Master Thesis

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Growth, lipid contents and bioactivities of the microalga *Tetraselmis chuii* in a low-cost, custom-made photobioreactor

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Growth, lipid contents and bioactivities of the microalga *Tetraselmis chuii* in a low-cost, custom-made photobioreactor

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Abstract

The decline of petroleum production has led the scientific world to research novel oil sources that could one day compete with fossil oil. So far, biofuels generated from oil producing crops, such as corn and rapeseed, have been used at a larger scale. However, this type of biofuel competes with food-crops for arable land and the yields are far lower than those needed for them to compete with fossil fuels. Microalgae may be a suitable source of biofuels. However, its high production costs have prevented the development of large scale production ventures for microalgal-based biofuels. In this thesis, the building of a low cost photobioreactor was carried out in order to lower the overall production costs of biodiesel from microalgae. The microalga *Tetraselmis chuii* was selected as feedstock, and its growth rate and lipid production were evaluated, along with its antioxidant potential. The microalgal batch reached the stationary phase in the photobioreactor after approximately 7 days, 3 times faster than under laboratorial conditions, and exhibited a maximum lipid content of 0.07 g/L, which represented 18% of its total dry weight. The GC-MS analysis of *T.chuii* lipid profile revealed a composition of 23.97%, 69.09% and 6.93% for saturated, monounsaturated and polyunsaturated fatty acids, respectively. The IC_{50}(7.97 mg/mL) of radical scavenging activity was determined using the ABTS assay only, as the microalgae presented a poor antioxidant activity by the DPPH assay, even at a concentration of 10 mg/mL. It has been concluded that even though the microalga used as feedstock significantly increased its lipid content and growth rate in a low-cost, custom-made photobioreactor, the bioactivities found in the extracts are still not sufficient to ensure the production of biodiesel at a competitive price as compared with fossil diesel.

Sammendrag

Nedgangen i råoljeproduksjonen har ført den vitenskapelige verden til å eksperimentere med nye olje kilder som en dag skal kunne konkurrere med petroleum baserte brensler. Til nå har det mest effektive vært biobrenselen generert fra oljeproduserende avlinger, som mais og raps, men disse konkurrerer med matavlingene om dyrkbar jord og gir utilfredsstillende ytelser, langt fra de verdiene som trengs for at de skal kunne konkurrere med petroleum basert brensel. Mikroalger kan være en passende kilde av biodrifstoff, men de er fortsatt ikke egnet til storskalaproduksjon på grunn av høye produksjonskostnader. I denne oppgaven ble byggingen av en lavpris photobioreaktor utført for å redusere de samlede produksjonskostnader for biodiesel fra mikroalger. Stammen *Tetraselmis chuii* ble valgt for dette eksperimentet, og veksthastighet og lipidproduksjon ble evaluert, sammen med de ”radicale scavenging activities” for DPPH og ABTS forsøk. Mikroalge-batchen kom til den stasjonære fasen i photobioreaktoren etter omtrent 7 dager, 3 ganger raskere enn i laboratoriet, og viste en maksimal verdi for lipidinnholdet på 0.07 g/L, som representerte 18% av sin totale tørrvekt. Den GC-MS analyse av *T. chuiis* lipid profil presenterte konsentrasjoner på henholdsvis 23.97%, 69.09% og 6.93% for mettede, enumettede og flerumettede fettsyrer. IC50 ble bestemt kun for ABTS forsøk, på 7,97 mg / ml, fordi mikroalgene presenterte en dårlig bioaktivitet for DPPH analysen, selv ved en konsentrasjon på 10 mg/ ml. Konklusjonen er at, selv om algene betydelig økte sitt fettinnhold og vekst i photobioreactoren, er fortsatt ikke biomaterialet funnet i ekstraktene nok til å sikre produksjon av biodiesel til en konkurransedyktig pris kontra petroleumbasert diesel.

Contents

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1 Introduction

The biggest concerns regarding the future of world energy are oil production, which is in decline, and the effects of fossil fuels on the atmosphere, in particular the impact that the CO_2 emissions have on the environment. Over 80% of the energy being consumed today comes from fossil fuels, namely oil, coal and natural gas, with oil representing the largest percentage in this group (Kirtay, 2009). This also has major geo-political implications, as oil production and oil reserves around the world are distributed unevenly, with the Middle East producing 32% of the world's oil and containing over 60% of the proven oil reserves (Hacisalihoglu *et al.,* 2009). According to the International Energy Agency's World Energy Outlook from 2010, the world oil production has begun its decline and, because of this, the prices of petroleum-based oils can only be expected to rise. The second issue that comes up when discussing the future of fossil fuels is the alarming rate at which CO_2 concentration has risen in the last few years. The Stern Report 'The Economics of Climate Change' and the 'Intergovernmental Panel on Climate Change AR4 Synthesis' report show that the current level of CO_2-e (e= equivalent contribution of all greenhouse gases) is over 450 ppm and concluded that this level was reached 10 years earlier than predicted (Schenk *et al.,*2010). Most of the nations have adopted between 10-20% CO_2 emission reduction targets that must be met until 2020, but it is the opinion of many that this undertaking will come to be a huge challenge, as over 98% of carbon emissions are from fossil fuel burning (Kirtay, 2009).

It is in such circumstances that the attention of the world is focused on finding new sources of renewable energy and CO_2-neutral fuels. Biofuels produced by plants or microalgae seem to be the only viable option for replacing liquid petroleum-based fuels necessary for heavy transportation. One of the main reasons for that is the simple production procedures and general availability of the feedstock, whereas for other fuels that do not have an impact on the atmosphere, such as hydrogen, the feedstocks currently used are composed almost entirely of

fossil fuels, which have a high H:C ratio, as the production is based on steam reforming of natural gas or petroleum refining (Renewable fuel – hydrogen, Sustainable Development Technology Canada, 2006; Gouveia, 2011).

The biofuels obtained from biomass can be classified according to the technology used in producing them into biofuels of the first, second and third generation. Until now the largest part of biofuels was produced from higher plants using first generation (sugar, starch and vegetable oil) and second generation technologies (lignocellulose) (Demain *et al.,*2005; Schaub *et al.,*2007), but these procedures require large amounts of arable land in order to produce enough biomass and, in the background of a constant rise in human population, the need for providing sufficient quantities of food means that we cannot spare any farm land for biofuel production. It is estimated that if all the arable land on earth were used for growing oil-producing crops, we would still be covering only half of the energy demands of today (Shenk, 2008).It is easy to conclude by these numbers that first and second generation biofuels do not show any important contributions to the future of global energy requirements. However, third generation biofuel technologies show much more promise, and in particular microalgae farming.

1.1 Microalgae – an alternative oil source

Microalgae are either prokaryotic or eukaryotic organisms that can be either autotrophic or heterotrophic and can be found in either fresh or salt water. They are unicellular, with their size varying from a few micrometres to a few hundred micrometres. Estimations are that there may be up to 800,000 species of microalgae, of which just 35,000 have been described, and

over 15,000 compounds originated from algal biomass have been determined (Cardozo *et al.,* 2007).

As they are photosynthetic organisms, microalgae convert water, sunlight and carbon dioxide into biomass, which includes a great variety of metabolites. These cells can produce up to 300 times more oil for biodiesel production than first generation oil-crops, on an area basis. Moreover, terrestrial oil-producing crops can only be harvested once or twice a year, whereas the harvesting cycle of microalgae can be as short as 1-10 days. In addition, microalgae can be grown on non-arable land (Shenk, 2008). Lipid (oil) levels in microalgae can go up to 40%, but usually are in the range of 15 to 25% (Rodolfi *et al,* 2009). Another advantage that microalgae have over terrestrial plants is the efficiency with which they capture solar energy and convert it to biomass; studies have shown that the photosynthetic efficiency of microalgae is in the range of 10 to 20% or even higher (Richmond, 2000; Huntley & Redalje, 2007), as opposed to terrestrial plants of which the fastest growing shows a yield of less that 0.5% for a typical mid-latitude region (200-300 W/m^2 solar energy) (UN Report, 2003; Lewis & Nocera, 2006). A more recent study (Wijffels & Barbosa, 2010) supports a maximum value of 9% of solar conversion to biomass efficiency and calculates the amount of land needed for the production of biodiesel from microalgae that could replace the entire petroleum-based diesel in Europe to a surface of 9.25 million ha, roughly the size of Portugal. The study considers a productivity of 40.000 litres per ha per year, with lipids representing 50% of the total dry weight of the biomass harvested, equalling 10 times more litres of biodiesel and 10 times less space than the most efficient first generation fuel plant available (Chisti, 2007; Mata *et al*., 2010). Taking all these into account, Chisti (2007) has concluded that microalgae seem to be the only source of biomass able to produce biodiesel that can compete with fossil liquid fuels.

1.2 Biochemical properties of microalgae

There are three main groups of research when referring to aquatic natural products: toxins, bio-products and chemical ecology. About 18,500 new products have been isolated and characterized from marine sources, starting from 1965 up to 2006, but it is believed that this value represents only 3% of all the existing marine compounds (Cardozo *et al*., 2006; Guedes *et al.*, 2011). Derived from these bio-compounds there are already some products in use (*Citarabina*, an anti-tumoral, and *Virabadina*, an anti-viral) or undergoing clinical studies (e.g. *Prialt*, an analgesic and *Yondelis*, an anti-tumoral) (Haefner, 2003).

In addition to lipids, microalgae also present a wide amount of chemical compounds that can be isolated and extracted. Microalgae currently are of interest in the industry and research departments regarding human nutrition, animal feed, aquaculture and biofertilizers (Pulz & Gross, 2004).

The main compounds with scientific and/or industrial importance that can be extracted from microalgae are:

- Antioxidants: These compounds have long been established to be beneficial for human health. They have been proven to control or prevent the growth of certain tumours, the incidence and severity of cardiovascular and degenerative diseases are lessened and the extension of one's lifespan has been reported. Carotenoids are the most important antioxidant group that can be found in microalgae (Guedes *et al.*, 2011). From this group of compounds, lutein is one of the most common and it can be found in several species of algae (e.g. *Dunaliella* sp., *Murielopsis* sp., *Scenedesmus almeriensis*), with temperature and irradiance determining a different production yield in each species (Hu *et al.,* 1998; Garcıa-Gonzalez *et al.,* 2005; Blanco *et al.,* 2007; Fernandez-

Sevilla*et al.,* 2010). Lutein has been recommended and even prescribed for the prevention of cancer and diseases related to retinal degeneration (Granado-Lorencio *et al.*, 2009).

- Anti-inflammatory compounds: Active compounds, such as astaxanthin (identified in *Haematococcus pluvialis*, *Chlorococcum* sp.) or sulphated polysaccharides (identified in *Porphyridium* sp.), are known to have anti-inflammatory action by suppressing cell-mechanisms that lead to these effects, such as reducing low density lipoprotein oxidation or inhibiting the spreading of immune cell recruitment towards inflammatory stimuli (Iwamoto *et al,*, 2000; Lee *et al.,* 2003; Matsui *et al.,* 2003). A more recent study (McNulty *et al.*, 2007) suggests that these compounds prevent lipid based oxidation and constrain the ability of hydrophilic biophores to be exposed and interact with reactive oxygen species and reactive nitrogen species in their microenvironment, the surplus production of which can lead to initiation and progression of atherosclerosis (Guedes *et al.*, 2011).
- Anti-microbial compounds: Several studies (Juttner, 2001; d'Ippolito *et al.,* 2004; Ward & Singh, 2005) have shown anti-microbial activity of compounds such as polyunsaturated fatty acids (in particular eicosapentaenoic acid) isolated from the microalgae species *Phaeodactylum tricornutum* and *Skeletonema costatum* (Naviner *et al.*, 1999; Desbois *et al.*, 2009), but the bio-mechanisms on which these appear to act against bacteria is only beginning to be understood.
- Anti-viral compounds: The main antiviral compounds that algae produce are highly sulphated polysaccharides which seem to exhibit a pleiotropic mode of action that is less likely to produce resistant mutants, as compared to other compounds that attack only one target through the viral life cycle, but the exact metabolic pathways that lead to these polysaccharides and the specific stage in which they act against virus replication has not yet been precisely determined (Guedes *et al.,* 2011).

- Anti-tumoural compounds: Several algal bio-compounds that seem to inhibit the development of cancer cells have been isolated. One has attracted a lot of attention in particular: the red ketocarotenoid astaxanthin, which presents a much more powerful anti-tumoural activity than other carotenoids (Ip & Chen, 2005; Guedes *et al*, 2011).
- Lipids: Polyunsaturated fatty acids (PUFA),and in particular eicosapentaenoic acid (EPA), are important for human and animal nutrition and health, as they confer flexibility, fluidity and selective permeability properties to cellular membranes, making them vital for brain development and function and beneficial for the cardiovascular system (de Urquiza *et al.,* 2000; Funk, 2001; Colquhoun, 2001). These fatty acids have been identified in many species of algae, such as diatoms, chrysophytes, cryptophytes and dinoflagellates (Cohen *et al.,* 1995; Behrens & Kyle, 1996).

1.3 Large scale production

Although we can find microalgae in almost every waterbody, the process of successfully growing them in order to get a satisfactory production of biomass viable for lipid extraction can prove quite difficult. This is because cell concentration is relatively low and so the amount of space needed to grow microalgae in order to get sufficient biomass is considerably large, which in turn brings up new issues related to finding economical solutions for maintaining growth parameters at optimal levels.

The main ways to grow algae for large-scale production at this moment are open pond systems (Terry & Raymond, 1985; Grima *et al.*, 1999) and closed systems such as photobioreactors (Grima *et al*., 1999; Tredici, 1999; Sánchez *et al*., 1999), both with their advantages and disadvantages.

1.3.1 Open-pond systems

This type of growing system for microalgae is most usually in a form of a raceway pond and consists of a closed loop channel, usually around 0.3 m deep, in which the water is mixed and circulated using a paddlewheel The flow is controlled and guided by baffles placed in the channel, especially in the curving sections of the raceway. They are either built in compacted earth or concrete and can be lined with white plastic. The flow begins in front of the paddle–wheel, where the culture is injected into the system, and the medium is afterwards collected at the back of the paddlewheel at the end of the raceway pond loop (Figure 1) (Chisti, 2007; Demirbas, 2010).

Open ponds are cheaper than closed systems, this being their main advantage and the reason many companies prefer to use this method for microalgae growth. However, they require more space, CO_2 and light than closed systems; they can easily get contaminated and overall present lower productivity. This is why the focus is now on closed system, photobioreactors, in which the culture can be better controlled and a single species of microalgae with high lipid content can be grown in a much smaller space than open ponds (Chisti, 2007).

