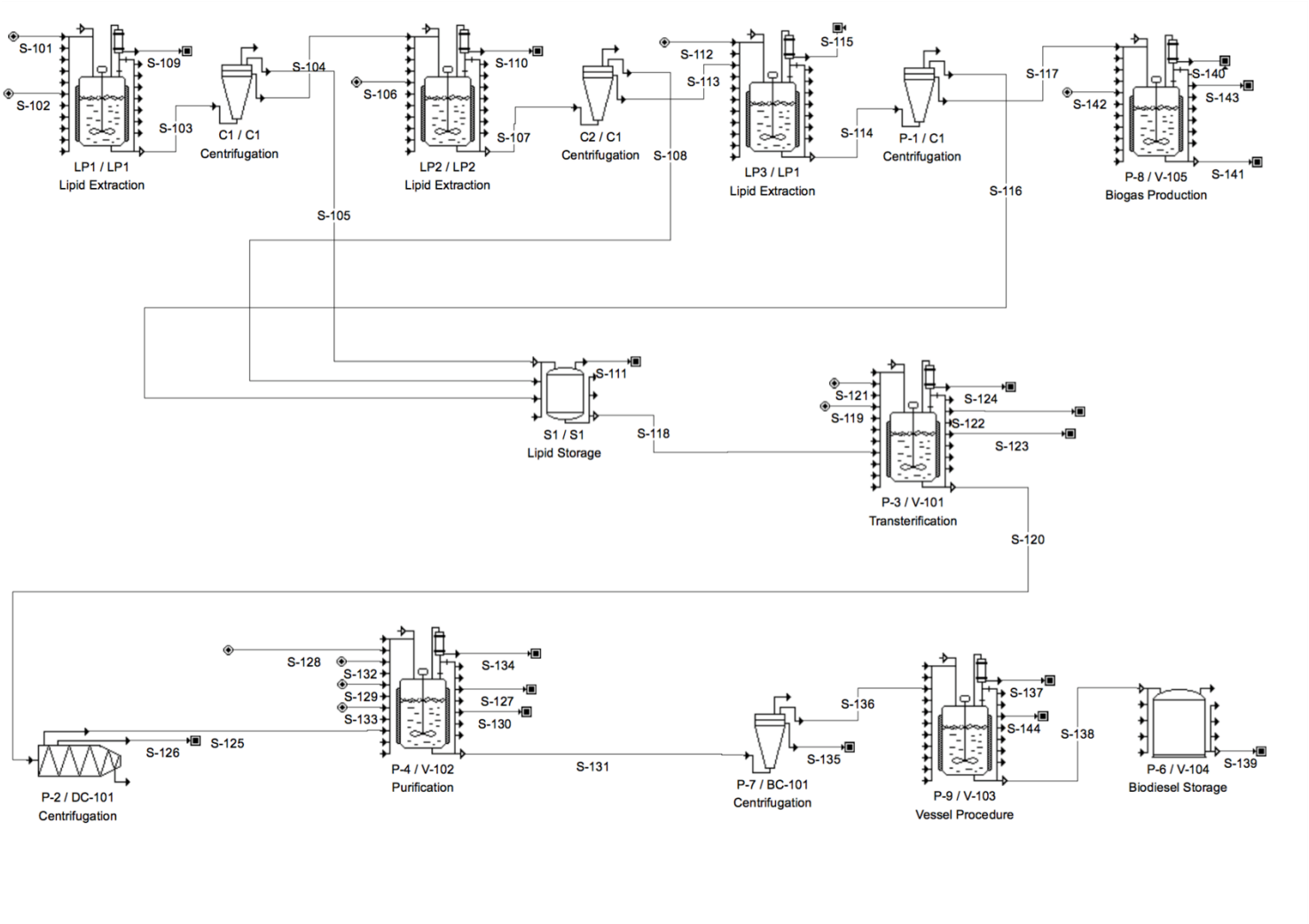

Figure 5.16 - Flowsheet of the biodiesel and biogas production.

