Universidade do Algarve

Physiology and biochemistry of the effect of abiotic stress on the autochthonous CTP4 strain, a candidate microalga for biofuel production in Algarve

Eunice Maria Filipe Silva Santos

Dissertation

Marine Biology Master

Supervisors: Prof. Dr. João Varela

Prof. Dr.ª Luísa Barreira

2014

Universidade do Algarve

Physiology and biochemistry of the effect of abiotic stress on the autochthonous CTP4 strain, a candidate microalga for biofuel production in Algarve

Eunice Maria Filipe Silva Santos

Dissertation

Marine Biology Master

Supervisors: Prof. Dr. João Varela

Prof. Dr.ª Luísa Barreira

2014

Physiology and biochemistry of the effect of abiotic stress on the autochthonous CTP4 strain, a candidate microalga for biofuel production in Algarve

Declaração de autoria de trabalho

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

_____

(Eunice Maria Filipe Silva Santos)

Agradecimentos

Este foi um ano difícil, com muito trabalho e pouco tempo. Pouco tempo para a família, para os amigos e até pouco tempo para tanto trabalho. Já o esperava, mas não foi por o esperar que se tornou mais fácil. Foi difícil, muito difícil mas consegui, conseguimos. Muito obrigada ao Professor João Varela e à Professora Luísa Barreira por tudo o que me ensinaram, por todo o apoio, e pela oportunidade de poder trabalhar neste grupo fantástico que é o MarBiotech. Obrigada a todo o grupo, agradecer a cada um de vós individualmente daria outra tese; agradeço por todos os momentos que passámos juntos, por me deixarem com um sorriso na cara mesmo quando ainda tinha uma noite de trabalho pela frente, lembrarei com alegria todos os momentos que passámos juntos e não esquecerei as amizades que travei.

Ao Hugo Pereira, o "nosso mini chef", agradeço por tudo o que fez por mim, muito do que aprendi, a nível laboratorial e não só, a ele o devo; agradeço-lhe ainda a preocupação, todos os emails de madrugada e as ligações via Skype ao fim de semana.

Agradeço à Dr.ª Filomena por todo o material disponibilizado, aos técnicos superiores André Mozes e Cláudia Florindo pela disponibilidade e paciência. Não posso deixar de fazer um agradecimento especial à técnica superior Vera Gomes por todo o material disponibilizado, por todos os momentos em que me ajudou a erguer a cabeça, por ter sempre um "cantinho" para utilizarmos e a *hotte* disponível.

À Sara Costa, não podia deixar de lhe agradecer por todo o companheirismo e por ser a minha ouvinte tantas e tantas vezes.

À Catarina Duarte, Carla Lourenço, ao Rúben, à Eva e ao Diogo, por nos terem acompanhado neste ano difícil, principalmente nesta reta final, obrigada por nos tirarem de casa. Talvez graças a esses poucos mas intensos momentos com vocês mantive até hoje a minha sanidade mental.

Ao Ivo Monteiro, não tenho palavras para lhe agradecer tudo o que fez por mim, todo o companheirismo, todas as noites sem dormir, todos os ensinamentos, esclarecimentos, toda a paciência quando eu sou uma chata. Obrigada por estares sempre aqui quando eu preciso de desabafar ou apenas de ralhar, ou chorar, só porque estou stressada. Bem sei que trabalhar comigo não é fácil, fico feliz por termos passado por isto juntos.

Por fim, mas não menos importante, a toda a minha família, aos meus pais por tornarem tudo isto possível, pela preocupação pelas noites mal dormidas: à minha mãe por me ouvir todas as noites naquelas conversas de 20 minutos ao telefone que me ajudavam a espairecer nas semanas em que as saídas de casa significavam apenas uma entusiasmante ida ao Algartalhos; e ao meu pai por acreditar em mim, mesmo crendo muita vezes que, nas suas palavras "isto era uma tonteira". Ao meu irmão, às minhas avós e à minha tia por se lembrarem de mim muitas vezes mas telefonarem poucas com receio de me incomodar. Não posso deixar de lembrar a minha tia-avó Maria do Rosário, sinto muito que não me tenha visto chegar aqui mas sei que, onde quer que esteja, está muito orgulhosa de mim. Desculpem pelos meses de ausência mas creio que valeu a pena. Avó Maria tenho a certeza que todas as tuas orações foram importantes para me ajudarem a chegar aqui.

Foi muito difícil mas conseguimos, inclua-se toda a gente mencionada acima, nada se faz sem sacrifício, nada se consegue sozinho e nada que valha a pena é atingido sem abdicar de algumas coisas.

Se hace camino al andar (Antonio Machado)

Abstract

Because of the limited stocks of fossil fuels it is imperative to find renewable sources of energy. Microalgal-based biofuels are promising alternative forms of energy. However, research to isolate highly productive microalgae is urgently needed in order to turn the production process into a feasible and profitable option. CTP4 is a green microalga originally isolated from effluents of a sewage treatment plant in Algarve that held promise to be used as feedstock for biofuel production, as it showed a lipid content of 20% of dry weight (DW) under non-inductive conditions and was able to grow in sewage. In the present work, evidence is shown that this microalga is highly resistant to stress, remaining viable at temperatures ranging from 5 ºC to 35 ºC and photon flux densities (PFD) of 100 to 400 µmol m^{-2} s^{-1}. In addition, lipid-induction experiments showed that CTP4 contains high lipid content values for biodiesel production ranging from 19.4% to 40.2 % of DW. The most successful lipid induction factor was light intensity (400 µmol m^{-2} s^{-1}) combined with nitrogen depletion at the 7^{th} day of induction (40.2% of DW). This higher lipid content was later confirmed by fluorescence microscopy using the lipid dye BODIPY 505/515. As the economical feasibility of microalgal biofuel production may be improved by a high-value co-product strategy, the bioactivity profile of CTP4 was determined. Antioxidant activity was evaluated by radical scavenging activity (RSA). All extracts showed a higher RSA at the maximum concentration tested (10 mg mL^{-1}), reaching 87.7% of inhibition with the methanol extract. Hexane extracts from algae under either nutrient depletion or nutrient supplementation showed medium anti-hyperglycaemic activity at the concentration of 1 mg mL^{-1}. Overall, this strain appears to be very promising for the production of biodiesel and bioactive compounds ensuring a profitable biodiesel production.

Keywords: biofuel; microalgae; lipid induction; antioxidant activity; anti-diabetic activity; FAME.

Resumo

Os combustíveis fósseis são recursos finitos. Como tal é imperativo encontrar fontes renováveis de energia. Os biocombustíveis de microalgas são uma promissora forma de energia alternativa. No entanto, é urgente encontrar e produzir microalgas mais produtivas para tornar o processo de produção numa opção viável e rentável. Recentemente o grupo MarBiotech isolou uma microalga verde, através de sorteamento de células ativada por fluorescência, mostrou ser capaz de crescer em efluentes de ETAR e possuir um conteúdo lipídico de 20% em condições não indutivas. Uma vez que esta estirpe ainda não identificada até ao género/espécie, ela foi denominada CTP4.

Neste trabalho, esta microalga foi submetida a stress abiótico, nomeadamente a temperaturas altas e baixas (35 ºC e 5 ºC, respetivamente) e a elevada intensidade luminosa (200 e 400 µmol m^{-2} s^{-1}) sob condições de depleção e suplementação de nutrientes. As culturas usadas como controlo foram crescidas a 20ºC e a uma densidade de fluxo de fotões (PFD) de 100 µmol m^{-2} s^{-1}.

Esta microalga foi cultivada à escala laboratorial, sendo o seu crescimento medido a cada dois dias através da concentração celular e do peso seco. De modo a tornar mais expedito a determinação do crescimento da cultura, a densidade ótica medida a 540 e 750 nm foi positivamente correlacionada com a concentração celular e com o peso seco. Assim, foram traçadas as curvas de calibração para cada correlação, tanto para a densidade ótica com a concentração celular, como para a densidade ótica com o peso seco. O conteúdo lipídico foi determinado através do método gravimétrico Bligh & Dyer (1959) e foi positivamente correlacionado com a fluorescência do vermelho do Nilo, de modo a facilitar a experiência da indução lipídica.

As culturas demoraram cerca de 11 dias a atingir a fase estacionária ($2{,}7x10^{6}$ células mL^{-1}), tendo o inóculo inicial uma concentração de $2x10^{5}$ células mL^{-1}.

Depois da obtenção das curvas de calibração, as culturas foram stressadas e cultivadas durante 7 dias num sistema de crescimento bifásico, após o início da fase estacionária, de modo a permitir que as células atingissem a máxima concentração celular possível. A acumulação de lípidos foi assegurada pela imposição de stress abiótico, nomeadamente variações da temperatura, intensidade luminosa e adição ou depleção de nutrientes. Um padrão registado em todos os ensaios foi o efeito da depleção de nutrientes, o que levou a uma maior acumulação de lípidos em todas as culturas (19,4-40,2%) quando comparadas com culturas com suplementação de nutrientes sob as mesmas condições de indução (8,3-24,4%). O fator de indução mais promissor foi a

intensidade luminosa a 400 µmol m^{-2} s^{-1}, sob depleção de nutrientes ao 7º dia (7,3%-40.2%). Estes resultados obtidos com o vermelho do Nilo foram depois comprovados por microscopia de fluorescência usando o fluorocromo solvatocrómico BODIPY 505/515. Como esperado, existe uma maior quantidade de gotas lipídicas nas culturas sob stress abiótico.

As microalgas têm a capacidade de produzir vários compostos bioativos de elevado valor. Assim sendo, a viabilidade económica da produção de biocombustíveis de microalgas pode ser melhorada por co-produtos de elevado valor, os quais poderiam ser extraídos da biomassa colhida. Deste modo, a produção de biocombustíveis e co-produtos de elevado valor poderia ser realizada de uma forma rentável e ecologicamente sustentável. Em consequência, a última parte do trabalho prende-se com o perfil bioativo e o processo de colheita da biomassa da microalga em estudo. Para isso, foram cultivadas 2 mangas de 100 L durante 18 dias, com suplementação e depleção de nutrientes e a sua colheita foi feita através da interrupção do arejamento por 48 horas e posterior centrifugação da restante biomassa. Os extratos foram preparados após liofilização do material colhido, com acetona, metanol, acetato de etilo e hexano, sendo posteriormente dissolvidos em DMSO às concentrações de 1, 5 e 10 mg mL^{-1} para a realização de ensaios relativos às atividades antioxidante e de 1 mg mL^{-1} para a atividade antidiabética.

A atividade antioxidante foi avaliada através da atividade sequestradora de radicais com radicais de ABTS. Todos os extratos mostraram uma atividade sequestradora de radicais elevada para a concentração máxima (10 mg mL^{-1}), atingindo 87,7% de inibição com o extrato de metanol a uma concentração de 10 mg mL^{-1}.

A atividade antidiabética foi investigada a partir da inibição da enzima α-amilase, responsável pela hidrólise do amido, reduzindo a absorção de glucose. Os extratos de hexano, tanto de culturas sujeitas a depleção como a suplementação de nutrientes mostraram valores de inibição superior a 1, o que sugere atividade inibitória. Por outro lado, todos os outros extratos apresentaram valores inferiores à unidade, representando uma atividade estimuladora da amilase.

Em conclusão, estes resultados indicam que a estirpe CTP4 é uma microalga promissora para a co-produção de biodiesel e compostos bioativos, os quais poderão ser utilizados para tornar rentável o processo de *scale-up* e produção em larga escala.

Palavras-chave: biocombustível; microalgas; indução lipídica; atividade antioxidante; atividade antidiabética; EMAG.

Abbreviations

A.I. – Amylase inhibition

ABTS – 2.2′-azino-bis (3-ethylbenzthiazoline-6- sulphonic) acid

ANOVA – Analysis of variance

BHT – Butylated hydroxytoluene

BODIPY – 4.4-difluro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene

CO_2 – Carbon dioxide

DAF – Dissolved air flotation

DIC – Differential interference contrast

DMSO – Dimethyl sulphoxide

e.g. – For example

FA – Fatty acids

FAME – Fatty acid methyl esters

FGB – First-generation biofuels

FITC – Fluorescein isothiocyanate

GC-MS – Gas chromatography with mass spectrometry

HC – Hydrocarbons

KNO_3 – Potassium nitrate

MUFA – Monounsaturated fatty acids

N^- – Nutrient depletion

n.d. – Not detected

N^+ – Nutrient supplementation

NIST – National Institute of Standards and Technology, U.S. Department of Commerce

NO_x – Nitrogen oxides

OD – Optical density

PAR – Photosynthetically active radiation

PBR – Photobioreactor

PFD – Photon flux density

PUFA – Polyunsaturated fatty acids

RSA – Radical Scavenging Activity

SFA – Saturated fatty acids

SGB – Second-generation biofuels

SO_x – Sulphur oxides

TAG – Triacylglycerol

TGB – Third-generation biofuels

Index

Agradecimentos II
Abstract V
Resumo VI
Abbreviations VIII
1. Introduction 15
1.1. Energetic demand – fossil fuels as energy sources 15
1.2. Biofuel 16
1.3. Microalgae as a feedstock for biofuel production 17
1.4. Lipids for biofuels production 19
1.4.1. Nutrient stress 20
1.4.2. Thermal stress 22
1.4.3. Light intensity stress 22
1.5. Microalgal biodiesel production 23
1.5.1. Microalgal cultivation and harvesting 23
1.5.2. Oil extraction 25
1.5.3. Transesterification 25
1.6. Biorefinery 26
1.7. CTP4 strain 28
2. Aims 29
3. Materials and methods 30
3.1. CTP4 growth 30
3.1.1. Growth of CTP4 cultures under non-stressed conditions 31
3.1.2. Growth of cultures under stress conditions 32
3.1.3. Cultivation in 100-L bags for bioactivity determination 33
3.2. Determination of growth parameters 34
3.2.1. Optical density 34
3.2.2. Cellular concentration 35
3.2.3. Dry weight 36
3.3. Determination of lipid content 36
3.4. Evaluation of nitrate concentration in the medium 37
3.5. Determination of the lipid profile 38
3.6. Microscopy 41

3.7. Bioactivity determination 41
3.7.1. Extract preparation 41
3.7.2. α-Amylase inhibitory activity 41
3.7.3. Antioxidant activity - Radical scavenging activity (RSA) 42
3.8. Statistical analyses 43
4. Results and discussion 44
4.1. CTP4 growth curves 44
4.1.1. Culture growth and productivity in a batch culture system 44
4.1.2. Growth calibration curves 45
4.2. CTP4 Nile red fluorescence calibration 46
4.3. CTP4 in a two-stage growth system resorting to culture manipulation 47
4.3.1. Growth and nitrate determination 47
4.3.2. Lipid content 48
4.3.2.1. Thermal stress 50
4.3.2.2. Light intensity 52
4.3.2.3. BODIPY 505/515 fluorescence microscopy 53
4.4. Fatty acid methyl esters (FAME) profile 56
4.5. Determination of biological activities 59
4.5.1. Amylase inhibitory activity 59
4.5.2. Antioxidant activity 61
5. Conclusions 63
6. References 64
7. Annex i

Figure index

Figure 1.1 - Renewable energy share of global final energy consumption in 2010 (REN 21, 2012)..... 16
Figure 1.2 - Estimates of the net energy density of several energy carriers (Guzzella & Sciarretta, 2007)..... 16
Figure 1.3 - Representation of algal growth rate in batch culture (solid line) and nutrient concentration (dashed line) which declines dramatically during the stationary phase and forward. During batch culture, microalgal growth proceeds in five phases: lag phase (1), exponential growth phase (2), linear growth phase (3), stationary growth phase (4), and decline (5). The highest growth rate is achieved from point 2 to 3 (Mata et al., 2010). 21
Figure 1.4 - a) Arial view of a raceway pound (Chisti, 2007), b) tubular PBR (Pereira, 2009)..... 24
Figure 1.5 - Transesterification process, where the triacylglycerol is named as "triglyceride". R1, R2 and R3 are hydrocarbon groups (Chisti, 2007) 26
Figure 1.6 - Example of a biorefinery scheme 26
Figure 3.1- CTP4 cultivation in 5-L plastic bottles under continuos air flow at control conditions to establish growth correlations. 32
Figure 3.2- Cultures under stress in 100 mL tubes in a climatic chamber. 33
Figure 3.3 – 100-L bags after settling for 1 day for biomass production, in order to perform bioactivity assys. A) Nutrient depletd, B) Nutrient enriched. 34
Figure 3.4 - 96-well plate containing algae culture from 5-L plastic bottles for spectrophotometry analysis. 35
Figure 3.5 - Neubauer chamber containing 10 µL of algal cell suspension 35
Figure 3.6 - Glass microfiber filters (VWR, 1.2 µm) containing 10 mL of algal culture and dried for 72 hours..... 36
Figure 3.7 - Gravimetric lipid determination after centrifugation..... 37
Figure 3.8 - GC-MS temperature for methyl ester separation (Adapted from Pereira, 2009)..... 39
Figure 4.1 - CTP4 growth at 20 ºC and PFD of 100 µmol m^{-2} s^{-1} in a batch culture system for 12 days with modified Algal culture medium. 44
Figure 4.2 – Relationship between cellular concentration and optical density measured at 750 nm ($p < 0.001$). 45
Figure 4.3 – Relationship between dry weight and optical density measured at 750 nm ($p < 0.001$)..... 46
Figure 4.4 – Relationship between Nile red fluorescence and total lipids with gravimetric method Bligh & Dyer, 1959 ($p < 0.001$). 47
Figure 4.5- Nitrate concentration (○) and dry weight (●) of cultures grown under nutrient depletion (left) and addition of nutrients supplied at day 11 (right)..... 47
Figure 4.6- Lipid content (% of dry weight) of the two-stage growth cultures at 20 ºC and 100 µmol m^{-2} s^{-1} with nutrient depletion (N^-) and supplementation (N^+). Bars show means and standard deviation of three replicates. Bars labelled with different letters are significantly different on a given day ($p < 0.05$). 49

Figure 4.7: Comparison of lipid content (% of dry biomass) of the two-stage growth cultures at 20 ºC, 5 ºC and 35 ºC with 100 µmol m^{-2} s^{-1} using under nutrient depletion (N^-) and supplementation (N^+). Bars show means and standard deviation of three replicates. Bars labelled with different letters are significantly different on a given day ($p < 0.05$). 51
Figure 4.8: Comparison of lipid content (% of dry biomass) of the two-stage growth cultures at 100, 200 and 400 µmol m^{-2} s^{-1} at 20ºC using nutrient depletion (N^-) and supplementation (N^+). Bars show means and standard deviation of three replicates. Bars labelled with different letters are significantly different on a given day ($p < 0.05$)..... 52
Figure 4.9 - Fluorescence microscopy photographs showing lipid bodies tagged with BODIPY in cultures grown at 20 ºC and 100 µmol m^{-2} s^{-1} of PFD (control); 5 ºC, 35 ºC, 200 and 400 µmol m^{-2} s^{-1} with nutrient supplementation (N^+) or nutrient depletion (N^-). Cells against a grey background correspond to merged DIC and BODIPY fluorescence micrographs. Cells against a black background are micrographs of BODIPY fluorescence alone. Scale bar – 5 µm. 54
Figure 4.11 - Amylase inhibitory activity from acetone. methanol. ethyl acetate and hexane extracts at 1 mg mL^{-1}. Acarbose served as positive control. Dash line is the value from which it is considered to occur inhibitory activity, n=6. 59
Figure 4.12- Amylase inhibitory activity from acetone. methanol. ethyl acetate and hexane extracts at 1 mg mL^{-1}. Acarbose served as positive control. Dash line is the value from which it is considered to occur inhibitory activity, n=6. 60
Figure 4.13 - Radical scavenging activity (% of inhibition) against $ABTS^{\bullet+}$ radical from acetone, methanol, ethyl acetate and hexane extracts at 1. 5 and 10 mg mL^{-1}, prepared from cultures grown under nutrient depletion, n=6. 61
Figure 4.14- Radical scavenging activity (% of inhibition) against $ABTS^{\bullet+}$ radical from acetone, methanol, ethyl acetate and hexane extracts at 1. 5 and 10 mg mL^{-1}, prepared from cultures grown under nutrient supplementation, n=6. 61

Table index

Table 3.1- Concentrated (1000 ×) Algal modified medium composition 31
Table 3.2- Calibration curve, correlation coefficient and retention time for each FAME. 40
Table 4.1- FAME profile and total saturated fatty acids (SFA) of microalgae grown under different culture conditions (high and low temperature and different light intensities) cultivated for..... 58
Table 7.1 - Effect of lipid induction using different temperatures (5 and 35 ºC) and different light intensities (200 and 400 µmol m^{-2} s^{-1}) with nutrient depletion (N^-) and supplementation (N^+) in g L^{-1} and percentage (%). Cultures grown at 20 ºC under 100 µmol m^{-2} s^{-1} of PFD were used as control., n=3. ii

1. Introduction

1.1. Energetic demand – fossil fuels as energy sources

Global economy is highly dependent on energy and its demand is continuously increasing due to economic growth and population rise (Patil *et al.*, 2008). Holding to current policies, by the year 2030 the world will need about 60% more energy than today (Patil *et al.*, 2008). In 2006, 80.9% of the total energy produced was from fossil fuels and this dependence on non-renewable sources of energy has contributed to the increase of atmospheric CO_2, leading to the global warming issue (Tanner, 2009). Non-renewable sources include fossil fuels, such as coal, petroleum and natural gas, and they have been used as primary sources of energy for power generation, transportation, agriculture and industrial activities for the last centuries (Demirbas & Demirbas, 2010). About 27% of the primary energy is used in the transportation sector, making it one of the fastest growing sectors (Patil *et al.*, 2008).