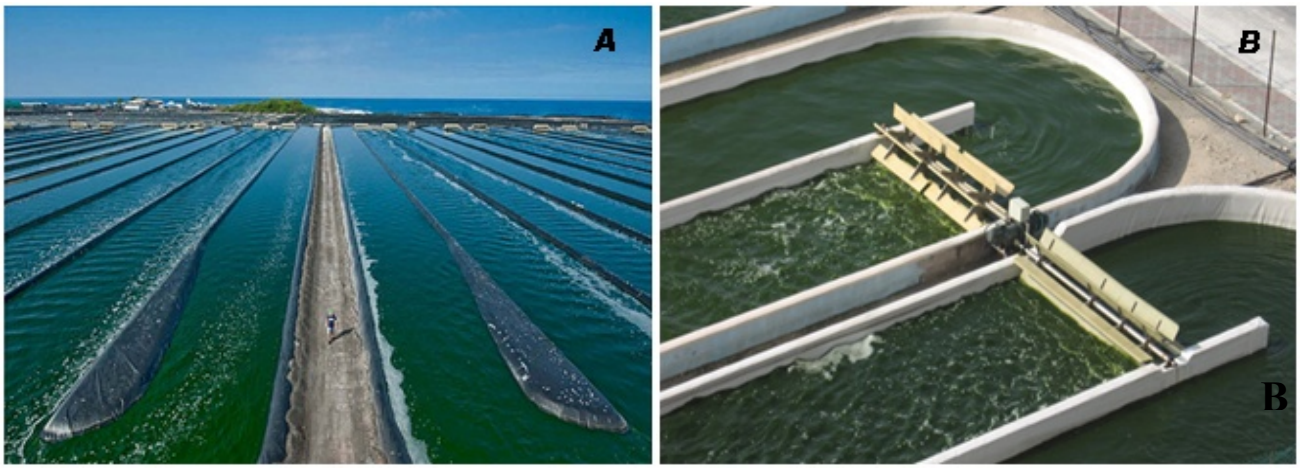

Figure 1.*Open pond systems used nowadays by different companies for large-scale production of microalgae: A) Cyanotech Corporation, USA; B) Seambiotic, Israel*

1.3.2 Photobioreactors

The scientific world has experimented with many types of photobioreactor designs, in order to find one that gives an optimum distribution of light and culture mixing, so that a maximum amount of biomass can be grown within the shortest time possible (Tredici, 1999). There are various possibilities of growing algae in photobioreactors, depending on whether it is for small-scale laboratory growth or large-scale biomass production. For the latter case, the designs are numerous: from thin flat airlift photobioreactors to column or tubular (either vertical or horizontal) photobioreactors (Figure 2) (Chisti, 2007; Chiu *et al.*, 2009).

The design of an airlift photobioreactor implies the use of thin areas where the culture is contained, usually made out of a transparent plastic material allowing more light to pass through, which according to some studies promotes the highest photosynthetic efficiency (Eriksen, 2008; Rodolfi *et al.,* 2009). The culture is stirred using a constant air flow, a method preferred because cell-damage associated with mechanical pumping is avoided and because the air-lift device combines the functions of a pump and a gas exchanger that removes the oxygen produced by photosynthesis (Prassone *et al.*). Its dimensions are usually in the range of a couple of centimetres in thickness and between 1 and 2 meters in height. However, this implies the use of large surface areas, which in turn presents problems in controlling culture temperature, carbon dioxide diffusion rate and the tendency for algae to adhere to the walls of the reactor (Demirbas, 2010).

A typical tubular photobioreactor is constructed of plastic or glass transparent tubes, generally with 0.1 m or less in diameter, to allow the sunlight to penetrate deep in the culture medium, which is circulated from a reservoir to the tubes and back (Chisti, 2007).The array of tubes, also called solar collector, is arranged spatially in order to maximize sunlight capture (Grima *et al*., 1999; Mirón *et al*., 1999). These tubes may either be arranged horizontally, in a fence-

like structure, or vertically, forming a helical coil. By maintaining a highly turbulent flow, biomass sedimentation can be avoided.

The high costs of closed systems consist mostly of the energy costs needed for the air supply and for the mechanism that mixes the culture inside the reactor so that all the cells pass through the peripheral zone. Cells at the periphery of the tube are subjected to higher light intensities than those at the centre of the reactor. This light/dark cycle, when used at a certain frequency, actually improves the growth of microalgae biomass as compared to the cells that just remain all the time exposed to sunlight (Demirbas, 2010). Constructing the photobioreactors is also an issue, as capital costs for these are much higher than for the open-pond systems (Gouveia, 2011), and because of this the main focus of the current work is trying a new low-cost photobioreactor design that could ultimately lead to minimize the capital and running costs of such a system and lead to profitable microalgae production.

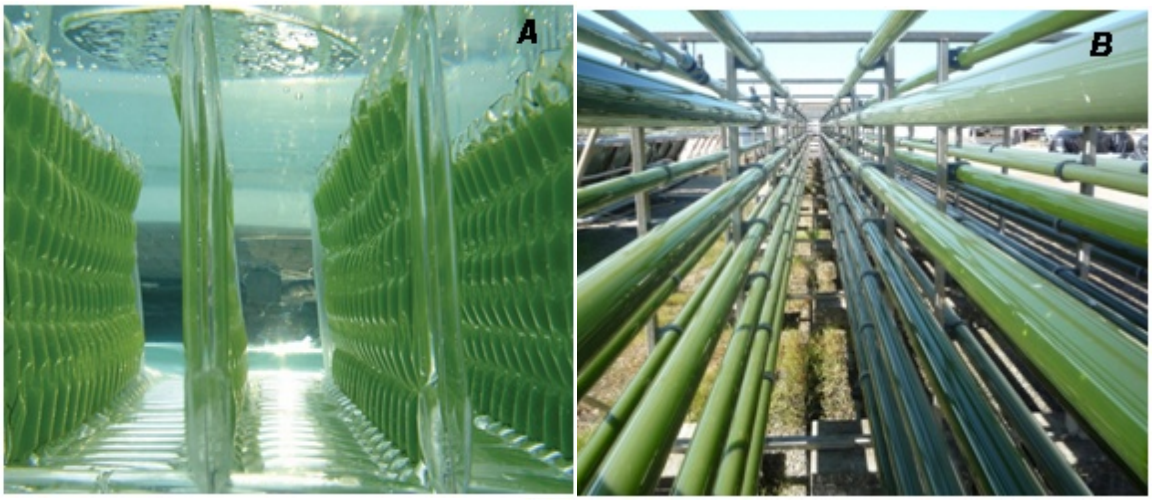

Figure 2.*Different designs of photobioreactors used for the large-scale production of microalgae biomass. A) Low-cost plastic photobioreactor (Proviron, Belgium); B) Tubular photobioreactor (Necton S.A., Portugal).*

1.4 Microalgae growth, harvesting, lipid-extraction and biofuel production

Depending on which microalga species is grown in the bioreactor, the operating parameters and the nutrients that must be supplied in order to obtain the highest amount of biomass vary significantly. Microalgae depend on light intensity and wavelength, concentration of CO_2 and of other nutrients (mainly nitrogen and phosphorus), vitamins and minerals, water, pH and temperature, which all must be adequately set (Williams, 2002).

After the completion of the algae's life cycle, harvesting can be performed by various methods: concentration through centrifugation (Haesman *et al.,* 2000), foam fractionation (Csordas & Wang, 2004), flocculation (Knuckey *et al.,* 1996; Poelman *et al.,* 1997), membrane filtration (Rossignol *et al.,* 2000) or ultrasonic separation (Bosman *et al.,* 2003). The optimum method is selected depending on the species involved and most often on the culture conditions. Still, all harvesting processes need a high level of attention since they present limitations, either regarding high energy costs or low recovery efficiency.

In order to recover lipids and other metabolic products from algae, cell disruption is often necessary and this can be performed either chemically or mechanically, or by a combination of the two. The efficiency of these methods also varies according to the species of microalgae harvested (Lee *et al*, 2010). In the case of large-scale production systems, the method usually used is a mechanical cell disruption technique, such as freezing, sonication or bead milling, followed by a solvent extraction (Chisti & Moo-young, 1986; Molina Grima *et al.,* 2004; Aresta *et al.,* 2005; Ferrentino *et al*, 2006).

The solvent extraction methods most used are the Bligh and Dyer (1959) protocol, which consist of a chloroform and methanol mix that is used to recover the corresponding lipid

fractions from the cells (Ferrentino *et al.*, 2006; Lee *et al.*, 2010). This procedure is normally applied to small-scale lipid extractions, whereas hexane/isopropanol techniques prevail in industrial scale lipid-extractions (Ferrentino *et al.*, 2007).

In order to transform the extracted lipids into biodiesel the main procedure used is base catalysed transesterification (Otera, 1993), which consists of the transformation of the triacylglycerols (TAG) into fatty acids methyl esters (FAME) using methanol, with glycerol as a by-product, and usually under the presence of a catalyst (Figure 3) (Ma & Hanna, 1999; McNeff *et al.*, 2008). The reason for the transesterification process is to reduce the viscosity of the oil found inside the microalgae to a value close to that of the petroleum diesel (Demirbas, 2007). Being derived from biomass, biodiesel does not contribute to the increase in atmospheric CO_2concentrations, and other emissions, except for NO_x, are lower than petroleum diesel (Laforgia & Ardito, 1994; Cardone *et al.*, 1998; Demirbas, 2010).

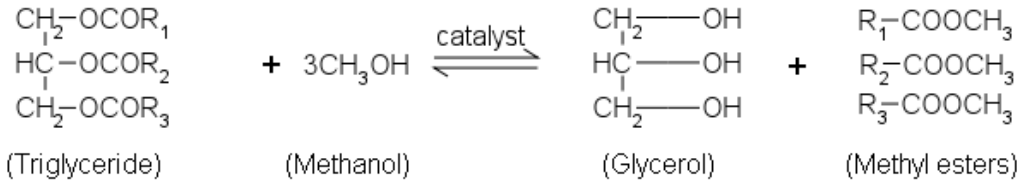

Figure 3.*Transesterification of triacylglycerols (also known as triglycerides) to biodiesel (methyl esters)*

Biodiesel is a variable mixture of saturated, monosaturated and polysaturated fatty acids, depending on the biomass feedstock used. Because saturated fatty acids present in large amounts can bring down the temperature at which the biodiesel crystallizes or polyunsaturated fatty acids can make the biodiesel more susceptible to oxidation, a balanced mix of the corresponding triacylglycerols must be used in the transesterification process, and microalgae seem to present such a balanced composition (Sheehan *et al.*, 1998; Mousdale,

2008). A thorough chemical analysis of the compounds isolated from a microalgae strain must be performed in order to fully evaluate its potential as a biodiesel source.

1.5 Algae-Based Biorefinery (ABB)

The main task after analysing all the steps needed to extract the biofuel from algae cells is to lower the costs of production so ultimately it will become profitable. The concept of an Algae-Based Biorefinery (ABB) thus appears as an optimum solution. This term has been used to describe the production of a wide range of chemicals and biofuels from biomasses through the integration of bio-processing and appropriate low environmental impact chemical technologies in a cost effective and environmental suitable manner (Figure 4) (Li *et al.,* 2008).The main goal of the ABB is extracting as many usable products from the microalgae as possible, and then recycling the unused biomass and wastewater in an attempt to create a closed loop cycle, so that the ABB would ultimately be able to sustain itself.

Several conversions technologies can be integrated so that the ABB could produce, besides a wide range of biofuels, such as biodiesel, aviation fuel, ethanol and methane, a lot of valuable co-products such as proteins and carbohydrates, natural oil dyes, anti-oxidants and polyunsaturated fatty acids (Gouveia, 2011). These products would contribute to keeping the overall price of the microalgae growth process low thus allowing the biodiesel to compete with the fossil fuels currently produced.

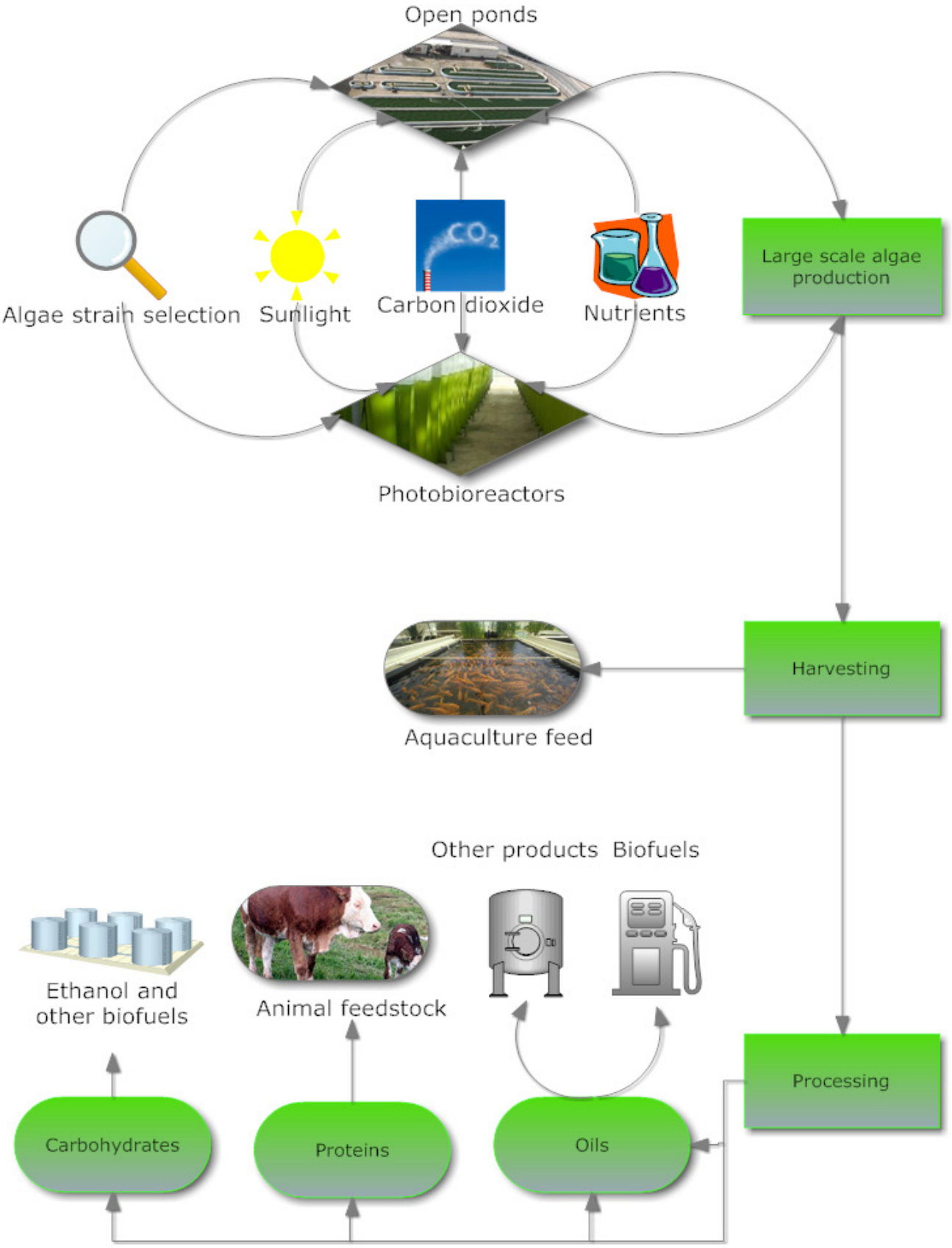

Figure 4.*A typical algae biorefinery flowchart, describing the processes involved from the growth of the microalgae to the products obtained at the end of the cycle*

1.6 *Tetraselmis*

The algae belonging to the genus *Tetraselmis* are unicellular green flagellates of cordiform, elliptical or almost spherical form that live in marine or fresh water environments. It has been determined to be one of the most productive microalgae in terms of biomass and lipid production, and a good source of biocompounds for dermatological or pharmaceutical use (Cardozo *et al.*, 2007; Huerlimann *et al.,* 2010; Guedes *et al.*, 2011; Custódio *et al.*, 2012).