Table 5.10 presents the waste streams of the process.

S-135 stream was a bentonite sludge from the centrifugation after the biodiesel purification. This sludge contains a considerable quantity of hexane with biodiesel. With other centrifugation the liquid phase can be recovered, and a forward evaporation step can separate the biodiesel from the hexane, which will improve the biodiesel yield, reducing the waste produced and the hexane can be recycled for other step of the process. The stream S-141 was the digestate from the anaerobic digestion, this sludge still had a high quantity of water, the forward step was a centrifugation to reduce the water content. This water had a high quantity of nutrients, and can be reused as nutrient supplements to the growth of the microalgae. This sludge can be recycled to the next anaerobic batch, and the excess can be upgraded to be used as fertilizer. The liquid waste of this biorefinery concept was mainly generated by the centrifugation done after the transesterification (S-126) and the water washes of the biodiesel after the transesterification (S-127 and S-130). To decrease the liquid waste and improve the production costs, the methanol from the S-126 can be separated and recycled.

Table 5.10 - Quantities of solid and liquid waste from the biorefinery concept.

Waste treatment / Disposal		
Solid Waste		
Stream name	**Main composition**	**Quantity (t)**
S-141	Inoculum sludge	711.5
S-135	Bentonite sludge	5.3
Liquid waste		
Stream name	**Main composition**	**Quantity (t)**
S-127	Water and contaminants	671.4
S-130		741.3
S-126		24.0

The energy required for this biorefinery concept is presented on table 5.11. The processes with the highest energetic cost were P-3; P-8 and P-9, mainly due to the high quantity of solvents to evaporate. The energetic cost for the evaporation of solvents on P-3 (transesterification, 70926 kWh) was one order of magnitude higher than in P-8 and P-9

(3758 and 5356 kWh, respectively), due to the quantity evaporated. The second most energy required step was the biogas production P-8 with (7176 kWh), this energy was required to the agitation and heating of the batch.

Table 5.11 - Energy required for the biorefinery.

Utility requirements			
Electricity			
Procedure name	**Equipment name**	**Task**	**Energy (kWh)**
LP1	LP1	Lipid extraction	103
C1	C1	Centrifugation	775
LP2	LP2	Lipid extraction	25
C2	C1	Centrifugation	767
LP3	LP1	Lipid extraction	101
P-1	C1	Centrifugation	767
P-3	V-101	Agitation	7
P-2	DC-101	Centrifugation	27
P-4	V-102	Agitation	58
P-7	BC-101	Centrifugation	445
P-8	V-105	Agitation	7176
Subtotal			10251
Heat transfer agent (Steam)			
Procedure name	**Equipment name**	**Task**	**Energy (kWh)**
LP1	LP1	Lipid extraction	1098
LP2	LP2	Lipid extraction	880
LP3	LP1	Lipid extraction	793
P-3	V-101	Solvent evaporation	70926
P-4	V-102	Batch Heating	126
P-9	V-103	Solvent evaporation	5356
P-8	V-105	Solvent evaporation	3758
Subtotal			82936
Heat transfer agent (Cooling Water)			
Procedure name	**Equipment name**	**Task**	**Energy (kWh)**
C1	C1	Centrifugation	387
C2	C1	Centrifugation	383
P-1	C1	Centrifugation	383
Subtotal			1154
Total			94341

For the energetic balance of this biorefinery to be positive the quantity of solvents used has to be reduced, mainly the quantity of methanol and hexane on the lipid extraction and transesterification. The energetic demand to vaporize the solvents used on the production process, represents 85% of the overall energy demand. If we subtract the demand of energy required to the evaporation steps, this biorefinery will have a positive balance (33844 kWh).

To improve the mass balance of this process the water used on the wash of the biodiesel, after the transesterification, should decrease. This reduction will lead to less waste to treat and a drop on the production cost of the biodiesel.