Several events can be related with the increase and speculative prices of fossil fuels, starting with the oil crisis in 1973, and followed by wars (Gulf and Middle East wars (Urry, J., 2008), reduced/limited availability, stricter governmental regulations in order to reduce emissions (Kyoto Protocol) and economic interests. In addition from being limited, non-renewable resources, petroleum and other fossil fuels have also had substantial impacts on the environment, such as the increase of many gases like carbon dioxide (CO_2), hydrocarbons (HC), nitrogen oxides (NOx), and sulphur oxides (SOx) (Demirbas & Demirbas, 2010). Atmospheric CO_2 levels measured at Mauna Loa (Hawaii, US), for example, showed an increase from 280 ppmv in 1958 to 390 ppmv in 2010 (Kumar *et al.*, 2011).

Because of all these limitations, researchers started to study viable, renewable sources of energy (Demirbas & Demirbas, 2010), especially for the transport sector - since they are in general composed of hydrocarbons (see 1.2) - and biofuels are seen as viable alternatives (Mata *et al.*, 2010). In 2010, it is estimated that 16.7% of the global consumption of energy was supplied by renewable sources, from which 0.7% were supplied by biofuels (Figure 1.1) (REN 21, 2012).

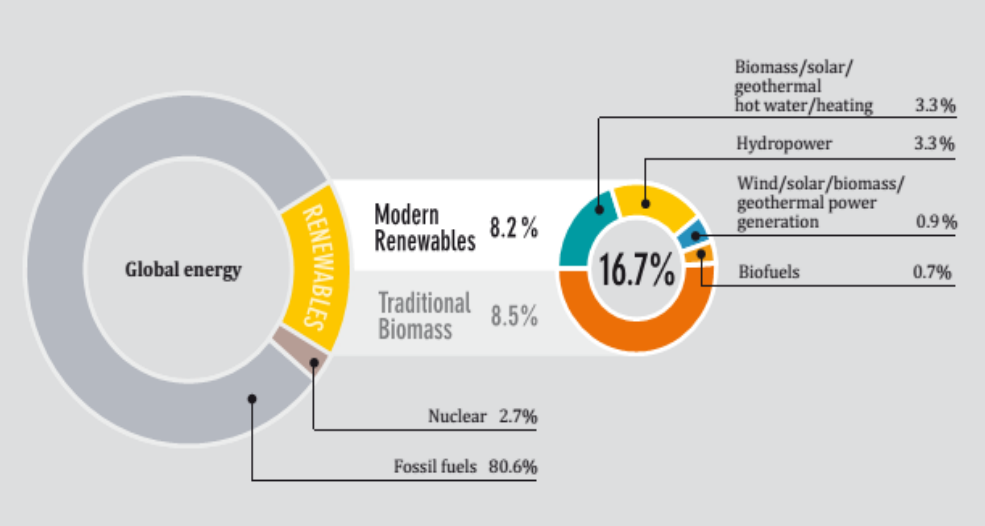

Figure 1.1 - Renewable energy share of global final energy consumption in 2010 (REN 21, 2012)

1.2. Biofuel

The term biofuel refers to liquid, gaseous or solid fuels, produced predominantly from natural sources such as biomass (Demirbas, 2009). In order to be viable when compared to fossil fuels, biofuels need to be environmentally friendly, competitive in an economically point of view, produced in quantities able to meet energy demand, and produce more energy than the one needed to produce them (Hill *et al.*, 2006).

Biofuels composed of hydrocarbons (diesel, gasoline, natural gas) have net mechanical propulsion energies significantly higher than currently existing batteries (Guzzella & Sciarretta, 2007; Figure 1.2). For heavy transportation as airplanes, ships and lorries, the most viable alternative is a biofuel made from hydrocarbons, as current electrical engines are not able to power heavy loads. Therefore, liquid fuels are the only viable alternative for heavy transport at this stage. The majority of existing engines are fuelled by hydrocarbons, and thus using biofuels that mimic the latter allow the use of the same engine components for both types of energy sources (Guzzella & Sciaretta, 2007).

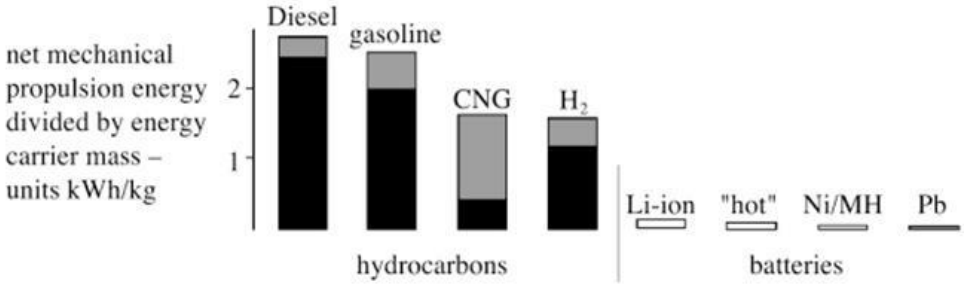

Figure 1.2 - Estimates of the net energy density of several energy carriers (Guzzella & Sciarretta, 2007)

According to Demirbas & Demirbas (2010), biofuels can be classified into four types, based on their production technologies: first-generation biofuels (FGB), second-generation biofuels (SGB), third-generation biofuels (TGB), and fourth-generation biofuels. FGB refer to biofuels made from sugar, starch, vegetable oils, or animal fats produced using conventional technologies. Their basic feedstocks are seeds or grains fermented or pressed, depending on the feedstock, which are converted into bioethanol (petrol additive/substitute) or biodiesel. SGB and TGB are also called advanced biofuels. SGB are made from wheat straw, corn, non-food crops, wood and energy crops, using advanced technology such as the Fischer–Tropsch process. TGB or algae fuel is a biofuel derived from algal lipids. There is also a fourth-generation fuel that converts vegetable oil and biodiesel into bio-gasoline using the most advanced technology, such as metabolic engineering and synthetic biology (Atsumi & Liao, 2008; Demirbas & Demirbas, 2010; Lee *et al*., 2008). Liquid biofuels are nowadays the most promising form of energy to replace petroleum-based fuels since they possess higher energy density when compared to hydrogen or electrical batteries and because their physicochemical properties are similar to those of petroleum-based fuels.

1.3. Microalgae as a feedstock for biofuel production

Microalgae are a diverse group of photosynthetic eukaryotic and prokaryotic microorganisms that can grow rapidly and live in harsh conditions due to their simple morphology, which can be unicellular and/or multicellular. They are present in marine and in freshwater environments. As photosynthetic organisms, microalgae convert sunlight, water and carbon dioxide into algal biomass (Chisti, 2007; Demirbas & Demirbas, 2010; Li *et al.*, 2008; Mata *et al.*, 2010). There are over 40,000 species of identified microalgae and it is suspected that a large number remains unidentified. Algae can be classified as Archaeplastida, which includes the green (Chlorophyta) and red (Rhodophyta) algae; Stramenopiles, which comprises taxa such as diatoms (Bacillariophyceae), brown (Phaeophyceae), yellow-green (Xanthophyceae) and golden (Chrysophyceae) algae; Alveolata (e.g. dinoflagellates); Hacrobia, a possible polyphyletic group containing haptophytes (e.g. *Isochrysis*) and cryptomonads (e.g. *Rhodomonas*); Excavata (e.g. euglenids); Rhizaria (e.g. chlorarachniophytes, which are photosynthetic amoebae) as well as cyanobacteria (Cyanophyceae) (Keeling, 2013).

Cyanobacteria are prokaryotic microalgae and are responsible for a large percentage of the photosynthetic production in open-ocean (Richmond, 2004).

Eukaryotic microalgae contain a well-defined nucleus, cell wall, chloroplast (containing chlorophyll and other pigments) and pyrenoid (responsible for carbon fixation. Several microalgae possess flagella (Kumar *et al*, 2011) and stigma, or eye-spot, which allows them to sense light direction and intensity (Wager, 2008).

Nowadays, microalgae are mainly used for food (food supplements - biomass from microalgae is dried and is sold in the human health food market in the form of powder or pressed in pills), animal feed, larvae feed in aquaculture and as biofertilizer (Demirbas & Demirbas, 2010; Tang *et al.*, 2011). However, there are other applications where microalgae hold enormous potential, namely: CO_2 sequestration, wastewater treatment, biomedical applications (production of bioactive metabolites) and biofuels production (Li *et al.*, 2008; Mata *et al.*, 2010).

Microalgae are capable of producing several types of renewable fuels as, for example, methane from anaerobic digestion of biomass, electricity from gasification and combustion of biomass, hydrogen from photobiological production and biodiesel from lipids (Marshall *et al.*, 2010).

Biodiesel production from microalgal lipids presents several advantages in comparison with other available feedstocks. For instance, microalgae have: i) high photosynthetic efficiency – it is estimated that the biomass productivity of microalgae can be 50 times higher than that of switchgrass, the most productive terrestrial plant (Garg *et al.*, 2012; Li *et al.*, 2008); ii) high lipid productivity – microalgae are more productive (usually 50 times higher) than any known terrestrial oleaginous plants; iii) consume less freshwater than land crops, for they can be grown in seawater and wastewater and the used water can still be reused after biomass harvesting; iv) high CO_2 fixation capacity – microalgae are able to tolerate high CO_2 concentrations and high temperatures in gas streams contributing for the mitigation of CO_2 through photosynthesis by the injection of flue gas into photobioreactors (Gouveia, 2011); and finally, v) microalgae do not require arable land to grow and thus they do not compete with crops for food and feed (Chisti, 2007; Hu *et al.*, 2008; Li *et al.*, 2008; Mata *et al.*, 2010; Rodolfi *et al.*, 2009).

However, biofuel production from microalgae has currently some disadvantages, the most important being the high production costs. There is limited light penetration in microalgal cultures leading to low biomass concentration, which in combination with the small size of algal cells makes the harvest of algal biomass relatively costly. Most strains

studied so far cannot be grown at high cell concentrations although they possess both high lipid content and high growth rates. Moreover the design of large-scale production systems has not yet been fully optimized (Lardon *et al.*, 2009; Li *et al.*, 2008). The cost of the final biodiesel product is currently about $4.92-2.89 per litter (Suali & Sarbatly, 2012), whereas the price of petro-diesel is currently in $0.69 per litter (Brent Crude oil in 25.04.14). Further technological developments are needed to overcome or minimize these problems in order for microalgae to become an important alternative source of energy (Chisti, 2007; Demirbas & Demirbas, 2010; Mata *et al.*, 2010).

In order to make the production of microalgal-based biofuels economically feasible is by diminishing the biomass production costs. One of the key factors to achieve that is by selecting a strain that fulfil some criteria: i) high growth rate (amount of biomass per unit of time and volume); ii) high lipid content; iii) good resistance to changes in the environmental conditions (temperature, light, nutrients, competition with bacteria); iv) easy to harvest; and v) production of co-products such as high-value bioactive compounds that can be used as pharmaceutical compounds, such as pigments and / or polyunsaturated fatty acids (PUFA) (Li *et al.*, 2008; Mata *et al.*, 2010).

1.4. Lipids for biofuels production

Microalgae are capable of accumulating and synthesizing great amounts of lipids and lipid levels of 20 to 50% of the dry biomass weight are common although in particular cases they can exceed 80% (Chisti, 2007).

The interest in microalgae for oil production relies on the fact that, besides having high lipid contents, biomass yields, and photosynthetic efficiency, the lipid synthesis by microalgae can be modulated by different growth conditions (Rodolfi *et al.*, 2008; Slocombe *et al.*, 2012).

Lipids produced by microalgae can be separated in two groups: structural lipids (polar lipids or membrane lipids) and storage lipids (non-polar). The first ones are important structural components of the cell membrane. Storage lipids are mainly produced in the form of triacylglycerols (TAG), which are usually synthesized in the presence of light, stored in cytosolic lipid bodies and used for polar lipid synthesis in the dark. TAG are composed by three fatty acids esterified with one molecule of glycerol and may include saturated, mono-saturated and polyunsaturated fatty acids.

During growth, the predominant lipids produced are structural lipids (such as phospholipids and glycolipids); however, when cultures reach the stationary-phase many

species accumulate TAG, which may reach 20-50% of the total biomass dry weight (Chisti, 2007; Day *et al.*, 2012, Hu *et al.*, 2008). Additionally, under stress conditions, many algae accumulate storage lipids in the form of TAG (Demirbas & Demirbas, 2010; Roleda *et al.*, 2013). Such stress conditions are generally environmental physical and/or chemical stimuli, which lead microalgae to substantially increase the synthesis and storage of TAG, followed by considerable changes in lipid composition (Hu *et al.*, 2008). Chemical stimuli can be variations in nutrient levels and salinity or alterations in the pH of the growth medium. Conversely, major physical stimuli are temperature and light intensity variations (Hu *et al.*, 2008).

1.4.1. Nutrient stress

Microalgal lipid content and fatty acid (FA) composition, cell growth and proliferation and cell division rate are affected by nutrient availability (Sharma *et al.*, 2012). In order to induce lipid accumulation, cultures need to be subjected to some depletion of essential nutrients, especially nitrogen, and limiting those nutrients is an effective way to induce lipid accumulation by letting the excess carbon to be stored in lipid molecules (Chi *et al.*, 2009; Granger *et al.*, 1993), mainly in the form of TAG (Solovchenko *et al.*, 2008).

Under nitrogen depletion, the increase in the fatty acid cell content results mainly from the synthesis of saturated and monounsaturated fatty acids. Growth-limiting conditions induced by different nutrient depletions result in an increase of the synthesis yield of PUFA, compensated by a severe decrease in their specific production rates (Granger *et al.*, 1993).

Nitrogen deprivation has been shown to be the most critical nutrient deprivation leading to the accumulation of lipids, especially TAG (Hu *et al.*, 2008; Solovchenko *et al.*, 2008) and is one of the first nutrients to be depleted during cultivation (Sharma *et al.*, 2012). Several authors reported increases in lipid content due to a reduction in the nitrogen levels in the medium (Illman *et al.*, 2000; Hu, 2006; Miao & Wu, 2006; Rodolfi *et al,.* 2009)

Even though nitrate depletion is one of the most successful strategies for lipid enhancement, a two-stage growth system is often used (Widjaja *et al.*, 2009). As seen in Figure 1.3, in a batch culture, nutrient depletion takes place during exponential growth phase (Mata *et al*, 2010). However, because nutrient depletion takes 2-5 days, this procedure leads to lower biomass and lipid productivity, as neither growth nor lipid

accumulation is optimized. Hence, an optimization of the cultivation conditions for high lipid production and biomass yield is needed (Chi *et al.*, 2009; Su *et al.*, 2011). A two-stage culture system, in which the growth phase and the lipid accumulation phase are distinct and fully optimized, may overcome this limitation (Chi *et al.*, 2009). In the first stage cells are grown with no restriction in nitrogen for maximum growth rates and in the second stage nitrate is withheld, maximizing lipid production (Su *et al.*, 2011). The sane authors state that a two-stage culture is effective for the increase of lipid production from microalgae and the results prove that transferring algal biomass from a nitrogen-replete phase to a nitrogen-depleted phase induces lipid production. According to Suali & Sarbatly (2012), during the first stage of cultivation, it is very important to provide high concentrations of nitrogen to support growth and reproduction of cells, but for the second stage this nutrient needs to be depleted so that in the second stage the fixated carbon can be converted into lipids.

In this system cells are allowed to reach maximum cell concentration and are later stressed (late exponential stage) to enhance the lipid content of cultures (Chi *et al*., 2009).

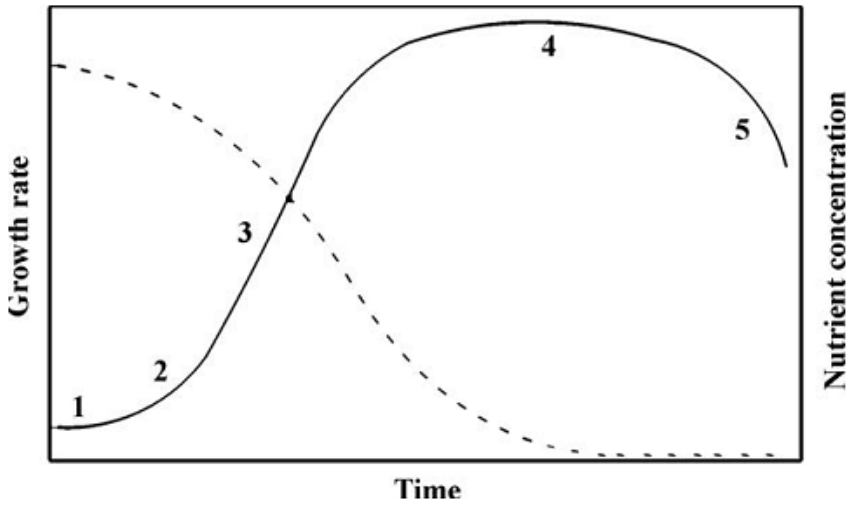

Figure 1.3 - Representation of algal growth rate in batch culture (solid line) and nutrient concentration (dashed line) which declines dramatically during the stationary phase and forward. During batch culture, microalgal growth proceeds in five phases: lag phase (1), exponential growth phase (2), linear growth phase (3), stationary growth phase (4), and decline (5). The highest growth rate is achieved from point 2 to 3 (Mata *et al*., 2010).

1.4.2. Thermal stress

Temperature was found to have a major effect on FA composition of algae. In many algae, variation in the concentration of several fatty acids was observed after both increasing and decreasing temperature (Hu *et al.*, 2008; Sharma *et al.*, 2012). Usually, lowering temperature leads to an increase in unsaturated fatty acids, whereas a temperature rise increments the saturation of fatty acids (Day *et al.*, 2013, Hu *et al.*, 2008). It has been proposed that these changes in the lipid profile alter the physical properties of membranes, especially fluidity, which increases due to higher FA unsaturation levels. Under this situation, FA cannot be packed as tightly as their saturated counterparts because of the bends caused by the presence of double bounds. Changes in membrane fluidity caused by temperature can thus be offset by changes in FA saturation, so that normal functions (e.g., ion permeability, photosynthetic and respiratory processes) can continue unaltered (Hu *et al.*, 2008; Sharma *et al.*, 2012; Somerville, 1995).

