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MICROALGAE BIOMASS AS A SOURCE OF NATURAL COMPOUNDS: CHEMICAL CHARACTERIZATION AND NEW APPROACHES FOR LIPID EXTRACTION AND CULTURE HARVESTING

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This thesis was considered adequate for the title of Doctor and was approved in its final form by the Postgraduate Program in Chemical Engineering at the Universidade Federal de Santa Catarina.

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This thesis is dedicated to my parents, who have always encouraged me on my studies.

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The dictionary is the only place that success comes before work.

(Vince Lombardi Jr.)

ABSTRACT

Microalgae have emerged as a source of a wide range of compounds, which enable the development of a variety of products for pharmaceutical, nutraceutical and cosmetic industries. The extraction of these natural compounds is essential for the commercialization of these high value compounds (HVPs) from microalgae. The success of the extraction depends on the affinity of the compounds with the solvent used in the process that should be suitable for the extraction of value compounds. In this context, this thesis is addressed to the extraction of compounds from the biomass from three marine microalgae species (*Phaeodactylum tricornutum*, *Nannochloropsis oculata* and *Porphyridium cruentum*), using subcritical *n*-butane for the obtainment of the crude extracts. In order to compare and evaluate the affinity of *n*-butane with the extracted compounds, were also applied to each microalgae biomass, organic solvent extraction through Soxhlet method (AOAC 963.15) and supercritical extraction using CO_2 (SC-CO_2) as solvent. For the obtainment of the biomass needed for the extraction experiments, the microalgae cultivation and the steps of harvesting, washing and drying of biomass were performed. With the obtainment of the dry biomass, the chemical composition was determined (moisture, ashes, proteins, carbohydrates, lipids and fatty acids profile) for each species. The proposed extraction methods were applied to the three different microalgae biomass, and the fatty acids composition of the extracts obtained by each method were compared. The same extracts obtained by SC-CO_2 and subcritical *n*-butane were evaluated regarding to their antioxidant activities using DPPH radical scavenging assay. The total carotenoids content was also quantified by UV-visible (UV-vis) and the results correlated. Furthermore, a case study was developed for the harvesting step at pilot scale, using the protozoan *Tetrahymena* as flocculant agent in microalgal cultures of *Chlorella sorokiniana* 8-C4 (australian local strain). From the results obtained from the chemical composition of each microalgae cultivated for the extraction experiments, these species were suggested for the enhancement of food products considering their respective nutritional benefits. Comparing the extraction methods used, subcritical *n*-butane extracted higher percentages of polyunsaturated fatty acids (ω3 and ω6) than supercritical CO_2, and in some cases reached higher affinity with these compounds than the organic solvent, showing it to be a suitable method for the extraction of high value fatty acids (i.e. eicosapentaenoic acid, docosahexaenoic acid, arachidonic acid). The extracts obtained by CO_2

and *n*-butane as solvents presented antioxidant activity values against the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), where the subcritical *n*-butane extract of *P. cruentum* presented the highest capacity to scavenge DPPH, showing value close to the synthetic antioxidant butylated hydroxytoluene (BHT). Moreover, the correlation between the antioxidant activity results and carotenoids content was considered significant (R^2=0.8021) between the obtained extracts, independent of the method used. In the case study about the application of the protozoan *Tetrahymena* as bioflocculant in microalgal cultures of *C. sorokiniana*, was possible to obtain an OD_{750} concentration profile along the flocculator developed for the assays. From the verified OD_{750} profile a gradient of suspended solids (algal cells) was demonstrated along the cylinder, however the expected efficiency was not achieved, suggesting the performance of more experimentation towards the optimization of this biomass pre-concentration process, which is still in early stages of research.

Keywords: Microalgae biomass. Extraction method. Chemical composition. Fatty acids composition. Antioxidant activity.

RESUMO EXPANDIDO

As microalgas têm emergido como fonte de vasta gama de compostos que possibilitam o desenvolvimento de grande variedade de produtos, desde biocombustíveis até produtos de alto valor agregado. A extração de compostos da biomassa é uma etapa essencial para a comercialização de produtos a partir das microalgas, e seu sucesso depende da afinidade dos compostos em questão com o solvente utilizado no processo. Neste contexto, a presente tese trata da extração de compostos da biomassa de três espécies de microalgas marinhas (*Phaeodactylum tricornutum*, *Nannochloropsis oculata* e *Porphyridium cruentum*) utilizando *n*-butano subcrítico como solvente na obtenção dos extratos brutos. Com o intuito de comparar e avaliar a afinidade do *n*-butano com os compostos extraídos, também foram aplicados à biomassa de cada espécie, extração com solvente orgânico através do método de Soxhlet (AOAC 963.15) e extração supercrítica utilizando CO_2 (SC-CO_2). Para a obtenção da biomassa necessária para os experimentos de extração, foram desenvolvidos os cultivos das microalgas e as etapas de separação, lavagem e secagem da biomassa. Com a obtenção da biomassa seca, foi determinada a composição química (umidade, cinzas, proteínas, carboidratos, lipídios e perfil de ácidos graxos) de cada espécie. Foram aplicados os três diferentes métodos de extração propostos, onde foi comparada a composição de ácidos graxos dos extratos obtidos por cada método de extração das três espécies produzidas. Os mesmos extratos obtidos via SC-CO_2 e *n*-butano subcrítico foram avaliados quanto as suas atividades antioxidantes utilizando o radical 2,2-difenil-1-picrilhidrazil (DPPH) como método de determinação. Os resultados de atividade antioxidante foram correlacionados com os valores conteúdos de carotenóides totais, quantificados por UV-visível (UV-vis). Além disso, foi desenvolvido um estudo de caso em escala piloto para a etapa de separação da biomassa, onde foi utilizado o protozoário *Tetrahymena* como agente floculador em culturas da microalga *Chlorella sorokiniana* 8-C4 (espécie local australiana). A partir dos resultados sobre a composição química da biomassa de cada microalga produzida para os experimentos de extração, espécies foram sugeridas para o enriquecimento de alimentos considerando seus respectivos benefícios nutricionais. Comparando-se os métodos de extração utilizados, *n*-butano subcrítico extraiu as maiores porcentagens de ácidos graxos poli-insaturados (ω3 e ω6) do que CO_2 supercrítico e em alguns casos obteve maior afinidade com estes compostos do que o solvente orgânico, mostrando-se um método adequado para a extração de ácidos graxos de

alto valor agregado (ácido eicosapentaenóico, ácido docosahexaenóico, ácido araquidônico, por exemplo). Os extratos obtidos através de SC-CO_2 e *n*-butano apresentaram diferentes níveis de atividade antioxidante frente ao radical DPPH, onde o extrato obtido da microalga *P. cruentum* utilizando *n*-butano como solvente apresentou a maior capacidade de sequestro do radical DPPH, apresentando valor mais próximo ao antioxidante sintético hidroxitolueno butilato (BHT). Ainda, a correlação entre os resultados de atividade antioxidante e conteúdo de carotenóides foi considerada significante (R^2 = 0.8021) entre os extratos obtidos, independentemente do método utilizado. No estudo de caso da aplicação do protozoário *Tetrahymena* como biofloculante em culturas da microalga *C. sorokiniana*, foi possível a obtenção de um perfil de concentração de OD_{750}, ao longo do floculador desenvolvido para os testes. A partir do perfil de OD_{750} constatado foi demostrado um gradiente de sólidos suspensos (células algais) ao longo do cilindro, no entanto a eficiência esperada no processo não foi atingida, o que sugere a realização de mais experimentações na busca da otimização deste processo de pré-concentração da biomassa, que ainda se encontra em estágio inicial de pesquisa.

Palavras-chave: Biomassa de microalgas. Método de extração. Composição química. Composição de ácidos graxos. Atividade antioxidante.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text.
I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of Universidade Federal de Santa Catarina - UFSC.

Publications during candidature

Original Research Papers:

Gisela Jakob, Evan Stephens, Rafael Feller, Melanie Oey, Ben Hankamer, Ian Ross. Triggered exocytosis of the protozoan *Tetrahymena* as a source of bioflocculation and a controllable dewatering method for efficient harvest of microalgal cultures. *Algal Research*, 2016, 13: p. 148-158. http://dx.doi.org/10.1016/j.algal.2015.11.011.

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Original Research Paper:

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

HVPs – High Value Products
AOAC – Association of Official Analytical Chemists
SC-CO_2 – Supercritical CO_2 extraction
DPPH - 2,2-diphenyl-1-picrylhydrazyl
PUFAs – Polyunsaturated Fatty Acids
EPA – Eicosapentaenoic acid
DHA – Docosahexaenoic acid
AA/ARA – Arachidonic acid
BHT – Butylated Hydroxytoluene
OD_{750} – Optical density at 750 nm
R&D – Research and Development
PBRs – Photobioreactors
CAPEX – Capital Expenditures
OPEX – Operational Expenditures
EPS – Extracellular Polymeric Substance
LA – Linolenic acid
ALA – α-Linolenic acid
GLA – γ-Linolenic acid
LC-PUFA – Long Chain Polyunsaturated Fatty Acid
ROS – Reactive Oxygen Species
Tc – Critical temperature
Pc – Critical pressure
SCO – Single Cell Oil
BBM – Bold Basal Medium
PSM – Paoleti Synthetic Medium
TDF – Total Dietary Fiber
AACC – American Association of Cereal Chemists
FAME – Fatty Acid Methyl Ester
GC – Gas Chromatography
ID – Inner diameter
IQN – Nutritional Quality Indexes
AI – Atherogenicity Index
TI – Thrombogenicity Index
H/H – Hypocholesterolemic/hypercholesterolemic ratio
SFA – Saturated Fatty Acid
MUFA – Monounsaturated Fatty Acid
DM – Dry Matter
SCP – Single Cell Protein
NPN – Non-protein nitrogen

GRAS – Generally Recognized as safe
FDA – Food and Drug Administration
WHO – World Health Organization
FAO – Food and Agriculture Organization
LDL – Low-density lipoproteins
P/S – Polyunsaturated and Saturated fatty acids ratios
SFE – Supercritical Fluid Extraction
OEC – Overall Extraction Curve
DW – Dry weight
TAGs – Triglycerides
AA% - Percent of Antioxidant Activity
EC_{50} – Effective Concentration at 50%
BHA – Butylated Hydroxyanisole
TEAC – Trolox Equivalent Antioxidant Capacity
FRAP – Ferric Reducing Antioxidant Power
ORAC – Oxygen Radical Absorbance Capacity
TRAP – Total Reactive Antioxidant Potential
HPLC – High Performance Liquid Chromatography
RO – Reverse Osmosis

SUMÁRIO

Chapter 1

1 INTRODUCTION

Microalgae are considered one of the most promising feedstock for developing a wide variety of high value products (HVPs) for pharmaceutical, nutraceutical and cosmetic industries (RYCHEBOSCH et al., 2014; KOLLER et al., 2014). This is mainly due to their capacity to accumulate carbohydrates, proteins, lipids and other intracellular valuable organic compounds (e.g. pigments, antioxidants, PUFAs, etc.) by the efficient use of solar energy, water, CO_2, and nutrients (RICHMOND, 2004). The extraction of these intracellular compounds is essential for the commercialization of HVPs from microalgae (HERRERO & IBÁÑEZ, 2015; HARUN et al., 2010). For this purpose, three marine microalgae strains *Phaeodactylum tricornutum*, *Nannochloropsis oculata*, and *Porphyridium cruentum* were selected for this study because of their high and diverse contents of PUFAs, carotenoids, and other antioxidant compounds.

Phaeodactylum tricornutum is a diatom with different cell morphotypes (fusiform, triradiate, and oval) enclosed within a cell wall comprised of silica (hydrated silicon dioxide) called a frustule. It grows rapidly and has high lipid content, about 20-30% dry weight under standard culture conditions. For commercial uses of *P. tricornutum*, eicosapentaenoic acid (EPA 20:5ω3) have been the focus as the main polyunsaturated fatty acid (PUFA) produced by this species (MATOS et al., 2016; MEDINA et al., 1998; KHOZIN-GOLDBERG et al., 2011). *P. tricornutum* has been also identified as a rich source of fucoxanthin (ten times more than in macroalgae) (KIM et al., 2012). Fucoxanthin, a major marine carotenoid, occurring especially in brown algae, has been found to have a number of therapeutic activities, including anticancer, antihypertensive, anti-inflammatory, and anti-obesity effects (KOLLER et al., 2014).

Nannochloropsis oculata specie has a diameter of about 2-3 µm and is able to build up a high range of carotenoids such as β-carotene, zeaxanthin, cantaxanthin, violaxanthin, and astaxanthin. *Nannochloropsis* sp. has been used as food additive for human nutrition in the form of supplements and nutraceuticals because of its ability to synthesize long-chain polyunsaturated fatty acids (PUFA) (RICHMOND, 2004; RYCKEBOSCH et al., 2014). When cultured in normal growth conditions the cells have an oil content of about 30%,

however various strains including *N. oculata* were shown to accumulate up to 60% of their overall biomass as lipids under nitrogen limitation (RODOLFI et al., 2008).

Porphyridium cruentum is a red microalga (Rodophyta), that contains phycoerythrin, an accessory pigment, which provides the characteristic of red color. *P. cruentum* cells excrete a sulfated polysaccharide (exopolysaccharide) which turns the culture viscous (ARAD & LEVY-ONTMAN, 2010). Sulfated polysaccharide form thermally reversible gels similar to agar and carrageenan, which are usually used in commercial applications as thickeners, stabilizers, and emulsifiers (OH et al., 2009). Most of the biomass is composed by carbohydrates (40-57%), the total lipids may reach 9-14% mostly composed of arachidonic acid (ARA 20:4-ω6) (RYCKEBOSH et al., 2004). The biomass also contains tocopherol, vitamin K and large amounts of carotenes mainly β-carotene (FUENTES et al., 200; WANG et al., 2007).

PUFAs, carotenoids and other antioxidant compounds provide bioactivity in human body and possess anti-inflammatory and antioxidant properties, which reduce the risk and prevent chronic diseases associated with oxidative stress such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative diseases and certain types of cancers (RAPOSO et al., 2015; KHOZIN-GOLDBERG et al., 2011). From this perspective new researches are being developed in the downstream process of microalgae production, from microalgae cultivation through to the obtainment of the final products. However, there are still some technological bottlenecks in this process, as the extraction step of the interested compounds (KIM et al., 2013; MATA et al., 2010; HARUN et al., 2010).