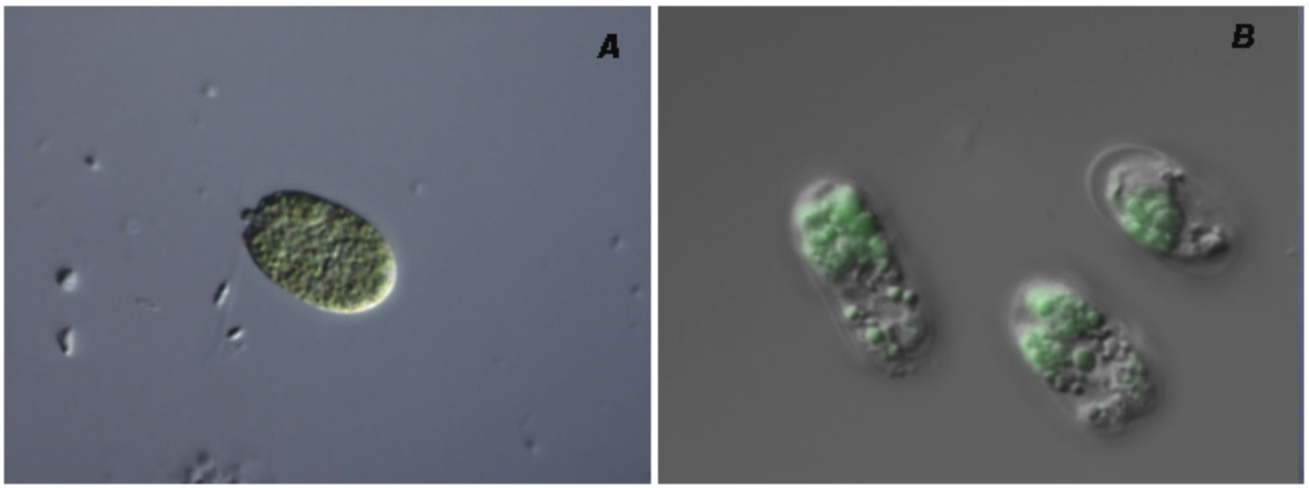

Figure 5.*Picture of the microalga* Tetraselmis chuii. *(A) Brightfield image with differential interference contrast (DIC). (B) Fluorescence microscopy merged with DIC of cells stained with BODIPY dye; green dots show the accumulation of lipid bodies.*

The species *Tetraselmis chuii* (Figure 5) belongs to the subgenus *Parviselmis* and, like other microalgae present in the same subgenus (such as *T. striata* or *T. suecica*), exhibits a pyrenoid matrix, not larger than 1.1 x 2 μm, having a cavity that is filled with a lobe of cytoplasm and that is always penetrated from the side facing the nucleus. The cells are 12-16 μm long and 7-10 μm broad and the flagella pit is covered with abundant slit hairs at the bottom. Two

dictyosomes are located around the flagellar base or in the upper lobes of the cell. It is a common microalga, found in tide pools and estuaries in Europe and North America (Hori *et al.,* 1986).

Its present use in aquaculture (Hemaiswarya *et al.*, 2010) and its potential as a source for anti-oxidant and anti-Alzheimer dietary supplements (Custódio *et al.,* 2012) make the *T.* chuii microalga an important prospect for industrial applications. These factors, together with its abundance in marine waters, which underlines its resilience, and because it has been previously studied in laboratory condition and proven to be a promising candidate for biodiesel production (Huerlimann *et al.*, 2010), have determined the choice of the *T.chuii* strain for the present study, in order to further assess its qualities and its capacity to produce biocompounds in an outdoor photobioreactor.

2 Objectives

In this work, a previously in-house designed low-cost photobioreactor will be constructed and its ability to maintain a contamination-free microalgae culture will be determined to test whether capital costs can be reduced and parameters for obtaining a maximum yield of microalgal biomass can be optimized, so that MBB can ultimately become economically feasible.

For building the bioreactor, the right kind of plastic material will have to be found. The plastic film will have to be strong enough to support the weight of the water inside the reactor, but thin and transparent enough to allow light to penetrate the reactor walls. The bioreactor will be placed in a water pond and the airflow will have to be well adjusted in order to keep the bioreactor afloat and to maintain the algae in suspension. Water loss via evaporation will be replaced each day, as it can have a significant impact on the culture volume and salinity, especially during the summer season.

Regarding the evaluation of the specific parameters of the culture, correlations will be made between simple measurements, such as optical density and Nile red fluorescence, and more complex ones, such as cell counting and gravimetric lipid determination, in order to assess the growth parameters, biomass production, and the total amount of lipids produced by the algal culture more easily.

GC-MS analysis will be used to determine if the microalgae grown in the bioreactor exhibited an adequate profile of fatty acids needed for biodiesel production. This test, alongside the ones that show the anti-oxidant bioactivities of the microalgae, will be taken into consideration when discussing the implementation of a profitable algae-based biorefinery.

3 Materials and methods

For this study, the *Tetraselmis chuii* LEOA strain used has been re-isolated by the MarBiotech laboratory from the University of Algarve from a previous sample obtained from the LEOA laboratory at the same university. In the first part, calibration curves of the growth parameters were established. For this purpose, algae were grown in a 5 L container and culture growth was observed over the course of 3 weeks. The process of inoculation was as follows: the container was filled with approximately 4 L of sea water (37ppm) and, in order to sterilize it, 4 mL of bleach were subsequently added and the container was connected to a Schego M2K3 air flow (Schego, Offenbach am Main, Germany), filtered using cellulose acetate membrane syringe filters with 0.2 µm pores and 25 mm diameter (VWR, Leuven, Belgium). After 7 hours, 4 mL of neutralizing solution $Na_2S_2O_3$ were added and the container reconnected to the airflow. After 3 more hours, the container was inoculated with 1 L of *T.chuii* from a previous culture. An aliquot of 5 mL of Algal growth medium was added to the batch. The composition of the ALGAL solution (per litre) was:

- 100 mL of micronutrients solution (136.4 mg $ZnCl_2$; 179.5 mg $ZnSO_4.7H_2O$; 197.8 mg $MnCl_2.4H_2O$; 242 mg $NaMoO_4.2H_2O$; 23.8 mg $CoCl_2.6H_2O$; 24.9 mg $CuSO_4.5H_2O$; 2.4 g EDTA; 492 mg $MgSO_4.7H_2O$);
- 700 mL of macronutrients solution (170 g $NaNO_3$ and 13.6 g KH_2PO_4);
- 200 mL of iron solution (5.4 g $FeCl_3.6H_2O$; 7.4 g of EDTA);

With the exception of cellular concentration and optical density, which were carried out from Day 0, the following tests were done from Day 2 onwards.

3.1 Cellular concentration

To determine the cellular concentration, a Neubauer counting chamber was filled with 10 µL of algal cell suspension on each side. Because *T.chuii* is a motile strain (presents flagella), cells were fixed beforehand by adding 1 µL of Lugol's solution in a 1 mL sample of algae culture. After cell counting, appropriate calculations were made to deduce the final concentration of cells. For the exponential growth phase, the microalgae growth rate was calculated using the formula $\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$, where X_2 and X_1 are the cellular concentrations for the days t_2 and t_1 respectively, and the division time was calculated according to the formula $t_g = \frac{0.6931}{\mu}$ (Guillard, 1973).

3.2 Optical density

Optical density constitutes another way of determining the amount of cells that can be found in an aqueous suspension, although other cells like bacteria or fungi, if present in considerable quantities in the microalgal culture, can affect the result. To assess the optical density, 300 µL of algal cell suspension, undiluted, was introduced in 6 wells of a 96 well plate and a BioTek Synergy 4spectrophotometer (BioTek, Winooski, Vermont, U.S.A.) was used to measure the absorbance at two wave lengths: 540 nm and 750 nm.

3.3 Dry weight

Glass microfiber filters,5 cm in diameter, and with a particle retention of 1.2 µm, were pre-washed by cleaning them with 10 mL distilled water in a Millipore filtration system (Millipore, Billerica, Massachusetts, U.S.A.) and left to dry for 1 day at 60ºC. In the day of the analysis, their weight was measured in a microbalance prior to filtering 10 mL of algal

culture and rewashed with 10 mL of distilled water. The filters were then dried again at 60ºC for at least 3 days or until a constant weight was achieved. Biomass dry weight was calculated by subtraction and results were expressed in mg/L of culture.

3.4 Nile red lipid determination

The experiments were carried out according to a modified protocol of Chen *et al.* (2009). A staining solution was prepared to achieve the final concentration of 1 µM of Nile red and 25% of DMSO. A 96 well plate was prepared with dilutions of the algal culture and salt water of 1:1, 1:2, 1:5, 1:10 and 1:20 at a volume of 250 µL to which 50 µL of the staining solution described above were added. Negative controls were prepared by checking the fluorescence of 250 µL of water mixed with 50 µL of Nile red staining solution and the natural fluorescence of distilled water and algal culture at volumes of 300 µL. The well-plate was introduced in the BioTek Synergy 4 spectrofluorometer (BioTek, Winooski, Vermont, U.S.A.) which was preheated at 37ºC and continued to mix for 10 min at 100 r.p.m. The fluorescence was then measured upon excitation at 530 nm and emission at 580 nm, according to the protocol by Chen *et al.* (2009).

3.5 Gravimetric lipid determination

A protocol developed by Bligh & Dyer (1959) and modified by the Centre of Aquaculture in Trondheim was used. Four tubes containing 50 mL of algae culture were centrifuged for 10 min at 8000 *g*. The spun down biomass was washed with 800 µL of distilled water and transferred to separate tubes. Afterwards, the following procedures were executed for each

tube: 1) 2 mL of methanol and 1 mL of chloroform were added and the solution mixed using an IKA T-10 basic Ultra-Turrax (IKA, Staufen, Germany) disperser for 1 minute at maximum power; 2) 1 mL of chloroform was added and the result mixed in the disperser for 30 s; 3) 1 mL of distilled water was added and the result mixed in the disperser for 30 s. The tubes were then centrifuged for 10 min at 5000*g*. Then 600 µL of the chloroform phase were transferred to the weighting tubes, which were pre-dried for 6 hours at 60 ºC and left to cool down for 3 hours in a desiccator. The tubes were then left at 60ºC overnight for the chloroform to evaporate. The resulting weight difference between the tubes before and after the chloroform phase was added and evaporated represents the amounts of lipids per 600 µL of chloroform phase from an algae culture of 50 mL.

3.6 Lipid productivity

The lipid productivity was calculated for the last day of the exponential phase of the culture using the formula $P = \left(DM * \mu * \frac{LC}{100}\right) * 1000$ ($mgL^{-1}day^{-1}$), according to Huerlimann *et al.* (2010), where DM is the total dry weight of the microalgae, LC is the percentage represented by the lipids of the total dry weight and µ is the growth rate of the culture.

3.7 Genetic identification of microalgae

As the vast biodiversity of the microalgae are usually misidentified when they are classified in terms of morphology alone, the identification of the microalgae used in the bioreactor was carried out by means of 18S rDNA sequencing.

Genomic DNA was extracted from a fresh sample of our algal strain using the E.Z.N.A DNA extraction kit (VWR International, Leuven, Belgium) according to the instructions provided

by the manufacturer. A PCR reaction was performed with the isolated DNA to amplify a partial fragment of the 18S gene, which is used to identify most microalgae (Olmos *et al.*, 2000;Pereira *et al*., 2011).Aliquots of 0.5 µL of the 18SUnivFor (10 µM) and 18SUnivRev (10 µM) primers were used to create a master mix composed of 12.8 µL of H_2O, 5 µL of Promega buffer solution (5x) (Promega, Madison, Wisconsin, U.S.A.), 2 µL of $MgCl_2$(25 mM), 0.5 µL of dimethyl sulphoxide (100%), 0.5 µL of deoxynucleotides (10 mM) and 0.2 µL of a Promega *Taq* DNA polymerase (5 U/µL).

The amplification procedure was carried out by preheating 3 µL of DNA and 22 µL of the master mix for 5 minutes at 94ºC and then exposing them to 35 cycles of 30 seconds at 95ºC, 30 seconds at 55ºC and 1 minute at 72ºC, after which the mix was incubated at 72ºC for 10 min. Upon amplification, the PCR product was stained with GelRed and passed through an agarose gel (1%), to ensure that the required fragment was isolated. It was then to sequencing at an in-house DNA sequencing facility equipped with an Applied Biosystems 3130XL DNA sequencer (Life Technologies BV, Porto, Portugal).

The sequencing results were visualized and edited in Geneious (Biomatters, version 4.8), after which it was exported, along with the other microalgal 18S DNA sequences downloaded from the GenBank database and stored into a FASTA file. The latter was uploaded to the website www.phylogeny.fr in order to construct two phylogenetic trees, one using a neighbour joining method – the BioNJ algorithm –, and another using a maximum likelihood method –the PhyML algorithm. The sequences were first aligned using MUSCLE v. 3.7 (Edgar, 2004) and curated with Gblocksv. 0.91b. For the BioNJ algorithm (version 3.66) and the PhyML algorithm (version 3.0), the bootstrapping procedure was selected using 1000 bootstraps.