Temperature also affects the total lipid content of some algae, although results are controversial. A few studies reveal significant increase of lipid content with increasing temperature in *Ochromonas danica* (Aaronson, 1973) and in *Nannochloropsis salina* (Boussiba *et al.*, 1987), whereas others showed no significant changes, regardless of the temperature change, such as in *Chlorella sorokiniana* (Patterson, 1970). In some cases this increase in lipid content can be caused by a decrease in temperature. Kalacheva *et al.* (2002) found that *Botryococcus braunii* cultures grown under temperatures lower than the optimal also showed an increase in the lipid content, specifically TAG. Hence, a general trend cannot be established (Hu *et al.*, 2008; Sharma *et al.*, 2012).

According to Elmoraghy *et al.* (2012), thermal stress appears to be the easiest to apply. Algae are grown at room temperature, until stationary growth phase, and are then stressed by exposure to a lower or higher temperature, in a two-stage growth system.

1.4.3. Light intensity stress

Light is a key factor to photosynthesis, without which no photoautotrophic life can be sustained. Microalgae have been reported to grow on different light intensities showing, in consequence, remarkable changes in their gross chemical composition, pigment content and photosynthetic activity (Richardson *et al.*, 1983). Furthermore, different light intensities and wavelengths have been reported to change the lipid

metabolism in microalgae, altering the lipid profile, generally because high light intensity leads to oxidative damage of polyunsaturated fatty acids as well as an increase in carotenoid content (Sharma *et al.*, 2012). Usually, low light intensities induce the formation of polar lipids, mostly those associated with the chloroplast membrane, whereas high light intensities decrease total polar lipid content with a simultaneous increase in the amount of neutral storage lipids, mainly TAG (Hu *et al.*, 2008). Light/dark cycles at different growth phases have also a significant effect on algal lipid composition (Sharma *et al.*, 2012). Brown *et al.* (1996) showed that diatom cultures of *Thalassiosira pseudonana* grown under a 24-h light or under 12:12 h strong light/dark photoperiods had a higher amount of TAG with saturated and monounsaturated fatty acids, when compared to cultures grown with less light. For microalgal-based biodiesel production high amounts of neutral storage lipids are needed, and thus one approach to obtaining lipids suitable for biodiesel might be the use of high light intensities.

1.5. Microalgal biodiesel production

1.5.1. Microalgal cultivation and harvesting

There are two major systems for microalgae high scale cultivation: open systems like ponds or raceways and closed systems such as photobioreactors (PBR) (Chisti, 2007; Mata *et al*, 2010).

A raceway consists in a closed loop recirculation channel (Figure 1.4 a), typically 0.3 m deep. The channels can be made of concrete or compact earth. A paddlewheel is used to mix the culture and baffles are placed to guide the flow around the bends in the channel. The culture is fed continuously, during daylight, in front of the paddlewheel after the circulation loop is completed. To prevent sedimentation the paddlewheel must operate continuously (Chisti, 2007).

Usually open culture systems are more durable, less expensive to build and operate, and have a large production capacity compared to closed systems (Mata, *et al.*, 2010). However, open systems use more energy to homogenize nutrients and water depth needs constant maintenance, as it cannot be lower than 15 cm in order to maintain correct flow and turbulence for microalgae to grow (Richmond, 2004). Ponds are more susceptive to weather conditions and they do not allow the control of water temperature, evaporation and lighting. They can produce large quantities of microalgae, but have the inconvenience of using more land area. There is also the problem of mass transfer limitations, because

atmosphere contains only 0.03 to 0.06% of CO_2, which can slow down microalgal growth (Mata *et al.*, 2010).

PBR are flexible systems and can be optimized according to the biological and physiological characteristics of the cultivated species. There are several types, such as cylindrical, flat panels, tubular (Figure 1.4 b) and disposable (Pereira, 2009) PBR. The walls of the reactors limit direct exchange of gases and contaminants between cultivated cells and the atmosphere and thus PBR offer the advantage of having a better control of culture conditions and growth parameters like pH, light, mixing and CO_2 than open systems, allowing for a ≈ 16-fold increase in microalgal concentration and an ≈ 8-fold increase in volumetric productivity, preventing evaporation, reducing CO_2 losses and offering a safer environment due to the walls that minimize invasion by competitive and predatory microorganisms (Mata *et al.*, 2010).

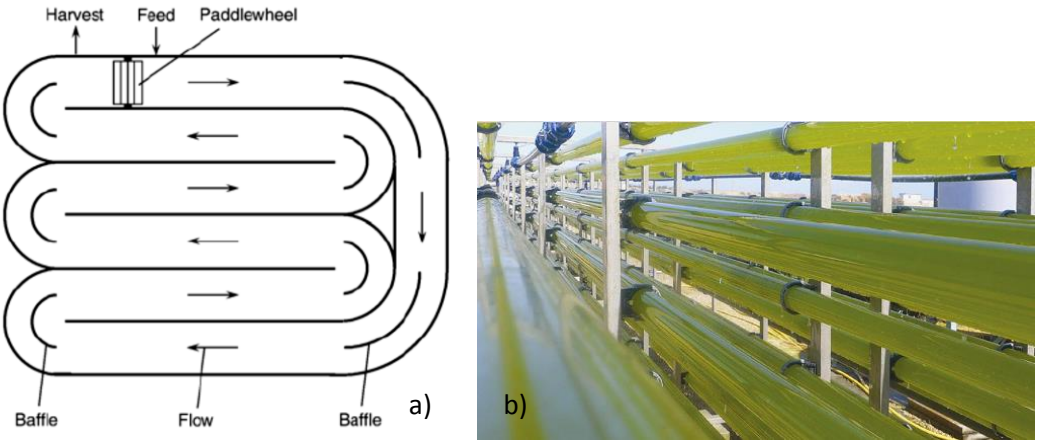

Figure 1.4 - a) Arial view of a raceway pound (Chisti, 2007), b) tubular PBR (Pereira, 2009).

Although PBRs seem to be more profitable than open systems, they still present some limitations that need to be solved such as overheating, bio-fouling, oxygen accumulation, difficulty in scaling up, high building and operating costs, and cell damage by shear stress (Hu *et al.*, 2008). In order to achieve high growth rates and also high oil yields it is essential to develop innovative large-scale culture systems. Hu *et al.* (2006) indicates that open raceway pounds are not very effective or sustainable for oil-rich biomass, even though higher biomass and oil production was accomplished in closed PBRs. In order to increase large-scale cultivation biomass and oil yields some novel cultivation systems are needed, also with the purpose of lowering the production costs (Hu *et al.*, 2008).

Harvesting microalgae can be difficult due to their small cell size and represent around 25% of the production costs due to expenses with electricity, reagents and equipment maintenance. Harvesting usually involves flocculation followed by harvesting by filtration, centrifugation, sedimentation or flotation (Suali & Sarbatly, 2012). The selection of the best harvesting technique is dependent on the properties of microalgae, such as density, size and value of the desired products (Gouveia, 2011).

1.5.2. Oil extraction

There are three methods to extract oil from algae: expeller/press, solvent extraction with hexane, and supercritical fluid extraction. The first is a simple method in which a press is used to extract 70 to 75% of the oils from the algal biomass. In solvent extraction, hexane is normally used because it is an inexpensive chemical. Supercritical fluid extraction is by far more efficient than traditional solvent separation methods, providing high product concentrations and purity by being selective (Demirbas & Demirbas, 2010).

1.5.3. Transesterification

Microalgae produce storage lipids in the form of TAG (Demirbas & Demirbas, 2010). TAG are composed by three fatty acids esterified with one molecule of glycerol. To make biodiesel, TAG are reacted with methanol or ethanol in a process called "transesterification". In this process fatty acid methyl esters (FAME) and glycerol are produced, the former being the feedstock that will become biodiesel upon proper downprocessing (Figure 1.5). This reaction is stepwise: first triacylglycerols are converted to diacylglycerols, and then to monoacylglycerols and the final process is their conversion to glycerol, producing one methyl ester molecule from each FA released from glycerol (Fukuda *et al*., 2001). Transesterification requires three molecules of alcohol for each mole of TAG to produce one molecule of glycerol and three molecules of FAME (biodiesel; Chisti, 2007).

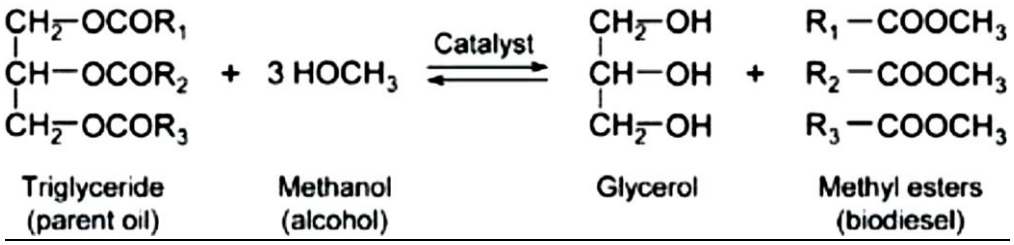

Figure 1.5 - Transesterification process, where the triacylglycerol is named as "triglyceride". R1, R2 and R3 are hydrocarbon groups (Chisti, 2007)

1.6. Biorefinery

Microalgae have the capacity to produce a variety of high-value bioactive compounds making them proper for use as pharmaceutical compounds, health foods, natural pigments and source of PUFA (Li *et al.*, 2008). The economical feasibility of microalgal biofuel production may therefore be significantly improved by a high-value co-product strategy, which, for example, would involve the cultivation of microalgae in a microalgal farming facility (allowing CO_2 mitigation), coupled with bioactive products extraction from harvested algal biomass, thermal processing (pyrolysis, liquefaction, or gasification) for energy production, and reforming/upgrading biofuels for different applications (Li *et al.*, 2008). This is the conceptual design of a "biorefinery" (Figure 1.6) in which biofuels and high-value co-product materials are produced, in a cost-effective and environmentally sustainable manner (Li *et al.*, 2008; Gouveia, 2011). Biorefineries can simultaneously produce biofuels and bio-based chemicals, heat, and power (Demirbas & Demirbas, 2010).

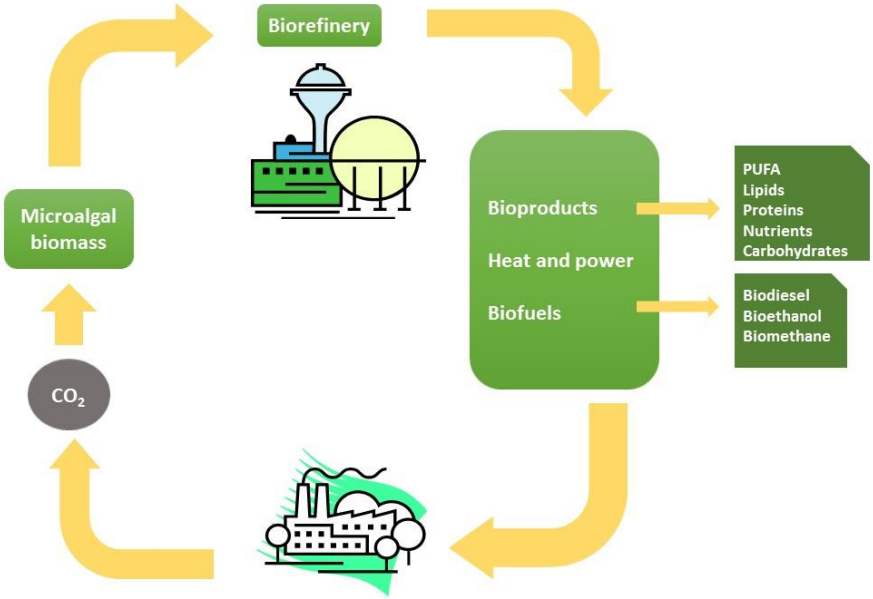

Figure 1.6 - Example of a biorefinery scheme

Marine organisms are sources of bioactive compounds with new structures and bioactivities (Bhadury & Wright, 2004), so over the last years they have been studied for this purpose (Blunt *et al*., 2014), including microalgae (Custódio *et al.*, 2012a; Plaza *et al.*, 2010; Rodríguez-Meizoso *et al.*, 2010). Finding bioactive products from harvested algal biomass can be very beneficial for a variety of uses, and they can serve as food preservatives or added to food and feed supplements as well as health products (Rodríguez-Meizoso *et al.*, 2010). Most used biologically active compounds from microalgae are carotenoids, polyphenols and PUFA. When subjected to environmental changes, such as salinity, temperature, nutrients or irradiation shifts, microalgae need to acclimate rapidly to the new conditions in order to survive, producing multiple unique secondary metabolites (Rodríguez-Meizoso *et al.*, 2010). Considering microalgal taxonomic diversity, several screenings for novel biological compounds with biomedical application have been carried out recently.

One of the possible approaches is to determine the antioxidant activity, as this can give an initial assessment of the presence of bioactive compounds that counteract oxidative stress. This type of stress has been associated with the progression of diseases like cancer, depression, heart disease and Alzheimer. This stress will occur if the unwanted free radicals are not eliminated efficiently. These free radicals are produced as byproducts of lipid degradation reactions, chemcial energy (ATP) generation and inflamatory processes (Wang *et al.*, 2008). Compounds with radical scavenging activity (RSA) are very important to protect organisms against oxidative stress, since prevention is the most efective way to avoid chronic diseases. Their consumption can prevent ageing and reduce the risk of developing degenerative diseases (Dillard & German, 2000). Additionally, there are some evidence that synthetic antioxidants such as butylated hydroxytoluene (BHT) are carcinogenic. Therefore the search for novel antioxidants is very important (Custódio *et al.*, 2011).

Another possible strategy is to screen for bioactivities that have never been researched in a given taxonomic group. Diabetes mellitus is a disease characterized by chronic hyperglycaemia with disturbance of carbohydrate, protein and fat metabolism resulting in defects in insulin secretion and/or action (Ali *et al*., 2006; Maritim *et al*., 2002) and has been increasing due to global population aging and obesity. One therapeutic approach is to ameliorate diabetes by retarding the absorption of glucose, inhibiting the carbohydrate-hydrolysing enzymes (α-amylase and α-glucosidase) in the digestive tract (Conforti *et al*., 2005). Acarbose, miglitol, voglibose are currently being

used to decrease carbohydrate hydrolysis and prolong its overall digestion time, causing a reduction in the rate of glucose absorption (Ali *et al*., 2006; Lee *et al*., 2010). However, these inhibitors have been associated with side effects as flatulence, diarrhoea and vomits and they can increase the incidence of renal tumours and hepatic injuries (Lee *et al*., 2010). Therefore, it is important to search for new inhibitors in novel taxa, such as microalgae. Due to their biodiversity it is possible that some strains or species contain bioactive compounds that could be used as co-products of microalgal harvested biomass in a biorefinery

1.7. CTP4 strain

Recently, water samples have been collected from Ria Formosa, Algarve, and an autochthonous microalga belonging to the *Chlorophyceae* family was isolated by means of fluorescent-activated cell sorting following a method developed by the MarBiotech research group (Pereira *et al.*, 2011). The purpose of this isolation was to find novel strains showing promising results for biodiesel production able to grow on wastewater as culture medium. This strategy would allow for not only wastewater bioremediation, but also to a decrease in biodiesel production costs. As the taxonomical classification of the strain down to the genus/species level is still unknown this strain will be referred to as CTP4 throughout this work.

2. Aims

This thesis is part of an on-going project to develop a microalgal strain for biodiesel production. With that purpose in mind, the main aim was to evaluate the influence of abiotic conditions on the lipid production and profile of CTP4, studying the effect of temperature and light intensity variations, under nutrient depletion and supplementation, on the lipid concentration and the fatty acid profile of CTP4.

The secondary aims were:

- Cultivate CTP4 in batch culture and assess its growth parameters to determine biomass production;
- Correlate gravimetric lipid determination with Nile red fluorescence to ascertain the total amount of lipids produced during the main experiment;
- Examine the viability of this strain for a biorefinery concept screening for biological active compounds via anti-diabetic and antioxidant assays.

3. Materials and methods

The experimental work was conducted in the MarBiotech group lab, located on the Centre of Marine Sciences at the University of Algarve, between 15/02/2013 and 31/08/2013.

The lab work was divided in 7 parts: i) Culture scale-up to 100-mL Erlenmeyer flasks; ii) Growth of cultures in Algal growth medium and determination of growth parameters; iii) Establishment of working relationships between growth parameters and optical density and between lipid concentration and Nile Red fluorescence; iv) Culture growth under stress conditions; v) Evaluation of nitrate concentration in the medium; vi) Determination of the lipid profile; vii) Microscopical observation of cells to evaluate lipid body accumulation; and viii) Scale-up of biomass production to 100-L bags for bioactivity determination.

All the material was sterilized in the autoclave at 120 ºC and all reagent solutions were prepared with Milli-Q water (Millipore, Billerica, MA, USA).

3.1. CTP4 growth

To obtain enough CTP4 cells for all experiments, cultures were transferred from solid medium (Petri dishes) to liquid medium (100-mL Erlenmeyers), with 50 mL of autoclaved seawater and 50 µL of modified concentrated Algal culture medium. This medium was based on a method described by Fábregas *et al*. (1984) and consists in three solutions: i) micronutrients solution, ii) macronutrients solution and iii) iron solution (Table 3.1). Erlenmeyers were closed with cellulose caps and kept at room temperature under continuous lighting at a photon flux density (PFD) of 100 µmol m^{-2} s^{-1} on an orbital shaker for 5 to 7 days at room temperature.

Table 3.1- Concentrated (1000 ×) Algal modified medium composition

Reagents	Concentration	Units
Micronutrients solution		
EDTA-Na	6.4	mM
$ZnCl_2$	1	mM
$ZnSO_4•H_2O$	1	mM
$MnCl_2•4H_2O$	1	mM
$Na_2MoO_4•2H_2O$	0.1	mM
$CoCl_2•6H_2O$	0.1	mM
$CuSO_4•5H_2O$	0.1	mM
$MgSO_4•7H_2O$	2	mM
Macronutrients solution		
$NaNO_3$	2	M
KH_2PO_4	100	mM
Iron solution		
$FeCl_3•6H_2O$	20	mM
EDTA-Na	20	mM

For the medium preparation, 200 mL of micronutrients solution, 700 mL of macronutrients solution and 100 mL of iron solution were added in order to prepare 1 L of Algal modified medium.

3.1.1. Growth of CTP4 cultures under non-stressed conditions

Cultures were transferred from the 100-mL Erlenmeyers to 5-L plastic bottles (Figure 3.1) and mixed with sterilized seawater to a final cell concentration of 1.5×10^4 cells mL^{-1}. Concentrated modified Algal culture medium was added in a 1/1000 proportion (Pereira *et al.*, 2011). CTP4 cultures to be used for further experiments were then grown for 11 days in a specialized growth chamber (Aralab Fitoclima s 600 PL clima plus 400) at 20 ºC under a continuous PFD of 100 µmol m^{-2} s^{-1}.

Figure 3.1- CTP4 cultivation in 5-L plastic bottles under continuos air flow at control conditions to establish growth correlations.