6. Conclusion

With the depletion of the fossil fuels and the growth of the world population, increasing the demand on basic commodities and fuel consumption, microalgae biomass can be a promising feedstock for the production of biofuels. To achieve a competitive biofuel price the production costs of the biomass should decrease, and a biorefinery should be implemented in order to reduce the wastes.

The *B. braunii* strain here researched revealed a good lipid content and suited for lipid extraction with low cost solvents. The biodiesel produced from this microalgae achieved a good quality, only failing on the density, viscosity and phosphorus specifications (EN 14214 and ASTM D6751). The heating value for this biodiesel sample was 39.42 MJ/Kg.

The anaerobic digestion of the defatted biomass proved to be more efficient than the raw biomass, mainly because of the disruption of the cell wall and the availability to be degraded by the bacteria consortium, this results meets with the biorefinery concept. Higher temperatures of incubation (35°C) showed higher biogas yields than at lower temperatures (25°C). The two inocula tested presented different availability for the different biomass digested. The one from Silves was more suited for the degradation of raw biomass and the one from Lagos for defatted biomass. Lastly, the inocula used were not optimized for the degradation of this type of biomass, to achieve better biogas yields one re-addition of biomass should be done after the biogas production ceased, consequently the time needed for the degradation would decrease.

For the production of 1000 Kg of biodiesel, 16.4 t of raw biomass would be necessary, which would lead to 2687 Kg of methane has a sub product. The net energy balance estimated for the proposed biorefinery was negative. The energy demand for the production was 94341 kWh and the revenue 48145 kWh. To decrease the production costs and have a positive energy balance, the energy required to evaporate the solvents should decrease, 85% of the energy cost was spent on this step.

With the optimization of the production processes (biodiesel and anaerobic digestion), this biorefinery could be more attractive to the production of these biofuels from this biomass.

7. References

Angelidaki, I., Sanders, W., 2004. Assessment of the anaerobic biodegradability of macropollutants. Rev. Environ. Sci. Bio/Technology 3, 117–129. doi:10.1007/s11157-004-2502-3

Antizar-ladislao, B., Turrion-Gomez, J., 2008. Second-generation biofuels and local bioenergy systems. Biofuels, Bioprod. Biorefining 2, 455–469. doi:10.1002/bbb

APHA, AWWA, WEF, 2005. Standard methods for the examination of water and wastewater, Centennial. ed. APHA,American Public Health Association; AWWA, American Water Works Association; WEF, Water Environment Federation, Washington, DC.

Ashokkumar, V., Rengasamy, R., 2012. Mass culture of Botryococcus braunii Kutz. under open raceway pond for biofuel production. Bioresour. Technol. 104, 394–399. doi:10.1016/j.biortech.2011.10.093

Bagi, Z., Ács, N., Bálint, B., Horváth, L., Dobó, K., Perei, K., Rákhely, G., Kovács, K., 2007. Biotechnological intensification of biogas production. Environ. Biotechnol. 76, 473–482. doi:10.1007/s00253-007-1009-6

Balasubramanian, R., Doan, T., Obbard, J., 2013. Factors affecting cellular lipid extraction from marine microalgae. Chem. Eng. J. 215–216, 929–936. doi:10.1016/j.cej.2012.11.063

Banerjee, A., Sharma, R., Chisti, Y., Banerjee, U.C., 2002. Botryococcus braunii: A renewable source of hydrocarbons and other chemicals. Crit. Rev. Biotechnol. 22, 245–279.

Bligh, E., Dyer, W., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Borowitzka, M.A., 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. J. Biotechnol. 70, 313–321.