3.8 Construction of the bioreactors

As the main goal was to create a cheap and easy to construct bioreactor, the structure was kept to a minimum necessary. The plastic material used was a transparent low-density plastic foil of 0.2 mm thickness, procured from Faplastal Lda. (Faro, Portugal). A double layer of the same material was used on the bottom side of the reactor and in a single layer on the topside, around a metallic structure of 1.2 m in diameter. A SealMaster sealer, with a 0.3 mm seal thickness, was used to seal the plastic foils around the metallic structure of the reactor and to create passageways for the two air-tubes on the bottom layer of the bioreactor, which were laid out in a spiral form. A plastic cap was fixed in the metal ring of the metallic structure of the bioreactor through which three incisions were made, two used to take out the air tubes and one to let the air escape. The air tubes were connected to an air supply which ensured a constant flow of air, filtered using a 1 μm polyester air filter (Supremo, Italy) .The reactors were placed in a salt water pond, of approximately 1 m in depth and averaging an area of approximately 1000 m^2, and secured with ropes (Figure 6), so that the water inside the pond can help regulate the temperature inside the bioreactor. Autoclaved water was added daily to replace the amount lost by evaporation, and to keep the volume inside the bioreactors at approximately 100 L.

A Thermochron DS1921G (Eclo, Leira, Portugal) temperature sensor was covered in a waterproof plastic foil and introduced into one of the bioreactors, and the data that it has recorded was collected at the end of the experiment. Salinity and pH values were measured in the laboratory using a VWR refractometer (VWR International, Leuven, Belgium) and a Cyberscan pH 510 meter (Eutech, Singapore) respectively, in order to assess the water-loss and CO_2 consumption.

Figure 6 *Low cost photobioreactors constructed throughout this work. The floatable bioreactors are maintained by a rope system and the air-supply allows a continuous shuffle of the culture.*

3.9 Assessment of the fatty acid composition by gas chromatography coupled with mass spectrometry

After the centrifugation of the algae culture, 0.1 g of biomass was weighted and introduced in a vial for further processing. Four replicas were made. A volume of 1.5 mL of the derivatization solution composed of a mixture of methanol and acetyl chloride (20:1) was added to the vials and the result was submerged in ice and homogenized with the help of an IKA T-10 basic Ultra-Turrax disperser in 3 cycles of 30 seconds each. An aliquot of 1 mL of hexane was added to each vial after which they were placed in a bath at 100 ºC for 1 hour. The solution was transferred to a centrifuge tube, along with 1 mL of distilled water, and spun down for 5 minutes at 1000 *g* in order to separate the phases. The hexane phase was removed to a new vial, 1 mL of hexane was added to the centrifuge tubes and the process was repeated until the resulted hexane phase was colourless. Anhydrous sodium sulphate was added and the

samples were filtered to another pre-weighed vial. Samples were dried under a light nitrogen flow, the vials were weighed and the pellet was resuspended in 100 µL of hexane for analysis. The GC-MS equipment used was an Agilent Technologies 6890 Network GC System (Agilent Technologies, Santa Clara, California, USA) and a 5973 Inert Mass Selective Detector equipped with a DB5-MS capillary column (25 m × 0.25 mm internal diameter, 0.25 µm film thickness) and the oven settings were as follows: 60ºC for 1 minute, 60ºC to 120ºC at 30ºC / minute, 120ºC to 250ºC at 5ºC / minute, 250ºC to 300ºC at 20ºC / minute, 300ºC for 2 minutes. The detection of the compounds was made by mass spectrometry, comparing the obtained mass spectra with the ones from the National Institute of Standards and Technology, U.S. Department of Commerce (NIST).

3.10 Evaluation of algal biomass bioactivities

In order to assess the antioxidant activity of *T.chuii*, the algal cells were freeze-dried and resuspended in methanol in the proportion of 1 g biomass / 40 mL of methanol, lysed using an IKA Ultra-Turrax disperser, extracted overnight with stirring at room temperature and filtered using 125 mm Ø filter paper. The yield of extraction was determined and the filtrates were dissolved in DMSO, in order to obtain a final concentration of 50 mg/mL, and were analysed in triplicate at the following concentrations: 1 mg/mL, 5 mg/mL and 10 mg/mL. The differences between the two anti-oxidant tests at different concentrations were analysed using a one-way ANOVA with a Tukey's post-hoc test in SPSS Statistics, version 17.0.

3.10.1 DPPH radical scavenging activity

A DPPH solution of 120 μM was made using 2.4 mg of 2,2-diphenyl-1-picrylhydrazyl and 50 mL of methanol. The samples (22 μL of each dilution) were suspended in 200 μL of the DPPH solution in a 96 well plate. The colour control was performed using the same sample volume and 200 μL of methanol. The negative and positive controls consisted of 200 μL of DPPH solution plus 22 μL of DMSO and 22 μL of BHT (Butylated hydroxytoluene, 1 mg/ml)) respectively. The absorbance was measured at 517 nm in a spectrophotometer and the radical scavenging activity was calculated using the formula:

$$\%\ of Radical Scavenging Activity\ (RSA) =$$

$$\frac{Absorbance\ of\ negative\ control - Absorbance\ of\ test\ sample}{Absorbance\ of\ negative\ control} \times 100$$

where

$$Absorbance\ of\ test\ sample$$

$$= Absorbance\ of\ test\ solution - Absorbance\ of color control$$

3.10.2 ABTS radical scavenging activity

An ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) solution was made by mixing 5 mL of distilled water, 0.0035 g of potassium persulphate and 0.0203 g of ABTS and kept at 5ºC in the dark for 12 hours.

The absorbance at 734 nm was read and the solution diluted with ethanol until a value of approximately 0.7 was obtained and then 10 µL of the sample was mixed with 190 µL of the resulted ABTS solution in a 96-well plate.

The negative control was made with the same amount of ABTS solution as the samples plus 10 µL of DMSO (100%) and the positive control with 10 µL of BHT (1 mg/ml). The 96 well-plate was read at 734 nm, 6 minutes after incubation in the dark. The radical scavenging activity was calculated using the formula:

$$\%\ of\ RSA = \frac{Absorbance\ of\ negative\ control - Absorbance\ of\ test\ sample}{Absorbance\ of\ negative\ control} \times 100$$

3.11 Statistical analysis

The data was analysed using SPSS Statistics (version 17.0) with an independent samples *t*-test for the growth parameters between the microalgae batch grown in the laboratory and the batch grown in the photobioreactor, with a confidence level of 95%. The anti-oxidant assays were subjected to variance analysis (one-way ANOVA with Tuckey's post-hoc test) with the same confidence level of 95%.

4 Results

4.1 Identification of the microalgae species by 18S rDNA sequencing

After the initial DNA extraction and gene amplification carried out according to the procedures described in the Materials and Methods section, the PCR product was stained with GelRed and electrophoresed in a 1% agarose gel, which confirmed that the PCR sequence had the same size as the targeted gene. In Figure 7, lanes B and C present DNA fragments of about 1000 bp., which matched the size of the product obtained in the positive control.

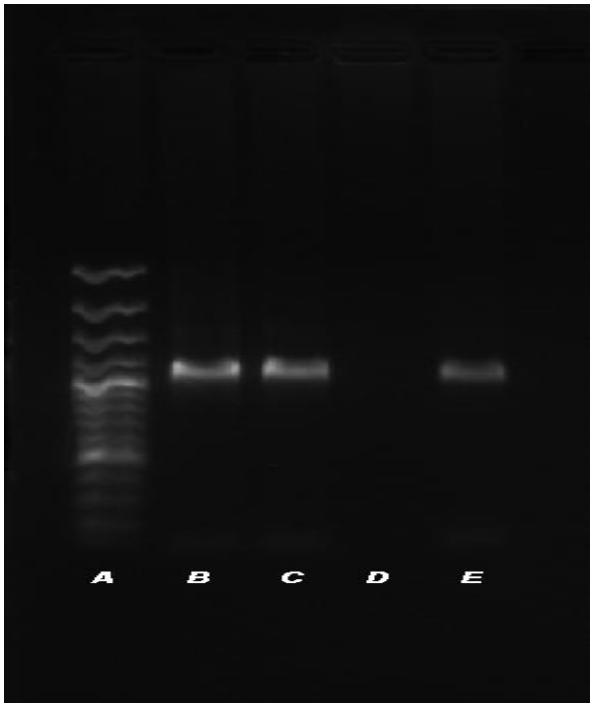

Figure 7 *Agarose gel electrophoresis stained with GelRed of PCR fragments amplified from microalgal genomic DNA: A) 100 bp DNA ladder, Fermentas, B)&C)Desired microalgae PCR product of the* T.chuii *strain, D) PCR negative control with genomic DNA omission, E) Positive control with a known DNA sample of* Nannochloris sp.

Upon amplification the DNA was sequenced and the obtained sequence of nucleotides was analysed by two distinct methods for phylogenetic analysis. The two phylogenetic trees built

using the online tool www.phylogeny.fr and using the 18SrDNA sequences downloaded from the BLAST tool of the NCBI nucleotide database, identified our algae strain as a *Tetraselmis chuii*(Figures 8 and 9) based upon the tree topology. For these analysis, the species selected to be compared alongside the one isolated in the laboratory are all from the *Tetraselmis* genus, an exception being the two *Chlorella vulgaris*18S rDNA sequences, which have been added to the phylogenetic tree as outgroups in order to have a rooted phylogenetic tree, more precisely to show a common node shared by the entire group of the *Tetraselmis* genus.

The first phylogenetic inference method used to identify our algal strain was the BioNJ algorithm (Figure 8). These results suggest that the microalgal strain used in this work belongs to the *Tetraselmis* genus, and more exactly corresponds to a *Tetraselmis chuii* green microalga: our isolated rDNA sequence, along with the two *T.chuii* DNA sequences downloaded from GeneBank, shared a significant common node, excluding all the other taxa here analysed. The two *Chlorella* strains are clustered in a single node with a branch support value of 1.0 from the other *Tetraselmis*18S rDNA sequences thus excluding the *Chlorella* genus as a probable taxonomical classification of the microalgae used in this work.

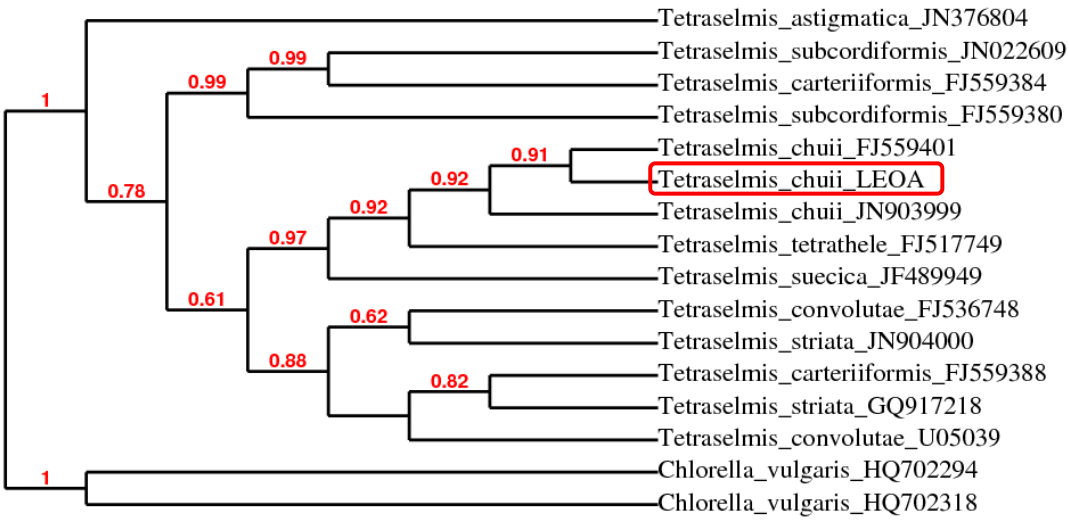

Figure 8. *Phylogenetic tree obtained using the Bio Neighbour Joining algorithm*

The second phylogenetic tree analysis, carried out with the PhyML algorithm (Figure 9), gave similar results: our algal strain is grouped in the same branch as the *T. chuii* DNA sequences downloaded from the NCBI nucleotide database, but they are sharing a common node with a *Tetraselmis tetrathele* and a *Tetraselmis suecica*, thus making it impossible to know for certain whether our species is *T.chuii*,*T. tetrathele* or *T. suecica* strain if the PhyML results were to be taken into account alone. Both *C. vulgaris* samples are also separated by one node, with a branch support value of 1, from all of the *Tetraselmis* genus taxa, confirming the result from the BioNJalgorithm.

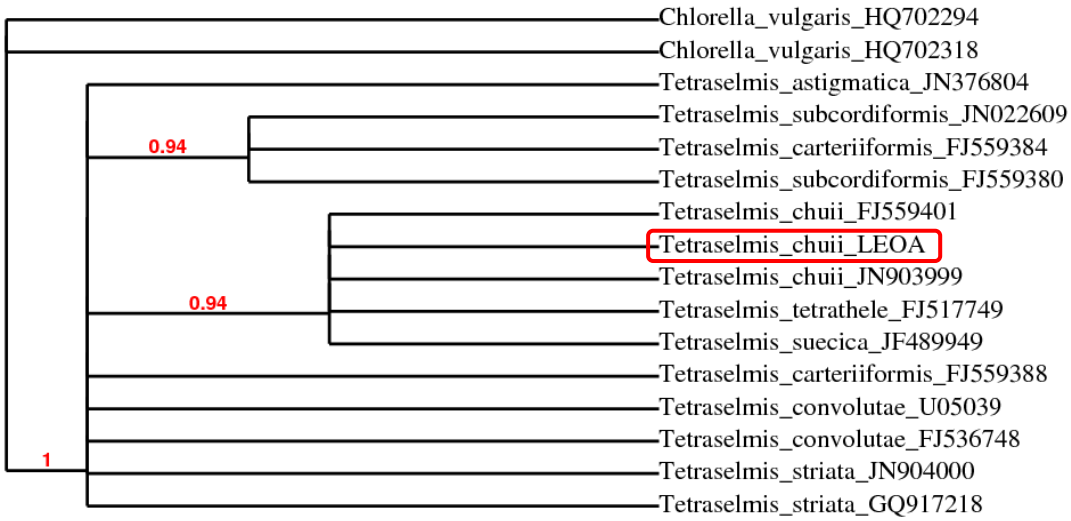

Figure 9.*Phylogenetic tree obtained using the PhyML algorithm*

4.2 Determination of the *T. chuii* culture growth curves at laboratory scale

T. chuii grown in batch cultures at laboratory scale presented a growth curve typical of microalgae cultures (Figure 10). The culture reached the stationary phase (1.9 x 10^6 cells/mL) approximately 18 days after the beginning of the culture (0.11 x 10^6 cells/mL), the specific

growth rate was calculated at 0.125(day^{-1}) and the doubling time was determined to be 5.5 (days).