3.1.2. Growth of cultures under stress conditions

After the stationary phase was reached, 80 mL of the CTP4 cultures grown to stationary phase were divided and transferred to 100 mL tubes, which were placed in different climatic chambers (Aralab Fitoclima s 600 PL clima plus 400) where light intensity, temperature and humidity can be monitored and controlled (Figure 3.2). Cultures were then submitted to light and temperature shifts for 7 days. For high light intensity stress, PFD of 400 and 200 µmol $m^{-2}s^{-1}$ (24 hours cycle) were applied to cultures pre-grown at 100 µmol m^{-2} s^{-1} (Liu, 2012). For the thermal stress, cultures were subjected to 5 ºC and 35 ºC using a starting inoculum grown at 20 ºC. All stresses were performed under two different conditions: nutrient depletion and nutrient repletion and all assays and conditions described were performed in triplicate. Another set of 6 tubes, used as controls, were incubated for the same 7-day time period at 20 ºC with 100 µmol m^{-2} s^{-1} of PFD, three of them under nutrient depletion and the other three under nutrient repletion. During stress induction, optical density and lipid content, using Nile red assay, were measured every day, in 2 mL aliquots of culture (Figure 3.2). The volume lost by evaporation was compensated with distilled water. At the end of the experiment the content of every tube was transferred to Falcon tubes, labeled and frozen for analysis of the fatty acid methyl esters (FAME) profile.

Figure 3.2- Cultures under stress in 100 mL tubes in a climatic chamber.

3.1.3. Cultivation in 100-L bags for bioactivity determination

For the bioactivity determination microalgae was grown in two 100-L cultivation bags in order to produce enough biomass for the analysis. At first 100-L bags were filled with seawater and sterilized with bleach (1 / 1000) and, after 24 h, neutralized with sodium thiosulphate. Afterwards CTP4 inoculum was added in a concentration of 2 x 10^4 and maintained for 11 days with aeration and constant PFD of 100 µmol m^{-2} s^{-1}. Once the stationary phase was reached, one of the cultures was supplemented with nutrients (Algal Modified medium) (Figure 3.3A), whereas the second culture no additional nutrients were added (Figure 3.3B) to induce nutrient stress and consequently lipid production. Nutrient depletion was chosen instead of the thermal or light intensity stresses since the latter are more challenging to apply and maintain in the available growing conditions. Cultures were maintained in these conditions for 7 days (same as growth under stress conditions in the 80 mL tubes).

After this period aeration was stopped, and cultures were allowed to settle down for 1 day, the upper medium layer with a lower cell concentration was then decanted and the concentrated biomass centrifuged in a Beckman Coulter Avanti J-25 High-Performance centrifuge for 5 min at 5000 *g* and lyophilised.

Figure 3.3 – 100-L bags after settling for 1 day for biomass production, in order to perform bioactivity assys. A) Nutrient depletd, B) Nutrient enriched.

3.2. Determination of growth parameters

The 5-L plastic bottles were cultivated from acclimation phase until stationary phase (day 11) and used to obtain calibration curves for the different growth parameters. Growth was measured every two days by means of optical density, cellular concentration and dry weight determination.

3.2.1. Optical density

Optical density was measured at 750 nm, in 96-well plates, with a BioTek Synergy 4 spectrophotometer using 300 μL per well of undiluted algal cell suspension (Figure 3.4). Seawater was used as a negative control.

Figure 3.4 - 96-well plate containing algae culture from 5-L plastic bottles for spectrophotometry analysis.

3.2.2. Cellular concentration

Cellular concentration was measured by optical microscopy, using Neubauer chambers with 10 µL of algal cell suspension (Figure 3.5). Cell counting was carried out four times per sample. As instructed by the cell chamber manufacturer each counting needs to reach between 30 and 300 cells in order to obtain significant cell numbers. In case of excess cell numbers (higher than 300), culture samples were diluted with seawater.

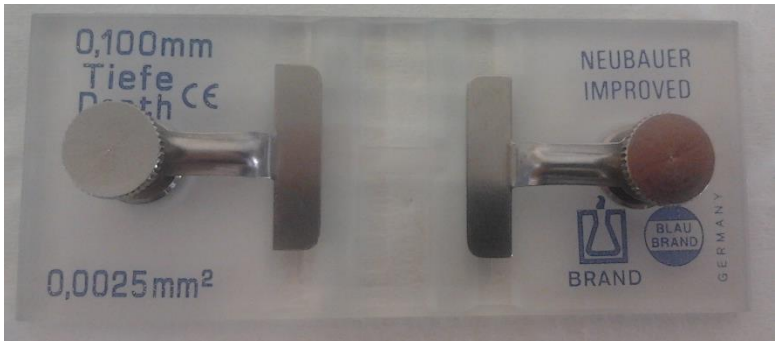

Figure 3.5 - Neubauer chamber containing 10 µL of algal cell suspension

Cellular concentration was calculated with the following equation:

$$Cellular\ concentration\ (cells.\ mL^{-1}) = \frac{No.of\ cells\ x\ 10^3}{Volume}$$

where *Volume* is the volume of counted squares (0.5 mm^3).

3.2.3. Dry weight

Glass microfiber filters (1.2 μm, VWR) (Figure 3.6) were pre-washed with 10 mL ammonium formate using a Millipore filtration system and left to dry for 24 hours at 50 ºC in an oven. In the day of the analysis, their weight was measured prior to filtering 10 mL of algal culture and rewashed with 10 mL of ammonium formate. The filters were again dried at 50 ºC for at least 72 hours or until constant weight was achieved. Biomass dry weight was calculated by subtraction of the initial and final weights divided by the volume filtered and results were expressed in mg L^{-1} of culture.

Figure 3.6 - Glass microfiber filters (VWR, 1.2 μm) containing 10 mL of algal culture and dried for 72 hours

.

3.3. Determination of lipid content

The lipid content of cultures was determined by the analysis of the fluorescence emitted by the lipids dyed with Nile red, using a modified method previously developed by Chen *et al.* (2009) and calibrated for the total lipids content determined by the gravimetric Bligh & Dyer (1959) method.

Briefly, a staining solution was prepared to achieve a final concentration of 1 μM of Nile red (9-diethylamino-5H-benzophenoxazine-5-one, $C_{20}H_{18}N_2O_2$) and 25% of DMSO. A 96-well plate was prepared with dilutions (1:1, 1:2, 1:5, 1:10 and 1:20) of algal culture with seawater in a final volume of 250 μL to which 50 μL of the staining solution described above was added. Negative controls were prepared by checking the fluorescence of 250 μL of Nile red staining solution mixed with 50 μL of seawater and the natural fluorescence of distilled water and algal culture at volumes of 300 μL. Fluorescence was read at 580 nm (excitation at 530 nm) in a BioTek Synergy 4 plate-

reader, preheated at 37 ºC and with continuous mixing for 10 min at 100-rpm (Enache, 2013).

The relationship between Nile Red fluorescence and lipid content was established using different dilutions of a sample previously analysed for lipid content by a protocol developed by Bligh & Dyer (1959) and modified by the Centre of Aquaculture in Trondheim. Four tubes containing 50 mL of algal culture were centrifuged for 10 min at 8000 *g*. The spun down biomass was then washed with 800 µL of distilled water and transferred to separate tubes. Afterwards, to each tube 2 mL of methanol and 1 mL of chloroform were added and the solution mixed using an Ultra-Turrax (IKA T-10 basic) disperser for 1 minute at maximum power. In subsequent steps, 1 mL of chloroform and, upon mixing with the disperser for 30 s, 1 mL of distilled water were added. After a second round of, the tubes were then centrifuged for 10 min at 5000 *g* (Figure 3.7). Then 600 µL of the chloroform phase were transferred to the tubes that had been pre-dried for 6 hours at 60 ºC and left to cool down for 3 hours in a desiccator. The tubes were left at 60 ºC overnight for the chloroform to evaporate. The resulting weight difference between the tubes before and after addition (and evaporation) of the chloroform phase represents the amounts of lipids per 50 mL of algae culture (Enache, 2013).

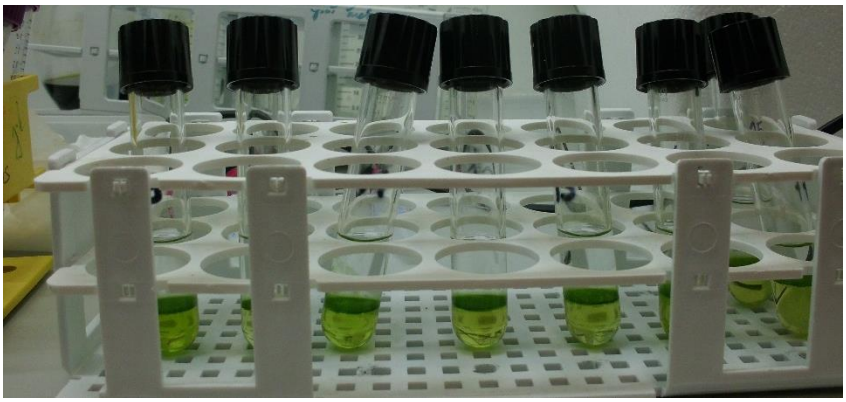

Figure 3.7 - Gravimetric lipid determination after centrifugation

3.4. Evaluation of nitrate concentration in the medium

For nitrate determination, samples were taken from the 5-L cultures and centrifuged for 5 min at 10,000 *g*. The supernatants were removed and buffered with an ammonium acetate buffer. The mixture was passed through a cadmium column to reduce nitrates to nitrites. The cadmium column was previously treated with 2% (w/v) copper sulphate and equilibrated with 1% (w/v) ammonium chloride buffer (pH 8.5). Nitrites

were determined using a modified Griess-Ilosvay method, based on the Griess reaction. Briefly, 200 µL of sulfanilamide solution (0.097 M in hydrochloric acid 37% v/v) were added to the samples, in order to form an azo compound, which then reacted with 200 µL of 3.9 mM N-(1-naftil)ethylenediamine, forming a pink aromatic amine, which was determined at 540 nm after 10 to 30 min. A correlation curve was established using potassium nitrate (KNO_3^-) standards at the concentrations of 0, 10, 20, 50, 70 and 100 mM.

3.5. Determination of the lipid profile

The lipid profile was determined by gas chromatography coupled with mass spectrometry (GC-MS) as described in Pereira (2009). Briefly, 0.1 *g* of biomass was aliquoted and centrifuged in triplicate. To this biomass a derivatization solution was added comprising methanol and acetyl chloride (20:1, v/v), immersed in ice, and homogenised with an Ultra-Turrax (IKA T-10 basic) disperser in 3 30-s cycles to achieve cell disruption. Afterwards, 1 mL of hexane was added and samples were kept in a bath at 90°C for one hour to complete the derivatization procedure. Samples were cooled on ice, transferred to new centrifuge tubes, and 1 mL of Milli-Q water (Millipore, Billerica, MA, USA) was added to each tube, vortexed for one minute and centrifuged at 1000 *g*, at 4 °C for 10 min to allow separation of the aqueous and organic phases. The organic phase was collected to a new tube, and 1 mL of hexane added to each centrifuge tube. This process was repeated until the added organic phase exhibited no colour. Anhydrous sodium sulphate was added in excess to remove residual water and samples were filtrated (0.45 µm). The samples were then dried with a gentle stream of nitrogen gas, weighted (total FAME fraction) and re-suspended in 500 µL of hexane.

The identification and quantification of the FAME was performed by GC-MS using an Agilent 6890 GC System Network coupled with a 5973 inert Mass Selective detector. The separation of the compounds was obtained by using a temperature program specific for FAMEs (Figure 3.8), starting at 60 ºC and maintaining this temperature during the first minute and then ramping up to 120 ºC in two min. The procedure was continued by increasing temperature up to 250 ºC within a time period of 26 min, rising 20 ºC in the next minute and ending 12 min later upon reaching 300 ºC.

An aliquot of l µL of each re-suspended sample was injected in an Agilent Tech column, DB-5MS (length: 25 m; internal diameter: 0.250 m; film: 0.25 µm), and the carrier gas was helium fed at a rate of 0.8 mL min^{-1}.

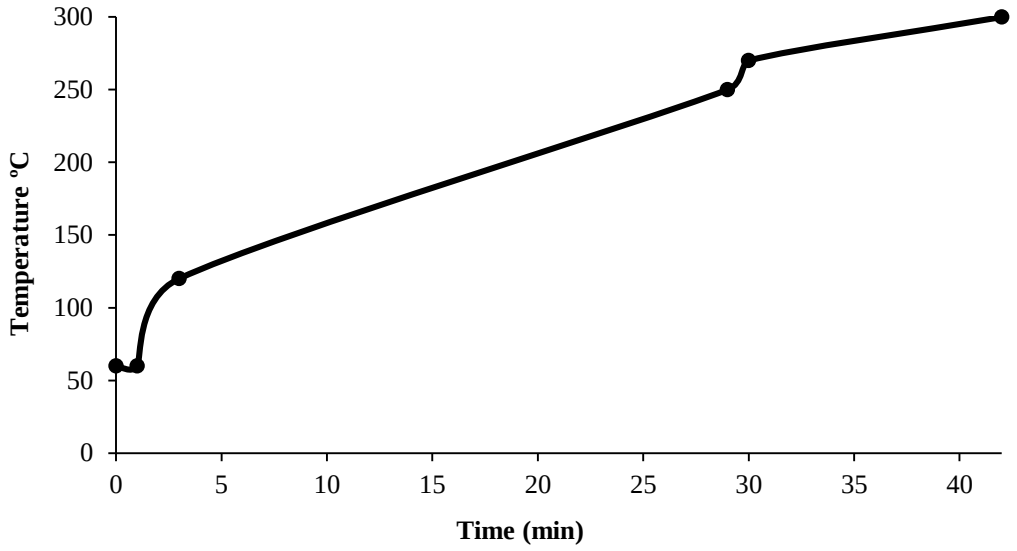

Figure 3.8 - GC-MS temperature for methyl ester separation (Adapted from Pereira, 2009)

A commercial solution composed of a mixture of 37 FAMEs (Supelco 37 FAME Mix) was used, which served for quantification purposes. Identification of different compounds was achieved by the comparison of the retention times with those of the standard and confirmed by comparison of the mass spectra obtained for the different peaks with those found in the NIST database (National Institute of Standards and Technology, U.S. Department of Commerce). FAME quantification was performed from calibration curves for the 37 methyl esters (Table 3.2). To quantify FAMEs that were not in the standard, the calibration curve from the most similar compound was used, accordingly to its chemical composition.

Table 3.2- Calibration curve, correlation coefficient and retention time for each FAME.

Compound	Calibration curve	r^2	Retention time (min)
C6:0	$y = 5.931x10^{7}x + 1.090x10^{5}$	0.989	3.147
C8:0	$y = 8.645x10^{7}x + 1.186x10^{5}$	0.993	4.723
C10:0	$y = 1.153x10^{8}x + 1.690x10^{3}$	0.993	7.248
C11:0	$y = 1.221x10^{8}x - 7.944x10^{4}$	0.996	8.945
C12:0	$y = 1.417x10^{8}x - 7.264x10^{4}$	0.995	10.856
C13:0	$y = 1.416x10^{8}x - 8.992x10^{4}$	0.996	12.870
C14:1	$y = 1.360x10^{8}x - 2.570x10^{5}$	0.998	14.647
C14:0	$y = 1.675x10^{8}x - 8.865x10^{4}$	0.995	14.920
C15:1	$y = 1.355x10^{8}x - 2.548x10^{5}$	0.998	16.947
C15:0	$y = 1.673x10^{8}x - 1.528x10^{5}$	0.997	16.947
C16:1	$y = 1.433x10^{8}x - 2.675x10^{5}$	0.997	18.498
C16:0	$y = 1.894x10^{8}x - 8.272x10^{4}$	0.994	18.942
C17:1	$y = 1.514x10^{8}x - 2.902x10^{5}$	0.998	20.145
C17:0	$y = 1.633x10^{8}x - 1.854x10^{5}$	0.997	20.847
C18:3n6	$y = 1.380x10^{8}x - 3.397x10^{5}$	1.000	21.747
C18:2n6	$y = 7.425x10^{7}x - 2.886x10^{5}$	0.999	22.070
C18:1n9 c	$y = 7.220x10^{8x} - 6.830x10^{5}$	0.996	22.210
C18:1n9 t	$y = 7.220x10^{8}x - 6.830x10^{5}$	0.996	22.332
C18:0	$y = 1.996x10^{8}x - 1.562x10^{5}$	0.995	22.709
C20:4n6	$y = 1.266x10^{8}x - 3.014x10^{5}$	0.999	24.936
C20:5n3	$y = 1.408x10^{8}x - 3.397x10^{5}$	0.999	25.027
C20:3n3/C20:3n6	$y = 1.320x10^{8}x - 3.045x10^{5}$	1.000	25.270
C20:2n6	$y = 1.518x10^{8}x - 2.969x10^{5}$	0.999	25.623
C20:1n9	$y = 3.605x10^{8}x - 5.001x10^{5}$	0.997	25.733
C20:0	$y = 2.145x10^{8}x - 2.823x10^{5}$	0.996	26.195
C21:0	$y = 1.928x10^{8}x - 2.734x10^{5}$	0.999	27.838
C22:6n3	$y = 1.221x10^{8}x - 3.793x10^{5}$	1.000	27.786
C22:2	$y = 1.396x10^{8}x - 3.122x10^{5}$	1.000	28.626
C22:1n9	$y = 1.788x10^{8}x - 3.320x10^{5}$	0.996	29.006
C22:0	$y = 2.126x10^{8}x - 2.665x10^{5}$	0.995	29.390
C23:0	$y = 1.997x10^{8}x - 2.032x10^{5}$	0.998	30.528
C24:1	$y = 1.694x10^{8}x - 2.270x10^{5}$	0.998	31.410
C24:0	$y = 2.179x10^{8}x - 1.647x10^{5}$	0.997	31.732

3.6. Microscopy

Live cells were stained with BODIPY 505/515 dye (4.4-difluro-1.3.5.7-tetramethyl-4-bora-3a.4a-diaza-s-indacene, Life Technologies Europe BV, Porto, Portugal) as described by Cooper et al (1999). Briefly, an amount of BODIPY sufficient to achieve a final concentration of 1µM was added, samples vortexed for 1 minute and incubated in the dark for 10 min. Fluorescence images were acquired with a Zeiss AXIOMAGES Z2 microscope, equipped with a coollSNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), using the 100x lens. For the fluorescence images, a Zeiss 38 He filter set was used (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) for fluorescein isothiocyanate (FITC) and the transmitted light images were acquired using differential interference contrast (DIC). Images were treated using Image J software (Research Service Branch, NIH, Bethesda, MD).

3.7. Bioactivity determination

3.7.1. Extract preparation

One gram of lyophilized biomass was extracted with 40 mL of four different solvents: hexane, ethyl acetate, acetone and methanol, and homogenized using an Ultra-Turrax disperser (IKA T10 basic) in three 2-min cycles. Extraction was achieved at room temperature with stirring, overnight. The extracts were filtered by means of a Whatman Nº 4 filter, evaporated in a rotary evaporator under a reduced pressure atmosphere, re-suspended in DMSO and stored at -20 ºC (Custódio *et al*., 2012a).

3.7.2. α-Amylase inhibitory activity

α-Amylase inhibitory activity was determined according to the method described by Kwon *et al*. (2008a). Each of the extracts (hexane, methanol, ethyl acetate and acetone) at the concentration of 1 mg mL^{-1} were mixed with 500 µL of 20 mM sodium phosphate buffer (pH 6.9) in 6 mM NaCl containing α-amylase at a concentration of 0.5 mg mL^{-1}. This mixture was pre-incubated at 25 °C for 10 min. After the pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) in 6 mM sodium chloride was added to each tube at 5-s intervals. The reaction mixtures were then incubated at 25 °C for 10 min. The reaction was stopped with 1 mL of dinitrosalicylic

acid colour reagent. The test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. A volume of 200 µL of distilled water were added to 50 µL of sample in a 96-well plate and the absorbance was measured at 540 nm (Biotek Synergy 4). The positive control used in this experiment was acarbose. The negative control consisted of a sample mixture containing 500 µL of DMSO. A colour control was also prepared by mixing extracts at the concentrations of 1 mg mL^{-1} with sodium phosphate buffer. Results were expressed as α-amylase inhibition activity (A.I.). The A.I. is defined as the ratio between the α-amylase activity of the negative control (enzyme alone) and that of the enzyme extract mixture. A.I. > 1 suggest α-amylase inhibitory activity and A.I. < 1 suggest α-amylase stimulatory activity (Correia *et al.*, 2004).