The two most useful methods found in the literature applied to microalgae biomass for extraction of intracellular compounds from microalgae, are extraction using organic solvents (hexane, chloroform, methanol, among others) and extraction using supercritical CO2 (SC-CO_2) (HALIM et al., 2012; CHENG et al., 2011; MEDINA et al., 1998). Each extraction method has its peculiarities and has influence on the chemical composition of the extracts. The use of organic solvents, for example, is widely applied in the extraction of compounds from microalgae biomass, however large amount of solvent is required, and its recovery requires high temperatures and can affect labile compounds, for example carotenoids (MENDES et al., 2003; HERRERO & IBÁÑES, 2015). Furthermore, their application may lead to contamination to the final product in the form of residues, and this may

limit their application for human health and nutrition products (HALIM et al., 2012; MICHALAK, et al., 2015). Supercritical CO_2 emerges as an alternative method applied to microalgae biomass, proving to be a green technology in the extraction of the compounds (HERRERO & IBÁÑEZ, 2006; CATCHPOLE et al., 2009; MILLAO & UQUICHE, 2016).
The use of pressurized gases as solvents, such as supercritical CO_2, has certain inherent characteristics, such as ease of solute recovery, solvent recycling and the possibility of directing separation by modifying the thermodynamic conditions of temperature and pressure (HALIM et al., 2011; CHENG et al., 2011; MENDES et al., 2003). The use of pressurized fluids such as *n*-butane to replace the CO_2 usage differs to work in the sub-critical region, instead supercritical, so compressed liquid, well below the critical point, with the main advantage to be operated at moderate pressures. The advantages of working at lower pressures are lower initial investment with equipment, lower maintenance cost and less risk of operation (NOVELLO et al., 2015; CAPELETTO et al., 2016). In this context, the present study aims to compare the effects of subcritical *n*-butane, as an innovative extraction method applied to microalgae biomass, with two well-established methods (Sohxlet and SC-CO_2) through analysis of the extracts obtained. The three marine microalgae strains used to produce the biomass needed for all the extraction experiments were physically and chemically characterized and the obtained extracts were analyzed in terms of lipid content, fatty acids profile, total carotenoids and antioxidant activity.

Apart of the main objective of the work, another technological bottleneck in the microalgae cultivation downstream process - the harvesting step, was also investigated in this work. A new harvesting method was tested using a biological approach from the laboratory to a pilot scale. The method consisted to use the protozoa *Tetrahymena* as flocculant agent to harvest the alga strain *Chlorella sorokiniana* isolated from freshwater habitat.

Chapter 2

2 LITERATURE REVIEW

2.1 MICROALGAE TECHNOLOGY

Microalgae are able to synthetize a huge range of natural compounds, and this fact is currently favoring extensive research focused on biology, physiology, engineering and their integration for microalgae cultivation to produce sustainable products such as biofuel, food, feed and high-value products (HVPs) (SHEEHAN et al., 1998; MATA et al., 2010; KOLLER et al., 2014). Microalgae are sunlight-driven cell factories able to utilize solar energy and fix carbon dioxide to produce organic compounds through the process of photosynthesis. Algae belong to many different and unrelated taxonomic groups that all contain chlorophyll *a*. It is unknown how many species of algae exist, with estimates ranging between several hundred thousand and several million different species (MOHEIMANI, et al., 2015; CHISTI, 2007; RICHMOND, 2004).

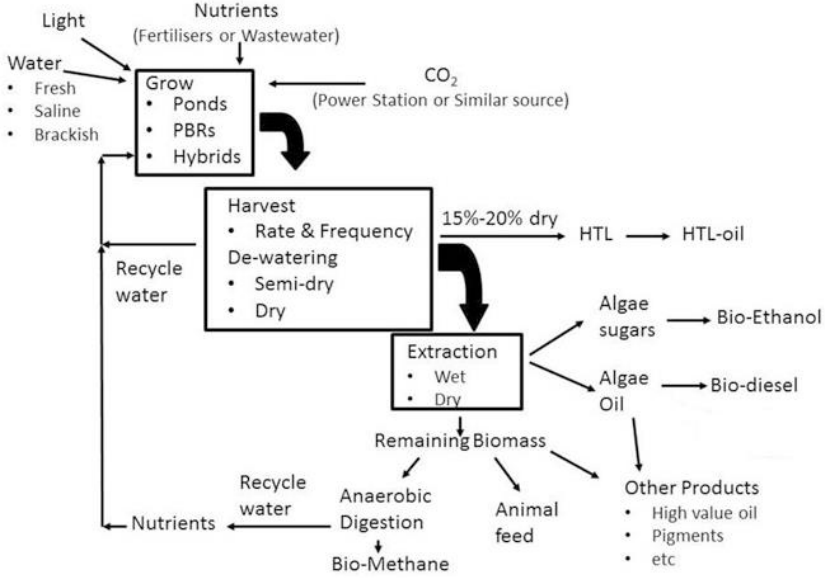
Microalgae mass cultures have been studied for almost 70 years, starting the 1940's as a potential source of human foods. Concerns about water pollution in the 1960's increased interested in the use of microalgae in wastewater treatment. The perception during the 1970's that fossil fuels would run out, made microalgae culture a focus of renewable fuel production R &D (BENEMANN & OSWALD, 1996). The potential attractive features of algae have been often listed, but the high cost of producing algae biomass means that algal biofuels as an economical, renewable and sustainable source of biofuels and bioenergy is still somewhat in the future (SHEEHAN, 1998; BOROWITZKA & MOHEIMANI, 2013).

Commercial interest in HVPs, specifically nutraceuticals, led during the 1960's, to the commercial development of the microalgae industry. It was in Japan that the first commercial production systems for microalgae were developed, several *Chlorella* cultivation facilities were established, using circular ponds, to produce a dried algal powder, sold as "health food" (BENEMANN & OSWALD, 1996). The next commercial algal production system was for *Spirulina*, a filamentous blue-green alga that has been a traditional food source in Mexico and Africa. Most recently commercialized was the production of green alga *Dunaliella salina*. Some strains of *D. salina*, growing at high salinities,

produce high levels of β-carotene, used as a food colorant and vitamin/antioxidant food supplement. Natural astaxanthin produced by *Haematococcus* has become a commercial reality through new and advanced technology. *Haematococcus* is used as a natural food color and pigment for fish feed, and has been approved as a dietary supplement ingredient in U.S. and in a number of European countries for human consumption (RICHMOND, 2004; BENEMANN & OSWALD, 1996; HARUN et al., 2010).

Despite estimates about the number of exiting species of microalgae (several million), only a small portion (several thousand) can be kept alive in culture, and only a handful of them have been successfully grown commercially (MOHEIMANI et al., 2015). A conventional microalgae production system consists of growth and cultivation of microalgae, biomass harvesting and dewatering and extraction/conversion of the biomass to the product of interest. Figure 2.1 shows a conceptual schematic diagram for the development of products from microalgae through carbon recycling.

Figure 2.1 Schematic diagram of microalgae downstream processing.



Source: Adapted from Fon Sing et al. (2013).

2.2 MICROALGAE CULTIVATION

Mineral nutrients supply and other growth requirements to algae in culture has been studied for a long time. Algae are capable of many kinds of trophy centered on both major forms of nutrition, namely autotrophy (phototrophy) and heterotrophy (phagotrophy), of which autotrophy is by far the most important (RICHMOND, 2004; BOROWITZKA & MOHEIMANI, 2013). Photoautotrophic organisms obtain their energy through the absorption of light for the reduction of CO_2 by the oxidation of substrates, mainly water, with the release of O_2. Most algae belong to this category, although they require inorganic mineral ions and minimal quantities of organic compounds for growth, such as vitamins. Heterotrophic organisms obtain their material (biomass synthesis) and energy needs from organic compounds produced by other organisms (external source) (RICHMOND, 2004; DERNER, 2006; ANDERSEN, 2005). Several algal species can be grow exclusively on organic substrates without the presence of light. Although the major forms of nutrition (autotrophy and heterotrophy), mixotrophic is a nutritional route category which combine the major forms of nutrition, where light and organic carbons are used as the energy source, while inorganic and organic carbons are used as the carbon source. Microalgae cultivation can be carried out in open systems (open ponds) or closed systems (photobioreactors). Some types of microalgae cultivation systems are described next (CHISTI, 2007; DEMIRBAS & FATIH DEMIRBAS, 2010).

2.2.1 Open systems

Cultivation of microalgae for commercial purposes is mostly carried out in open systems. The main reason for this is that large (commercial) open ponds are easier and less expensive to build and operate, and more durable than large closed reactors (SHEEHAN et al., 1998; BOROWITZKA & MOHEIMANI, 2013). Many types of ponds have been designed for microalgae cultivation, however, only two major designs are used for commercial production of microalgae: circular ponds with agitation provided by a rotating arm, and raceway ponds constructed as an endless loop, in which the culture is circulated by paddle wheels (MATA et al, 2010; DEMIRBAS & FATIH DEMIRBAS, 2010; JUNYING, 2013). Figure 2.2 shows two different types of open systems.

Figure 2.2 Open systems for microalgae cultivation. Raceway pond (left) and open tanks (right), both located at Laboratório de Cultivo de Algas (LCA), Florianópolis - SC, Brazil.

Source: Author.

A main disadvantage of outdoor algae ponds is the exposure to a variety of environmental factors affecting culture growth. Open systems are limited by key growth parameters including light-intensity, temperature, pH and dissolved oxygen concentration. Contamination by predators is another issue involved with open ponds. Contamination can limit the cultivation system to algal strains which can only grow under high selective conditions, e.g., *Chlorella spp*, *Nannochloropsis*, and *P. tricornutum* (high nutrient concentrations), *Spirulina* (high pH), *Dunaliella salina* (high salinities) (MOHEIMANI et al, 2015; HARUN et al., 2010).

2.2.2 Closed systems

Photobioreactors (PBRs) offer a closed culture environment for microalgae, which is protected from invading microorganisms. PBRs in general provide better control of cultivation conditions, yield higher productivity and reproducibility, reduce contamination risk, and allow greater selection of algal species used for cultivation (MATA et al., 2010; JUNYING et al., 2013; SCOTT et al., 2010). Compared to open systems, PBRs have higher efficiency and biomass concentration, shorter harvest time, and higher surface-to-volume ratio (BOROWITZKA & MOHEIMANI, 2013; DEMIRBAS & FATIH DEMIRBAS, 2010). Many configurations of PBRs have been devised and built. These closed systems consist of numerous designs: tubular, cylindrical, conical, flat-plated, etc. Figure 2.3 shows two examples of PBRs.

Figure 2.3 Closed systems (PBRs) used for microalgae cultivation. Horizontal tubular PBR (left), located at Laboratório de Cultivo de Algas, Florianópolis – SC, Brazil and bag system (right), located at Solar Biofuel Research Centre, Brisbane – QL, Australia.

Source: Author.

Despite various configurations, several basic design features must be considered when building a PBRs: how to provide light, how to circulate the algae, which materials to use for construction, how to provide CO_2 and remove O_2, and how to control pH and temperature. Since phototrophic microorganisms are highly diverse in their morphology, nutritional and light requirements, and resistance to stresses, PBRs cannot be designed to adapt all organisms and all conditions (CHISTI, 2007; RICHMOND, 2004; HARUN et al., 2010).

2.3 HARVESTING OF MICROALGAL BIOMASS

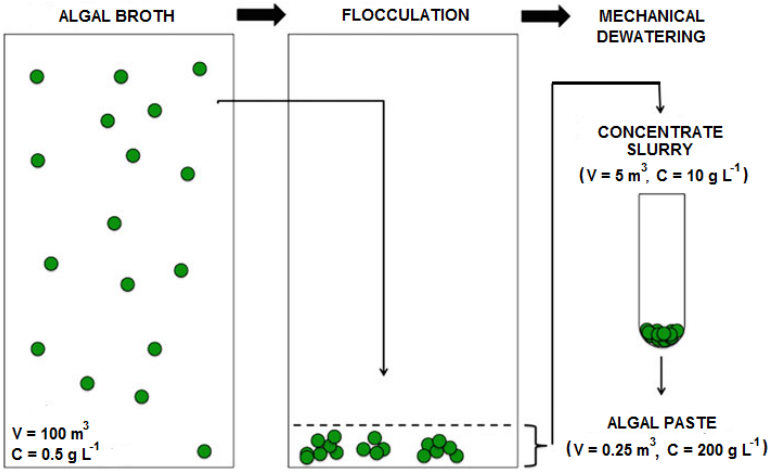
Harvesting is the next step in the downstream processing of algal biomass. This step represents 20–30% of the total operating costs (OPEX), and decreasing the costs and energy inputs in the algae harvesting processes is a key factor for the economic viability of a sustainable full-scale production of microalgal biomass (MOHEIMANI et al., 2015; BARROS et al., 2015; MOLINA GRIMA et al., 2003). The main challenge is collect small cells (2-20 µm) present in very dilute cultures (less than 1 g L^{-1}) (BARROS et al., 2015; UDUMAN et al., 2010).

At present, there is no microalgal harvesting method that is both economically viable and efficient. The selection of an appropriate

harvesting procedure must consider that microalgal biomass must be further processed (e.g., for human consumption). Therefore, these procedures must not be toxic or irrepairably contaminate microalgal biomass. It is also desirable that the selected harvesting method allows the recycling of the culture medium (BARROS et al., 2015; AL-HATTAB et al., 2015; VANDAMME et al., 2013).

Due to the large volumes of algae cultures, several authors (MOHEIMANI et al., 2015; BENEMANN & OSWALD, 1996; BARROS et al., 2015; SCHLESINGER et al., 2012) have suggested that low-cost harvesting of microalgae can be achieved by means of a two-stage harvesting process (Figure 2.4) in which the biomass is pre-concentrated by flocculation prior to final dewatering using a mechanical method such as centrifugation or filtration (more energy intensive methods). Minimising the total volume treated that is a key requirement. In the first step the biomass is concentrated to a 1-2% biomass slurry, and in the second step to a 10-20% biomass slurry obtaining a paste of 100-200 g dry matter L^{-1} slurry. This paste is then suitable for further processing or drying (MOHEIMANI et al., 2015; MOLINA GRIMA et al., 2003; UDUMAN et al., 2010).