Borowitzka, M.A., Moheimani, N.R., 2013. Sustainable biofuels from algae. Mitig. Adapt. Strateg. Glob. Chang. 18, 13–25. doi:10.1007/s11027-010-9271-9

Cherubini, F., 2010. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Convers. Manag. 51, 1412–1421. doi:10.1016/j.enconman.2010.01.015

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126–131. doi:10.1016/j.biotechadv.2007.02.001

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306. doi:10.1016/j.biotechadv.2007.02.001

Clark, J.H., Budarin, V., Deswarte, F.E.I., Hardy, J.J.E., Kerton, F.M., Hunt, A.J., Luque, R., Macquarrie, D.J., Milkowski, K., Rodriguez, A., Samuel, O., Tavener, S.J., White, R.J., Wilson, A.J., 2006. Green chemistry and the biorefinery: a partnership for a sustainable future. Green Chem. 8, 853–860. doi:10.1039/b604483m

Debowski, M., Zielinski, M., Grala, A., Dudek, M., 2013. Algae biomass as an alternative

substrate in biogas production technologies — Review. Renew. Sustain. Energy Rev. 27, 596–604. doi:10.1016/j.rser.2013.07.029

Demirbas, A., 2008. Relationships derived from physical properties of vegetable oil and biodiesel fuels. Fuel 87, 1743–1748. doi:10.1016/j.fuel.2007.08.007

Deppenmeier, U., Müller, V., Gottschalk, G., 1996. Pathways of energy conservation in methanogenic archaea. Arch Microbiol 165, 149–163.

Dogan-Subasi, E., Demirer, G., 2016. Anaerobic digestion of microalgal (Chlorella vulgaris) biomass as a source of biogas and biofertilizer. Environ. Prog. Sustain. Energy 35, 936–941. doi:10.1002/ep

El-Mashad, H.M., Zhang, R., 2010. Biogas production from co-digestion of dairy manure and food waste. Bioresour. Technol. 101, 4021–4028. doi:10.1016/j.biortech.2010.01.027

Fang, J., Chiu, H., Wu, J., Chiang, Y., Hsu, S., 2004. Fatty acids in Botryococcus braunii accelerate topical delivery of flurbiprofen into and across skin. Int. J. Pharm. 276, 163–173. doi:10.1016/j.ijpharm.2004.02.026

Fassinou, W.F., 2012. Higher heating value (HHV) of vegetable oils , fats and biodiesels evaluation based on their pure fatty acids ' HHV. Energy 45, 798–805. doi:10.1016/j.energy.2012.07.011

Finnveden, G., Hauschild, M., Ekvall, T., Guinée, J., Heijungs, R., Hellweg, S., Koehler, A., Pennington, D., Suh, S., 2009. Recent developments in life cycle assessment. J. Environ. Manage. 91, 1–21. doi:10.1016/j.jenvman.2009.06.018

Fitzpatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A., 2010. A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. Bioresour. Technol. 101, 8915–8922. doi:10.1016/j.biortech.2010.06.125

Francisco, É., Neves, D., Lopes, E., Franco, T., 2010. Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. J. Chem. Technol. Biotechnol. 85, 395–403. doi:10.1002/jctb.2338

Frigon, J.C., Matteau-Lebrun, F., Abdou, R., McGinn, P.J., O'Leary, S.J.B., Guiot, S.R., 2013. Screening microalgae strains for their productivity in methane following anaerobic digestion. Appl. Energy 108, 100–107. doi:10.1016/j.apenergy.2013.02.051

Gangadhar, K.N., Pereira, H., Diogo, H.P., Borges dos Santos, R.M., Prabhavathi Devi, B.L.A., Prasad, R.B.N., Custódio, L., Malcata, F.X., Varela, J., Barreira, L., 2016. Assessment and comparison of the properties of biodiesel synthesized from three different types of wet microalgal biomass. J. Appl. Phycol. 28, 1571–1578. doi:10.1007/s10811-015-0683-5

Guinée, J., Heijungs, R., Huppes, G., Zamagni, A., Masoni, P., Buonamici, R., Ekvall, T., Rydberg, T., 2011. Life cycle assessment : Past, present, and future. Environ. Sci. Technol. 45, 90–96.