3.7.3. Antioxidant activity - Radical scavenging activity (RSA)

The radical scavenging activity (RSA) was evaluated against 2.2'-azino-bis (3-ethylbenzthiazoline-6- sulphonic) acid ($ABTS^{\bullet+}$) radical.

The ABTS radical scavenging assay was based on the method described by Wang *et al.* (2008). A stock solution of 7.4 mM $ABTS^{\bullet+}$ was generated by mixing 5 mL of Milli Q water, 0.0203 g of ABTS and 0.0035 g of potassium persulphate and kept at 5 ºC for 16 h in the dark. The $ABTS^{\bullet+}$ solution was then diluted with ethanol to obtain an absorbance of 0.7 at 734 nm (Biotek Synergy 4). An extract sample of 10 µl at the concentration of 1 mg ml^{-1} was then mixed with 190 µl of $ABTS^{\bullet+}$ solution in 96-well plates. After 6 min of incubation, the absorbance was measured at 734 nm (Biotek Synergy 4). Results were expressed as RSA (%) relative to a control containing the same solvent of the sample. BHT (1 mg ml^{-1}) was used as the positive control (Custódio *et al.*, 2012b). Radical scavenging activity was assessed according to the following formula:

$$\%\ of\ RSA = \frac{Abs_{\ negative\ control} - (Abs_{\ test\ solution} - Abs_{\ colour\ control})}{Abs_{\ negative\ control}} \times 100$$

where Abs stands for absorbance at 734 nm.

3.8. Statistical analyses

Data was analysed using Statistica v. 7.0 using one-way ANOVA for growth and lipid correlations with a confidence level of 99%. Lipid content and bioactive assays were subjected to variance analysis (factorial ANOVA with Tuckey's post-hoc test) with confidence level of 95%. Graphs were plotted using Excel v. 2013 and SigmaPlot v. 11.0.

4. Results and discussion

4.1. CTP4 growth curves

CTP4 strain was grown for 12 days in a batch culture at laboratory scale. Microalgal growth was measured every two days by means of optical density, cellular concentration and dry weight.

4.1.1. Culture growth and productivity in a batch culture system

CTP4 follows the usual pattern for microalgae growth, with a small lag phase under control conditions (20 ºC; 100 µmol m^{-2} s^{-1}), since it starts to grow immediately at day one (Figure 4.1). This stage is followed by an exponential growth phase starting between the second or third day and a stationary phase (Figure 4.1; Mata *et al*., 2010). CTP4 cultures took about 11 days to reach stationary phase ($2.7x10^6$ cells mL^{-1}) with a starting inoculum concentration of $2x10^5$ cells mL^{-1}. The highest productivity was 0.11 g L^{-1}, achieved from day 5 to 7. The specific growth rate and the doubling time were 0.34 $days^{-1}$ and 2.03 days, respectively.

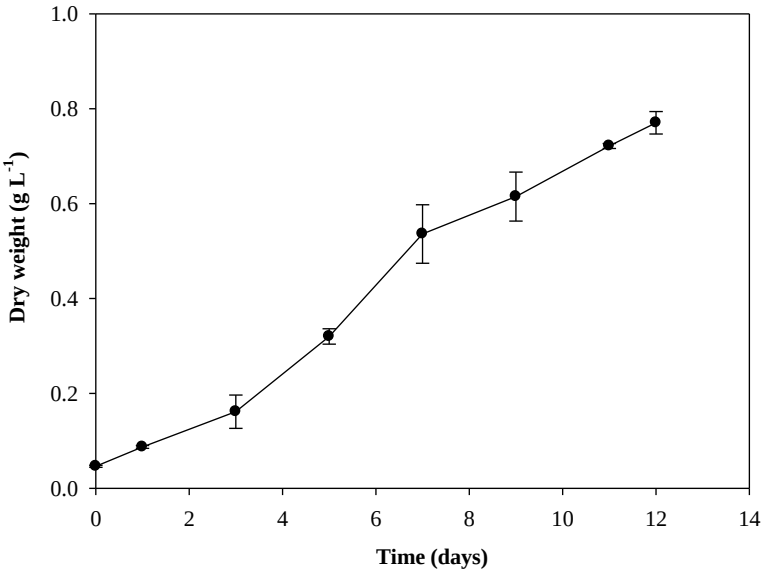

Figure 4.1 - CTP4 growth at 20 ºC and PFD of 100 µmol m^{-2} s^{-1} in a batch culture system for 12 days with modified Algal culture medium.

4.1.2. Growth calibration curves

Cell counting is a precise but time-consuming method. Therefore, calibration curves were established between the optical density measured at 750 nm (OD_{750}), cellular concentration determined by cell counting and dry weight. These correlations are species-specific since the relationship between OD and dry weight depends on numerous aspects, such as the size and shape of the cells, the wavelength of incident light and particle opacity (Rocha *et al*., 2003). Similarly, the correlation between OD and cell concentration depends on the cultures conditions, cell age, illumination, and culture media (Rocha *et al*. 2003). Figure 4.22 and Figure 4.3 present the relationship between OD_{750} and cell concentration and the relationship between the OD_{750} and dry weight, respectively. In both cases, significant exponential relationships were obtained ($p < 0.001$). For cellular concentration vs. OD_{750} and for dry weight (g L^{-1}) vs. OD_{750} linear relationships expressed as $y = 0.0157x$ ($r^2 = 0.85$) and $y = 0.6183x$ ($r^2 = 0.95$) were obtained, respectively. Therefore, the obtained equations were thereafter used to estimate cellular concentration and dry weight of the cultures from the optical density values.

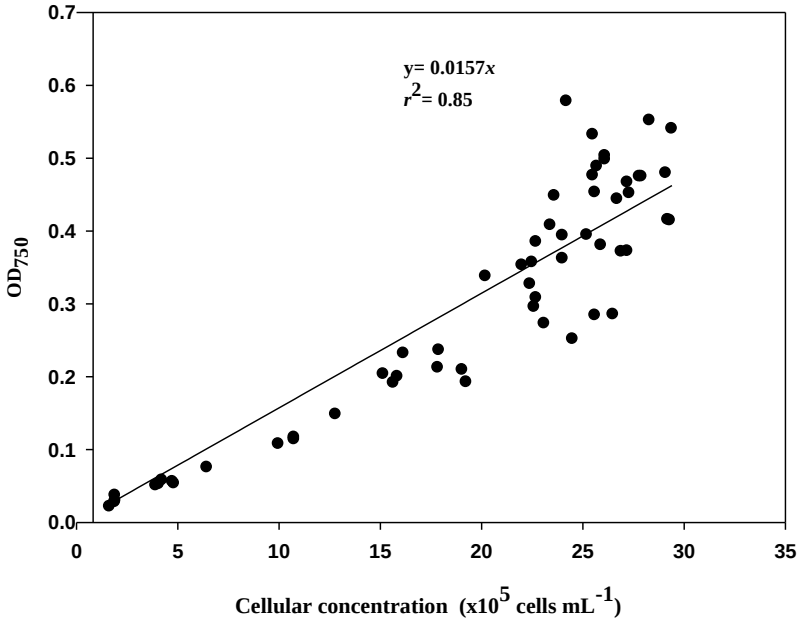

Figure 4.2 – Relationship between cellular concentration and optical density measured at 750 nm ($p < 0.001$).

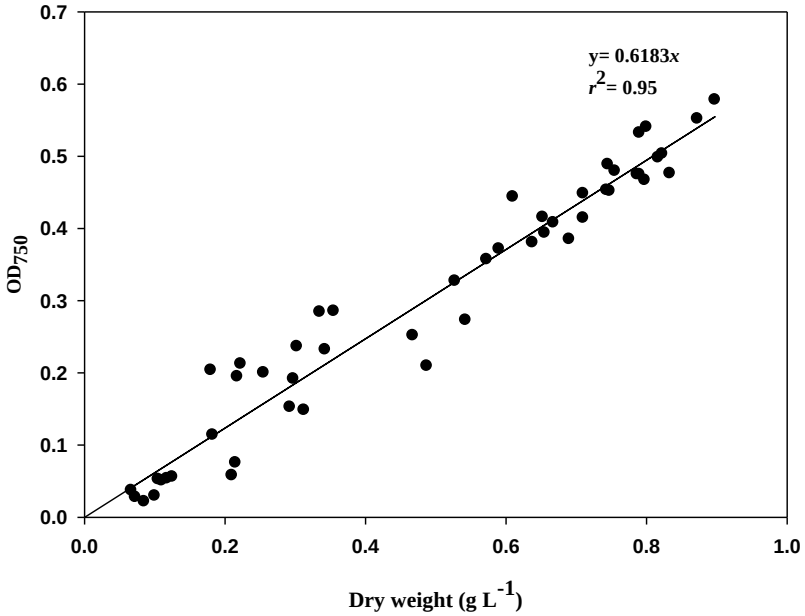

Figure 4.3 – Relationship between dry weight and optical density measured at 750 nm ($p < 0.001$).

4.2. CTP4 Nile red fluorescence calibration

Since the Nile red fluorescence method is fast, reliable and easy to carry out to quantify neutral lipids *in vivo*, especially for *Chlorophyceae* (Chen *et al*., 2009), a calibration curve was established between the fluorescence of Nile red and the total lipids estimated by the gravimetric Bligh & Dyer method. Similarly to the previous calibration curves, a significant linear relationship ($p < 0.001$) was obtained between lipid content using the gravimetric method and Nile red (Figure 4.4).

Traditionally the analysis of lipid content in biological samples is achieved by solvent extraction or gravimetric determination (Bligh & Dyer, 1959). However, these techniques are quite time consuming and a significant amount of biomass is needed, making them unfeasible if a large number of samples must be processed (Chen *et al*., 2009; Elsey *et al.*, 2007). Nile Red is a lipophilic dye that binds mostly to neutral lipids inside the cells (Liu *et al*., 2008). Significant correlations between the fluorescence of Nile red-stained cells and the lipid content in microalgae have previously been reported in several microalgae as *Chlorella vulgaris* (Liu *et al.*, 2008), *Nannochloropsis* sp. and *Tetraselmis* sp (Elsey *et al*., 2007) and *Botryococcus braunii* (Lee *et al*., 1998).

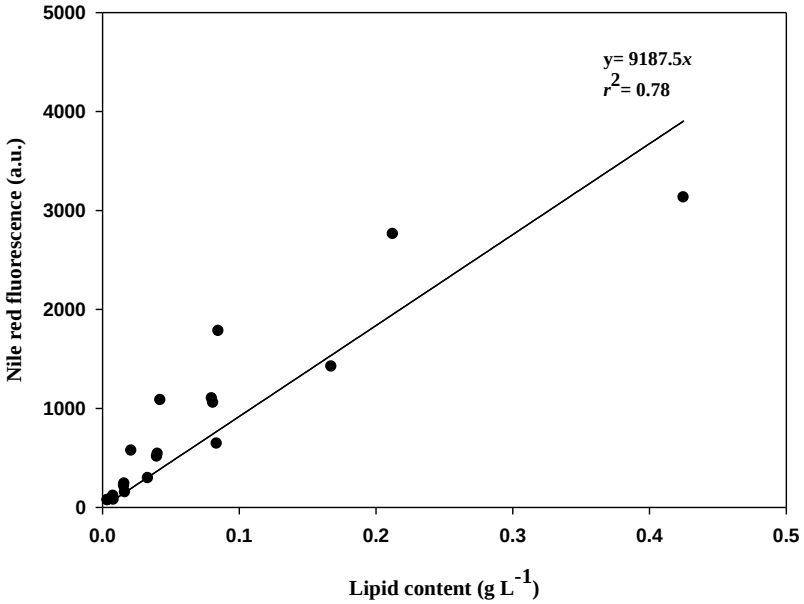

Figure 4.4 – Relationship between Nile red fluorescence and total lipids with gravimetric method Bligh & Dyer, 1959 ($p < 0.001$).

4.3. CTP4 in a two-stage growth system resorting to culture manipulation

4.3.1. Growth and nitrate determination

Nitrate concentration was measured in samples collected every day, for 17 days, in cultures grown under nutrient depletion or supplementation. Results are presented in Figure 4.5.

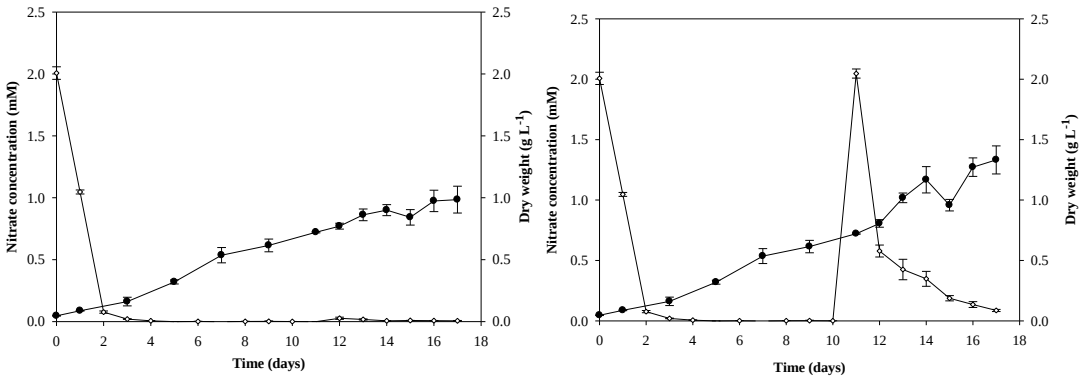

Figure 4.5- Nitrate concentration (○) and dry weight (●) of cultures grown under nutrient depletion (left) and addition of nutrients supplied at day 11 (right).

Modified Algal medium was added to both samples at day zero and, in the culture grown under nutrient supplementation, the same medium was re-added at day 11. Nitrate

concentration decreases from 2.0 to close to 0 mM in two days when added at day zero. In cultures where nutrients were supplemented at day 11, the fall is less dramatic after the supplementation, leading to a higher biomass value than in the non-supplemented culture ($p < 0.05$). Only at day 18 the nitrate values were similar to the ones found at the beginning of the experiment. This higher biomass suggests that nitrogen availability limits growth. However, it is possible that growth was also limited by light availability, as high culture concentrations may prevent full light penetration as compared to diluted cultures.

Nitrogen is the nutrient that has the strongest effect on lipid metabolism in microalgae and its depletion is often used to impose nutrient limitation, probably because it is the one that limits microalgal growth the most (Sharma *et al*., 2012). According to Converti *et al*. (2009), lack of nitrogen limits protein synthesis, increasing lipid accumulation. Taken together with the ease of applying this stress, nitrogen depletion is perhaps the strategy that should be applied to increase overall lipid productivity in most cases (Sharma *et al*., 2012).

4.3.2. Lipid content

To avoid the metabolic shift (from carbohydrate or proteins to lipid synthesis) related with lipid content, which consequently leads to lower cell division and productivities (Gouveia, 2011), a two-stage growth system was implemented.

The effect of lipid induction using different temperatures and light intensities with and without nutrient supplementation is presented in Figure 4.6, Figure *4.7*7 and**Erro! A origem da referência não foi encontrada.**8; the values in percentage and in g L^{-1} for each condition are presented in the annex (Table 7.1).

Cultures were grown for 11 days, after which one of the cultures (20 ºC and 100 µmol m^{-2} s^{-1}) was supplemented with algal modified medium (N^+), whereas the other was subjected to nutrient deprivation (N^-). From this point on, days 11-17 of the full experiment will be referred to as days 1-7 of the last stage of the two-stage growth system, respectively.

Figure 4.6 shows the lipid content levels of cultures grown with nutrient depletion and supplementation. Cultures with nutrient addition decreased their lipid content, exceeding the initial value (7.3%) only on day 5 ($p < 0.05$). Nutrient-deprived cultures decreased their lipid value on day 2, overcoming the initial value on day 3. After day 3, the daily lipid content of cultures grown in the nutrient deprived medium (N^-) was always

significantly higher ($p < 0.05$) than that of cultures grown in the nutrient supplemented medium (N^+).

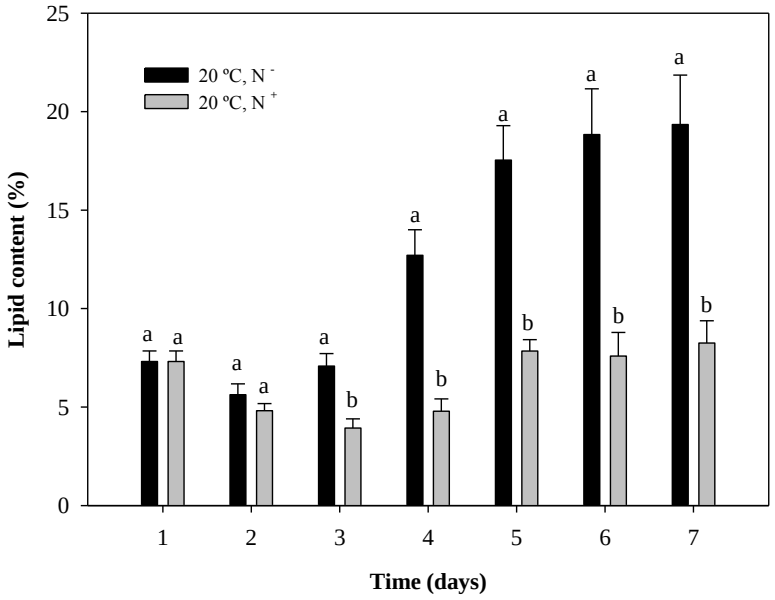

Figure 4.6- Lipid content (% of dry weight) of the two-stage growth cultures at 20 ºC and 100 µmol m^{-2} s^{-1} with nutrient depletion (N^-) and supplementation (N^+). Bars show means and standard deviation of three replicates. Bars labelled with different letters are significantly different on a given day ($p < 0.05$).

It is clear that nutrient deprivation led to an induction of the lipid content. On day 7, N^- cultures showed a lipid content of 19.4% of dry weight (DW) while the N^+ cells displayed less than half of that value (8.3% of DW). Nutrient limitation can therefore be considered an efficient environmental stimulus for increased lipid accumulation in CTP4 (Hu *et al*., 2008; Rodolfi *et al*., 2009; Sharma *et al*., 2012). Hu *et al*. (2008) state that from all nutrients, nitrogen is the one that affects lipid metabolism the most. Various species have shown lipid accumulation as a reaction to nitrogen starvation, particularly in the form of TAGs. Illman *et al.* (2000) studied five *Chlorella* strains and under nitrogen starvation all of them revealed a significant increase in lipid content when compared to the control conditions. The greatest increases were observed in *Chlorella emersonii* (from 29% to 63%), *Mychonastes homosphaera* (formerly known as *Chlorella minutissima*; up to 57% of lipids) and *Chlorella vulgaris* (from 18% to 40%). Converti *et al*. (2009) reported that *Chlorella vulgaris* and *Nannochloropsis oculata* increased their lipid content when subjected to a 75% nitrogen concentration reduction from 5.9% to 15.3% and 7.9% to 15.9%, respectively.

Nitrogen limitation can induce three major alterations: i) reduction of the thylakoid membrane; ii) activation of acyl hydrolase; and iii) increased phospholipid hydrolysis. These changes can lead to an increase of the intracellular content of long chain acyl-CoA. Also, nitrogen limitation might activate diacylglycerol acyltransferase that catalyses TAG synthesis (Hu *et al*., 2008; Xin *et al*., 2010).