Figure 2.4 Two-stage process for harvesting microalgae. In the first step, a dilute microalgal suspension is pre-concentrated by flocculation followed by sedimentation. The microalgal slurry is concentrated 20 times (10 g L^{-1} to 5 m^3 volume) and is then further dewatered using a mechanical method such as centrifugation or filtration.



Source: Adapted from Muylaert et al. (2015).

2.3.1 Current algae flocculation methods

Flocculation may be initiated through the use of inorganic coagulants, organic coagulants (often polymers) or by using electrofloculation, autoflocculation and bioflocculation procedures (LEE et al., 2013; VALERO et al., 2015; WAN et al., 2015; SALIM et al., 2011; PRAJAPATI et al., 2014; JAKOB et al., 2016). The method involves the destabilization of the microalgal cells in suspension by reducing or neutralising the cell surface charge (coagulation), followed by the aggregation of the destabilised microalgae cells (flocculation) (HOWE et al., 2012; HADJOUDJA et al., 2010).

A wide variety of metal salts, such as $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, $Fe_2(SO_4)_3$ have been used as inorganic flocculants in industries such as water treatment and mining (BARROS et al., 2015; VANDAMME et al., 2013). When dissolved in water, aluminium or ferric iron forms metal hydroxides that are positively charged and can induce flocculation. Metal salts work well for harvesting microalgae, but the doses required for effective flocculation are very high (usually >100 mg L^{-1}). Even higher doses are required in seawater compared to freshwater. Another disadvantage is that the use of metal salts results in contamination of the harvested biomass with metals (MOHEIMANI et al., 2015; SCHLESINGER et al., 2012).

Organic coagulants have generally received the most attention in the recovery of microalgae as they can interact more easily with the negative charge of the microalgae cell providing no contamination in the final product (VANDAMME et al., 2013; HADJOUDJA et al., 2010). Some of these biopolymers are relatively rare in nature; examples are poly-γ-glutamic acid, a polymer produced by *Bacillus subtilis*, and flour from the seeds of the *Moringa oleifera* tree. A well-known example is chitosan, which is prepared from chitin by de-acetylation of the acetylamine groups, leaving amine groups that are protonated, and thus positively charged at low pH. Chitosan is an effective flocculant for microalgae but only at low pH. (MOHEIMANI et al., 2015; WAN et al., 2015).

Autoflocculation induced by pH adjustment is another alternative. Increasing pH to 11-12 induces flocculation in *Chlorella* (AL-HATTAB et al., 2015). This works for some, but not all species, for example it did not produce flocculation in *Chlamydomonas* (SCHLESINGER, et al., 2012). It may require the addition of considerable amounts of pH-altering compounds to alter the pH (e.g. relatively large amounts of

NaOH) which is considered as uneconomic and can cause cell damage and death thus harming the quality of the biomass (VANDAMME et al., 2010).

Electroflocculation has been used for waters and wastewaters treatment with relative low energy consumption (HOWE et al., 2012; POELMAN et al., 1997). Therefore, it could be a promising microalgae harvesting technique offering an attractive alternative to conventional ones (LEE et al., 2013; VALERO et al., 2015; VANDAMME et al., 2011). The electroflocculation process offers some advantages, such as no direct addition of chemicals, versatility and selectivity. The main disadvantages of this technology are the energy input, the need to regularly replace the anode and the possibility of contamination of the biomass by increased doses of the electrode metal ions (UDUMAN et al., 2010; MATOS et al., 2013).

Biological approaches are emerging techniques that can lead to further reduction of operational costs (MOHEIMANI et al., 2015; BARROS et al., 2015; SALIM et al., 2011; JAKOB et al., 2016). Bioflocculation is a process characterized by aggregation of cells to form flocs on the presence of extracellular polymeric substances (EPS - typically composed of carbohydrate, protein, lipid or mixed chemistries of these) in the medium that induce the aggregation of the algae cells to form flocs (VANDAMME et al., 2013; LEE et al., 2009). The success of bioflocculation depends on the production of EPS from another organism (e.g. bacteria or fungi) and the attachment of the microalgae cells onto the polymers. Bacterial and fungal biomass has been used as sources of EPS for bioflocculation (SALIM et al., 2011; PRAJAPATI et al., 2014; LEE et al., 2010). This is usually because they produce filamentous hyphae in the case of fungi, or else because they secrete extracellular polymeric substances (EPS) which act as a naturally produced polymer, acting similarly to exogenously added polymers (LEE et al., 2009; GUTZEIT et al., 2005). These systems are a realistic prospect in terms of cost and energy balance but typically take a long time for flocculation to occur (e.g. overnight) which is problematic for an effective harvesting in a continuous process for example (LEE et al., 2010). Below Table 2.1 shows some advantages and disadvantages for the most commonly used harvesting process.

Table 2.1 Advantages and disadvantages of the current harvesting methods. First step dewatering means pre-concentration step and second step dewatering means mechanical methods.

	Harvesting method	Advantages	Disadvantages
First step dewatering	Chemical flocculation	• Simple and fast method • Low energy requirements	• Chemical flocculants may be expensive and toxic to microalgal biomass • Recycling of culture medium is limited
	Bioflocculation	• Inexpensive method • Allows culture medium recycling • Non-toxic to microalgal biomass	• Poorly understood • Extensive time required for effective flocculation
	Electroflocculation	• Applicable to a wide variety of microalgal species • Attractive for marine algal strains	• High energetic and equipment costs • Costs associated with electrodes replacement
Second step dewatering	Filtration	• Allows the separation of shear sensitive species • Attractive to filamentous species	• Membrane should be regularly cleaned • Costs associated with membrane replacement and pumping
	Centrifugation	• Fast method • High recovery efficiency • Suitable for most of microalgal species	• Expensive method • High energy requirement

Source: Adapted from Barros et al. (2015), Vandamme et al. (2013), and Muylaert et al. (2015).

The optimization of a pre-concentration step before the mechanical dewatering process is the most promising approach towards lowering microalgal harvesting costs. At the same time, environmental sustainability must be considered. Regarding biologically based harvesting methods, better understanding and control of flocculation processes could improve their performance and reduce operational costs.

Nevertheless, little is known about which molecules have these properties, which microorganisms produce them and how they do it (MOHEIMANI et al., 2015; BARROS et al., 2015; JAKOB et al., 2016; LEE et al., 2010).

2.3.2 *Tetrahymena* as a harvesting method

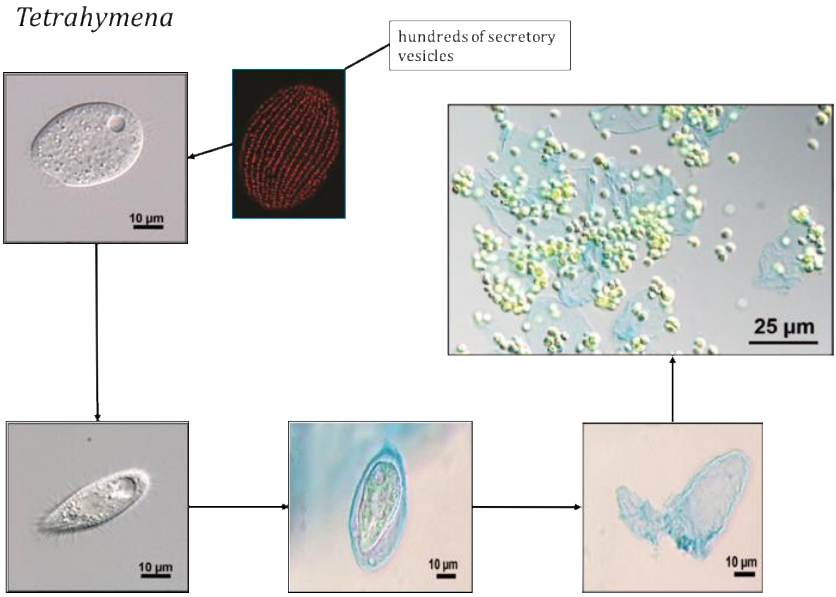
Tetrahymena cells, such as wild algal species, rotifers, fungi, viruses and another protozoan are common invaders of the microalgae open pond cultures, which eat or parasitise the desired strain (RICHMOND, 2004). In case of *Tetrahymena*, it typically feeds on bacteria rather than on the microalgae themselves (JAKOB et al., 2016). Also, bacteria are found in open pond cultures, which compete for nutrients and in some cases, produces cofactors which are beneficial to the algae (e.g. vitamins B1 and B12) (SALIM et al., 2011; LEE et al., 2009; GUTZEIT et al., 2005). Many of these common invaders produce EPS, which may lead in spontaneous algal bioflocculation, but the reasons remain poorly understood. The nature of the organisms, the timing of the events and, in particular, the biochemical signals responsible for inducing this spontaneous bioflocculation have until now been obscure (VANDAMME et al., 2013; JAKOB et al., 2016).

These phenomena were observed at the Solar Biofuels Research Centre (SBRC) in Brisbane Australia (JAKOB et al., 2016). Variable flocculation was noticed between different open pond cultures of a *Chlorella species*, which was attributed to the presence of a ciliate. The actual initiation of flocculation appeared to be triggered by the addition of small amounts of ammonium hydroxide, which was dosed into the systems as a pH-modifier and nitrogen source. The wild strain was isolated, identified to the genus level as a *Tetrahymena* species and kept in stock cultures.

To investigate these phenomena, the authors used *Tetrahymena* cells as a controlled flocculation reagent for the microalga *Chlorella sorokiniana* (local strain 8_C4). The process involves growth of *Tetrahymena* cells growth to high cell density and subsequent starvation (TURKEWITZ et al., 1999). After this shift in culture media, *Tetrahymena* synthesize dense granular vesicles, making them competent to perform exocytosis. The *Tetrahymena* cells were added to the algae culture and triggered by adding a variety of chemical substances to the starved ciliate. Triggered exocytosis of EPS leads, through electrostatic, hydrophobic and physical interactions to

biological flocculation, functioning as a binding substance between the algae cells (Figure 2.5).

Figure 2.5. Bioflocculation mechanism using the single cell organism of the genus *Tetrahymena*.



Source: Adapted from Jakob et al. (2016).

Investigations were conducted towards optimising the biomass recovery efficiency and resource minimisation. It was found that for all triggering stimuli examined, flocculation is satisfactory at ratios of 1:400 to 1:60 *Tetrahymena* to algae cells (JAKOB et al., 2016).

Although the biological advantage of exocytosis for *Tetrahymena* has not been well established, possibilities include that it forms a defensive capsule against predators, another is that it generates a substrate upon which bacteria grow (or are trapped) and which then provides a food source for the *Tetrahymena* (ROSATI & MODEO, 2003). Even though these functions of the EPS release in *Tetrahymena* have been poorly understood, the triggering of exocytosis in *Tetrahymena* has suggested a suitable controllable dewatering process for microalgal cultures.

2.4 COMPOSITION OF MICROALGAL BIOMASS

Microalgae biomass contain high levels of proteins, carbohydrates, and lipids; are capable to synthesize long-chain fatty acids, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linolenic acid (LA), and arachidonic acid (AA) (KOLLER et al., 2014; BATISTA et al., 2013; RYCKEBOSCH et al., 2014; MATOS et al., 2016). A wide range of biomolecules synthetized by microalgae are found to be potentially employed as antioxidant compounds, such as pigments including carotenoids, phenolic compounds, sulfated polysaccharides, and vitamins (AHMED, et al., 2014; GOIRIS et al., 2012; RODRIGUEZ-GARCIA & GUIL-GUERRERO, 2008; HAJIMAHMOODI et al., 2009). Because of their biomass chemical composition, microalgae have been found to provide a useful application in enhancing the nutritional value of foods. They also have potential applications as functional foods which are able to provide additional physiological and pharmacological benefits for human health (MATOS et al., 2016; RAPOSO & MORAIS, 2015; GOIRIS, et al., 2015).

2.4.1 Proteins

The component quantitatively most important of most microalgae biomass are proteins, which can represent up to 50% dry weight (VEGA & VOLTOLINA, 2007). High quality digestible proteins are found in *Chlorella* and *Spirulina* biomass, currently natural food products commercially available. The high protein content of various microalgal species was one of the main reasons for considering these organisms as an unconventional source of food (RICHMOND, 2004; BENEMANN & OSWALD, 1996). Most of the published works in the literature on concentrations of algal proteins are based on estimations of crude protein (BATISTA et al., 2013; MATOS et al., 2016, FELLER et al., 2014). This estimate is obtained by hydrolysis of the algal biomass, estimation of the total nitrogen and subsequent multiplication of the value by the factor 6.25 (Kjeldahl's method) (AOCS, 1995). This N-to-P factor assumes that the protein source contains 16 % N and does not consider the other nitrogenous constituents, e.g. nucleic acids, amines, glucosamides, and cell wall materials consisting of nitrogen. As a rule of thumb about 10% of the nitrogen found in microalgae consists of non-protein nitrogen (MATOS et al., 2016; VEGA & VOLTOLINA, 2007; LOURENÇO et al., 2004).

2.4.2 Carbohydrates

In most of the cases the whole microalgal biomass is used as food or food supplement. This means that in addition to the protein other components of the algal biomass such as carbohydrates, fibers, etc. will affect the overall value and digestibility of the algal product (RICHMOND, 2004). Carbohydrates of microalgae can be found in the form of starch, cellulose, sugars, and other polysaccharides, as storage products or cell wall components (BATISTA et al., 2013; MATOS et al., 2016). If the overall digestibility of the carbohydrates of the used algae is good, there seems to be no limitation in using the dried algae biomass as a whole (RICHMOND, 2004). The carbohydrate content in microalgae can vary widely, between 10 and 50% of the biomass dry weight, depending on the culture conditions (BATISTA et al., 2013; CAMPOS et al., 2010). Carbohydrates are divided into structural, being main components of cell walls, and as energy storage, mainly accumulating in chloroplasts, with higher accumulation rates under stress conditions (VEGA & VOLTOLINA, 2007).