Gunaseelan, N., 1997. Anaerobic digestion of biomass for methane production: A review. Biomass and Bioenergy 13, 83–114.

Guzzella, L., Sciarretta, A., 2005. Vehicle propulsion systems: Introduction to modeling and optimization. Springer.

Harun, R., Davidson, M., Doyle, M., Gopiraj, R., Danquah, M., Forde, G., 2011. Technoeconomic analysis of an integrated microalgae photobioreactor , biodiesel and biogas production facility. Biomass and Bioenergy 35, 741–747. doi:10.1016/j.biombioe.2010.10.007

Harun, R., Singh, M., Forde, G.M., Danquah, M.K., 2010. Bioprocess engineering of microalgae to produce a variety of consumer products. Renew. Sustain. Energy Rev. 14, 1037–1047. doi:10.1016/j.rser.2009.11.004

Huang, G., Chen, F., Wei, D., Zhang, X., Chen, G., 2010. Biodiesel production by microalgal biotechnology. Appl. Energy 87, 38–46. doi:10.1016/j.apenergy.2009.06.016

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

ISO 14040, Environmental management — Life cycle assessment — Principles and framework

Kafle, G.K., Kim, S.H., 2013. Anaerobic treatment of apple waste with swine manure for biogas production: Batch and continuous operation. Appl. Energy 103, 61–72. doi:10.1016/j.apenergy.2012.10.018

Kafle, G.K., Kim, S.H., 2012. Kinetic study of the anaerobic digestion of swine manure at mesophilic temperature : A lab scale batch operation. J. Biosyst. Eng. 37, 233–244. doi:http://dx.doi.org/10.5307/JBE.2012.37.4.233

Kaparaju, P., Serrano, M., Thomsen, A., Kongjan, P., Angelidaki, I., 2009. Bioethanol , biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour. Technol. 100, 2562–2568. doi:10.1016/j.biortech.2008.11.011

Kim, S., Dale, B.E., 2004. Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy 26, 361–375. doi:10.1016/j.biombioe.2003.08.002

Knothe, G., 2014. A comprehensive evaluation of the cetane numbers of fatty acid methyl esters. Fuel 119, 6–13. doi:10.1016/j.fuel.2013.11.020

Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process. Technol. 86, 1059–1070. doi:10.1016/j.fuproc.2004.11.002

Knothe, G., Gerpen, J. Van, Krahl, J., 2005. The biodiesel handbook. AOCS Press, Champaign, Illinois.

Lantz, M., Svensson, M., Björnsson, L., Börjesson, P., 2007. The prospects for an expansion of biogas systems in Sweden — Incentives , barriers and potentials. Energy Policy 35, 1830–1843. doi:10.1016/j.enpol.2006.05.017

Lee, J.Y., Yoo, C., Jun, S.Y., Ahn, C.Y., Oh, H.M., 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. 101, S75–S77.

doi:10.1016/j.biortech.2009.03.058

Leschine, S.B., 1995. Cellulose degradation in anaerobic environment. Annu. Rev. Microbiol. 49, 399–426.

Levine, F., Kayea III, R. V., Wexler, R., Sadvary, D.J., Melick, C., Scala, J., 2014. Heats of combustion of fatty acids and fatty acid esters. J. Am. Oil Chem. Soc. 91, 235–249. doi:10.1007/s11746-013-2367-0

Mata, T., Martins, A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy Rev. 14, 217–232. doi:10.1016/j.rser.2009.07.020

M.D. Guiry in Guiry, M.D. &, G.M. 2016. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http: //www.algaebase.org; searched on 08 December 2015.