The results shown in Figure 4.6 were thereafter used as the lipid content of cells grown under control conditions that would be compared to those of cells under thermal stress and high light.

4.3.2.1. Thermal stress

Considering the experiment conducted at a suboptimal temperature (Figure 4.7), in nutrient-depleted conditions there was no significant differences ($p > 0.05$) between cells grown under control (20 ºC) and at 5 ºC except at day 7 when cells at 5 ºC contained higher lipid levels ($p < 0.05$) as compared with control cells.

Regarding nutrient-supplemented experiments, cultures grown at 5 ºC displayed a higher lipid content ($p < 0.05$) than control cells from day 6 onwards.

The highest amount of lipids attained at 5 ºC (day 7) was similar for N^+ and N^- cultures ($p > 0.05$), suggesting that low temperatures seem to relieve the inhibition of lipid accumulation caused by nutrient supplementation.

In Kalacheva *et al.* (2002) cultures grown under temperatures lower than optimal showed also an increase in the lipid (TAG) content. *Botryococcus braunii* was cultivated at 25 ºC (optimal growth temperature) and 18 ºC (suboptimal). The values of TAG for optimal growth temperature were only about 2% while under suboptimal conditions neutral lipids varied between 7.6 and 9.3% after 13 days of cultivation.

This lipid increase observed at low temperatures can be explained as a mechanism to keep cells functioning (Somerville, 1995). At low temperatures microalgae tend to increase the degree of fatty acid unsaturation (Thompson, 1996) in order to maintain membrane fluidity (Harwood & Guschina, 2009). According to Thompson *et al*. (1992) the total fatty acid composition of cells that have been exposed to low temperature is a balance between an increase in membrane fatty acid unsaturation (increasing membrane fluidity) and chlorosis (leading to TAG accumulation). The balance between these two processes possibly will vary according to the algal species, leading to a distinct fatty acid profile in response to environmental stress. The effect of temperature on the fatty acid profile is discussed ahead.

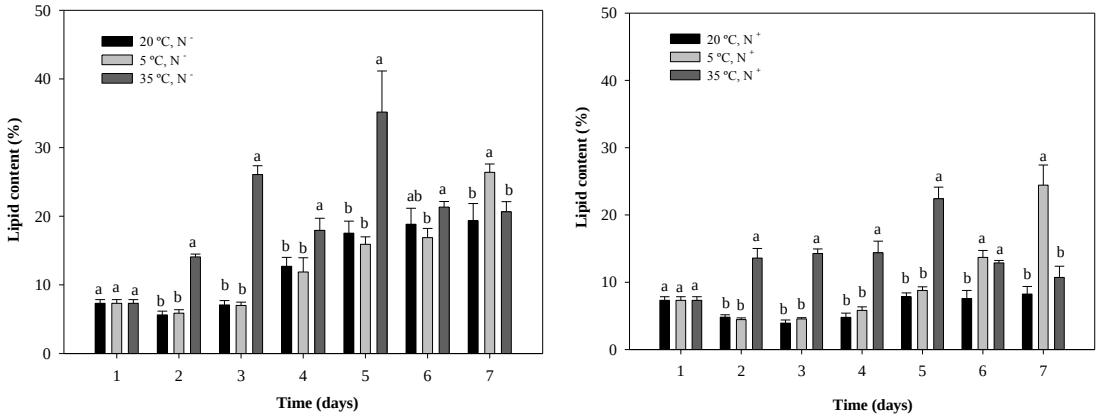

Figure 4.7: Comparison of lipid content (% of dry biomass) of the two-stage growth cultures at 20 ºC, 5 ºC and 35 ºC with 100 µmol m^{-2} s^{-1} using under nutrient depletion (N^-) and supplementation (N^+). Bars show means and standard deviation of three replicates. Bars labelled with different letters are significantly different on a given day ($p < 0.05$).

The results obtained at supra-optimal temperature (35 ºC) are presented in Figure 4.7. This treatment resulted in a lipid induction significant higher ($p < 0.05$) as compared with the control condition from day 2 onwards except for day 6 for N^- cultures and at days 2-5 for N^+ cultures. Contrary to other lipid-inductive stresses, which in general had their maximum lipid values at day 7, cultures grown at 35 ºC peaked their lipid content at day 5, with N^- cultures displaying the highest value (35.2%). More detailed data for temperature induction with g L^{-1} values are given in the annex (Table 7.1).

Taken together, these results suggest that heat stress induces lipids faster than light or nutrient deprivation but it may decrease cell viability afterwards. This was confirmed by the observation of high amounts of lysed cells at days 6 and 7 (data not shown) in samples viewed by fluorescence microscopy and lipid body staining with BODIPY 505/515. Lipids released by lysed cells may also be stained by Nile red but will generate a lower fluorescence level because Nile red fluoresces about 40 times less in water due to its low solubility (1 µg mL^{-1}; Fowler & Greenspan, 1985). In fact, the lipids were there but they were most probably externalized, preventing Nile red dye from being an effective lipid dye.

The best lipid induction with temperature was achieved at 35 ºC under nutrient depletion, leading to a lipid content of 35.2% of DW. According to Hu *et al*. (2008) and Sharma *et al.* (2012) and the studies discussed therein, a general trend of lipid increase with an increase or decrease of temperature cannot be established. In this case, both increase and decrease in temperature led to an increase in lipid content. Our results are in accordance with Aaronson (1973) who grew *Ochromonas danica* at temperatures ranging

from 15 to 30 ºC with 5-degree increments and in all increments this phytoflagellate increased the total lipid content.

4.3.2.2. Light intensity

Lipid content in cultures under different PFD are represented in Figure 4.8. Upon comparing cells at 100 µmol m^{-2} s^{-1} or 200 µmol m^{-2} s^{-1}, no significant differences ($p > 0.05$) were observed during the first two days for both N^- and N^+ cultures. Again nutrient depletion was the condition with the highest lipid values. At day 6, N^- cultures exposed to 200 µmol m^{-2} s^{-1} reached a lipid content of 34.5% of DW, almost doubling the control value (18.8% of DW). Supplemented cultures also exhibited an increase in lipid content but with a significantly lower value (15.5% of DW at day 7).

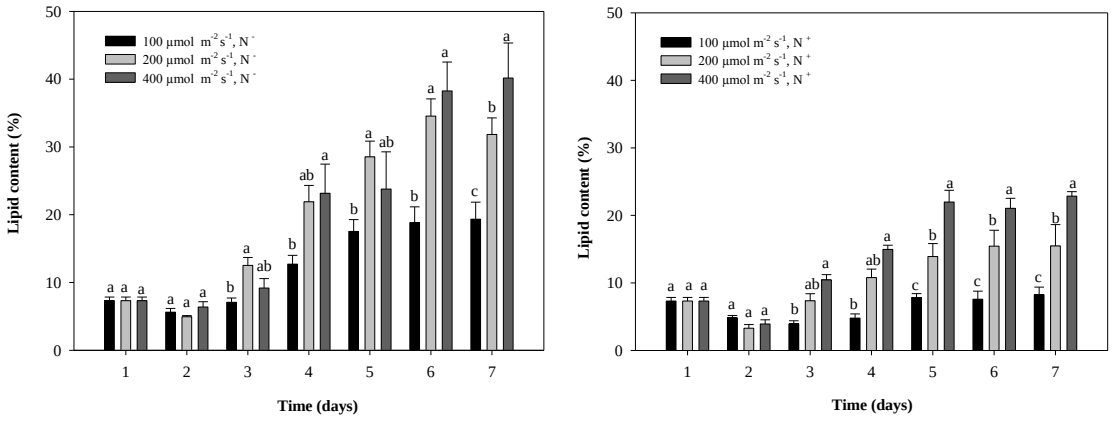

Figure 4.8: Comparison of lipid content (% of dry biomass) of the two-stage growth cultures at 100, 200 and 400 µmol m^{-2} s^{-1} at 20ºC using nutrient depletion (N^-) and supplementation (N^+). Bars show means and standard deviation of three replicates. Bars labelled with different letters are significantly different on a given day ($p < 0.05$).

Cells under N^- conditions and a PFD of 400 µmol m^{-2} s^{-1} exhibited significant differences ($p < 0.05$) on days 4, 6 and 7 when compared to control cells also under nutrient-depletion, and from day 3 onwards for nutrient-supplemented cultures. This condition appears to yield the highest lipid contents, showing an increase from 7.3% (day 1) to 40.2% of DW (day 7), a 2-fold increase compared to the control value (19.4% of DW). More detailed data regarding light induction expressed in g L^{-1} are given in the annex (Table 7.1).

Hu *et al*. (2008) concluded that usually high light tends to increase the amount of TAG. This conclusion is corroborated by several studies. Khotimchenko & Yakovleva (2005) showed that *Tichocarpus crinitus* grown under a 24-h photoperiod had a higher

amount of TAG at 70-80% of the incident photosynthetically active radiation (PAR), when compared to cultures grown at 8-10% PAR. According to the same authors, this alga has low tolerance to high irradiance, as the highest PAR tested was high enough to induce photoinhibition. Apparently, the ability of synthesizing high amounts of TAG is very important for the survival of *T. crinitus* under light stress conditions to counteract photooxidation. Brown *et al.* (1996) showed that *Thalassiosira pseudonana* cultures grown up to stationary phase under a 24-h light photoperiod at 100 µmol m^{-2} s^{-1} presented a 2-fold increase of the TAG levels (45%) compared to cultures cultivated at 50 µmol m^{-2} s^{-1}. Ruangsomboon (2012) also studied the effect of light intensity on the lipid content in *Botryococcus braunii* KMITL 2. Four different light intensities were tested (0.3, 87.5, 200 and 538 µmol m^{-2} s^{-1}) and the highest amount of lipids (450 mg L^{-1}) was observed in cultures cultivated at the highest PFD.

4.3.2.3. BODIPY 505/515 fluorescence microscopy

The lipid content values obtained with the Nile red dye were confirmed by fluorescence microscopy using BODIPY 505/515. There is a higher amount of lipid droplets in cultures under abiotic stress (Figure 4.9), which seem to corroborate the values shown in Figures 4.6, 4.7 and 4.8.

In **Erro! A origem da referência não foi encontrada.**9 it is possible to observe an increase in the amount of lipid droplets in CTP4 cells both under thermal stress and high light intensity. However, the highest number of lipid bodies was attained at 400 µmol m^{-2} s^{-1} with nutrient depletion, confirming the lipid contents determined previously.

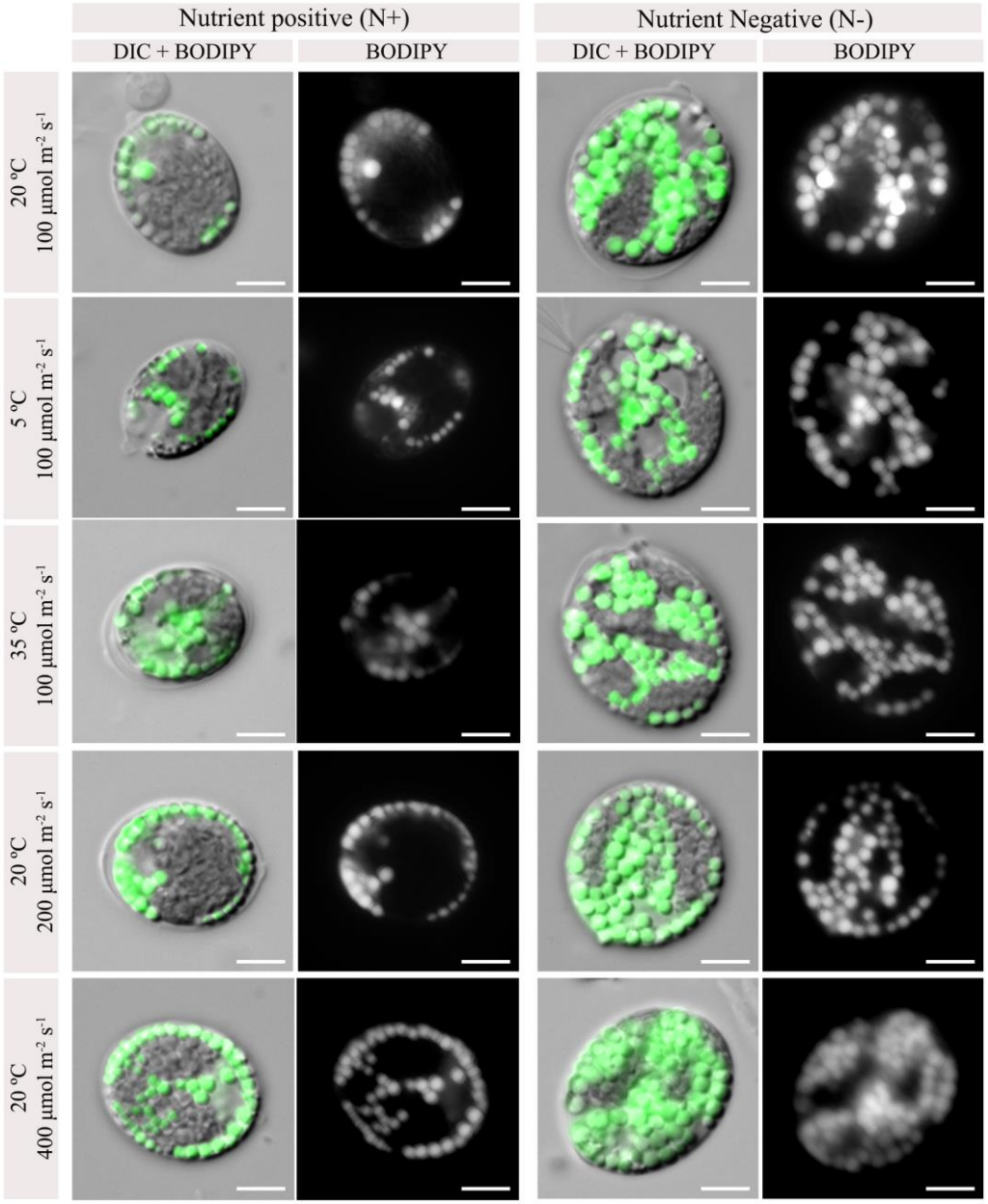

Figure 4.9 - Fluorescence microscopy photographs showing lipid bodies tagged with BODIPY in cultures grown at 20 ºC and 100 µmol m^{-2} s^{-1} of PFD (control); 5 ºC, 35 ºC, 200 and 400 µmol m^{-2} s^{-1} with nutrient supplementation (N^+) or nutrient depletion (N^-). Cells against a grey background correspond to merged DIC and BODIPY fluorescence micrographs. Cells against a black background are micrographs of BODIPY fluorescence alone. Scale bar – 5 µm.

Overall, Figure 4.9 confirms that nutrient depletion is a major factor for lipid induction in CTP4 cells, as all cultures under nutrient depletion appear to have more lipid droplets. These pictures are consistent with the values from lipid induction observed previously (Fig. 4.6) as well as in other microalgae (Lynn *et al*., 2000).

Interestingly, unlike cells under thermal stress, cells exposed to high light appear to accumulate most of the lipid droplets at the periphery of the cell. This may be a defence mechanism to prevent photoinhibition. With excess light, production of ATP and NADPH is increased because photosynthesis is controlled by light intensity. In order to maintain cell function these chemicals need to be consumed in order to liberate the major electron acceptor for photosynthesis – $NADP^+$ (Sharma *et al.*, 2012). High amounts of photosynthetically produced ATP and NADPH are needed for TAG synthesis. Consequently, lipid biosynthesis would help to convert excess light to chemical energy and prevent photooxidative damage of cells (Khotimchenko & Yakovleva, 2005). Some algae use the excess lipids not just as a growth reserve but also to directly absorb part of the incoming light, preventing photoinhibition (Thompson, 1996). Notwithstanding, these lipids alone would not be very efficient protecting the cell against excess light. Carotenoids are often produced by microalgae upon environmental stimuli (Guedes *et al.*, 2011) and act as a shield to absorb excess light and thus avoid photooxidative damage (Thompson, 1996). Rabbani *et al.* (1998) studied β-carotene synthesis driven by TAG deposition in *Dunaliella bardawil* and concluded that under normal conditions the production of β-carotene is not as active as under stress conditions that stimulate TAG biosynthesis, such as high light or nutrient starvation. This pigment is accumulated in recently formed TAG droplets inside the plastids and the formation of both TAG and β-carotene are interdependent, as the overproduction of β-carotene is inhibited when TAG synthesis stops. As carotenoids are known to absorb energetic wavelengths as blue light, the concomitant induction of lipid droplets containing carotenoids is perhaps a mechanism also present in CTP4 cells that may provide protection against photoinhibition (Ben-Amotz *et al*., 1989) and thus allow them to withstand high light stress. However, further research is needed to ascertain whether carotenoids do accumulate in the observed lipid droplets.

4.4. Fatty acid methyl esters (FAME) profile

The FAME profile of strain CTP4 under the different lipid induction conditions is represented in Table 4.1. The main fatty acids detected were the hexadecadienoic (C16:2), palmitoleic (C16:1), palmitic (C16:0), linoleic (C18:2), oleic (C18:1*c*), elaidic (C18:1*t*) and stearic (C18:0) acids.

For all conditions, the dominant FAME were C16:0 and C18:2. C18:0 was not detected in most conditions, namely at 5 ºC, 200 µmol m^{-2} s^{-1} both under N^- and N^+ and at 400 µmol m^{-2} s^{-1} under N^+.

At 5 ºC, total saturated fatty acids (SFA) increased, when compared to control cells, in both nutrient depleted and supplemented cultures (Table 4.1). According to Day (2013) and Hu *et al.* (2008), lowering temperature usually leads to an increase of unsaturated fatty acids. However, in CTP4, which is able to tolerate temperatures as low as 5 ºC, lipid accumulation appears not to be blocked by nutrient supplementation, which could mask any changes of saturation in membrane lipids if the saturation of FA present in TAG are not affected by temperature.

At 35 ºC there was no significant ($p > 0.05$) increase in the saturated fatty acids. Day (2013) and Hu *et al.* (2008) also state that a temperature upshift may increase fatty acid saturation. As 35 °C also stimulates lipid droplet accumulation in CTP4, it is thus possible that under these conditions a masking effect is also at play and overall lipid saturation does not reflect what is taking place with the saturation levels of phospholipids. However, other explanations are possible. CTP4 may also have other mechanisms to maintain membrane fluidity besides changes in fatty acid saturation. For example, lipid rafts, sterols and membrane proteins are known to modulate membrane fluidity as well (Lingwood & Simons, 2010). These possibilities are reinforced by the results of Renaud *et al.* (2012), who have studied the fatty acid composition of four Australian microalgae grown until late exponential phase at five different temperatures. There was no general relationship between percentage of SFA and temperature for all species studied, indicating that changes in temperature are not always accompanied by different levels of FA saturation. However, Sushchik *et al*. (2003) showed that both *Chlorella vulgaris* and *Botryococcus braunii* decreased the unsaturated FA with increasing temperature, these results being observed by other researchers (Hu *et al*., 2008; Sharma *et al*., 2012; Somerville, 1995). The explanation for these increases is the same as for low temperature, except that by lowering temperature, the FA unsaturation increases in order to maintain

membrane fluidity and keep the cell working as normally (Sushchik, *et al*., 2003). Conversely, increasing temperature would lead to decreased levels of unsaturated FA (Thompson, 1996). The total fatty acid composition of cells that have been exposed to low/high temperature is a balance between an increase in membrane fatty acid unsaturation/saturation (increasing membrane fluidity) and chlorosis (leading to TAG accumulation). The balance between these two processes is species specific, resulting in a distinct fatty acid profile in response to environmental stress (Thompson *et al*., 1992).