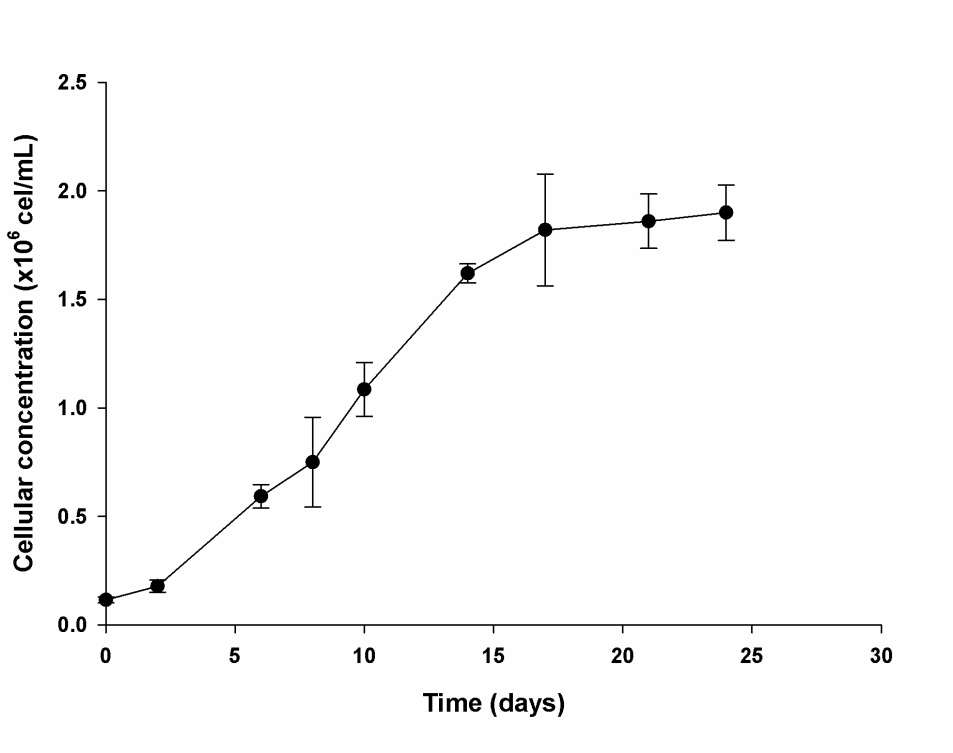

Figure 10.*Growth curve of* T. chuii *LEOA at laboratory scale*

In order to quickly assess the productivity of the bioreactor, tests that give the main characteristics of the culture must be made in an accurate and efficient way. For that reason, two conversion factors were established using the laboratory batch in order to simplify the work process. To assess the growth of the cultures, a correlation was determined between optical density and dry weight (Figure 11), and another between optical density and cellular concentration (Figure 12). The obtained linear correlations are highly significant with an r^2 of 0.969 for dry weight vs. optical density and an r^2value of 0.904 for cellular concentration vs. optical density.

In both cases, the linear correlations between the two parameters were valid throughout the entire growth period. Even though the optical density vs. cellular concentration correlation presented a slightly sigmoidal pattern, the linear correlation had a highly significant result and

was preferred to simplify the determination of the cellular concentration. The significant correlation coefficient between these measurements allowed for the estimation of the dry weight and cellular concentration from the measured optical density values.

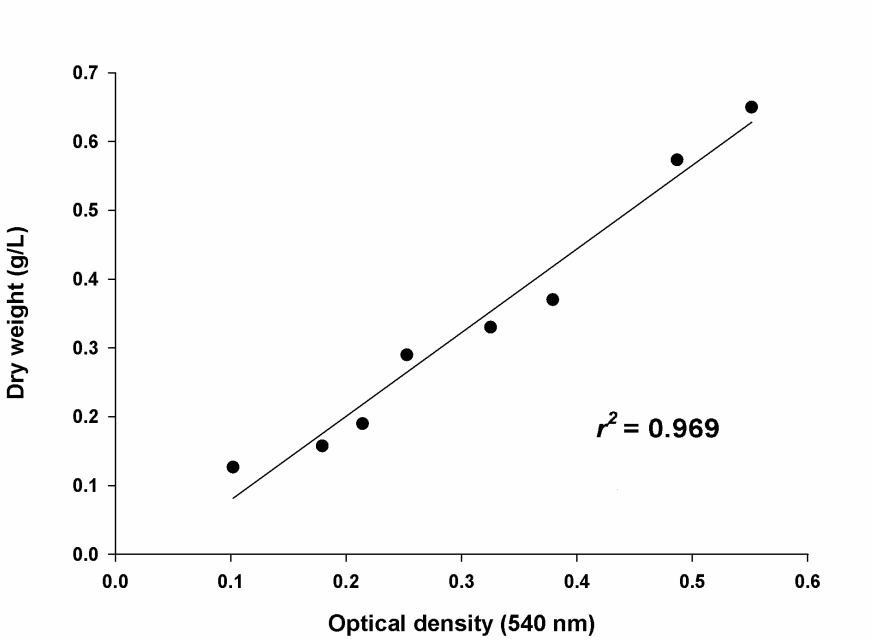

Figure 11. *Correlation between optical density and dry weight of* T. chuii *LEOA*

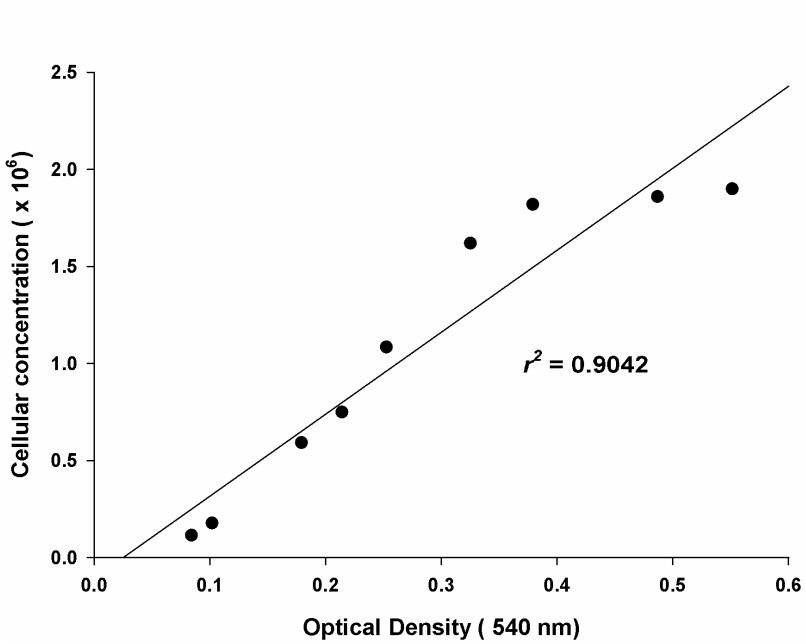

Figure 12. *Correlation between optical density and cellular concentration of the* T. chuii *LEOA*

Based on previous studies, which described the use of the Nile red dye to measure the lipid contents of microalgae, a correlation was also established between the values obtained from a modified version of the Nile red method (Chen *et al.,*2009) and the gravimetric method for lipid determination (Figure 13).

The different values were divided into groups referring to the different phases of the microalgal growth period at which they were measured in order to better determine the lipid concentration produced by the microalga, as this differs along the culture's life stages: lag phase (day 0 to day 2), early exponential phase (day 2 to day 5), exponential phase (day 5 to day 15), late exponential phase (day 15 to day 21), stationary phase (day 21 to day 25). Even though the values are grouped corresponding to the different growth phases, all of them were used in order to perform the correlation. Once more, the obtained correlation is significant with an r^2 of 0.823. This correlation coefficient, together with the linear trend presented during the entire growth period, made possible the use of a regression equation in order to assess the total lipids by the Nile red dye fluorescence of *T. chuii* cells.

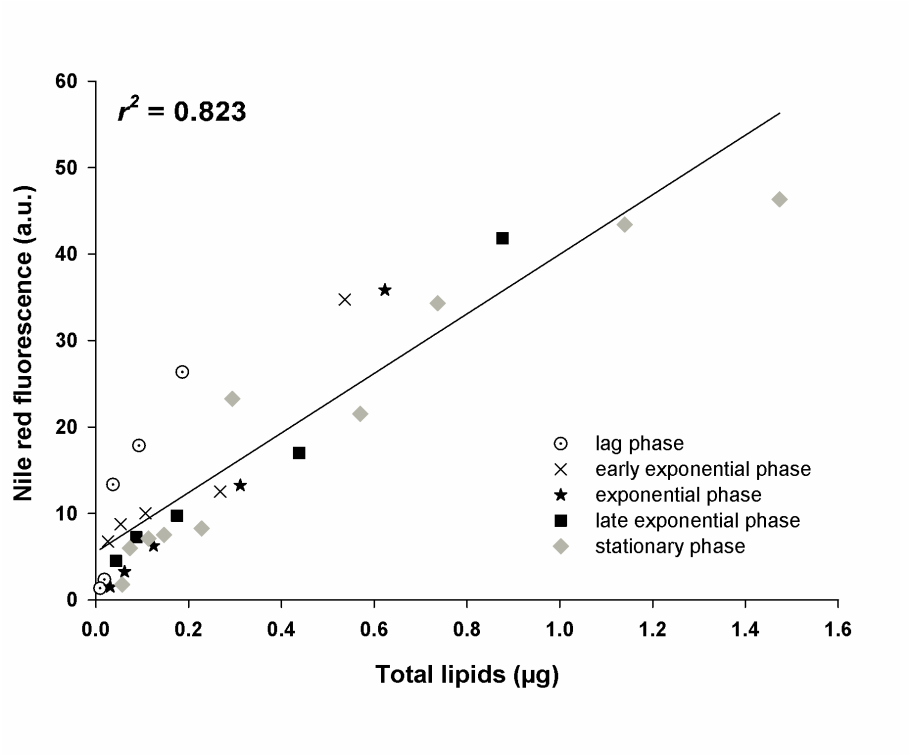

Figure 13 *Correlation between Nile red fluorescence and total lipids determined by the gravimetric method of the* T.chuii *grown in the laboratory*

The lipid productivity was calculated for day 21, which corresponds with the end of the exponential phase of the growth curve and when the harvesting of the microalgae would normally take place, at a value of 4.66 mg/L/day.

4.3 Characteristics of the microalgae grown in the photobioreactors

The photobioreactors were submerged in the pond and filled with 85 L of salt water at a salinity of 37 ppm, sterilized using the same method as in the laboratory batch, but with a quantity of 85 mL of bleach and $Na_2S_2O_3$ respectively. The inoculation was made with 15 L of laboratory grown *T. chuii* at concentrations of approximately 3 x 10^6 cells/mL.

Water samples were taken from the bioreactors daily at approximately 10:00 a.m. and analysed immediately. The following results represent the mean of only two replicate photobioreactors since one of them failed during the experiment due to, most probably, a fault in the plastic structure or in the sealing.The relatively large standard deviations that were measured between the bioreactors are probably due to the contamination of one of them, where there were noticeable lower values of cellular concentration, which in term affected the dry weight and the lipid percentage.

4.3.1 Physical and chemical measurements

The temperature sensor recorded the water temperature in the bioreactor (that presented the highest value of cellular concentration) every hour for the entire period of the trial. The sensor was later collected and the data recorded by it was downloaded and plotted (Figure 14).

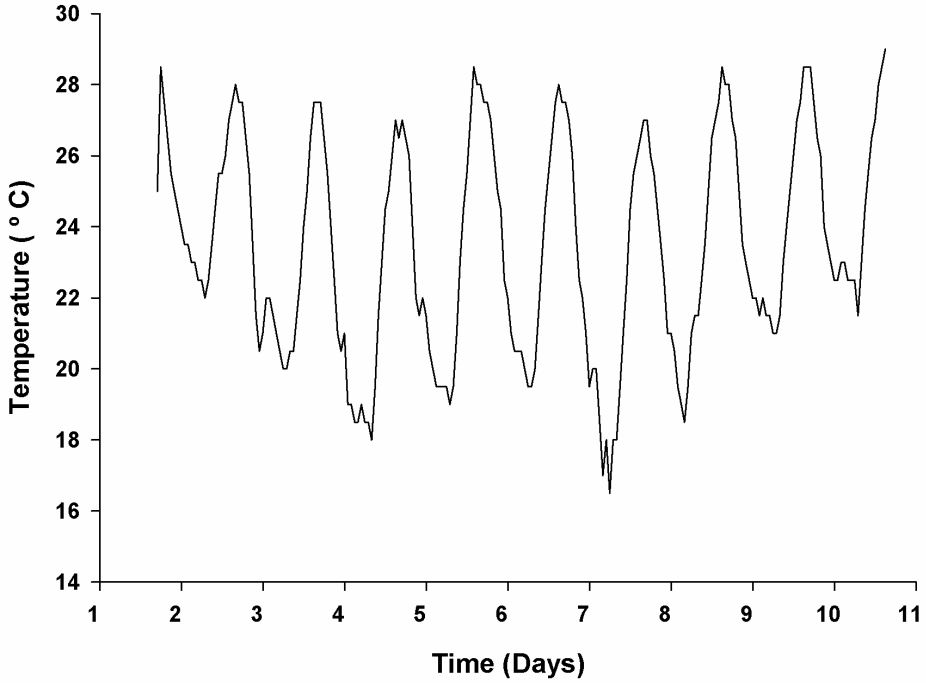

Figure 14. *Temperature variation recorded by the sensor inside one of the low-cost, custom-made photobioreactors constructed in this work*

Due to the fact that pH and salinity could also influence the growth and lipid content of the microalgae, these parameters were also measured during the experiment. Results are shown for the same bioreactor that had the water temperature measured (Figure 15).

The pH never attained values detrimental for microalgal growth, below 7 or over 12 (Thornton *et al*. 2010), and varied between 8.4 and 9.2 throughout the experiment, falling after the first day from a maximum value of 9.2 towards 8.4 in the third day, and then imitating a constant increase each day, whilst the salinity rose steadily during the microalgae growth period by almost 30%. Although autoclaved water was added to the photobioreactors to compensate for water evaporation, the amount of water added was probably under-estimated since the salinity still increased.

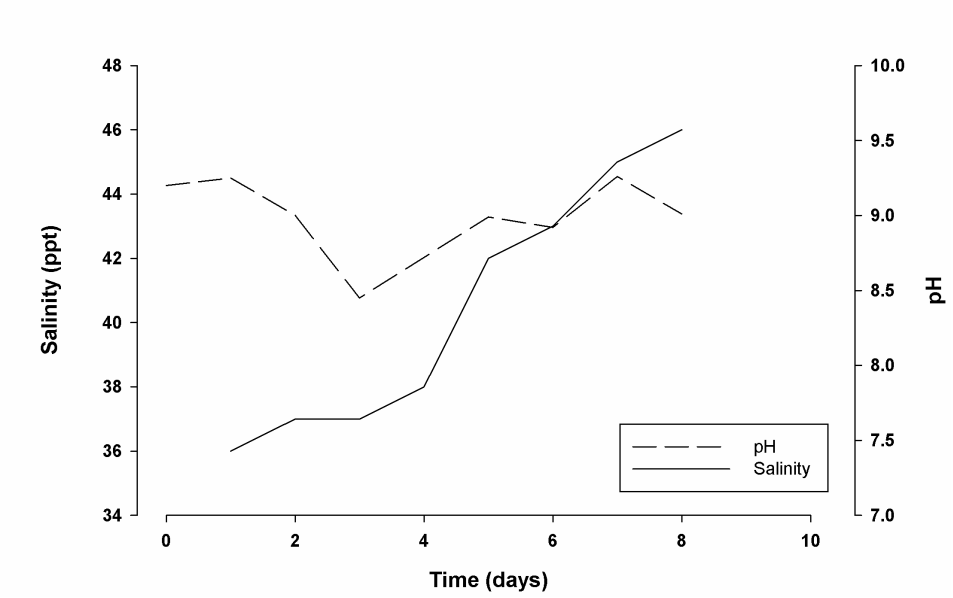

Figure 15.*Salinity and pH values inside the photobioreactor*

4.3.2 Growth curves

In case of the bioreactors, the cellular concentration growth curve is presented in Figure 16 superimposed on the laboratory growth curve presented in Figure 10.