2.4.3 Lipids

Lipids and fatty acids are constituents of all plant cells, where they function as membrane components, as storage products, as metabolites, and as sources of energy (MOHEIMANI et al., 2015; BOROWITZKA & MOHEIMANI, 2013; ANDERSEN, 2005). Lipids can be defined as any biological molecule which is soluble in an organic solvent, and called commonly total lipids. Lipids can be classified according to their polarity. Non-polar lipids (neutral lipids) comprise triglycerides and free fatty acids, and polar lipids which can be further sub-categorized into phospholipids and glycolipids. Neutral lipids are used primarily in the microalgal cells as energy storage, while polar lipids pack in parallel to form bilayer cell membranes (VEGA & VOLTOLINA, 2007; MEDINA et al., 1998; HALIM et al., 2012).

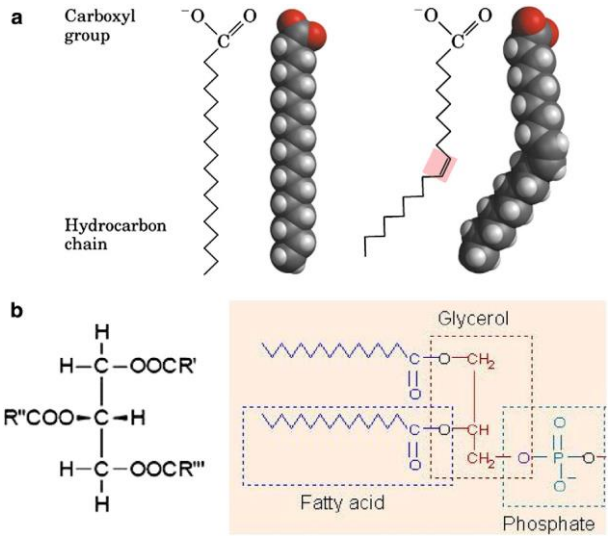
There are also some types of neutral lipids that do not contain fatty acids, such as hydrocarbons, sterols, phenolic compounds, pigments (carotenoids and chlorophylls). These lipid fractions are soluble in organic solvents (hence fitting the definition of lipids). Many of them are considered high value products, such as carotenoids, while triglycerides are desirable for commercial-scale biodiesel production (RICHMOND, 2004; HALIM et al., 2012). Microalgae lipid content is affected by life cycle and cultivation conditions, such as medium

composition, temperature, illumination intensity, and light/dark cycle (MATA et al., 2010; HARUN et al., 2010; ANDERSEN, 2005; JUNYING et al., 2013). Many microalgae can contain more than 50% of dry weight lipid content under controlled conditions. Nitrogen starvation is most influential on lipid storage and lipid fractions as high as 70–85% of dry weight were reported (MOHEIMANI et al., 2015; DEMIRBAS & FATIH DEMIRBAS, 2010; CHIU et al., 2009).

2.4.4 Fatty acids

A fatty acid molecule consists of a hydrophilic carboxylate group attached to one end of a hydrophobic hydrocarbon chain (Figure 2.6). Fatty acids are constituents of lipid molecules (both neutral and polar) and designated based on the total number of carbon atoms, and double bonds along the hydrocarbon chain (SHEEHAN et al., 1998; BOROWITZKA & MOHEIMANI, 2013). Saturated fatty acids have no double bond, while unsaturated fatty acids consist of at least one double bond. When the carboxylate end of the fatty acid molecule is bonded to an uncharged head group (e.g. glycerol), a neutral lipid molecule is formed (e.g. triacylglycerol). On the other hand, the association of a fatty acid molecule to a charged head group (e.g. glycerol and phosphate complex) forms a polar lipid molecule (e.g. phospholipid) (MEDINA et al, 1998; HALIM et al., 2012).

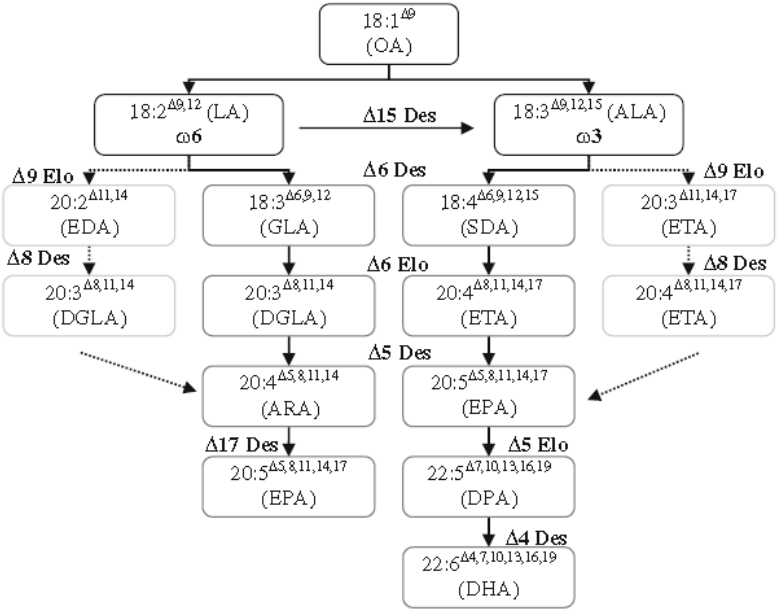
Figure 2.6 Fatty acid chains and lipid molecules. (a) Saturated fatty acid (C18:0) on the left and unsaturated fatty acid (C18:1) on the right. (b) Triacylglycerol (neutral lipid) on the left and phospholipid (polar lipid) on the right. R′, R″, R‴ in the triacylglycerol molecule represent fatty acid chains.



Source: Adapted from Halim et al. (2012).

Beyond short-chain (C_8-C_{18}) fatty acids, microalgae also synthetize a group of essential fatty acids, the polyunsaturated fatty acids (PUFAs), such as omega-3 (ω3) and omega-6 (ω6). Linoleic acid (LA, 18:2 ω6) and α-linolenic acid (ALA, 18:3 ω3), are precursors for the long-chain (C_{20}-C_{22}) PUFA (LC-PUFA) of the ω6 group and of the ω3 group (KHOZIN-GOLDBERG et al., 2011; BUCY et al., 2012). In general, the LC-PUFA biosynthesis pathways (Figure 2.7) in the endoplasmic reticulum are initiated by Δ12 desaturation of the chloroplast-derived oleic acid (OA, $18{:}1^{\Delta 9}$, ω9), producing LA ($18{:}2^{\Delta 9,12}$, ω6). Subsequently, LA may be further desaturated by a Δ15 (ω3) desaturase, generating ALA ($18{:}3^{\Delta 9,12,15}$ ω3). These fatty acids are further converted via the common ω6 and ω3 pathways, which are initiated with the Δ6 desaturation of LA or ALA, respectively (KHOZIN-GOLDBERG et al., 2011).

Figure 2.7 Pathways for the biosynthesis of LC-PUFA in microalgae.



Source: Khozin-Goldberg et al. (2011).

Important health benefits are associated with LC-PUFA and particularly with eicosapentaenoic acid (EPA, 20:5 ω 3), docosahexaenoic acid (DHA, 22:6 ω 3), and Arachidonic acid (ARA, 20:4 ω6) (KOLLER et al., 2014; RYCKEBOSCH et al., 2014; MATOS et al., 2016; RAPOSO & MORAIS, 2015). LC-PUFA producing microalgae are mainly marine planktonic species, belonging to different phyla. Literature shows that the most promising species belong to the *Bacillariophyta* (e.g. *Chaetoceros, Phaeodactylum, Skeletonema, Thalassiosira*), *Chlorophyta* (e.g. *Tetraselmis*), *Cryptophyta* (e.g. *Cryptomonas, Rhodomonas*), *Haptophyta* (e.g. *Isochrysis, Pavlova*), *Heterokontophyta* (e.g. *Nannochloropsis*) and *Rhodophyta* (e.g. *Porphyridium*) (KOLLER et al., 2014; BATISTA et al., 2013; MATOS et al., 2016; KHOZIN-GOLDBERG et al., 2011). An adequate dietary intake of PUFAs is vital for healthy nutrition and brain function, reducing the occurrence of cardiovascular disease, risk of cardiac death and mental illness, which enable PUFAs to be commercialized for

pharmaceutical and therapeutic industries (MATOS et al., 2016; RAPOSO & MORAIS, 2015).

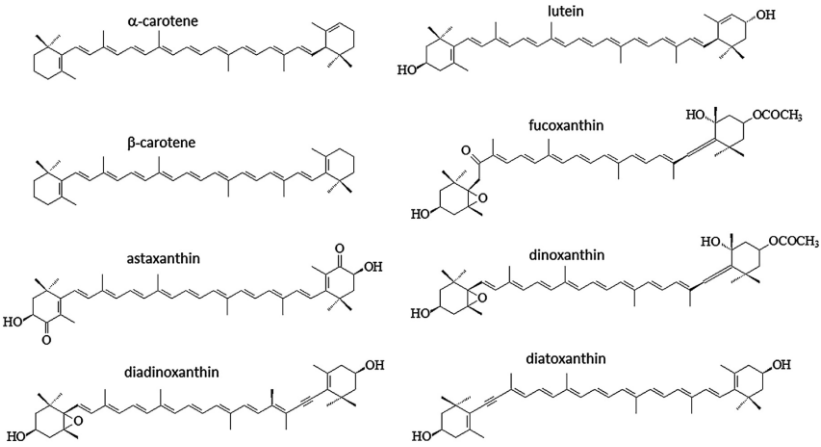
2.4.5 Antioxidant compounds

During the photosynthesis, microalgae absorb solar light and CO_2 which is converted into biomass, and at the same time, generating molecular oxygen. As, oxygen is easily activated by ultraviolet radiation or heat from sunlight into toxic reactive oxygen species (ROS), plants and algae able to minimize the concentration of these reactive species (AHMED et al., 2014; GOIRIS et al., 2015). Since, microalgae have developed a protective mechanism against reactive oxygen species (ROS) which consists in the preparation of numerous of efficient antioxidant compounds, they have emerged as a potential source of natural antioxidants, increasingly being used in food supplements and functional foods (HAJIMAHMOODI, et al., 2009).

There are a number of reports on the evaluation of antioxidant activity in microalgae and cyanobacteria belonging to the genera *Botryococcus* (RAO et al., 2006), *Chlorella* (WU et al., 2005), *Dunaliella* (HERRERO, et al., 2006), *Nostoc* (LI et al., 2007), *Phaeodactylum* (GUZMAN et al., 2001), *Spirulina* (MIRANDA et al., 1998), etc. These studies have shown that microalgal bioactive compounds possesses anti-inflammatory, antiviral, antimicrobial, antihelmintic, cytotoxic, immunological, and enzyme inhibition properties. The scavenger capacity of microalgal antioxidant compounds bring them up as the potential alternative substances against oxidation-associated conditions like chronic diseases, inflammation, skin UV-exposure and prevention of cardiovascular disorders, ageing related diseases such as Alzheimer and certain types of cancer (RAPOSO & MORAIS, 2015; GOIRIS et al., 2015a).

An important class of antioxidants from microalgae are carotenoids (Figure 2.8). Carotenoids are lipophilic pigments that provide protection to the photosynthetic apparatus by dissipating excess energy and harvesting light (AHMED et al., 2014; GOIRIS et al., 2015). They play an important role in quenching ROS generated during photosynthesis, especially singlet oxygen (VARELA et al., 2015; GOIRIS et al., 2015a).

Figure 2.8 Chemical structures of some carotenoids occurring in microalgae.



Source: Goiris et al. (2015).

Carotenoids are a wide group of lipophylic isoprenoids whose colours ranged from yellow to reddish brown (AHMED et al., 2014). These compounds are synthesized by all photosynthetic organisms, present in the pigment-protein complexes of the thylakoid membranes of chloroplasts, where they fulfill a dual function. As mentioned above, they are indispensable in light harvesting and energy transfer during photosynthesis and in the protection of the photosynthetic apparatus against photooxidative damage (VARELA et al., 2015; SARANYA, et al., 2014). Lycopene, synthesized by stepwise desaturation of the first 40-carbon polyene phytoene, is the precursor of all carotenoids found in algae. Two major groups of carotenoids can be distinguished into carotenes and xanthopylls based on their chemical structure. The carotenes are hydrocarbons whereas the xanthopylls have oxygenated functional groups making them more polar compounds than carotenes. Although certain carotenoids like β-carotene, violaxanthin and neoxanthin occur in most algal classes, other carotenoids are restricted to only a few algal classes (RICHMOND, 2004; AHMED et al., 2014; GOIRIS et al., 2015).

Microalgae are already commercially produced as a source of carotenoid antioxidants (e.g. *Haematococus* for astaxanthin, and *Dunaliella* for β-carotene) for use as additives in food and feed applications, as well as for use in cosmetics and as food supplements (GOIRIS et al., 2012). Recently, there has been interest in fucoxanthin,

a carotenoid available in brown algae and diatoms, due to claims that it can induce apoptosis in human cancer cells and that it possesses anti-inflammatory, anti-oxidant, anti-diabetic and anti-obesity properties (VARELA et al., 2015; ZHAO et al., 2014).

There are other antioxidants found in microalgae. For example, ascorbic acid or vitamin C, present in both cytosol and chloroplast, has vital role in the elimination of hydrogen peroxide and scavenges superoxide, hydroxyl radical, and lipid hydroperoxides (GOIRIS et al., 2012; RODRIGUEZ-GARCIA & GUIL-GUERRERO, 2008; HAJIMAHMOODI, et al., 2009). Tocopherols, located in the lipid bilayers of cell membranes, where the most active antioxidant form is α-tocopherol, acts as an antioxidant through its ability to quench both singlet oxygen and lipid peroxides (GOIRIS et al., 2015). Polyphenols, which comprise a structurally diverse group of components, including simple phenols, phenolic acids, flavonoids, tannins, and lignans. These compounds can inhibit lipid oxidation in different ways, that is, by directly scavenging HOCl, singlet oxygen, lipid peroxyl, superoxide and hydroxyl radicals, by metal chelation or by inhibiting lipoxygenase (RICHMOND, 2004; GOIRIS et al., 2012; HAJIMAHMOODI, et al., 2009).