Regarding to light intensity, both 200 and 400 µmol m^{-2} s^{-1} had similar results with an increase in PUFA C16:2 and C18:2, comparing with the control (20 ºC, 100 µmol m^{-2} s^{-1}).

Brown *et al.* (1996) showed that *Thalassiosira pseudonana* grown up to stationary phase under 24-h light at 100 µmol m^{-2} s^{-1} had a higher amount of TAG with saturated and monounsaturated fatty acids, when compared to cultures grown with half that PFD. In CTP4 the opposite was observed: this strain produced higher amounts of TAG with increased light exposure but there was a decrease in saturated and monounsaturated fatty acids.

Bold values in Table 4.1 represent a significant ($p < 0.05$) increase in FAME relative quantity from nutrient supplemented to nutrient-depleted cultures. This increment was observed in all conditions in C16:0, except for the highest light stress (400 µmol m^{-2} s^{-1}) where there was no significant ($p > 0.05$) increment in any FAME from N^+ to N^-. Guarnieri *et al.* (2011) had similar results with *Chlamydomonas reinhardtii* with an increase in C16:0 in cultures grown under nitrogen deprivation.

For the production of biodiesel, the lipid profile of the CTP4 strain under lipid-inductive conditions appears to be adequate. FAME composition should be a mix of mostly saturated and monounsaturated fatty acids, as FAME with 4 or more double bonds are more likely to have reduced oxidative stability during storage. This profile is more suitable for biodiesel production because it has long-chain carbons from C16 to C18 (Chisti, 2007).

Table 4.1- FAME profile and total saturated fatty acids (SFA) of microalgae grown under different culture conditions (high and low temperature and different light intensities) cultivated for 17 days with nutrient starvation (N^-) and supplementation (N^+). Bold values represent an increase in FAME quantity from N^+ to N^-. Values in the table are represented in percentage (%), *n*=6 ($p < 0.05$).

FAME	Conditions									
	20 ºC 100 µmol m^{-2} s^{-1}		**5 ºC 100 µmol m^{-2} s^{-1}**		**35 ºC 100 µmol m^{-2} s^{-1}**		**20 ºC 200 µmol m^{-2} s^{-1}**		**20 ºC 400 µmol m^{-2} s^{-1}**	
	N^-	**N^+**	**N^-**	**N^+**	**N^-**	**N^+**	**N^-**	**N^+**	**N^-**	**N^+**
C16:2	5.60±0.37	5.68±0.18	1.45±0.36	1.52±0.67	4.01±0.48	4.54±0.35	8.35±0.88	8.92±0.83	8.54±0.54	8.77±0.34
C16:1	6.34±0.26	8.93±0.49	2.43±0.77	2.35±0.38	10.07±0.70	10.45±0.45	2.56±0.56	3.27±0.81	2.98±0.20	3.00±0.36
C16:0	**43.90±0.63**	38.10±1.17	**54.61±1.81**	49.66±3.36	**43.98±1.75**	39.74±1.19	**36.41±2.49**	33.67±2.22	33.71±1.52	32.67±0.57
C18:2	29.09±1.15	31.84±1.32	22.69±1.78	27.58±1.71	26.82±1.91	30.36±1.12	41.77±1.70	42.37±1.22	42.91±1.04	43.93±0.27
C18:1*c*	14.02±0.67	13.94±0.30	18.07±0.73	19.00±0.86	14.05±0.88	13.10±0.45	10.42±0.61	10.35±0.55	10.66±0.42	10.17±0.22
C18:1*t*	0.61±0.14	1.19±0.09	0.65±0.12	1.29±0.13	0.86±0.06	1.13±0.18	0.40±0.15	1.26±0.52	0.89±0.41	0.96±0.21
C18:0	0.51±0.05	0.32±0.09	n.d.	n.d.	0.71±0.08	0.67±0.11	n.d.	n.d.	0.31±0.07	n.d.
Σ SFA	44.41±0.63	38.42±1.18	54.61±1.81	49.66±3.36	44.69±1.75	40.41±1.19	36.41±2.49	33.67±2.22	34.02±1.52	32.67±0.57
Σ MUFA	20.97±0.74	24.06±0.58	21.15±1.07	22.63±0.94	24.99±1.12	24.69±0.66	13.38±0.85	14.87±1.84	14.53±0.62	14.13±0.47
Σ PUFA	34.68±1.21	37.52±1.33	24.13±1.82	29.11±1.84	30.83±1.97	34.90±1.17	50.12±1.92	51.30±1.47	51.45±1.17	52.69±0.44

4.5. Determination of biological activities

One of the objectives of this work was to turn microalgal biodiesel production economically feasible with bioactive compounds extracted from harvested algal biomass. Considering microalgal taxonomic diversity, several screenings for novel biological compounds with biomedical application have been carried out recently (Plaza, Herrero, Cifuentes & Ibáñez, 2009).

For the analysis of CTP4, biological activities were analysed on the biomass collected from the cultivation of two 100-L bags. Solvents were chosen according to their polarity.

4.5.1. Amylase inhibitory activity

Amylase inhibition (A.I.) values of the four extracts from four different solvents (acetone, methanol, ethyl acetate and hexane) are represented in Figure 4.10 and 4.12. Inhibition values were compared with those of acarbose (inhibitor of α-glucosidase and α-amylase, Kwon *et al*., 2008b). Inhibition values above one suggest amylase inhibitory activity and below one suggest amylase stimulatory activity (Correia *et al*., 2004).

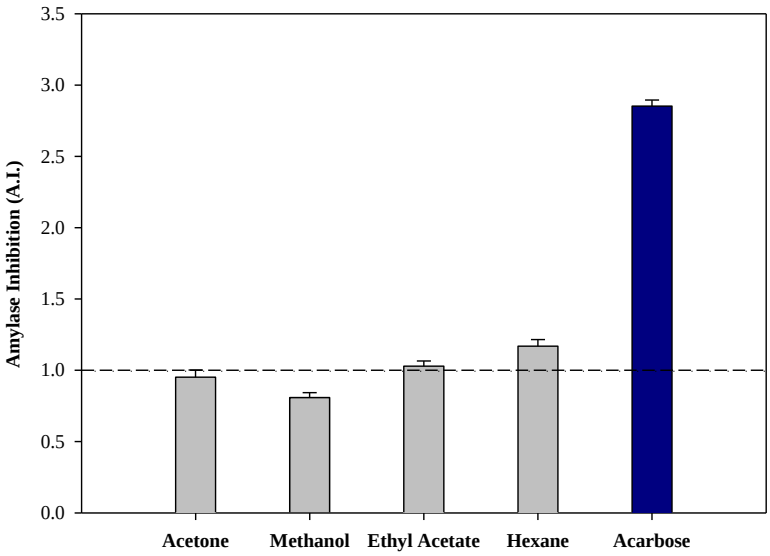

Figure 4.10 - Amylase inhibitory activity from acetone. methanol. ethyl acetate and hexane extracts at 1 mg mL^{-1}. Acarbose served as positive control. Dash line is the value from which it is considered to occur inhibitory activity, *n*=6.

In the Figure 4.11 the A.I. of extracts obtained from CTP4 cultures under nutrient depletion. Acetone, methanol and ethyl acetate extracts are not significantly different (*p*

> 0.05) than 1, and thus they do not exhibit inhibitory activity at the concentration of 1 mg mL^{-1}. The only extract showing inhibitory activity corresponded to hexane ($p < 0.05$) (1.2 A.I.). However, it was not very effective as compared to the positive control (acarbose).

Under nutrient supplementation (Figure 4.12), again all extracts except hexane (1.2 A.I.) yielded values that were not significantly different ($p > 0.05$) than 1. In other words, there is inhibitory activity (> 1.0) at the concentration of 1 mg mL^{-1} only for the hexane extract ($p < 0.05$). Once more hexane values were not very effective when compared to acarbose.

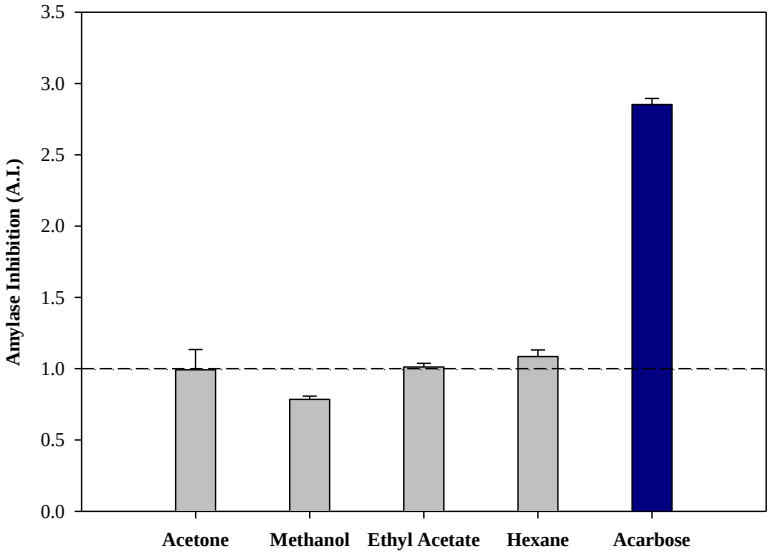

Figure 4.11- Amylase inhibitory activity from acetone. methanol. ethyl acetate and hexane extracts at 1 mg mL^{-1}. Acarbose served as positive control. Dash line is the value from which it is considered to occur inhibitory activity, n=6.

The use of acarbose as inhibitor of α-glucosidase and α-amylase has the strong inconvenient of producing side effects, such as abdominal distention, flatulence and diarrhea. These symptoms are probably caused by an excessive inhibition of the pancreatic α-amylase causing abnormal bacterial fermentation of undigested carbohydrates in the colon (Kwon *et al*., 2008b). For this reason it is very important to search for an alternative for acarbose. Apparently CTP4 extracts of cells under either N^- and N^+ conditions do not seem to be an alternative to this drug. The only extract that has some inhibitory activity, the hexane extract (1.1 and 1.2 A.I.), it is apparently not very promising.

4.5.2. Antioxidant activity

Antioxidant activity was evaluated by radical scavenging activity (RSA) against 2.2'-azino-bis (3 ethylbenzthiazoline-6-sulphonic) acid (ABTS•$^{+}$) radical. Results were expressed as percentage of inhibition relatively to a blank containing the solvent of each extract (Figure 4.12 and Figure 4.13).

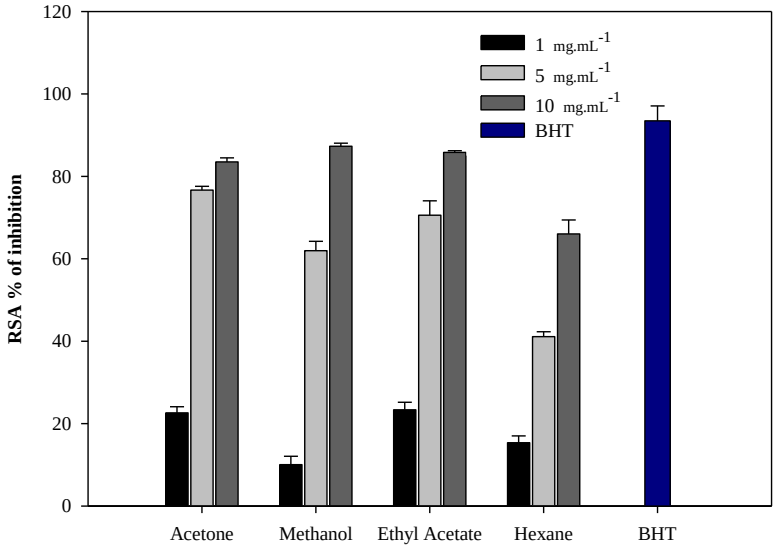

Figure 4.12 - Radical scavenging activity (% of inhibition) against ABTS•$^{+}$ radical from acetone, methanol, ethyl acetate and hexane extracts at 1. 5 and 10 mg mL^{-1}, prepared from cultures grown under nutrient depletion, *n*=6.

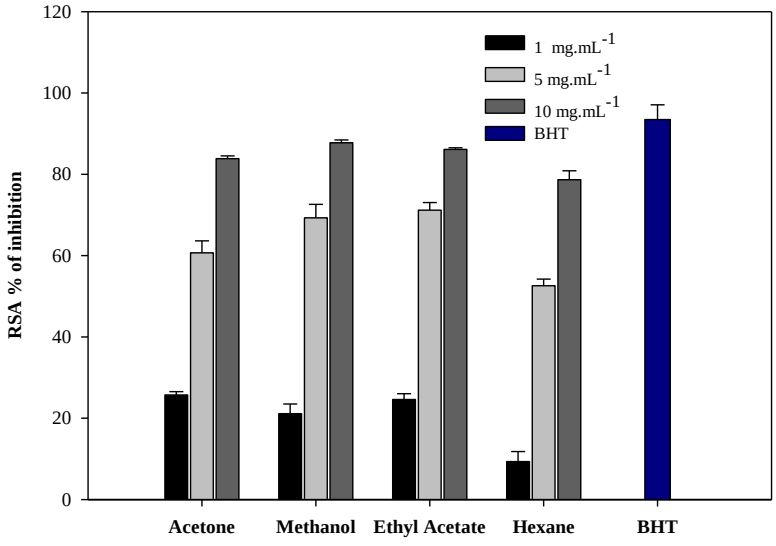

Figure 4.13- Radical scavenging activity (% of inhibition) against ABTS•$^{+}$ radical from acetone, methanol, ethyl acetate and hexane extracts at 1. 5 and 10 mg mL^{-1}, prepared from cultures grown under nutrient supplementation, *n*=6.

The antioxidant activity presented a dose-dependent response. All extracts showed a higher RSA for the maximum concentration tested (10 mg mL^{-1}), between 66% and 87.7% of inhibition.

This data indicates that microalga CTP4 has antioxidant compounds and the cultivation without nutrient supplementation it is not significantly different ($p < 0.05$) from the culture without it. Therefore simple cultivation without enrichment should be adopted.

In recent years there has been a growing interest for new antioxidants from natural sources (Custódio *et al*., 2012b), since some of the synthetic antioxidants used for industrial processing (butylated hydroxyl anisole – BHA, E320; butylated hydroxytoluene – BHT, E321) have carcinogenic activity and can be toxic (Wichi, 1998; Williams *et al*., 1999). The results here presented indicate that CTP4 is a potential source of bio-compounds with radical scavenging activity which play a very important role in the protection against oxidative stress and with the potential to reduce the risk of oxidative-induced diseases such as neurodegenerative diseases and prevent ageing (Dillard & German, 2000).

Lipid induction values, along with bioactive potential for antioxidant activities against $ABTS^{\bullet+}$ enables the use of this strain for a large-scale biorefinery. It is now important to perform additional assays (such as DPPH) in order to evaluate the full potential of CTP4 radical scavenging activity. It would also be interesting to perform other studies of bioactive activities such as anti-tumoural and anti-microbial assays, since several authors have carried out these assays in microalgae with promising results (Gerwick *et al*., 1994; Plaza *et al*., 2010). This will be important to take advantage of all the potential of lipid induction conditions tested in this experimental work, for instance anti-microbial activity has been attributed to long-chain unsaturated fatty acids (C16-C20) that were also found in this study.

These new perspectives regarding the establishment of a sustainable microalgal-based biorefinery could be open, turning the biofuel production in an economically feasible alternative for petroleum-based fuels.

5. Conclusions

CTP4 microalga is a highly resistant strain that can support a wide range of temperatures, from 5 ºC to 35 ºC, as far as we know; has a great resistance to high light intensity (100 to 400 µmol photons m^{-2} s^{-1}) and it is easy to grow at laboratory scale. Another important aspect about the potential of this strain is that this is an autochthonous microalga from Ria Formosa, Algarve; meaning this strain is already adapted to the conditions of this region, this aspect makes it very easy to grow and manipulate in this region.

A standard pattern observed in all assays was the effect of nutrient depletion, which led to a higher accumulation of lipids in all cultures (19.4-40.2%) when compared to cultures with nutrient supplementation under the same growth conditions (8.3-24.4%), except for low temperature (5 ºC) where the difference between nutrient treatments was supressed. The most successful induction factor was light intensity, namely at 400 µmol m^{-2} s^{-1} with nutrient depletion at day 7 (40.2%).

The major conclusion from this experiment is that nutrient depletion was found to be the best induction method for lipid accumulation; and temperature and light inductions enhance the nutrient depletion effect. This strain responded very well to lipid induction, especially to high light intensities; also without aeration this microalga sinks relatively easy, which may result in lower costs during harvesting.

The antioxidant activity assay against $ABTS^{\bullet+}$ also revealed great potential. For future investigation it will be very interesting to see whether the same result is obtained with cultures grown at pilot and industrial scale, searching for other bioactivities and/or added-value compounds. All of these factors together make CTP4 a good candidate for biodiesel production and biorefinery use.

6. References

Aaronson, S. (1973) Effect of incubation temperature on the macromolecular and lipid content of the phytoflagellate *Ochromonas danica*. *Journal of Phycology*. **9**: 111-113.

Ali, H., Houghton, P. J. & Soumyanath, A. (2006) α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes, with particular reference to *Phyllanthus amarus*. *Journal of Ethnopharmacology*. **107**: 449-455.

Atsumi, S. & Liao, J. C. (2008) Metabolic engineering for advanced biofuels Production from *Escherichia coli*. *Current Opinion in Biotechnology* **19**: 414-419.

Barsanti, L. & Gualtieri, P. (2006) *Algae: Anatomy, Biochemistry, and Biotechnology*. Taylor & Francis. USA.

Ben-Amotz, A.; Shaish, A. & Avron, M. (1989) Mode of action of the massively accumulated β-Carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation. *Plant Physiology*. **91**: 1040-1043.

Bhadury, P. & Wright, P. C. (2004) Exploitation of marine algae: biogenic compounds for potential antifouling applications. *Planta*. **219**: 561-578.

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

Blunt, J. W.; Copp, B. R.; Keyzers, R. A.; Munro, M. H. G.; Prinsep, M. R. Marine natural products. *Natural Product Reports*. **31**: 160-258.

Boussiba, S.; Vonshak, A.; Cohen, Z.; Avissar, Y.; Richmond, A. (1987) Lipid and biomass production by the halotolerant microalga *Nannochloropsis salina*. *Biomass*. **12**: 37-47.

Brown, M. R.; Dunstan, G. A.; Norwood, S. J.; Miller, K. A. (1996) Effects of harvest stage and light on the biochemical composition of the diatom *Thalassiosira pseudonana*. *Journal of Phycology*. **32**: 64-73.

Chen, W.; Zhang, C.; Song, L.; Sommerfeld, M.; Hu, Q. (2009) A high Throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods*. **77**: 41-47.

Chi, Z.; Liu, Y.; Frear, C.; Chen, S. (2009) Study of a two-stage growth of DHA-producing marine algae *Schizochytrium limacinum* SR21 with shifting dissolved oxygen level. *Applied Microbiology and Biotechnology*. **81**: 1141-1148.

Chisti, Y. (2007) Biodiesel from microalgae. *Biotechnology Advances*. **25**: 294-306.

Coesel, S. N.; Baumgartner, A. C.; Teles, L. M.; Ramos, A. A.; Henriques, N. M.; Cancela; L.; Varela, J. C. (2008) Nutrient limitation is the main regulatory factor for carotenoid accumulation and for Psy and Pds steady-state transcript levels in *Dunaliella*

salina (Chlorophyta) exposed to high light and salt stress. *Marine Biotechnology* **10**: 602-611.

Conforti, F.; Statti, G.; Loizzo, M. R.; Sacchetti, G.; Poli, F.; Menichini, F. (2005) In vitro antioxidant effect and inhibition of α-amylase of two varieties of *Amaranthus caudatus* seeds. *Biological and Pharmaceutical Bulletin*. **28**: 1098-1102.