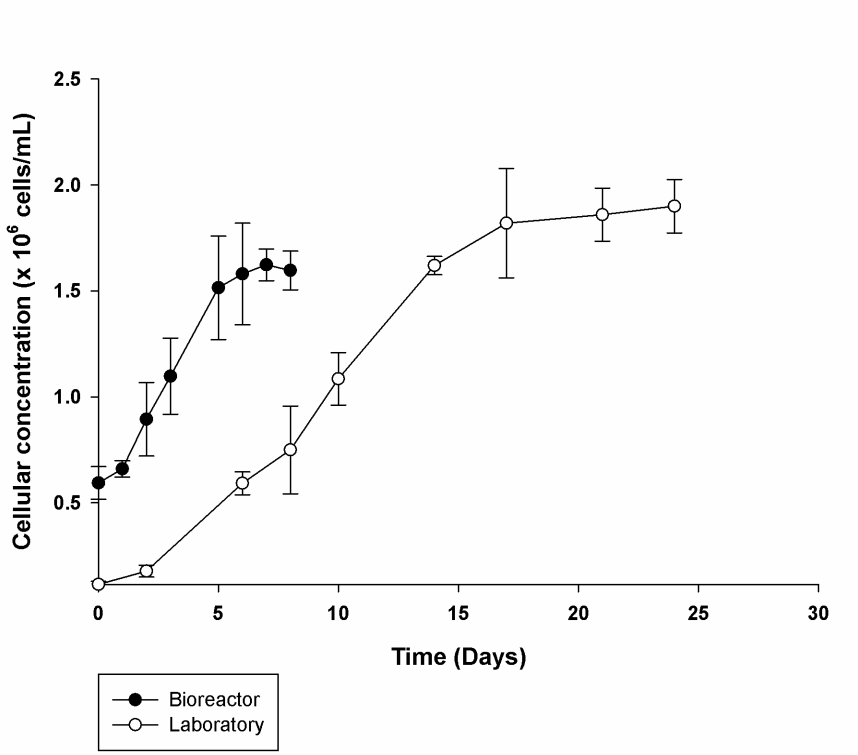

Figure 16.*Comparison between the cellular concentration of* T. chuii *LEOA in the laboratory and in the photobioreactor*

The cellular concentration values were determined using the regression equation calculated from the correlation previously established between the optical density and the cellular concentration of the microalgae grown in the laboratory. The plot in Figure 16 shows, in comparison to the laboratory grown batch, a very short lag phase, as the microalgae appeared to enter the early exponential phase at the beginning of the experiment and start the exponential phase of the growth at day 1. The late exponential phase was reached at day 5 and the culture stabilized upon reaching the stationary phase at day 7.The specific growth rate was calculated at 0.21 day^{-1}, for the microalgae grown in the bioreactor, and the division time had a value of 3.17 days.

The other relevant values determined for the bioreactor algal culture included the dry weight, the total lipids content and, based on these two, the lipid content in percentage. The dry weight was also calculated with the help of the correlation done in the laboratory batch

between the optical density and the dry weight of the culture. The dry weight followed the same growth trend of cellular concentration. That is, on the fifth day, the dry weight stabilized at 0.4 g L^{-1} (Figure 17).

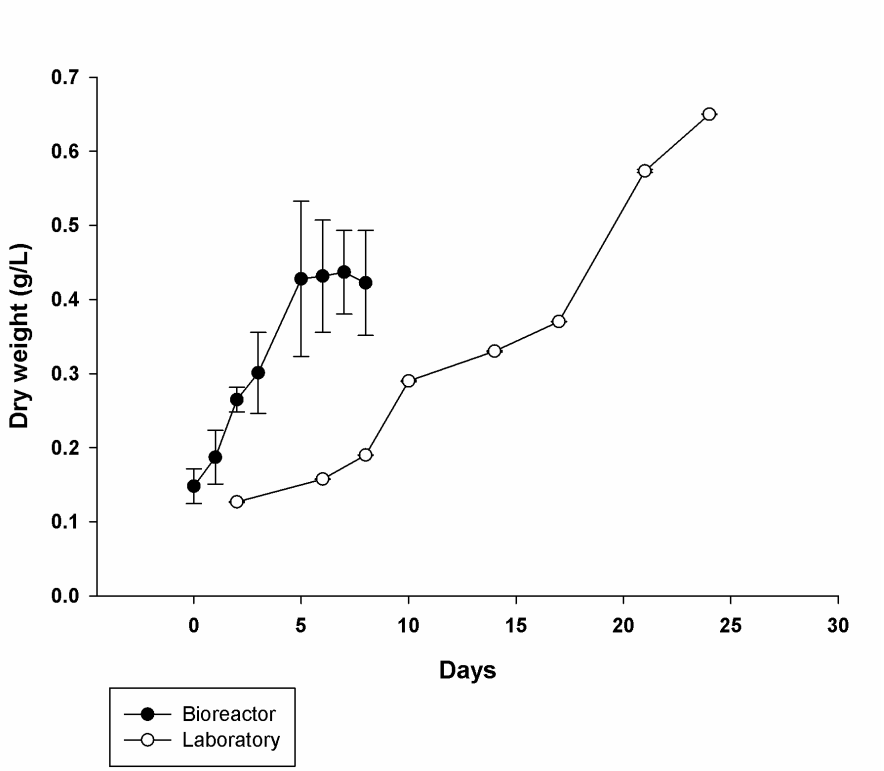

Figure 17. *Growth curve of* T. chuii *LEOA cultivated in the photobioreactor superimposed on the laboratory growth curve, showing the variation of the produced biomass (expressed as dry weight) during the time of the experiment. The individual values were determined according to the regression equation established from the optical density-dry weight correlation of the microalgae batch carried out in the laboratory.*

The total lipids were determined using the Nile red fluorescence method and the lipid content was deduced by calculating the percentage of lipids in the total dry weight of the culture for each day (Figure 18). The total lipids concentration remained stable during the first day at nearly 0.02 g L^{-1}. From day 1 onwards, lipid content exhibited a linear increase as the cellular

concentration did and continued to increase, reaching a value of 0.07 g L^{-1} at day 7, which coincided with the beginning of the stationary phase of the growth curve, and stabilized in the last day of the culture at a value of 0.06 g L^{-1}.

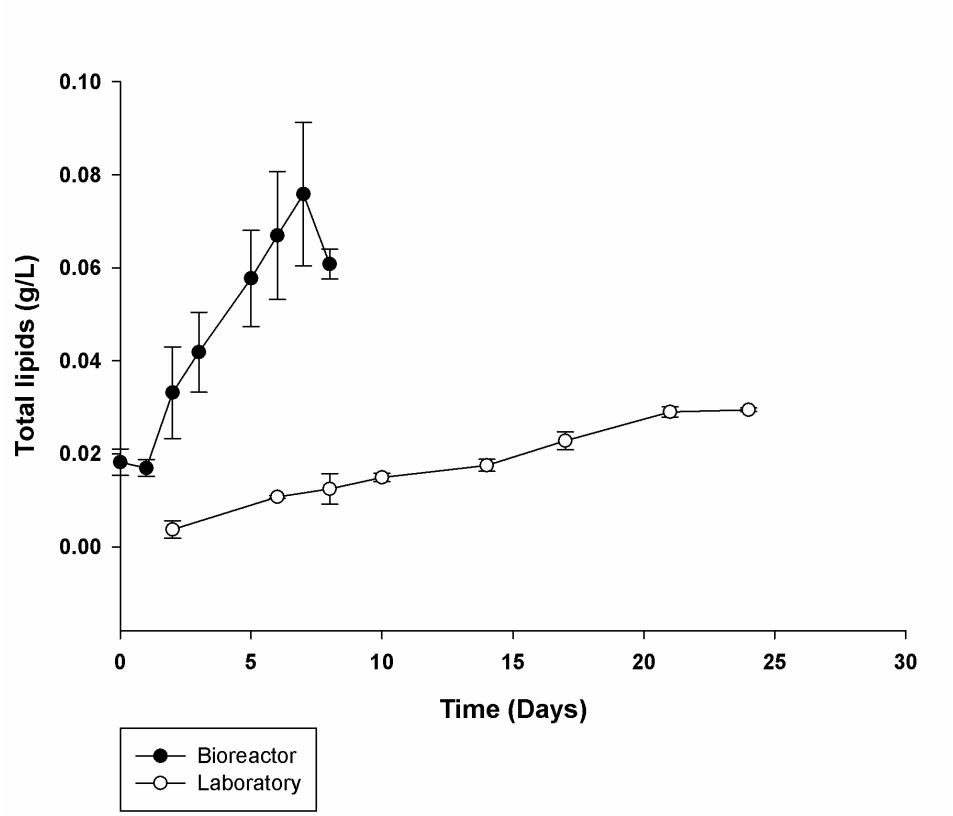

Figure 18. *Variation of the total lipid concentration of* T. chuii *LEOA grown in the laboratory and in the photobioreactor. Total lipids were determined according to the relationship between the Nile red fluorescence and lipid concentration measured by the gravimetric method established earlier.*

When expressed as a percentage of dry weight of biomass (Figure 19), the total lipids curve showed a decrease after the first day, down to 10%, and then presented two linear growth phases, between day 1 and 3, and another between days 5 and 7. It is also possible that the plateau in the middle could be an experimental error and the linear growth would be constant

between day 1 and 7.Upon reaching day 7, the total lipids represented almost 18% of the total dry weight, which then decreased to approximately 12% at day 8, the final day of the experiment.

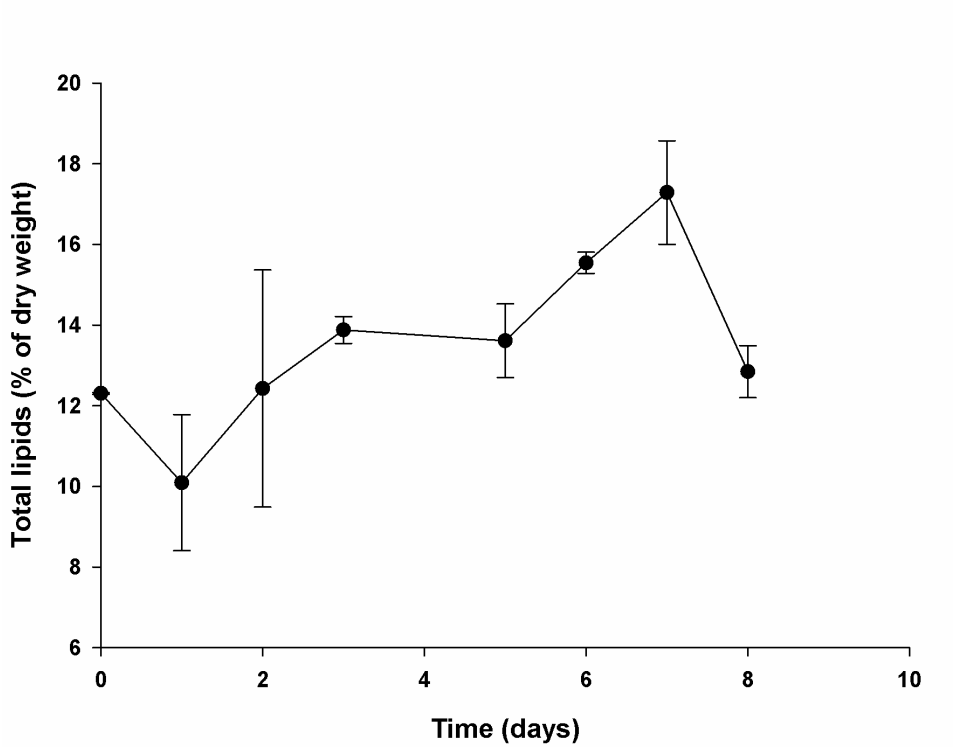

Figure 19. *Total lipid percentage of* T. chuii *LEOA grown in the photobioreactor.*

The lipid productivity was calculated for day 7 of the growth cycle, the last day of the exponential phase, at a value of 16.46 mg/L/day.

4.3.3 Assessment of fatty acid contents

Figure 20 presents the chromatogram obtained by GC/MS analysis of the oil extracted from the microalgal biomass cultivated in the photobioreactor upon derivatization.