Metzger, P., Largeau, C., 2005. Botryococcus braunii: a rich source for hydrocarbons and related ether lipids. Appl. Microbiol. Biotechnol. 66, 486–496. doi:10.1007/s00253-004-1779-z

Mussgnug, J.H., Klassen, V., Schlüter, A., Kruse, O., 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. J. Biotechnol. 150, 51–56. doi:10.1016/j.jbiotec.2010.07.030

Naik, S.N., Goud, V. V, Rout, P.K., Dalai, A.K., 2010. Production of first and second generation biofuels: A comprehensive review. Renew. Sustain. Energy Rev. 14, 578–597. doi:10.1016/j.rser.2009.10.003

Neumann, P., Torres, A., Fermoso, F.G., Borja, R., Jeison, D., 2015. Anaerobic co-digestion of lipid-spent microalgae with waste activated sludge and glycerol in batch mode. Int. Biodeterior. Biodegrad. 100, 85–88. doi:10.1016/j.ibiod.2015.01.020

Ohara, H., 2003. Biorefinery. Appl. Microbiol. Biotechnol. 62, 474–477. doi:10.1007/s00253-003-1383-7

Pacini, H., Silveira, S., Filho, A.C. da S., 2013. The european biofuels policy : from where and where to ? Eur. Energy J. 3, 17–36.

Passos, F., Gutiérrez, R., Brockmann, D., Steyer, J.P., García, J., Ferrer, I., 2015. Microalgae production in wastewater treatment systems, anaerobic digestion and modelling using ADM1. Algal Res. 10, 55–63. doi:10.1016/j.algal.2015.04.008

Passos, F., Hernández-Mariné, M., García, J., Ferrer, I., 2014. Long-term anaerobic digestion of microalgae grown in HRAP for wastewater treatment. Effect of microwave pretreatment. Water Res. 49, 351–359. doi:10.1016/j.watres.2013.10.013

Peña, E., Medina, A., Callejón, M., Sánchez, M., Cerdán, L., Moreno, P., Grima, E., 2015. Extraction of free fatty acids from wet Nannochloropsis gaditana biomass for biodiesel production. Renew. Energy 75, 366–373. doi:10.1016/j.renene.2014.10.016

Pereira, H., 2009. Desenvolvimento e optimização de um meio de cultura para produção de biomassa algal em larga escala. Master Thesis. Universidade do Algarve.

Pereira, H., Amaro, H.M., Katkam, N.G., Barreira, L., Guedes, A.C., Varela, J., Malcata,

F.X., 2013a. Microalgal biodiesel, in: Kennes, C., Veiga, M. (Eds.), Air Pollution Prevention and Control: Bioreactors and Bioenergy. Wiley, pp. 399–430.

Pereira, H., Barreira, L., Custódio, L., Alrokayan, S., Mouffouk, F., Varela, J., Abu-salah, K.M., Ben-hamadou, R., 2013b. Isolation and fatty acid profile of selected microalgae strains from the red sea for biofuel production. energies 6, 2773–2783. doi:10.3390/en6062773

Pereira, H., Barreira, L., Mozes, A., Florindo, C., Polo, C., Duarte, C. V, Custódio, L., Varela, J., 2011. Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae. Biotechnol. Biofuels 4, 61. doi:10.1186/1754-6834-4-61

Ramos, M., Fernández, C.M., Casas, A., Rodríguez, L., Pérez, Á., 2009. Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour. Technol. 100, 261–268. doi:10.1016/j.biortech.2008.06.039

Razon, L.F., Tan, R.R., 2011. Net energy analysis of the production of biodiesel and biogas from the microalgae: Haematococcus pluvialis and Nannochloropsis. Appl. Energy 88, 3507–3514. doi:10.1016/j.apenergy.2010.12.052

Rebitzer, G., Ekvall, T., Frischknecht, R., Hunkeler, D., Norris, G., Rydberg, T., Schmidt, W., Suh, S., Weidema, B., Pennington, D., 2004. Life cycle assessment Part 1: Framework , goal and scope definition , inventory analysis , and applications. Environ. Int. 30, 701–720. doi:10.1016/j.envint.2003.11.005

Rebitzer, G., Hunkeler, D., 2003. Discussing a framework. Life Cycle Costing LCM Ambitions, Oppor. Limitations 8, 253–256.