Converti, A.; Casazza, A. A.; Ortiz, E. Y.; Perego, P.; Del Borghi, M.. (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing*. **48**: 1146–1151.

Correia, R.; McCue, P.; Vattem, D.; Magalhães, M.;. Macêdo, G.; Shetty, K. (2004) Amylase and Helicobacter pylori inhibition by phenolic extracts of pineapple wastes bioprocessed by *Rhizopus oligosporus*. *Journal of Food Biochemistry*. **28**: 419-434.

Custódio, L.; Escapa, a. L.; Fernandes, E.; Fajardo, A.; Aligué, R.; Alberício, F.; Neng, N.; Nogueira, J. M. F.; Romano, Anabela. (2011) Phytochemical profile, antioxidant and cytotoxic activities of the carob tree (*Ceratonia siliqua* L.) germ flour extracts. *Plant Foods for Human Nutrition*. **66**:78-84.

Custódio, L.; Ferreira, A.; Pereira, H.; Silvestre, L.; Vizetto-Duarte, C.; Barreira, L.; Rauter, A.; Alberício, F.; Varela, J. (2012b) The marine halophytes *Carpobrotus edulis* L. and *Arthrocnemum macrostachyum* L. are potential sources of nutritionally important PUFAs and metabolites with antioxidant, metal chelating and anticholinesterase inhibitory activities. *Botanica Marina*. **55**: 281–288.

Custódio, L.; Justo, T.; Silvestre, L.; Barradas, A.; Vizetto-Duarte, C.; Pereira, H.; Barreira, L.; Rauter, A.; Alberício, F.; Varela. J. (2012a) Microalgae of different phyla display antioxidant, metal chelating and acetylcholinesterase inhibitory activities. *Food Chemistry*. **131**: 134–140.

Day, J.; Slocombe, S.; Stanley, M. (2012) Overcoming biological constraints to enable the exploitation of microalgae for biofuels. *Bioresource Technology*. **109**: 245–251.

Demirbas, A. & Demirbas, M. F. (2010) *Algae Energy: Algae as a New Source of Biodiesel*. Springer. NY.

Demirbas, A. (2009) Political, economic and environmental impacts of biofuels: A review. *Applied Energy*. **86**: 108–117.

Dillard, C. J. & German, J. B. (2000) Phytochemicals: nutraceuticals and human health. *Journal of the Science of Food and Agriculture*. **80**: 1744-1756.

Elmoraghy, M.; Webster, T.; Farag, I. (2012) Microalgae Lipid Triggering by Cooling Stressing. *Journal of Energy and Power Engineering*. **6**. 1918-1924.

Elsey, D.; Jameson, D.; Raleigh, B.; Cooney, M. (2007) Fluorescent measurement of microalgal neutral lipids. *Journal of Microbiological Methods*. **68**: 639–642.

Enache, V. (2013) *Growth, lipid contents and bioactivities of the microalga Tetraselmis chuii in a low-cost, custom-made photobioreactor*. MSc Thesis. Universidade do Algarve.

Fábregas, J.; Abalde, J.; Herrero, C.; Cabezas, B. V.; Veiga, M. (1984) Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. *Aquaculture*. **42**: 207-215.

Fowler, S. D.& Greenspan, P. (1985) Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: comparison with Oil red O.

Fukuda, H.; Kondo, A.; Noda, H.(2001) Biodiesel fuel production by transesterification of oils. *Journal of Bioscience and Bioengineering*. **92**:405-416.

Garg. S.; Li, Y.; Wang, L.; Schenk, P. (2012) Flotation of marine microalgae: Effect of algal hydrophobicity. *Bioresource Technology*. **121**: 471–474.

Gerwick, W. H.; Roberts, M. A.; Proteau, P. J.; Chen, J.-L. (1994) Screening cultured marine microalgae for anticancer-type activity. *Journal of Applied Phycology*. **6**: 143-149.

Gouveia, L. (2011) *Microalgae as a Feedstock for Biofuels*. Springer.

Granger, L. M.; Perlot, P.; Goma, G.; Pareilleux, A. (1993) Effect of various nutrient limitations on fatty-acid production by *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology*. **38**: 784-789.

Guarnieri, M. T.; Nag, A.; Smolinski, S. L.; Darzins, A.; Seibert, M.; Pienkos, P. T. (2011) Examination of triacylglycerol biosynthetic pathways via *de novo* transcriptomic and proteomic analyses in an unsequenced microalga. *Plos One*. **6**: e25851.

Guedes, A. C.; Amaro, H. M.; Malcata, F. X. (2011) Microalgae as Sources of Carotenoids. *Marine Drugs*. **9**: 625-644.

Guzzella & Sciaretta (2007) *Vehicle propulsion systems.* Springer.

Harwood, J. L. & Guschina, I. A. (2009) The versatility of algae and their lipid metabolism. *Biochimie*. **91**: 679-684.

Hill, J.; Nelson, E.; Tilman, D.; Polasky, S.; Tiffany, D. (2006) Environmental, economic and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of Sciences of the United States of America*. **103**: 11206-11210.

Hu, Q. (2006) PSA abstracts. *Journal of Phycology*. **42**: 1-48.

Hu, Q.; Sommerfeld, M.; Jarvis, E.; Ghirardi, M.; Posewitz, M.; Seibert, M.; Darzins, A. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant Journal*. **54**: 621-639.

Hu, Q.; Zhang, C. W.; Sommerfeld, M. (2006) Biodiesel from algae: lessons learned over the past 60 years and future perspectives. Juneau, Alaska: Annual Meeting of the Phycological Society of America. **pp.** 40-41.

Illman, A. M.; Scragg, A. H.; Shales, S. W. (2000) Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme and Microbial Technology*. **27**: 631-635.

Kalacheva, G. S.; Zhila, N. O.; Volova, T. G.; Gladyshey, M. I. (2002) The effect of temperature on the lipid composition of the green alga *Botryococcus*. *Microbiology*. **71**: 286-293.

Keeling, P. J. (2013) The number, speed, and impact of plastid endosymbioses in eukaryotic evolution. *Annual Review of Plant Biology*. **64**:27.1–27.25.

Khotimchenko, S. V.; Yakovleva, I. M. (2005) Lipid composition of the red alga *Tichocarpus crinitus* exposed to different levels of photon irradiance. *Phytochemistry*. **66**: 73-79.

Kumar, K.; Dasgupta, C. N.; Nayak, B.; Lindblad, P.; Das, D. (2011) Development of suitable photobioreactors for CO_2 sequestration addressing global warming using green algae and cyanobacteria. *Bioresource Technology*. **102**: 4945-4953.

Kwon, Y. I.; Apostolidis, E.; Shetty, K. (2008a) In vitro studies of eggplant (*Solanum melongena*) phenolics as inhibitors of key enzymes relevant for type 2 diabetes and hypertension. *Bioresource Technology*. **99**: 2981–2988.

Kwon, Y. I.; Apostolidis, E.; Shetty, K. (2008b) Inhibitory potential of wine and tea against α-amylase and α-glucosidase for management of hyperglycemia linked to type 2 diabetes. *Journal of Food Biochemistry.* **32**: 15-31.

Lardon, L.; Hélias, A.; Sialve. B.; Steyer, J-P.; Bernard, O. (2009) Life-Cycle Assessment of Biodiesel Production from Microalgae. *Environmental Science & Technology*. **43**: 6475-6481.

Lee, S. J.; Yoon, B. D.; Oh, H. M. (1998) Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnology Techniques*. **12**: 553-556.

Lee, S. K.; Chou, H.; Ham, T. S.; Lee, T. S., Keasling, J. D. (2008) Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Current Opinion in Biotechnology*. **19**: 556-563.

Lee, S.-H.; Park, M.-H.; Heo, S.-J.; Kang, S.-M., Ko, S.-C.; Han, J.-S.; Jeon, Y.-J. (2010) Dieckol isolated from *Ecklonia cava* inhibits α-glucosidase and α-amylase in vitro and

alleviates postprandial hyperglycemia in streptozotocin-induced diabetic mice. *Food and Chemical Toxicology*. **48**: 2633-2637.

Li, X.; Hu, H. Y.; Gan, K.; Sun, Y. X. (2010) Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus sp*. *Bioresource. Technology*. **101**: 5494-5500.

Li, Y.; Horsman, M.; Wu, N.; Lan, C. Q.; Dubois-Calero, N. (2008) Biofuels from Microalgae. *Biotechnology Progress*. **24**: 815–820.

Lingwood, D. & Simons, K. (2010) Lipid rafts as a membrane-organizing principle. *Science*. **327**: 46-50.

Liu, J.; Cheng, Y.; Hu, G.; Li, F. (2012) Effects of Light Intensity on the Growth and Lipid Accumulation of Microalga *Scenedesmus sp*. 11-1 under Nitrogen Limitation. *Applied Biochemistry and Biotechnology*. **166**: 2127-2137.

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

Lynn, G.; Kilham, S.; Kreeger, A.; Interlandi, J. (2000) Effect of nutrient availability on the biochemical and elemental stoichiometry in the freshwater diatom *Stephanodiscus minutulus* (bacillariophyceae). *Journal of Phycology*. **53**: 265-270.

Maritim, A. C.; Sanders, R. A.; Watkins III, J. B. (2003) Diabetes, oxidative stress, and antioxidants: a review. *Journal of Biochemical and Molecular Toxicology*. **17**: 24-38.

Marshall, J. S; Huang, Y. (2010) Simulation of light-limited algae growth in homogeneous turbulence. *Chemical Engineering Science*. **65**: 3865–3875.

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

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

Patil, V.; Tran, K-Q.; Giselrød, R. (2008) Towards sustainable production of biofuels from microalgae. *International Journal of Molecular Sciences*. **9**. 1188-1195.

Patterson, G. (1970) Effect of temperature on fatty acid composition of *Chlorella sorokiniana. Lipids*. **5**: 597-600.

Pereira, H. (2009) *Desenvolvimento e optimização de um meio de cultura para produção de biomassa algal em larga escala*. Master Thesis. Universidade doAlgarve.

Pereira, H.; Barrera, 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. *Biotechnology for Biofuels*. **4**: 61.

Plaza, M.; Herrero, M.; Cifuentes, A. & Ibáñez, E. (2009). Innovative natural functional ingredients from microalgae. *Journal of Agriculture and Food Chemistry*. **57**: 7159-7170.

Plaza, M.; Santoyo, S.; Jaime, L. García-Blairsy Reina, G.; Herrero, M.; Señoráns, F. J.; Ibáñez, E. (2010) Screening for bioactive compounds from algae. *Journal of Pharmaceutical and Biomedical Analysis*. **51**: 450-455.

Rabbani, S.; Beyer, P.; Lintig, J. V.; Hugueney, P.; Kleinig, H. (1998) Induced β-carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. *Plant Physiology*. **116**: 1239-1248.

REN 21 (2012) *Renewables 2012. Global status report.* Renewable Energy Policy Network for the 21st Century.

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

Richardson, K.; Beardall, J.; Raven, J.A. (1983) Adaptation of unicellular algae to irradiance: An analysis of strategies. *The New Phytologist*. **93**: 157-191.

Richmond, A. (2004) *Handbook of microalgal culture: biotechnology and applied phycology*. Blackwell Science Ltd. Oxford.

Rocha, J.; Garcia, J.; Henriques, M. (2003) Growth aspects of the marine microalga *Nannochloropsis gaditana*. *Biomolecular Engineering*. **20**: 237-242.

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. *Biotechnology and Bioengineering*. **102**: 100-112.

Rodríguez-Meizoso, I.; Jaime, L.; Santoyo, S.; Señoráns, F.J.; Cifuentes, A.; Ibáñez, E. (2010) Subcritical water extraction and characterization of bioactive compounds from *Haematococcus pluvialis* microalga. *Journal of Pharmaceutical and Biomedical Analysis*. **51**: 456-463.

Roleda, M.; Slocombe, S.; Leakey, R.; Day, J.; Bell, E.; Stanley, M. (2013) Effects of temperature and nutrient regimes on biomass and lipid production by six oleaginous microalgae in batch culture employing a two-phase cultivation strategy. *Bioresource Technology*. **129**: 439-449.

Ruangsomboon, S. (2012) Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2. *Bioresource Technology*. **109**: 261-265.

Sharma, K. K.; Schuhmann, H.; Schenk, P. M. (2012) High Lipid Induction in Microalgae for Biodiesel Production. *Energies.* **5**: 1532-1553.

Slocombe, S.; Zhang, Q.; Black, K; Day, J; Stanley, M. (2012) Comparison of screening methods for high-throughput determination of oil yields in micro-algal biofuel strains. *Journal of Applied Phycology*. **25**: 961–972.

Solovchenko, A. E.; Khozin-Goldberg, I.; Didi-Cohen, S.; Cohen, Z.; Merzlyak, M. N. (2008) Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga *Parietochloris incisa*. *Journal of Applied Phycology*. **20**: 245-251.

Somerville, C. (1995) Direct tests of the role of membrane lipid composition in low-temperature induced photoinhibition and chilling sensitivity in plants and cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*. **92**: 6215-6218.

Su, C.-H.; Chien, L.-J.; Gomes, J.; Lin, Y.-S.; Yu.,Y.-K.; Liou, J.-S.; Syu, R.-J. (2011) Factors affecting lipid accumulation by *Nannochloropsis oculata* in a two-stage cultivation process. *Journal of Applied Phycology*. **23**: 903-908.

Suali, E.; Sarbatly. R. (2012) Conversion of microalgae to biofuel. *Renewable and Sustainable Energy Reviews.* **16**. 4316-4342.

Sushchik, N. N.; Kalacheva, G. S.; Zhila, N. O.; Gladyshev, M. I.; Volova, T. G. (2003) A temperature dependence of the intra- and extracellular fatty-acid composition of green algae and cyanobacterium. *Russian Journal of Plant Physiology*. **50**: 374-380.

Tang, H.; Chen, M.; Garcia, M. E. D.; Abunasser, N.; Simon, N. K. Y.; Salley, S. O. (2011) Culture of microalgae *Chlorella minutissima* for biodiesel feedstock production. *Biotechnology and Bioengineering*. **108**: 2280-2284.

Tanner, S. (2009) *Biofuels of the third generation – Do microalgae solve the energy problem*? Term Paper in Major Course Biogeochemistry and Pollutant Dynamics. ETH. Zürich.

Thompson, G. A. (1996) Lipids and membrane function in green algae. *Biochimica et Biophysica Acta*. **1302**: 17-45.

Thompson, P. A.; Harrison, P. J.; Whyte, J. N. C. (1992) Effects of variation in temperature. II. On the fatty acid composition of eight species of marine phytoplankton. *Journal of Phycology*. **28**: 488-497.

Urry, J. (2008) Climate change. travel and complex futures. *The British Journal of Sociology*. **59**: 261-279.

Wager, H. (2008) On the Eye-spot and Flagellum in *Euglena viridis*. *Journal of the Linnean Society of London. Zoology*. **27**: 463-481.

Wang, D.; Wang, L. J.; Zhu, F. X.; Zhu, J. Y.; Chen, X. D.; Zou, L.; Saito, M.; Li, L. (2008) In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). *Food Chemistry*. **107**: 1421-1428.

Wichi, H. P. (1998) Enhanced tumor development by butylated hydroxyanisole (BHA) from the prospective of effect on for estomach and esophageal squamous epithelium. *Food and Chemical Toxicology*. **26**: 717-723.

Widjaja, A.; Chien, C.-C.; Ju, Y.-H. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *Journal of the Taiwan Institute of Chemical Engineers*. **40**: 13-20.

Williams, G. M. M., Iatropoulos, J.; Whysner, J. (1999) Safety assessment of butylated hydroxyanisole and butylated hydroxyltoluene as antioxidant food additives. *Food and Chemical Toxicology*. **37**: 1027-1038.

Xin, L.; Hong-ying, H.; Ke, G.; Ying-xue, S. (2010) Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. *Bioresource Technology*.**101**: 5494-5500.

7. Annex

Table 7.1 - Effect of lipid induction using different temperatures (5 and 35 ºC) and different light intensities (200 and 400 µmol m^{-2} s^{-1}) with nutrient depletion (N^-) and supplementation (N^+) in g L^{-1} and percentage (%). Cultures grown at 20 ºC under 100 µmol m^{-2} s^{-1} of PFD were used as control., *n*=3.

Days	**Conditions**									
	20 ºC 100 µmol m^{-2} s^{-1}		**5 ºC 100 µmol m^{-2} s^{-1}**		**35 ºC 100 µmol m^{-2} s^{-1}**		**200 µmol $m^{-2}s^{-1}$ 20 ºC**		**400µmol $m^{-2}s^{-1}$ 20 ºC**	
	N^-	N^+	N^-	N^+	N^-	N^+	N^-	N^+	N^-	N^+
11	0.053±0.004	0.053±0.004	0.053±0.004	0.053±0.004	0.053±0.004	0.053±0.004	0.053±0.004	0.053±0.004	0.053±0.004	0.053±0.004
	7.32%±0.534	7.32%±0.534	7.32%±0.534	7.32%±0.534	7.32%±0.534	7.32%±0.534	7.32%±0.534	7.32%±0.534	7.32%±0.534	7.32%±0.534
12	0.043±0.004	0.039±0.002	0.040±0.002	0.029±0.002	0.126±0.002	0.127±0.009	0.045±0.001	0.032±0.004	0.047±0.005	0.037±0.005
	5.63%±0.553	4.81%±0.360	5.87%±0.507	4.45%±0.254	14.08%±0.409	13.60%±1.418	4.95%±0.163	3.29%±0.544	6.39%±0.767	3.92%±0.612
13	0.061±0.004	0.040±0.005	0.053±0.001	0.031±0.000	0.272±0.013	0.180±0.006	0.138±0.008	0.098±0.013	0.085±0.013	0.145±0.010
	7.08%±0.639	3.93%±0.469	7.00%±0.486	4.56%±0.174	26.08%±1.273	14.27%±0.679	12.53%±1.165	7.36%±1.040	9.18%±1.394	10.45%±0.780
14	0.114±0.010	0.056±0.005	0.098±0.014	0.047±0.002	0.191±0.017	0.196±0.017	0.257±0.027	0.159±0.016	0.232±0.038	0.233±0.005
	12.70%±1.303	4.79%±0.623	11.88%±2.067	5.82%±0.518	17.94%±1.768	14.39%±1.722	21.91%±2.412	10.79%±1.246	23.17%±4.297	14.97%±0.620
15	0.148±0.010	0.075±0.004	0.111±0.004	0.064±0.004	0.334±0.042	0.289±0.017	0.320±0.025	0.204±0.025	0.222±0.047	0.328±0.010
	17.54%±1.751	7.84%±0.579	15.91%±1.094	8.79%±0.535	35.17%±5.996	22.41%±1.720	28.55%±2.304	13.90%±1.931	23.79%±5.485	21.98%±1.727
16	0.184±0.016	0.097±0.014	0.128±0.006	0.119±0.009	0.213±0.005	0.190±0.002	0.411±0.006	0.258±0.035	0.410±0.021	0.366±0.005
	18.84%±2.324	7.59%±1.199	16.88%±1.343	13.69%±1.041	21.33%±0.818	12.87%±0.374	34.55%±2.542	15.44%±2.367	38.26%±4.262	21.05%±1.482
17	0.191±0.013	0.110±0.012	0.193±0.008	0.203±0.024	0.223±0.015	0.171±0.026	0.402±0.030	0.273±0.051	0.467±0.056	0.435±0.005
	19.35%±2.511	8.25%±1.127	26.40%±1.212	24.43%±2.994	20.65%±1.460	10.72%±1.662	31.85%±2.437	15.48%±3.155	40.17%±5.168	22.85%±0.668