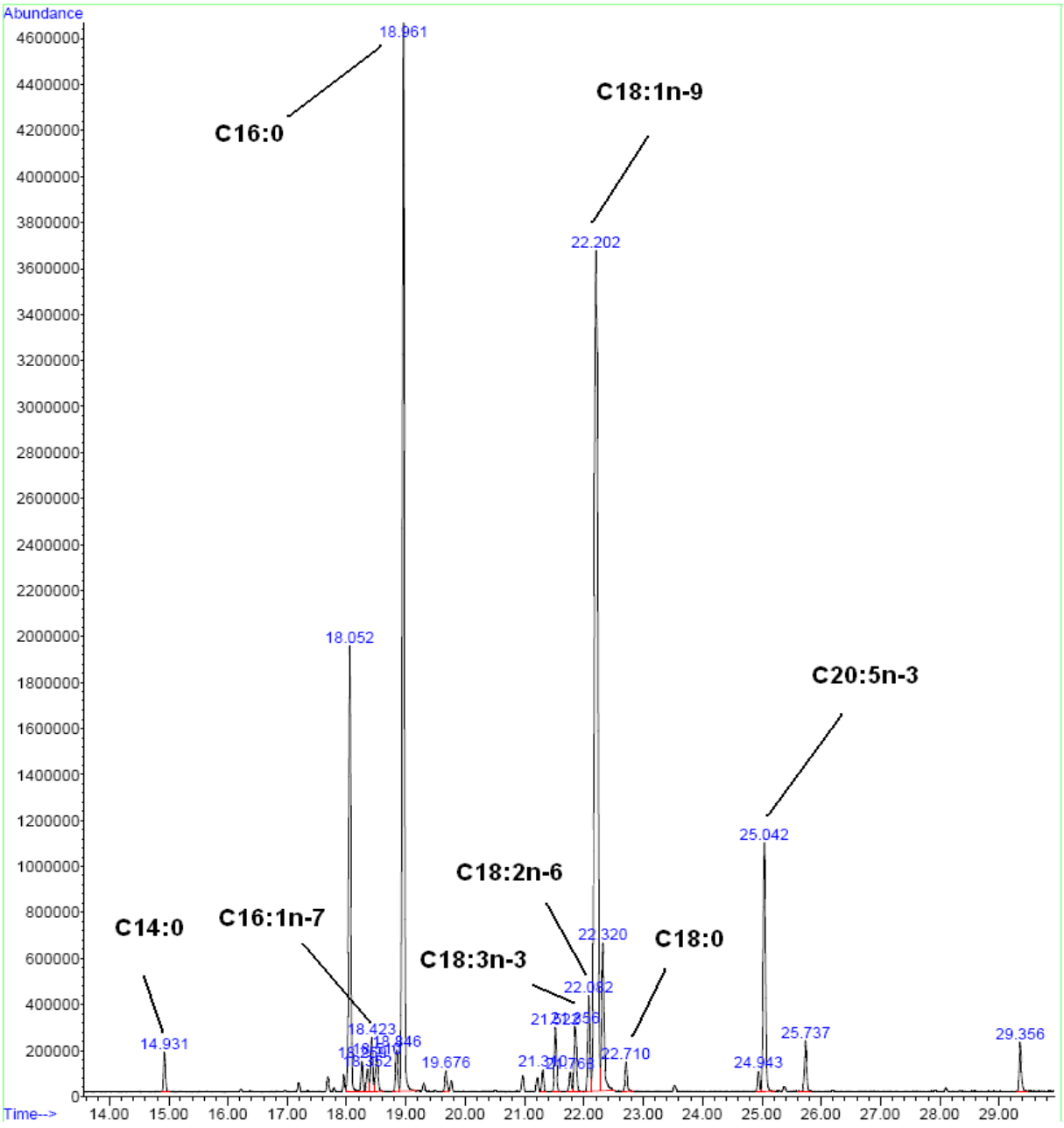

Figure 20 *Chromatogram of the GC/MS analysis showing the different peaks and their corresponding methyl esters recorded from the methanolic* T. chuii *extracts from the photobioreactor*

Eight fatty acid methyl esters (FAME) were identified and quantified in the lipid extracts of microalgal biomass (Figure 21). Palmitic acid (C16:0) and oleic acid (C18:1n-9) were the most abundant fatty acids (FA) and accounted for almost 90% of the total lipids present in the samples. Eicosapentaenoic acid (EPA, C20:5n-3) corresponded to nearly 5% of the total FAME. Although at lower percentages(less than 5% of the total FAME) myristic (C14:0), palmitoleic (C16:1n-7), linoleic (C18:2n-6), linolenic (C18:3n-3) and stearic (C18:0) acids were also detected in the samples.

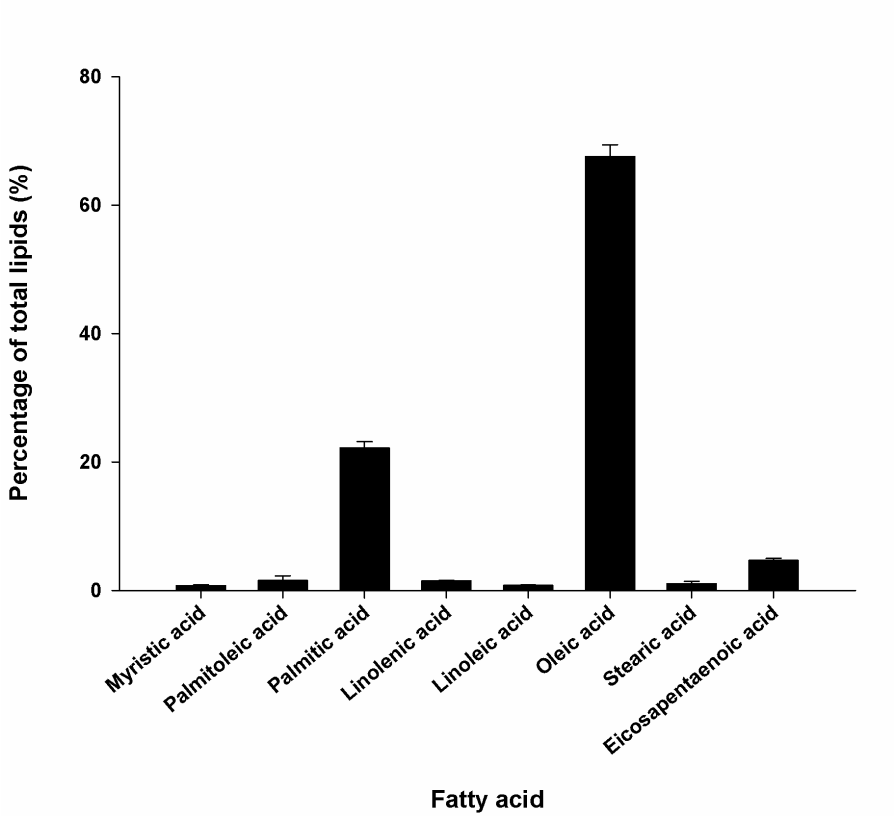

Figure 21. *Lipid composition of the* T. chuii *LEOA methanol extracts grown in the photobioreactor, according to the GC-MS analysis*

Overall, regarding the chemical composition of FAME, biodiesel should consist mainly of a mix of saturated and monounsaturated fatty acids, since polyunsaturated fatty acids are more prone to oxidation (Demirbas, 2009). The lipid content of the *T.chuii* culture consisted mainly of saturated fatty acids (myristic, palmiticand stearic acids) and monounsaturated fatty acids

(palmitoleic and oleic acids), corresponding to 23.97% and 69.09% of the total FAME composition respectively (Figure 22).The detected polyunsaturated fatty acids (linoleic, linolenic and eicosapentaenoic acids) presented a much lower percentage, 6.93% of the total FAME.

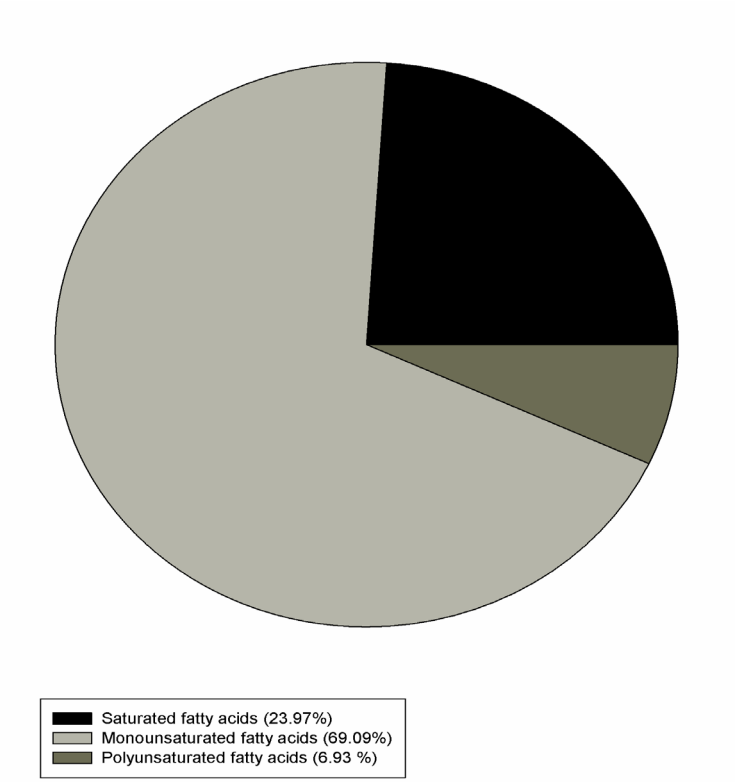

Figure 22. *Concentrations of saturated, monounsaturated and polyunsaturated fatty acids, expressed as % of the total FAME detected using the GC-MS in the methanolic extracts of the* T. chuii *LEOA grown in the photobioreactor*

4.3.4 Antioxidant activity of *Tetraselmis chuii* LEOA

The antioxidant activity of *T. chuii* LEOA was evaluated by measuring the radical scavenging capacity of the microalgal methanolic extracts towards the DPPH and ABTS radicals. The results of both assays are presented below (Figure 23).

The DPPH scavenging activity of the extracts increased with the extract concentration reaching values slightly higher than 30% of inhibition at 10 mg/mL (Figure 16B).The IC_{50} was not determined as the bioactivity was considered poor even for the extract with 10 mg/mL concentration, which did not reach 50% of inhibition. Concerning the scavenging capacity towards the ABTS radical, *T. chuii* extracts showed a higher RSA for the maximum extract concentration tested (Figure 16A; $p< 0.05$). Once more, the activity increased with the extract concentration, reaching 60% of inhibition in the 10 mg/mL extract. The IC_{50} value for the ABTS assay was calculated at a concentration of 7.97 mg/mL for the methanolic microalgal extract.

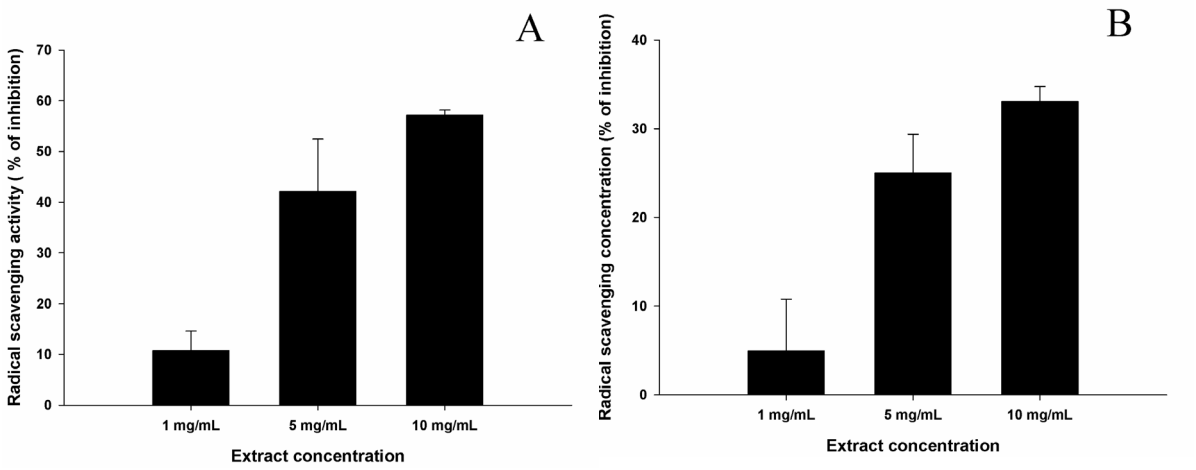

Figure 23.*Radical scavenging activity (% of inhibition) of the methanolic extracts of* T. chuii *LEOA biomass harvested from the bioreactor. A)Radical scavenging activity towards the ABTS radical; B).Radical scavenging activity towards the DPPH radical*

5 Discussion

According to the results of the 18S rDNA sequencing of our microalgae, it is clear that our selected species belongs to the *Tetraselmis* genus and, as shown by two different phylogenetic inference tools, is most likely *Tetraselmis chuii* because of the close relation with the other *T. chuii* DNA sequences analysed. Hori *et. al* (1986) place *Tetraselmis chuii* in the subgenus *Parviselmis* of the genus *Tetraselmis*, along with *T. striata*, *T. levis*, *T. suecica* and *T.alacris*. Our results are supported to some extent by this classification, as *T. suecica* is located on the same branch as the *T. chuii* strains in the PhyML phylogenetic tree and at a distance of 2 nodes in the BioNJ phylogenetic tree. Other studies that dealt with the phylogenetic analysis of the genus *Tetraselmis* using the 18S rDNA gene sequences also present results that can further confirm the validity of ours: Lee & Hur (2009), using a ClustalW2 alignment program and a Neighbour joining algorithm for building the phylogenetic tree, has found no differences between *T.carteriiformis FJ559388* and 11 other strains of *T.striata,* which explains why in the present paper these two species are clustered together on the same branch. More similarities with our phylogenetic tree are represented by: *T.carteriiformis FJ559384,* which is also grouped on the same branch with *T.subcordiformis FJ559380*; *T.tetrathele FJ517749* and *T.suecica,* which are branched together with *T.chuii*, and *T.convulatae,* which is found on the same branch with a *T.striata* strain. Another study (Arora *et al.*) constructed a phylogenetic tree where the two *T.chuii* strains used in the present paper as references (*FJ559401* and *JN903999*) were separated by *T.tetrathele FJ517749* and two other *T.suecica* strains by a single node with a branch support value of 0.99. The strain *T.carteriiformis FJ559384* is found together on the same branch with *T.subcordiformis FJ55938*, and a *T.convulatae* strain is branched together and grouped into the *T.striata* clade of the tree. Such phylogenetic analyses lead to the conclusion that the simple morphological identification of microalgae is usually not enough, and that species are easily misidentified, as in the case of

T.carteriiformis FJ559388.These two aforementioned studies underline the results of the phylogenetic trees constructed in the present work and firmly establish our microalgae strain as *Tetraselmis chuii*.

The microalgae grown in batch under laboratorial conditions presented a growth rate of 0.12, similar to the results obtained by Huerlimann *et al.* (2010), where a batch of *Tetraselmis sp.* exhibited growth rates of 0.14, 0.19 and 0.24 day-1,depending on the culture medium that was used.

There were important differences between the algae cultures grown in laboratory conditions and in the photobioreactor, which may affect the outcome of the desired biorefinery process. Firstly, the amount of time needed for the algae grown in the bioreactor to reach the stationary phase was about 3 times shorter than in laboratory conditions. The maximum cellular concentration in the bioreactor was, however, 10-15% lower than in the laboratory. The microalgae batch grown in the bioreactor presented a growth rate of 0.218 day^{-1}, significantly higher than the one calculated for the laboratory batch, of 0.125 day^{-1} ($p< 0.05$), and a significantly lower division time of 3.17 days compared to the value for the microalgae grown in laboratory conditions 5.50 days ($p< 0.05$).

Secondly, and perhaps the most important feature, the amount of total lipids per unit volume and the percentage of lipids per unit mass (dry weight) reached in the stationary phase was around 2–3 times higher in the bioreactor, a significant difference from the laboratory batch ($p< 0.05$). These two facts lead to the possibility of having small cultivation periods with a high lipid productivity, which would make *T. chuii* a good candidate for biodiesel production using this type of photobioreactor. The idea is that harvesting can be undertaken daily, removing a part of the culture and replacing with new culture media (seawater supplemented with Algal), allowing the harvesting of a significant quantity of biomass every day. Inside the reactor such a semi-continuous culture the microalgae could be maintained close to or at a

physiological state similar to a late exponential growth stage in a batch culture. This stage of the microalgae growth is optimal for harvesting, as it can be seen in Figures 18 and 19: the lipids are at the highest point, and by reducing the cell count and adding new nutrients and fresh water the maximum lipid yield can be obtained from the existing microalgae while maintaining the concentration of algal biomass inside the photobioreactor relatively constant at the beginning of each new day.