2.5 EXTRACTION OF LIPIDS FROM MICROALGAE BIOMASS

Extraction has been defined as a material transportation process of one phase to another for the purpose of separating one or more substances (RICHMOND, 2004; BOROWITZKA & MOHEIMANI, 2013). Therefore, the extraction of lipid fraction, i.e., the phase which is soluble in the solvent used, is separated from the cells that comprise the biomass. During the dissolution extract and solvent form a molecular mixture (HALIM et al., 2012).

The lipid fraction (extract) obtained from microalgal biomass after extraction process is comprised of a wide range of biomolecules used in different biological processes (KOLLER et al., 2014; GOIRIS et al., 2015). These biomolecules present little functional or structural relationship with lipids such as sterols, lipophilic vitamins, pigments, etc. In the extraction process these molecules are also extracted and quantified as total lipids (crude lipids) (MEDINA et al., 1998; HALIM et al., 2012). Extraction of these compounds from microalgae requires attention to their polarity. The polarity of a compound is related to the distribution within the algal cell and association with lipid and non-lipid

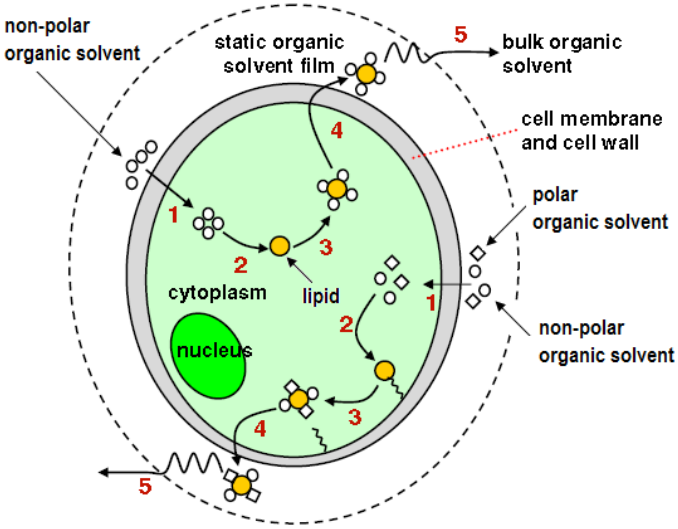
components (MOHEIMANI et al., 2015; RICHMOND 2004; MEDINA et al., 1998).

2.5.1 Extraction using organic solvent

The use of organic solvents in the extraction of lipids from microalgal biomass is widely used, since the solubility of these compounds in certain solvents is quite high (MOHEIMANI et al., 2015; DEMIRBAS & FATIH DEMIRBAS, 2010; MERCER & ARMENTA 2011). Organic solvents, such as hexane, chloroform and methanol are widely used and shown to be effective in this process. In addition, a suitable solvent should be insoluble in water, preferentially solubilize the compound of interest, have a low boiling point to facilitate its removal after extraction, and have a considerably different density than water (BOROWITZKA & MOHEIMANI, 2013; MERCER & ARMENTA, 2011). Also, for process cost-effectiveness, it should be easily sourced, as well as inexpensive and reusable. Due to these qualities, hexane is typically the solvent of choice for large scale extractions (HALIM et al., 2012; CHENG et al., 2011).

The solvent extraction theory, based on the laws of thermodynamics, has been explained by Kates (KATES, 1986). During dissolution, two separate substances (e.g. the lipid and the solvent) form a molecular mixture. Because dissolution involves mixing of two substances, an increase in their disorder (i.e. a positive entropy change) occurs. Dissolution involves two endothermic processes and one exothermic. First, lipid molecules separate into isolated molecules; second, the separated lipid molecules are dispersed into solvent and energy is required to dissociate the solvent molecules. These are endothermic processes. In the third process, which is exothermic, the dispersed lipid molecules interact with neighboring solvent molecules. A better understand about this mechanism is shown in Figure 1.9, and described next.

Figure 2.9 Schematic diagram of the proposed organic solvent mechanism divided in 5 steps.



Source: Adapted from Halim et al. (2012).

When a microalgal cell is exposed to a non-polar organic solvent, such as hexane or chloroform, the organic solvent penetrates through the cell membrane into the cytoplasm (step 1) and interacts with the neutral lipids (step 2) to form an organic solvent-lipids complex (step 3). This organic solvent–lipids complex, driven by a concentration gradient, diffuses across the cell membrane (step 4) and the static organic solvent film surrounding the cell (step 5) into the bulk organic solvent.

In the case of neutral lipids associated as complex with polar lipids (i.e. phospho-glycolipids), these are linked via hydrogen bonds to proteins in the cell membrane (RICHMOND, 2004; BOROWITZKA & MOHEIMANI, 2013). The van der Waals interactions formed between non-polar organic solvent and neutral lipids in the complex are inadequate to disrupt these membrane-based lipid–protein associations. On the other hand, polar organic solvent (such as methanol or isopropanol) can disrupt the lipid–protein associations by forming hydrogen bonds with the polar lipids in the complex (MEDINA et al., 1998; KATES, 1986). The mechanism in which the non-polar/polar organic solvent mixture extracts membrane-associated lipid complexes is also proposed in lower half of Figure 2.9. Table 2.2 shows some

examples of organic solvents used and the compounds expected into the obtained extract, according to affinity.

Table 2.2 Examples of organic solvents used in the extraction process of microalgal biomass and compounds expected.

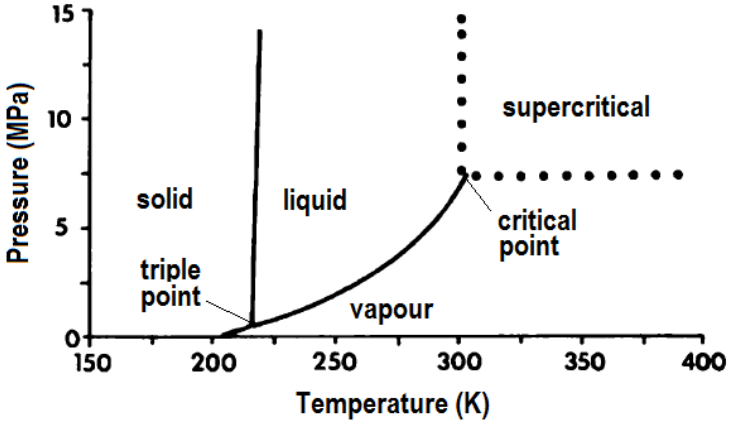
Polarity	Solvent	Compound
Non-polar	Chloroform, hexane	Hydrocarbons, pigments (carotenoids, clorophylls, phycobilins) sterols, triglycerides, free fatty acids
Polar	Methanol, isopropane	Phospholipids and glycolipids.

Source: Adapted from Medina et al. (1998).

2.5.2 Extraction using supercritical CO_2 (SC-CO_2)

Carbon dioxide is the most common solvent used for supercritical fluid extraction, mainly due to its advantageous physicochemical properties near the critical point and its chemical inertness (HALIM et al., 2011; YEN et al., 2015). The SC-CO_2 extraction is a clean and emerging technology that has the potential for replacing organic solvent extraction. This extraction method has shown wide acceptance for extracting high value products from microalgae biomass. This is due to the selectivity of the method, no solvent residue occurrence, rapid separation and preservation of thermolabile products (MERCER & ARMENTA, 2011; REVERCHON & MARCO, 2006; HERRERO & IBÁÑEZ, 2015). The fluid is called supercritical because it is above its critical temperature (Tc) and its critical pressure (Pc) (MENDES et al., 2003; JACQUES, 2005). When the temperature and pressure of a fluid are increased above their critical values (Tc and Pc), the fluid goes to the supercritical region (Figure 2.10).

Figure 2.10 Pressure-temperature phase diagram for CO_2.



Source: Adapted from Mendes et al. (2003).

In this state, compared to liquid, the fluid shows highest diffusion coefficient and lower viscosity and density according to Table 2.3. The lower viscosities and higher diffusion rates of the supercritical fluid make it suitable for the extraction of compounds present in solid matrix.

Table 2.3 Range of thermophysical properties of gas, supercritical fluid and liquid.

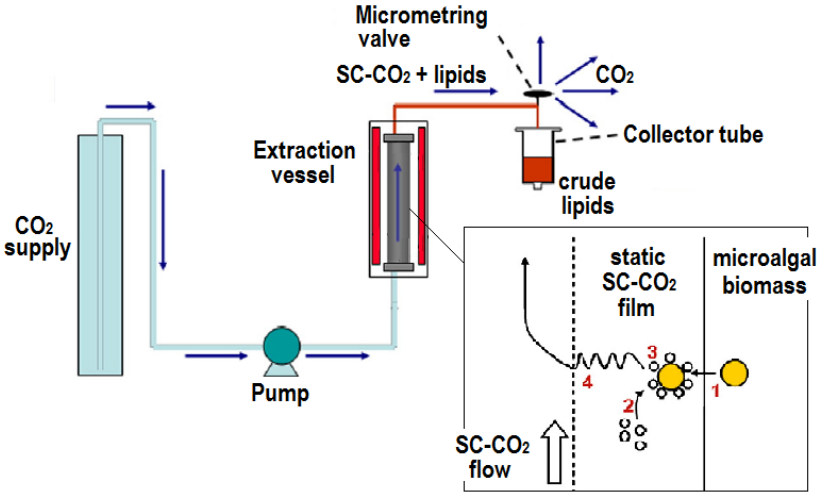
Fluid state	Density ($kg\ m^{-3}$)	Diffusion coefficient ($m^2\ s^{-1}$)	Viscosity ($N\ s\ m^{-2}$)
Gas	1 – 100	$10^{-4} – 10^{-5}$	$10^{-5} – 10^{-4}$
Supercritical	250 – 800	$10^{-7} – 10^{-8}$	$10^{-4} – 10^{-3}$
Liquid	800 – 1200	$10^{-8} – 10^{-9}$	$10^{-3} – 10^{-2}$

Source: Adapted from Mendes et al. (2003).

These properties are affected by changes in temperature and pressure. The increased pressure leads to increased density (which favors the process yield), on the other hand, lower diffusivity and higher viscosities are produced (which disadvantage yield). The temperature presents an opposite effect than pressure, where an increase in temperature leads a reduction in density, an increase in diffusivity and viscosity decrease. It must be noted also that the increase in temperature leads an increase in the lipid fraction vapor pressure, and increasing the

extraction yield (JACQUES, 2005; HERRERO & IBÁÑEZ, 2015; CHENG et al., 2011). Thus, the effect of temperature and pressure is complex, and its understanding is of fundamental importance for the analysis of the extraction process using supercritical fluids (HALIM et al., 2012). Operational variables that influence the performance of the supercritical extraction include, pressure, temperature and fluid flow rate (HERRERO & IBÁÑEZ, 2006; HALIM et al., 2011). The extraction system using supercritical fluids, basically consists of four components: gas supply; high pressure pump; extraction vessel and collector. Figure 2.11 shows a supercritical extraction system using CO_2 as solvent.

Figure 2.11 Schematic diagram of a laboratory-scale SC-CO_2 extraction system, and the proposed SC-CO_2 extraction mechanism.



Source: Adapted from Halim et al. (2012).

During the lipid extraction process, the microalgal biomass are packed inside the extraction vessel. The fluid travels on the surface of the biomass and the lipid fraction is desorbed from the microalgal biomass. Immediately upon dissolution, the SC-CO_2 encloses the lipids to form a SC-CO_2 -lipids complex. The complex, driven by concentration gradient, diffuses across the static SC-CO_2 film and enters the bulk SC-CO_2 flow. The SC-CO2–lipids mixture, then leaves the extraction vessel to enter the collection vessel, where a micrometring valve is used to rapidly depressurize the incoming fluid. Upon complete

depressurization, the SC-CO_2 returns to gaseous state and the extracted crude lipids precipitate in the collector vessel. As crude lipids derived from supercritical fluid extraction are free from any extraction solvent, it does not need to undergo an extraction solvent removal step (HALIM et al., 2012; HERRERO & IBÁÑEZ, 2006; CHENG et al., 2011; MENDES et al., 2003).

2.5.3 Extraction using subcritical fluids

Apart from carbon dioxide, other compressed solvents can be used for the obtainment of solvent free extracts, for example: ethane, propane, *n*-butane, dimethyl ether, etc. (FREITAS et al., 2008; CATCHPOLE et al., 2009; CAPELETTO et al., 2016; GOTO et al., 2015). The use of these gases as compressed fluids to replace carbon dioxide usage differs from the work in the subcritical region, as compressed liquids (at least one order of magnitude below the critical point) afford the significant advantage of operating at moderate pressures, which lowers initial investment (CAPEX) and reduces maintenance costs and operational risks (OPEX) (LIPKIN et al., 1942; OLDS et al., 1944; NOVELLO et al., 2015). Despite the advantages of using, at subcritical compressed conditions, substances which are normally gaseous at room conditions, few examples have been reported in the literature. Catchpole et al. (2009) briefly reviewed the state-of-the-art in the extraction and fractionation of selected lipids using near critical fluids, reporting that propane and dimethyl ether can be used without co-solvents to extract polar lipids and other complex lipids from a range of raw materials, including dairy powders, eggs, biomass from fermentation, and wet marine biomass. Goto et al. (2015) demonstrated that the use of SC-CO_2 together with liquefied dimethyl ether (DME) as clean extraction processes for bioactive compounds (lipids and carotenoids) from wet algae. Using *n*-butane as a subcritical solvent, Novello et al. [86] found that palmitic (C16:0) and oleic (C18:1) fatty acids were the major components observed in *n*-butane extracts of *Carapa guianensis* seeds. Moreover, Capeletto et al. (2016) achieved high extraction yields and higher antioxidant activity using subcritical *n*-butane compared to SC-CO_2 from *Campomanesia xanthocarpa* seeds.