Richmond, A., 2004. : biotechnology and applied phycology, 1st ed. Blackwell Science Ltd, Oxford.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection , induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100–112. doi:10.1002/bit.22033

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U., Mussgnug, J., Posten, C., Kruse, O., Hankamer, B., 2008. Second generation biofuels: High-Efficiency microalgae for biodiesel production. BioEnergy Res. 1, 20–43. doi:10.1007/s12155-008-9008-8

Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol. Adv. 27, 409–416. doi:10.1016/j.biotechadv.2009.03.001

Sydney, E.B., Sturm, W., Carvalho, J., Thomaz-Soccol, V., Larroche, C., Pandey, A., Soccol, C.R., 2010. Potential carbon dioxide fixation by industrially important microalgae. Bioresour. Technol. 101, 5892–5896. doi:10.1016/j.biortech.2010.02.088

Uggetti, E., Sialve, B., Trably, E., Steyer, J., 2014. Integrating microalgae production with anaerobic digestion: a biorefinery approach. Biofuels, Bioprod. Biorefining 8, 516–529. doi:10.1002/bbb

Varela, J., Pereira, H., Santos, E., Monteiro, H., Tocha, C., Custódio, L., Barreira, L.,

2014. bio tecnologia. biotecnologia 2, 8–10.

Vergara-Fernández, A., Vargas, G., Alarcón, N., Velasco, A., 2008. Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. Biomass and Bioenergy 32, 338–344. doi:10.1016/j.biombioe.2007.10.005

Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L., 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresour. Technol. 99, 7928–7940. doi:10.1016/j.biortech.2008.02.044

Weiland, P., 2003. Production and energetic use of biogas from energy crops and wastes in Germany. Appl. Biochem. Biotechnol. 109, 263–274. doi:10.1385/ABAB:109:1-3:263

Wiley, P.E., Campbell, J.E., Mckuin, B., 2011. Production of biodiesel and biogas from algae: A review of process train options. Water Environ. Res. 83, 326–338. doi:10.2175/106143010X12780288628615

Yamaguchi, K., Nakano, H., Murakami, M., Konosu, S., Nakayama, O., Kanda, M., Nakamura, A., Iwamoto, H., 1987. Lipid composition of a green alga, Botryococcus braunii. Agric. Biol. Chem. 51, 493–498. doi:10.1080/00021369.1987.10868040

Yeesang, C., Cheirsilp, B., 2011. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresour. Technol. 102, 3034–3040. doi:10.1016/j.biortech.2010.10.013

Yoo, C., Jun, S.-Y., Lee, J.-Y., Ahn, C.-Y., Oh, H.-M., 2010. Selection of microalgae for lipid production under high levels carbon dioxide. Bioresour. Technol. 101, S71–S74. doi:10.1016/j.biortech.2009.03.030

Zhong, W., Zhang, Z., Luo, Y., Qiao, W., Xiao, M., Zhang, M., 2012. Biogas productivity by co-digesting Taihu blue algae with corn straw as an external carbon source. Bioresour. Technol. 114, 281–286. doi:10.1016/j.biortech.2012.02.111

Zhu, L., 2014. The combined production of ethanol and biogas from microalgal residuals to sustain microalgal biodiesel: A theoretical evaluation. Biofuels, Bioprod. Biorefining 8, 7–15. doi:10.1002/bbb

Zhu, L.D., Hiltunen, E., Antila, E., Zhong, J.J., Yuan, Z.H., Wang, Z.M., 2014. Microalgal biofuels: Flexible bioenergies for sustainable development. Renew. Sustain. Energy Rev. 30, 1035–1046. doi:10.1016/j.rser.2013.11.003