These results coincide with conclusions of other articles, supporting that algae accumulating higher lipid contents are found in environments and microclimates under frequent changes between optimal and suboptimal growth conditions, caused by wide temperature oscillation and high salinity (Schenk *et al.*, 2008; Rodolfi *et al.*, 2008; Converti *et al.*, 2009; Bruton *et al,* 2009).

The difference in lipids percentage between the laboratory culture and that grown in the photobioreactor can thus be explained by the stress factors to which the microalgae in the latter have been exposed. In contrast to the laboratory batch, where the temperature and light intensity were constant, the microalgae in the bioreactor experienced daily light intensity and temperature variations, which could have contributed to stressing them and in consequence improving their lipid production. This higher amount of lipids could be observed also by the much lighter green-yellowish colour of the algae, in comparison to the laboratory culture, which was of a dark green colour.

The results obtained in our bioreactor correspond to the ones published by Chisti (2007), where a batch of *Tetraselmis suecica* has been found to exhibit lipid contents between 15 and 23 percent of the total dry weight, but it must be taken into account that this level of lipids per dry weight is relatively small when compared to other green algae such as *Chlamydomonas reinhardtii*, *Dunaliella salina*, or different *Chlorella* species that can reach up to 60 percent of lipids per dry weight (Scott *et al.*, 2010).

In the case of large-scale microalgae production, the culture would have to be harvested at the peak of its lipid production, which usually corresponds to the last days of the exponential phase. The maximum lipid productivity was 16.46 mg L^{-1} day^{-1} (measured at the late exponential phase) for the microalgae grown in the photobioreactor, significantly different ($p < 0.05$) and almost 4 times higher than for the microalgae grown under laboratory conditions, which was just 4.66 mg L^{-1} day^{-1}. Both values, however, are lower than the ones presented by Huerlimann (2010), which obtained values of 18 and 22 mg L^{-1} day^{-1} for the cultures of *Tetraselmis* sp. grown using different mediums in carboys of 15 L inside the laboratory, and kept at a constant temperature of 24º C and a 12h light-dark stage, or when compared to Rodolfi *et al.*(2009) who obtained a lipid productivity of 43 mg L^{-1} day^{-1} within permanently illuminated 250 mL flasks, kept at a constant temperature of 25º C and with CO_2 enriched air. Taking into account the differences between the growing methods used in the current study and those used by Huerlimann and Rodolfi, the values presented by the named authors should be used only to characterize the *Tetraselmis* genus in terms of lipid productivity under various growth conditions and to determine the maximum obtainable yield.

This current trial was conducted in early June, when the night/day variation in temperature is comparable to the one recorded by the sensor. The fact that the bioreactor was floatable and suspended in a pond helped to reduce the temperature variations and thus the temperature inside the photobioreactor was maintained below 30ºC at all times, oscillating between 16 ºC and 28 ºC. The laboratory batches were not exposed to such variations, so temperature can be taken into consideration when speaking of stress factors that affected the microalgae. The floatability of these reactors and the possibility of being immersed in water is therefore a major advantage, as this is the cheapest way to help cool down a photobioreactor.

The pH varied around between 8.4 and 9.2 and no attempts were made to control them, as their influence on the culture growth was considered insignificant. These pH values are

explainable by the photosynthesis process, as algae consume the CO_2 dissolved in the medium, which leads to a pH increase, and because the water samples were taken in the morning, higher pH values would be expected later during the day. The variation could also be a consequence of the release of certain nutrients during the algae growth period, nutrients that affect the waters pH (Uusitalo, 1996). The salinity exhibited a rise towards the end of the growth period due to water evaporation. However, this increase coincided with the rise of total lipids within the culture, and according to Ghezelbash *et al.* (2008), salinities between 40 and 50 ‰, combined with a strong lightsource of 6500 lux (day-light is approximated at values between 10000 and 25000 lux), can explain the increase in biocompounds, such as carotenoids and proteins, produced by the algae and the loss of chlorophyll pigments.

The GC-MS analysis of the lipid profile of our microalgae culture gave different results than those published in current literature. In Meng *et al.*(2009) *T. chuii* presented higher values of some of the unsaturated fatty acids (in particular linolenic and linoleic acids) but also lower concentration of oleic acid than in the current work. The saturated palmitic and stearic fatty acids presented comparable values in both studies.

In a study that undertook the determination of the fatty acid composition of 10 different microalgae species (Pratoomyot *et al.*, 2005), a culture of *Tetraselmis chuii* was grown using the F/2 medium (Guillard, 1975) under a constant temperature of 28 ºC and a salinity of 30 ‰. The culture displayed 17.07% of SFAs, comparable to the present study, but much lower values for MUFAs (18.86%), due to the fact the oleic acids concentrations recorded were almost 6 times lower. It showed also much higher values for PUFAs (44.23%), mainly caused by a 10 times higher concentration of linolenic acid.

Compared to the values published by Huerlimann *et al.*(2010), where the microalga *T.chuii* was selected as the best choice for large scale culture for biomass and lipid production from 3 other microalgae (*Nannochloropsis* sp., *Isochrysis* sp. and *Rhodomonas* sp.) tested in different

growth media, some fatty acids measured in this paper are present at lower concentrations. For instance, palmitic acid is 20-30% lower than what was reported by Huerlimann and colleagues, and the polyunsaturated linoleic and linolenic acids are almost ten times lower. These authors reported, however, lower concentrations of eicosapentaenoic acid and oleic acid (C18:1n-9), which were present in concentrations of up to 2 times higher in the current study.

The low quantity of PUFAs inside the microalgal extracts represents a main advantage for biodiesel production, as the polyunsaturated fatty acids can make the fuel more susceptible to oxidation, but on the other hand they are necessary to a certain extent in the biodiesel composition to help maintain a low melting point (Schenk *et al.*, 2008).

According to Wittcoff *et al*. (2004), different derivatives can be obtained from crude biofuel. Depending on the number of carbons that compose each fatty acid methyl esters, different biofuels can be obtained. Industrially this separation is performed by fractionated distillation. The most suitable esters for biodiesel production range from C_{15}-C_{25} as far as the size of the carbon chain is concerned. The "ideal mix" of fatty acids for biodiesel production has been suggested to be C16:1, C18:1 and C14:0 in the ratio of 5:4:1 (Schenk *et al.*, 2008), but that could not be obtained from the current batch since the C18:1 was present in concentrations almost 30 times higher than C16:1 and C14:0 taken together.

According to the EN 14214 standard for FAME for diesel engines, the linolenic acid percentage should be lower than 12, a standard that the *T.chuii* methanolic extracts meet, but the percentages of all other polyunsaturated methyl esters with 4 or more double bonds should be less than 1, which makes the sole use of the *T.chuii* obtained in this study unsuitable for biodiesel production due to the highest percentage of the highly unsaturated fatty acid, EPA, which accounted for 4.71% of the total FAMEs analysed. However, this composition of fatty acids must not be viewed as a significant limitation, as there are methods for reducing or

increasing the percentage of particular FAMEs. Firstly, the fact that other studies, which used different growing methods for the *T.chuii* batch, presented different concentrations of FAMEs (Pratoomyot *et al.*, 2005; Meng *et al.*, 2009; Huerlimann *et al.*, 2010) can reinforce the idea that certain growth parameters and stress factors can influence the production of certain fatty acids, thus helping achieve a mix more suitable for biodiesel production. The partial catalytic hydrogenation of the microalgae oil is another possibility, and can reduce its extent of unsaturation and its content of fatty acids with 4 or more double bounds (Jang *et al.*, 2005; Dijkstra, 2006), but this would also increase the production costs. A much more promising approach to solving the problem regarding the high concentrations of certain FAMEs would be through genetic modification of microalgae, a important method used for altering biochemical pathways of the cells in order to produce more quantities of a specific fatty acid (Schenk *et al.*, 2008; Beer *et al.*, 2009; Meng *et al.*, 2009; Huang *et al.*, 2010), but this is a difficult process and would imply a higher cost.

The bioactivities that our algae extract were tested for showed low radical scavenging activity for the DPPH assay even at high concentrations of 10 mg/mL, with an average maximum RSA of 33%.The values were slightly lower, but still comparable, to the results obtained by Custódio *et al.* (2012), where *T. chuii* exhibited an RSA of 45% in the methanol extract, and emphasizes the fact that this particular microalgae strain is not suitable for being used as a dietary antioxidant supplement. However, better results were achieved with the ABTS assay, where the *T. chuii* batch showed an average RSA value of 57%. Taking into account the fact that the algae were not grown in laboratory conditions as in the article mentioned above, this could explain the observed differences in RSA. There were significant differences between the ABTS and the DPPH assays at concentrations of 10 mg/mL ($p< 0.05$), but at concentrations of 1 mg/mL and 5 mg/mL no significant differences were obtained between the two antioxidant tests ($p> 0.05$). Because the same strain of *T.chuii* was used in the present paper and in the paper by Custódio *et.al* (2012), the present results are reasons to speculate

that our microalgae batch exhibited lower antioxidant activity because of the growth conditions and the stress it was subjected to, and that higher RSA values for the ABTS and DPPH assays can be obtained in the laboratory using the parameters described in the aforementioned study. The results also contradict Ghezelbash *et al.* (2008), which showed that, under stress conditions related to increased salinity and a strong light source, *T. chuii* increases the production of carotenoids, but because the DPPH assay is not the best at measuring carotenoid RSA (Muller *et al.*, 2011), additional antioxidant assays should be performed, such as the oxygen radical absorbance capacity (ORAC) assay, the Folin-Ciocalteu method or the Trolox equivalent antioxidant capacity (TEAC) assay (Prior *et al*, 2005) before drawing a final conclusion on the antioxidant properties of *T. chuii* LEOA under outdoor growth conditions. Although this strain did not present a strong anti-oxidant activity, Custódio *et. al* (2012) demonstrated other biological activities, such as AChE inhibition, an enzyme which is linked with the development of Alzheimer's disease, and iron chelating activity, a metal which has also been implicated in the development of neurological diseases, however the lower concentrations of antioxidants present in the microalgae batch grown in the bioreactor could also indicate that the overall production of bio-active compounds could be inhibited.

The large standard deviations obtained between the two photobioreactors can reinforce the idea that growing a microalgae strain, such as *T. chuii,* in open ponds systems is not a feasible option because of the competition with other microalgae or micro-organisms, which can, in the least, lower the productivity of the strain if not surpass them altogether in cellular concentration values.

6 Conclusions

In order to maintain a low cost biofuel production, taking into account the high overall prices of production of biodiesel from microalgae in comparison to petroleum based fuels, the biorefinery concept must be implemented. By definition, a biorefinery should, in parallel to the biofuel production, be able to create bio-based chemicals, heat and power.

Biorefineries can be classified according to their technologies and the by-products they produce into: first generation biorefineries (FGBR), second generation biorefineries (SGBR), third generation biorefineries (TGBR) and fourth generation biorefineries, with TGBR referring to those who use algae as feedstock (Demirbas, 2010). After oil removal from the microalgae, the remaining biomass can be further processed to methane or livestock feed, burned for energy generation, used as a fertilizer or biocompounds can be extracted for pharmaceutical, cosmetics or other such purposes (Wang *et al*., 2008).

At this point it can be stated that the result from the bioactivities along with the lipid percentage and profile found in this microalgae batch does not give a promising prospect for the biorefinery, and thus not a viable option in producing biodiesel. Despite these drawbacks regarding *T.chuii* LEOA strain, the photobioreactor proved to be successful in the growing of the microalgae at a higher rate than in the laboratory.

The implementation of such bioreactors in a larger scale would imply initial investments for quality equipment, as there were problems encountered during the experiments in this work regarding the quality of the plastic and the sealing, which resulted in the contamination of several algae cultures. The reactors should be constructed of a higher density plastic, but still sufficiently transparent to allow enough sunlight to pass through and also lightweight enough to maintain the floatability of the bioreactor. The thermo-sealing is still the most adequate way of constructing the bioreactor, as glues could prove toxic and interfere with the growth of

the microalgae. The air tubing should also be improved, as there were portions on the backside of the bioreactors to which algae attached because of improper airflow.

Although the bioreactors showed the desired effect, that of growing microalgae with high lipid contents in a relatively short period of time, it must be taken into account that these high lipid values, when compared to the ones obtained in the laboratory, are likely due to stress factors and more experiments should be performed in order to determine which one of these has an influence on lipid production, or whether it is a combination of two or more such parameters.

The lipid profile of the *T. chuii* strain grown during this study was slightly different from other studies regarding this microalga, presenting a higher concentration of oleic acid (C18:1), but the low concentrations of palmitoleic (C16:1) and myristc (C14:0) acids do not help in achieving the 5:4:1 ratio of C16:1, C18:1 and C14:0, which is considered optimal for biodiesel production (Schenk *et al.*, 2008). The high concentration of eicosapentaenoic acid, a PUFA with more than four double bonds, is to be considered another drawback since this PUFA is present at a concentration not allowed by the international standards dealing with biodiesel from bio-feedstock. Other studies dealing with the growth and lipid contents of *Tetraselmis sp.* also obtained high overall concentrations of PUFA's (Pratoomyot *et al.*, 2005; Huerlimann *et al.*, 2010). It can be concluded, that without certain techniques such as the partial catalytic hydrogenation of the microalgae oil or genetic engineering of the microalgae, techniques used to lower the content of the polyunsaturated fatty acids, the lipid contents of *T.chuii* alone are not suitable for biodiesel production.

The procedures that can be used on the lipid extract in order to transform them to better suit the biodiesel industry bring the focus back on trying to establish a growth technique to maximize the lipid and bio-compounds contents. In the present case, the average lipid concentration compared to other microalgae strains makes the *T. chuii* strain an unsuitable

candidate for a large-scale bio-refinery implementation though further studies should be conducted. Even though the *T. chuii* LEOA strain showed promising results against free radicals in the ABTS assay, more antioxidant tests should be performed in order to fully assess microalgae radical scavenging activity. Moreover, this strain should be studied also for different biological activities, namely anti-bacterial, anti-viral, anti-inflammatory, anti-tumoral, among others, since microalgae are a known source of this bio-compounds (Guedes *et al.*, 2011).

Taking into account the huge biodiversity of microalgae, more rigorous research should be undertaken in order to find a species, which can provide higher lipid contents, and promising results regarding different bioactivities in order to obtain a multi-purpose microalgal biomass, an essential starting point for the establishment of sustainable microalgal-based biorefineries for the manufacture of biodiesel and ancillary products.

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