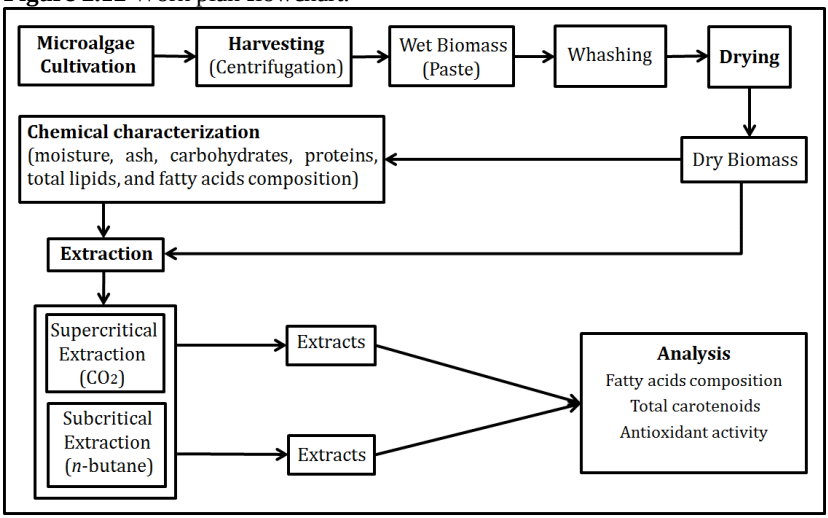
Besides these literature findings, the application of *n*-butane as solvent for the extraction of compounds from microalgae biomass has not been found. The use of subcritical *n*-butane applied to microalgal biomass can be a good choice. Its critical pressure is relatively low compared to that of carbon dioxide and present dielectric constants

(polarity) of 1.8, quite similar to carbon dioxide (1.6) (NOVELLO et al., 2015). Since, liquid *n*-butane exhibits low compressibility and very low solubility in water, the literature indicates that it has a hydrostatic behavior working as mechanical piston fluid that increases the system pressure changing favorably the extraction of non-polar components. Moreover, subcritical *n*-butane is a suitable method for large scale application, because it is plenty available, cheaper and it can be used in much lower pressure compared to carbon dioxide (CAPELETTO et al., 2016; NOVELLO et al., 2015).

2.6 STRATEGY AND AIMS OF THE STUDY

The content of this thesis is an investigation about the chemical composition of three marine microalgae with new approaches for lipids extraction, and harvesting of microalgal culture. In order to guide this study, the flowchart below (Figure 2.12) was created and followed based on the idea conceived and the structure needed. A survey was conduct to identify available collaborations for the development of this work and to define the methods to be used in each step of the downstream process.

Figure 2.12 Work plan **f**lowchart.



Source: Author.

The marine microalgae (*Phaeodactylum tricornutum*, *Nannochloropsis oculata*, and *Porphyridium cruentum*) used in this

study were grown at Laboratório de Cultivo de Algas (LCA), Universidade Federal de Santa Catarina (UFSC), Brazil, under supervision of Professor Roberto Bianchini Derner. The centrifugation, washing, and drying steps were developed partly at LCA and partly at Laboratório de Biotecnologia de Alimentos (BIOTEC/UFSC). The chemical characterization of each microalgae biomass was performed at Laboratório de Físico Química (LABCAL/UFSC). The supercritical extractions (SC-CO_2) were performed at Laboratório de Termodinâmica e Extração Supercrítica (LATESC/UFSC). The subcritical extractions (*n*-butane) were performed at Universidade do Alto Uruguai e das Missões (URI) and Universidade Federal de Santa Maria (UFSM) under the supervision of Professor José Vladimir de Oliveira and Marcus Vinícius Tres. The antioxidant activities of the biomass extracts were performed at LATESC, and the determination of total carotenoids was performed at Laboratório de Morfogênese e Bioquímica Vegetal (LMBV/UFSC). The case study on harvesting of microalgal culture was developed under the supervision of Dr. Ian Ross and Professor Ben Hankamer at Institute for Molecular Bioscience (IMB) and Solar Biofuel Research Centre (SBRC), The University of Queensland (UQ), Australia.

The main objective of this work was to evaluate the application of subcritical *n*-butane as an alternative extraction method for microalgal biomass, comparing with two others well-established extraction methods. In order to achieve this objective, specific objectives were proposed and some hypotheses were raised:

- To characterize the microalgae biomass composition;
- To obtain the lipid fraction (extract) from the microalgae biomass using the different extraction methods;
- To analyze the fatty acids composition, total carotenoids, and DPPH scavenging capacity of the extracts obtained;
- To compare the methods regarding to the compounds extracted.
- To investigate the viability of bioflocculation as a microalgal harvesting method.

Hypotheses: since subcritical *n*-butane is an innovative method, not found in the literature, applied to microalgae biomass, we hypothesized:

- Subcritical *n*-butane may extract more rich-lipids (PUFAs) from the microalgae biomass than the other methods tested;

- Subcritical *n*-butane extracts may present higher antioxidant activities than SC-CO_2 extracts;
- Subcritical *n*-butane method may extract more carotenoids than SC-CO_2.

2.7 STRUCTURE OF THIS THESIS

Chapter 3 presents the chemical composition of the three marine microalgae (*Phaeodactylum tricornutum*, *Nannochloropsis oculata*, and *Porphyridium cruentum)* used in this study, and *Chlorella vulgaris*, *Spirulina platensis*, and *Nannochloropsis gaditana* on behalf of the doctorate student Ângelo Paggi Matos from the Department of Food Science and Technology – UFSC. The main objective of this paper was to compile information on the biochemical composition of microalgae, which is needed for screening the optimal species for specific food applications (rich in protein, carbohydrates, PUFA, etc.). This chapter was published as an original research paper in Journal of American Oil Chemist's Society.

In chapter 4, a comparison of subcritical *n*-butane extraction with two well established methods (Soxhlet, and SC-CO_2) on lipid extraction yields, with a focus on the fatty acids composition of the extracts was performed. For this purpose, three marine microalgae strains (*Phaeodactylum tricornutum*, *Nannochloropsis oculata*, and *Porphyridium cruentum*) were selected because of their high and diverse contents of PUFAs ω3 and ω6. This chapter was submitted as an original research paper for Algal Research.

For chapter 5, the extracts obtained from the three marine microalgae biomass using *n*-butane and SC-CO_2 as solvents were analyzed regarding to antioxidant activity using free radical scavenging assay and total carotenoids content. All extracts possessed the ability to scavenging DPPH at various degrees. A correlation between the antioxidant activity and total carotenoid content of the supercritical CO_2 and *n*-butane microalgae extracts was determined. This chapter is being prepared as a manuscript for a research paper.

Chapter 6 presents the technical feasibility of a biological flocculation approach using the protozoan *Tetrahymena* as a harvesting method for microalgal cultures at pilot scale. This work was developed during the doctorate internship at The University of Queensland, Australia. It was a scale up of a previous work developed at laboratory scale and published as a research paper on Algal Research, entitled: "*Triggered exocytosis of the protozoan Tetrahymena as a source of*

bioflocculation and a controllable dewatering method for efficient harvest of microalgal cultures".

Chapter 7 presents a general discussion, with an overview of the main contributions that this thesis made for the extraction and harvesting step processes, the larger bottlenecks in the downstream process of microalgae cultivation. At the end of the chapter, suggestions for future work are presented.

Chapter 3

CHEMICAL CHARACTERIZATION OF SIX MICROALGAE WITH POTENCIAL UTILITY FOR FOOD APPLICATION

The work presented in this chapter was developed in collaboration with the doctorate student Ângelo Paggi Matos from the Department of Food Science and Technology – UFSC. It is a reproduction of the research paper published in Journal of American Chemist's Society (Volume 93, 27 May 2016, Pages 963-972; doi: 10.1007/s11746-016-2849-y), only the abstract, and acknowledgements were omitted. Part of this work was also presented at AOCS – 106th Annual Meeting & Industry Showcases in Orlando, USA.

The microalgae species *Chlorella vulgaris*, *Spirulina platensis*, and *Nannochloropsis gaditana*, were provided by Ângelo P. Matos and the species *Phaeodactylum tricornutum*, *Nannochloropsis oculata*, and *Porphyridium cruentum* provided by Rafael Feller. These authors contributed equally to this manuscript.

3.1 INTRODUCTION

Microalgae are a group of eukaryotic organisms and photosynthetic cyanobacteria able to accumulate sugars, carbohydrates, proteins, lipids and other valuable organic substances by the efficient use of solar energy, CO_2, and nutrients. These microorganisms convert inorganic substances such as carbon, nitrogen, phosphorus, sulfur, iron, and trace elements into organic matter (green, blue-green, red, brown, and other colored biomass) (BATISTA et al., 2013).

Microalgae are considered one of the most promising feedstock materials for developing a sustainable supply of commodities, including both food and non-food products. Microalgae have also great potential as they produce natural compounds, that could be used as functional food ingredients (RYCKEBOSCH et al., 2014; DRAAISMA et al., 2013). Currently, edible oils, proteins and carbohydrates are consumed in a variety of food products, which contain ingredients from both plant and animal origin. In this regard, microalgae can be used to enhance the nutritional value of foods. When using screening methodologies to identify valuable compounds (pigments, antioxidants, polyunsaturated fatty acids, etc.) in microalgae, knowledge of the chemical composition is a first requirement (BATISTA et al., 2013; HENRIKSON, 2009). The microalgae are an extremely diverse collection of organisms with a large

variation in chemical compositions, but this diversity is not yet fully explored (BOROWITZKA, 2013). For this reason, the nutritional content of algal biomass is sometimes poorly defined and for most species, including well-studied species like *Spirulina*, there is little consensus on their biochemical composition of different algal species.

Several fatty acids are synthesized by humans, but there is a group of essential fatty acids, the polyunsaturated fatty acids (PUFAs), which the human body cannot produce: omega-3 (ω-3) and omega-6 (ω-6). Therefore, both ω-3 and ω-6, which are necessary for human health, are entirely derived from the diet and nutrition experts have recommended that an ω3/ω6 fatty acids ratio of ≤ 1:5 is desirable (BOROWITZKA, 2013; FERNANDES et al., 2014; ARMENTA & VALENTINE, 2013). Since the Western diet contains massive quantities of ω-6, ω3/ω6 ratios of up 1:25 have been reported in the literature, which has been recognized to be undesirable, and most people consume thus a PUFA-deficient diet (FAO/WHO, 2008; MARTINS et al., 2006). Therefore, nutritionists emphasize the need to consume seafood (notably fish) and green vegetables to prevent an array of disorders, especially cardiovascular diseases (FAO/WHO, 2008; KLEINER et al., 2014). As reported by Armenta and Valentine (2013), single cell oils (SCO) containing long chain polyunsaturated fatty acids (LC-PUFA) such as EPA/DHA acids derived from algae are considered a promising oil alternative to oils from fish and land based plant sources.

The development of novel foods based on microalgal biomass is an exciting tool for providing nutritional supplements with biologically active compounds (*e.g.*, antioxidants, PUFAs-ω3) (LEMAHIEU et al., 2013). Depending on species/strain, environmental conditions and harvesting/processing methods, algal biomass after oil extraction may be a highly attractive source of essential dietary amino acids, fatty acids, sugars, vitamins, minerals, carotenoids and other health-promoting nutrients that are well suited as human food or feed additives for terrestrial livestock and aquatic animals (TIBBETTS et al., 2015). Although the potential for algal products/co-products for nutritional applications has long been recognized, so far it has had limited commercial success with only a few species (*e.g. Spirulina*, *Chlorella*) occupying niche markets (BRENNAN & OWENDE, 2010). Advancing our knowledge of the biochemical composition of algae is a key requirement for realizing the potential of algal products/co-products.

The main objective of the present study was to compile information on the biochemical composition of microalgae, which is

needed for selecting the optimal species for specific food applications (rich in protein, carbohydrates, PUFA, etc.). The microalgae species *Chlorella vulgaris*, *Spirulina platensis*, *Nannochloropsis gaditana*, *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Porphyridium cruentum* were mass cultivated in artificially illuminated photobioreactors to produce sufficient biomass quantities to be able to determine their chemical composition and nutritional value.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Acquisition of algal biomass

Freshwater species *Chlorella vulgaris* and *Spirulina platensis* were obtained from the Laboratory of Biochemistry Engineering, Federal University of Rio Grande. Marine species *Nannochloropsis gaditana* (clone 130) was kindly supplied by Banco de Micro-organismos Marinhos Aidar & Kutner (BMA&K), Oceanographic Institute at the University of São Paulo. Microalgae *C. vulgaris*, *S. platensis* and *N. gaditana* were cultured in the Laboratory of Food Biotechnology, Federal University of Santa Catarina. Microalgae were cultivated through the inoculation of microalgal cultures (monospecific algal cultures) in appropriate growth media: for *C. vulgaris*, Bold Basal Medium (BBM) (NICHOLS, 1973) was used, *S. platensis* cells were grown in Paoletti Synthetic Medium (PSM) (FERRAZ et al., 1985), and *N. gaditana* cells were cultured in previously autoclaved (121°C/15 min) seawater enriched with F/2 media (GUILLARD, 1975) with salinity of about 3.5%. Cultures of *C. vulgaris*, *S. platensis* and *N. gaditana* were developed in inverted conical photobioreactors (5 L capacity) with the respective growth media and scaled-up to 100 L capacity fiber photobioreactors (0.50 m diameter, 0.90 m length). The photobioreactors were placed under a photoperiod of 12:12 h light/dark at room temperature (25 ± 2°C), at a light intensity of 216 µmol photons m^{-2} s^{-1} provided by cool-white fluorescent lamps. Air saturated with CO_2 was continuously pumped into the photobioreactors. When the microalgal culture reached stationary growth phase, the entire culture broth (100 L) was harvested by continuous centrifugation at 4,000 rpm for approximately 1 h. The concentrated microalgal pellet was then transferred to a dish and dried in a dehydrator at 45 ± 5ºC for 24 h before analysis.

Marine algal species *N. oculata*, *P. tricornutum* and *P. cruentum* were cultured in the Laboratory of Algae Cultivation, Federal University

of Santa Catarina. The cultures were developed in two open tanks and a glass fiber cylinder that we custom designed and built. After cultures were grown in 5 L capacity Erlenmeyer flasks, mass cultures of *N. oculata* and *P. tricornutum* were scaled-up in 500-L capacity open tanks (1.60 m diameter and 2.75 m length) containing F/2 media. Cool-white fluorescent lamps were placed over the tanks under a constant light intensity of 100 µmol photons m^{-2} s^{-1}. The alga *P. cruentum* mass culture was scale-up in 180 L capacity glass fiber cylinder (0.50 m inner diameter and 1.00 m length) containing F/2 media. The surface of the cylinder was exposed continuously to 10 cool-white light lamps (100 µmol photons m^{-2} s^{-1}). The tanks and cylinder were constantly CO_2 aerated (40 L min^{-1}) and maintained at room temperature (22 ± 1ºC) using air conditioners. Salinity in the experiments was 3.5%. As soon as the cultures reached the stationary growth phase, the entire culture broth was harvested by continuous centrifugation and concentrated to biomass slurry, which was washed with isotonic ammonium formate (ZHU & LEE, 1997) and centrifuged at 3500 rpm for 35 min. Finally, the wet biomass was transferred to a dish and dried in a dehydrator (45 ± 5ºC, 24 h) before further analysis.

3.2.2 Biomass composition analysis

For each of the six microalgae species, triplicate samples of dried biomass were analyzed to determine moisture, ash, dietary fiber, carbohydrate, protein, lipid contents and fatty acids composition.

3.2.2.1 Moisture

Moisture was determined by drying the sample in an oven at 105°C for 3-4 h (until constant weight) (AOAC, 2005). The average moisture values were used to calculate the chemical composition as a percentage of total dry matter.

3.2.2.2 Mineral content

Total ash content was determined by heating the samples to 550ºC for 5 h using a carbolite muffle furnace (IAL, 2005).

3.2.2.3 Dietary fiber

Total dietary fiber (TDF) content was determined with a total dietary fiber analysis kit (Megazyme International Ireland Ltd, Wicklow, Ireland) (AOAC, 2005), which includes enzymatic hydrolysis

with α-amylase, protease and amyloglucosidade and is approved by the AACC (Method 32-05-01) and the AOAC (Official Method 985.29). Duplicate samples (approximately 1 g) were suspended in 50 mL phosphate buffer and submitted to enzymatic hydrolysis by incubating with 50 μL of α–amylase at 100°C for 30 min. The pH was adjusted to 7.5, 100 μL of protease was added and samples were incubated at 60°C for 30 min. Next, the pH was adjusted to 4.5, 200 μL of amyloglucosidade was added and the samples were incubated at 60°C for 30 min. Finally, fiber was precipitated with 95% ethanol at 60°C, filtered through fritted glass crucibles with a Celite filter and the residue in the crucible was dried in an oven at 105°C, cooled in a desiccator and weighed.

3.2.2.4 Protein content

Total nitrogen was determined by the Kjeldahl method after acid digestion (AOAC 991.20), ammonium addition, steam distillation and titration with 0.1 N HCl (AOAC, 2005). Protein content was calculated using a nitrogen-to-protein conversion factor of N x 4.78 (LOURENÇO et al., 2004).

3.2.2.5 Lipid content

After acid digestion with 4.0 N HCl for 6 h, intracellular lipids were extracted with petroleum ether by the Soxhlet method (AOAC 963.15), concentrated in a rotary evaporator, dried in an oven and weighed (AOAC, 2005).

3.2.2.6 Carbohydrates

The total carbohydrate content of each sample was calculated according to: (100% – (moisture + ash + protein + lipid + fiber)) (ANVISA, 2003.

3.2.3 Fatty acids composition

Fatty acids composition was determined after converting the fatty acids to their corresponding fatty acids methyl esters (FAME), which were determined by gas chromatograph using a GC-2014 (Shimadzu, Kyoto, Japan), equipped with split-injection port, flame-ionization detector and 105 m-long Restek capillary column (ID = 0.25 mm) coated with 0,25 μm of 10% cyanopropylphenyl and 90% biscyanopropylsiloxane. The injector and detector temperatures were

both 260°C. The oven temperature was initially set at 140°C for 5 min, programmed to increase at 2.5°C min^{-1}, and held at 260ºC for 30 min. The injection volume was 1µL, and the split ratio was 10:1. Nitrogen was used as the carrier gas (flow rate was 2.2 mL min^{-1}) at a constant pressure of 130.3 kpa. Fatty acid methyl esters were identified by comparison with the retention time of individual standards (Sigma, St. Louis, USA). For long-chain fatty acids (>C19) a correction factor for the quantification was used (AOCS, 1995). The proportions of the individual acids were calculated by the ratio of their peak area to the total area of all observed acids and expressed as mass percentage.

3.2.4 Lipids nutritional quality indexes (IQN)

The nutritional quality of the lipid fraction can be assessed by three separate indexes that are calculated based on the concentration of saturated fatty acids (lauric C12:0, myristic C14:0, palmitic C16:0 and stearic C18:0), monounsaturated fatty acids (MUFA, oleic C18:1ω9), and polyunsaturated fatty acids (linoleic C18:2ω6, linolenic C18:3ω3, arachidonic C20:4ω6 and eicosapentaenoic C20:5ω3) (ULBRICHT & SOUTHGATE, 1991; SANTOS-SILVA et al., 2002) according to:

(1) Atherogenicity index (AI) = [(C12:0 + (4 x C14:0) + C16:0)] / (ΣMUFA + Σω6 + Σω3)

(2) Thrombogenicity index (TI) = (C14:0 + C16:0 + C18:0) / [(0.5 x ΣMUFA) + (0.5 x Σω6 + (3 x Σω3) + (Σω3/Σω6)]

(3) Fatty acids hypocholesterolemic/hypercholesterolemic ratios (H/H) = (C18:1ω9 + C18:2ω6 + C20:4ω6 + C18:3ω3 + C20:5ω3) / (C14:0 + C16:0)

3.2.5 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) using STATISTICA Software (version 7.0) from StatSoft Inc. A *P*-value of <0.05 was considered statistically significant, and if significant differences were observed, treatment means were pairwise compared with the Tukey test.

3.3 RESULTS AND DISCUSSION

3.3.1 Composition of algal biomass

The chemical compositions of the six microalgae species are shown in Table 3.1. The moisture content in dried microalgal ranged from 1.4 to 12.6%, with *P. cruentum* and *S. platensis* having relatively high moisture values (12.6% and 10.0%, respectively). Ash contents were higher in biomass of the marine species *P. tricornutum* and *P. cruentum* (16.1% and 15.9%, respectively). Zhu and Lee (1997) have shown that washing the wet biomass with a 0.5 M ammonium formate solution greatly reduces the salt content of dry matter (DM) of these marine algal species. In fact, ash levels in non-washed biomass of marine *P. tricornutum* and *P. cruentum* were 40.7% and 33.2%, respectively. The diatom *P. tricornutum* has a high inorganic content due to the fact that its cell wall is covered by silica. The species *N. gaditana* and *S. platensis* had intermediate total ash contents (12.3-11.6%), while *N. oculata* and *C. vulgaris* were found to have the lowest ash contents (8.5-7.3%, respectively).

Table 3.1 Chemical composition of six microalgal biomass. Data represent the mean % ± SD (n=3).

Composition (%)	*C. vulgaris*	*S. platensis*	*N. gaditana*	*N. oculata*	*P. tricornutum*	*P. cruentum*
Dry matter	93.8 ± 0.3^{a}	89.9 ± 0.5^{d}	94.7 ± 0.4^{ab}	95.9 ± 0.1^{b}	98.6 ± 0.2^{e}	87.4 ± 0.3^{c}
Moisture	6.2 ± 0.5^{b}	10.1 ± 0.4^{d}	5.3 ± 0.3^{ab}	4.1 ± 0.2^{a}	1.4 ± 0.3^{c}	12.6 ± 0.4^{e}
Total ash	7.3 ± 0.2^{a}	11.6 ± 0.3^{b}	12.3 ± 0.2^{ac}	8.5 ± 1.7^{bc}	16.1 ± 0.2^{c}	15.9 ± 0.3^{c}
Total fiber	5.6 ± 0.4^{a}	8.5 ± 0.5^{b}	14.1 ± 0.3^{c}	13.0 ± 0.4^{c}	13.2 ± 0.3^{c}	18.3 ± 0.2^{d}
Total protein	41.4 ± 0.4^{a}	42.8 ± 0.1^{a}	41.6 ± 0.3^{a}	42.1 ± 0.1^{a}	39.0 ± 0.1^{b}	35.4 ± 0.9^{c}
Total lipid	12.8 ± 0.1^{a}	5.5 ± 1.2^{b}	8.1 ± 0.1^{b}	15.6 ± 1.1^{c}	14.9 ± 0.4^{c}	5.3 ± 0.3^{b}
Carbohydrates	26.7 ± 1.2^{a}	21.5 ± 0.5^{b}	18.6 ± 0.3^{bc}	16.7 ± 0.6^{bc}	15.4 ± 0.5^{cd}	12.5 ± 0.6^{d}

Values in the same row with different superscript letters are significantly different ($p < 0.05$)

Dry matter (protein, carbohydrate + fiber and lipids) is the major component in all algae studied and differences among species were small. Protein is the most abundant component followed by carbohydrate + fiber, and lipids. Algal biomass from the species studied contains on average 40 g protein (range 35.4-42.8%), 18 g carbohydrate (range 12.5-26.7%), 12 g fiber (range 5.6-18.3%) and 10 g lipid (range 5.3-15.6%) per 100 g of biomass DM. The total dry matter accounted for an average of 93.3% of total DM (range 87.4-98.6%), in good agreement with Matos et al. (2014), who reported a total biomass DM of 81.3-94.5% for *C. vulgaris*, using similar methods. In general it is common that total dry matter of microalgae is found to be less than 100% (TIBBETTS et al., 2015).

Using a nitrogen-to-protein (N-to-P) conversion factor of N x 4.78 (LOURENÇO et al., 2004), the protein content in *S. platensis* (42.8%) was calculated to be significantly higher than in *N. oculata* (42.1%), *N. gaditana* (41.6%) and *C. vulgaris* (41.4%), whereas the lowest levels were found in *P. tricornutum* and *P. cruentum* (39.0% and 35.4%, respectively, $P<0.05$). Among the six microalgae, *Spirulina* has been most extensively used as a source of single cell protein (SCP) and was even carried by astronauts during space travel. Many microorganisms (algae, bacteria, fungi yeast/filamentous) can be used as a source of SCP, but due to their low nucleic acid content and high level of essential amino acids, algae are preferred over fungi and bacteria as a source of SCP for human consumption (MATOS et al., 2014). The non-protein nitrogen (NPN) content in microalgae has been reported to range from 4 to 40% depending upon species, season and growth phase (TIBBETTS et al., 2015; LOURENÇO et al., 2004; ANUPAMA & RAVINDRA, 2000). The N-to-P factor assumes that the protein source contains 16% N and does not take into account the often high content of NPN found in microalgae. Our findings on protein content in the microalgal biomass are in accordance with data from Tibbetts et al. (2015), who reported that the N-to-P factor of N x 6.25, which has been historically applied for microalgae, is incorrect and should be avoided (LOURENÇO et al., 2002). It should be noted that the estimates for the crude protein include other nitrogen compounds, *e.g.*, nucleic acids, amines, glucosamides, and cell wall materials, which in general are expected to account for around 10% of the total nitrogen found in microalgae (VONSHAK, 2002).

The carbohydrate and dietary fiber contents in the algal biomass samples were found to be very diverse, varying between 12.5-26.7% and 5.6-18.3%, respectively. *P. cruentum* has the highest and well-balanced, carbohydrate (12.5%) and dietary fiber (18.3%) content (Table 3.1). Actually, *P. cruentum* showed the highest carbohydrate + fiber content (30.8%) which could be associated with its composition in sulfated polysaccharides (exopolysaccharide) (REBOLOSSO, et al., 2000; COHEN, 1990). Of the species studied, *Chlorella* and *Spirulina* were found to contain the lowest amounts of fiber (5.6% and 8.5%, respectively), which is important for human use, since low fiber values suggest an easily digestible biomass (VONSHAK, 2002; REBOLOSSO, et al., 2000). It is important to note that, while the fiber fraction in most terrestrial plants is generally comprised of cellulose, hemicellulose and lignin, the fiber in microalgae contain no lignin and has low hemicellulose levels. This makes it easier to digest, and *Chlorella* and *Spirulina* are in fact widely used as a dietary supplement for human consumption. Moreover, *Chlorella* is recognized as safe food ingredient, with GRAS (Generally Recognized as Safe) status by the US FDA (Food and Drug Administration) (ANUPAMA & RAVINDRA, 2000). The other microalgae (*N. gaditana*, *N. oculata*, *P. cruentum*, and *P. tricornutum*) have high fiber contents, which has been an argument against the use of marine microalgae in human nutrition, such as SCP (ANUPAMA & RAVINDRA, 2000; VONSHAK, 2002). While all microalgae produce hydrocarbons as energy and carbon stores, some microalgae have a preference for carbohydrate rather than lipid accumulation and these species are gaining attention as potential feedstocks for bioethanol production (LI et al., 2014). We found that *C. vulgaris* biomass contains the highest carbohydrate content (~26.7%), in agreement with Tibbetts et al. (TIBBETTS et al., 2015). These authors reported that *Chlorella* sp. (and similar species like *Scenedesmus*, *Chlamydomonas* and *Tetraselmis*) typically produce large amounts of carbohydrate as energy and carbon reserves. As a result, it has been proposed that the use of carbohydrate-rich algal biomass (for example *C. vulgaris*) as feedstock for bioethanol production may be advantageous over conventional feedstocks by providing increased hydrolysis efficiency, higher fermentable yields and reduced production costs (BAEYENS et al., 2015; LEE et al., 2015).

With regard to the algal intracellular lipids, total lipid contents vary from 5.3% to 15.6% of dry matter. Although this can be considered as narrow range, statistical differences were observed between lipid

contents among species (P<0.05). The marine algae *P. cruentum* and *S. platensis* have relatively low, statistically equal values for lipid contents (5.3% and 5.5%, respectively) and similar lipid contents values were reported by Rebolloso-Fuentes et al. (2000) for *P. cruentum* (6.3%) and by Henrikson et al. (2009) for *S. platensis* (4.5-7.0%). The algae *N. gaditana* and *C. vulgaris* had low to moderate lipid content (8.1% and 12.8%, respectively), while *P. tricornutum* and *N. oculata* showed the highest lipid content (14.9% and 15.6%, respectively) (Table 3.1), with an interesting composition in terms of PUFA-ω3 (Table 3.2). Our findings are consistent with those of Franz et al. (2013), who reported a lipid content for *P. tricornutum* and *N. oculata* of about 15.6% and 16.1%, respectively. Since the algal cultures studied were not subjected to nutrient starvation (N-deficient) or any other mechanism that can induce high lipid productivity, it is not surprising that the lipid content was relatively low compared to protein and carbohydrate + fiber. If the fatty acids profile of that lipid would be nutritionally attractive, these products could potentially be marketed as single cell oil (SCO). However, modified cultivation/processing protocols could easily be employed to enhance lipid accumulation (NETO et al., 2013; WONG & FRANZ, 2013).

3.3.2 Fatty acids composition

The microalgal lipid fraction was analyzed in terms of its fatty acids composition by identifying the main fatty acids, as well as the proportion of total saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) ω3 and ω6 fatty acids. Fifteen fatty acids, ranging from of C12:0 to C22:6 ω-3, were identified and quantified as percentage of the total fatty acid content of the algal samples (Table 3.2).

Table 3.2 Fatty acids composition of biomass of six microalgae.

Fatty acid	*C. vulgaris*	*S. platensis*	*N. gaditana*	*N. oculata*	*P. cruentum*	*P. tricornutum*
C12:0	-	90 ± 6	89 ± 6	108 ± 8	43 ± 3	77 ± 4
C14:0	34 ± 2	95 ± 5	890 ± 11	540 ± 12	22 ± 1	560 ± 23
C16:0	**1930 ± 28**	**3420 ± 34**	**5130 ± 35**	**1940 ± 36**	**2950 ± 21**	**1360 ± 41**
C18:0	67 ± 10	35 ± 5	160 ± 8	26 ± 1	78 ± 2	24 ± 2
Other SFA	125 ± 17	340 ± 13	344 ± 12	227 ± 9	344 ± 12	400 ± 11
Σ SFA	2156 ± 54	3930 ± 47	6613 ± 41	2840 ± 15	3437 ± 26	2420 ± 25
C15:1	280 ± 13	390 ± 9	69 ± 2	85 ± 2	150 ± 8	280 ± 6
C16:1	150 ± 4	560 ± 12	**1900 ± 11**	**2240 ± 18**	140 ± 4	**1550 ± 13**
C18:1	128 ± 6	45 ± 1	445 ± 8	280 ± 11	210 ± 10	129 ± 5
Other MUFA	131 ± 2	110 ± 5	192 ± 7	600 ± 6	17 ± 2	260 ± 6
Σ MUFA	690 ± 16	1105 ± 16	2606 ± 21	3205 ± 37	517 ± 11	2219 ± 17
C16:4 ω3	-	30 ± 2	-	-	50 ± 1	20 ± 1
C18:3 ω3 (ALA)	**2820 ± 41**	130 ± 8	34 ± 1	15 ± 2	20 ± 1	92 ± 2
C20:5 ω3 (EPA)	-	38 ± 1	-	**2973 ± 24**	697 ± 21	**2753 ± 16**
C22:6 ω3 (DHA)	-	62 ± 1	-	43 ± 5	-	80 ± 5
Σ PUFA-ω3	2820 ± 41	260 ± 7	34 ± 1	3031 ± 10	767 ± 12	2945 ± 34
C18:2 ω6 (LA)	**1030 ± 22**	150 ± 14	300 ± 10	290 ± 7	**1040 ± 54**	250 ± 8
C18:3 ω6 (GLA)	280 ± 10	**1920 ± 42**	120 ± 4	150 ± 3	18 ± 2	34 ± 2
C20:4 ω6 (AA)	28 ± 2	38 ± 2	-	-	**3705 ± 26**	83 ± 4
C22:5 ω6	-	38 ± 3	-	-	182 ± 7	115 ± 7
Σ PUFA-ω6	1338 ± 36	2146 ± 41	420 ± 8	440 ± 10	4945 ± 21	482 ± 12
SFA+MUFA+PUFA	7004 ± 55	7441 ± 64	9673 ± 78	9516 ± 102	9666 ± 89	8066 ± 82

Data are expressed as mg/100g ± SD (n=3). Numbers in bold indicate contents of the major fatty acid in the microalgal biomass. SFAs not shown in the table: *Tridecanoic acid* C13:0, *Pentadecanoic acid* C15:0, *Behenic acid* C22:0. MUFAs not shown in the table: *Margaroleic acid* C17:1, *Erucic acid* C22:1, *Nervonic acid*, C24:1.

The results showed that the marine species (*P. tricornutum* and *N. oculata*) contain high concentrations of PUFAs-ω3, predominantly C20:5 ω3 (EPA) and C22:6 ω3 (DHA) along with substantial amounts of C16:1 (monounsaturated fatty acid) and C16:0 (saturated fatty acid). In contrast, marine *P. cruentum* has relatively high concentrations of PUFAs-ω6, predominantly C20:4 ω6 (AA), whereas the freshwater algae species *C. vulgaris* and *S. platensis* contain high concentrations of C18:3 ω3 (ALA) and C18 ω6 (GLA) PUFAs, respectively. *N. gaditana* contains a high concentration of saturated fatty acids (SFA), predominantly C16:0 with a low level of PUFAs. These results indicate that freshwater algae *C. vulgaris* and marine species *N. oculata*, *P. tricornutum* and *P. cruentum* followed the same PUFA > SFA relative pattern, while *S. platensis* and *N. gaditana* show the opposite pattern, SFA > PUFA.

3.3.2.1 Saturated fatty acids

Palmitic acid (C16:0) is the most abundant SFA (1.3-5.1 g/100g) followed by myristic acid (C14:0, 22-890 mg/100g) and stearic acid (C18:0, 24-160 mg/100g) (Table 3.2). The sum of all identified SFAs ranged from 29% to 68% of the total fatty acid content. We found that *N. gaditana* contains 68% SFA, mainly comprised of palmitic acid (C16:0), followed by *S. platensis* with 52% SFA, also mainly C16:0, which is in agreement with values reported in the literature for *N. gaditana* (SELVAKUMAR & UMADEVI, 2014) and *S. platensis* (HENRIKSON, 2009). *P. cruentum* and *C. vulgaris* have intermediate SFA contents (35% and 30%, respectively), *N. oculata* and *P. tricornutum* the lowest SFA content.

3.3.2.2 Monounsaturated fatty acids

The highest monounsaturated fatty acids (MUFA) content was found in *N. oculata*, representing ~34% of total fatty acid content, followed by *P. tricornutum* (28%) and *N. gaditana* (27%), whereas *S. platensis*, *C. vulgaris* and *P. cruentum* contain very low concentrations of MUFAs, on average about 10%. The main MUFAs detected in all species studied are: C15:1, C16:1 and C18:1 ω-9, with a high variation in composition among the different species. Palmitoleic acid (C16:1) is the main MUFA found in all marine species studied, ranging from 1.9 to 2.2 g/100g, except for *P. cruentum* that has a very low content of 0.14 g/100g (Table 3.2). These results are consistent with those obtained by

Oh et al. (2009), who demonstrated that *P. cruentum* contains low levels of palmitoleic acid and higher levels of arachidonic acid (AA, C20:4ω6). It has been reported that the marine *Nannochloropsis* species and *P. tricornutum* have a high concentration of palmitoleic acid suggesting that these algae have a tendency to produce unsaturated fatty acids (ZHU & DUNFORD, 2013; MATOS et al., 2015; MITRA et al., 2015; RYCKEBOSCH et al., 2012).

Regarding the WHO/FAO recommendation about the amount of MUFAs in the human diet, the Expert Consultation stated that there is convincing evidence that replacing SFA (C12:0-C16:0) as well as carbohydrates with MUFA reduces LDL and increases HDL cholesterol concentrations. Our data suggest that the marine species *N. oculata* and *P. tricornutum* have optimal compositions for the above purposes because these two species have the lowest SFA content (28-29%), a low carbohydrate content (15.4-16.7%) and a high MUFA content (28-34%).

3.3.2.3 Polyunsaturated fatty acids

Among the polyunsaturated ω-3 and ω-6 fatty acids (PUFAs), α-linolenic (ALA, C18:3 ω3) and eicosapentaenoic (EPA, C20:5 ω3) acids are the predominant PUFAs-ω3, while γ-linolenic (GLA C18:3 ω6) and arachidonic (AA, C20:4 ω6) acids made up most of PUFAs-ω6. High concentrations of PUFAs-ω3 were observed in *C. vulgaris* (40%), *P. tricornutum* (~37%) and *N. oculata* (~32%) of the total fatty acids, while high concentrations of PUFAs-ω6 were found in *S. platensis* (29%) and *P. cruentum* (51%) of the total fatty acids. *N. gaditana* cells contain very low concentrations of PUFA (5%) and has the least favorable ω3/ω6 ratio (0.08) among all algae (Table 3.3).

Chlorella vulgaris contained 60% PUFA, with a high proportion of ω3 acids. In fact, except for *S. platensis*, *P. cruentum* and *N. gaditana*, all other microalgae showed a ω3/ω6 ratio of ≥ 2.0 (Table 3.3). Indeed, in cyanobacteria (*e.g.*, *S. platensis*) the unsaturated double bonds are preferentially in the ω6 position while in Chlorophyceae they are mainly in the ω3 position (BATISTA et al., 2013). *S. platensis* is rich in γ-linolenic acid (GLA, C18:3 ω6) (1.9 g/100g). Gamma linolenic acid is as a precursor of C_{20} eicosanoids (prostaglandins, leukotrienes and thromboxanes) and has been associated with beneficial health effects, such as a reduction in LDL (low-density lipoproteins), anti-inflammatory effects, stimulation of the apoptosis of cancer cells, and reduction in pain and inflammation associated with rheumatoid arthritis (MARTIN et al., 2006). The species *S. platensis* is a well-known source

of GLA, since in cyanobacteria this fatty acid plays the same role as α-linolenic acid (ALA, C18:3 ω3) in algae and higher plants (HENRIKSON, 2009). Although PUFA levels are relatively high for all species (>25% of total fatty acid), except for *N. gaditana* cells, it consists of medium-chain PUFA (*e.g.*, C_{16} and C_{18}) and is devoid of long-chain (LC) PUFA (*e.g.*, C_{20} and C_{22}). This is generally typical for freshwater microalgae/cyanobacteria and make them poor sources of nutritionally-essential LC-PUFA, arachidonic acid (AA, C20:4 ω6), eicosapentaenoic acid (EPA, C20:5 ω3) and docosahexaenoic acid (DHA, C22:6 ω3). For this reason, *N. oculata* and *P. tricornutum* are more interesting for nutrition applications, because they are able to synthesize high amounts of EPA and DHA acids, and thus can be used to enrich functional foods with ω-3 fatty acids. Algal oils and single cell oil (SCO) sources of LC-PUFAs are now becoming available (to provide EPA+DHA+AA) (RYCKEBOSCH et al., 2014; ARMENTA & VALENTINE, 2013). In addition, an advantage of a SCO from algae is that it usually contains a significant amount of natural antioxidants (*e.g.* carotenoids and tocopherols), which can protect ω-3 fatty acids from oxidation, hence making this oil less prone to oxidation than oil derived from plants and marine animals (LI et al., 2007).

As shown in Table 3.2, *C. vulgaris* and *P. cruentum* have the same PUFA content (~60%). However, α-linolenic acid (ALA, C18:3 ω3) in *C. vulgaris* contributes to an increased ω3/ω6 ratio of 2.10, while arachidonic acid (AA, C20:4 ω6) in *P. cruentum* causes a low ω3/ω6 ratio of 0.15. Consequently, *C. vulgaris* has a favorable ω3/ω6 ratio that is about 15-fold higher than that of *P. cruentum*, but the large amount of AA (3.7 g/100g) in *P. cruentum* makes this marine alga a potential source for the production of arachidonic acid. Since the metabolic breakdown of AA leads to an increased production of prostaglandin E_2, which belongs to a class of hormone-like substances that participate in a wide range of bodily functions, thromboxane and leukotriene, the importance of this fatty acid to human-cell functioning is evident (RAPOSO et al., 2014).

The marine microalgae *N. oculata* and *P. tricornutum* contain 37% and 43% PUFA, respectively, with a highly ω3/ω6 ratio of 6.50. These microalgae are also, rich in EPA and contain a small quantity of DHA. The species *N. oculata* contains 3.0 g EPA and 43 mg DHA, and *P. tricornutum* 2.7 g EPA and 80 mg DHA per 100 g microalgal biomass. According to Borowitzka (2013) the main market of these oils is infant formula and an oil rich in both DHA and EPA from a strain of

Schizochytrium has recently reached the market. Currently, there is no other commercial production of EPA-rich oils from microalgae, but Aurora Algae has announced a product from marine eustigmatophyte *Nannochloropsis*. According to American Heart Association a daily intake of 500 mg EPA + DHA and 800-1000 mg of ALA per day are recommended for the primary prevention of coronary heart disease (ISSFAL, 2004). Our results indicate that the marine species *N. oculata* and *P. tricornutum* are potential sources of EPA, with an average of 2.8 g/100g whereas *C. vulgaris* has a robust ALA (2.8 g/100g) producing profile. These microalgae have therefore an enormous potential for application in the development of health food products, such as single cell oil (SCO).

3.3.3 Lipids nutritional quality indexes

The nutritional quality of lipid profiles observed in the algae species was evaluated by different indexes as shown in Table 3.3. Foods with polyunsaturated and saturated fatty acids (P/S) ratios below 0.45 are considered by the FAO/WHO to be undesirable in the human diet, because of their potential to induce increases in blood cholesterol. The P/S ratios in all six algae are above 0.45, ranging from 0.46 in *N. gaditana* to 2.35 in *P. tricornutum*.

Table 3.3 Nutritional quality indexes of the lipid fraction in the biomass of six microalgae.

Species	P/S	ω3/ω6	H/H	AI	TI
C. vulgaris	2.25	2.10	2.04	0.42	0.21
S. platensis	0.89	0.12	0.66	1.10	1.46
N. gaditana	0.46	0.08	0.12	1.70	3.82
N. oculata	2.33	6.88	1.44	0.63	0.22
P. cruentum	1.79	0.15	1.90	0.49	0.60
P. tricornutum	2.35	6.10	1.72	0.65	0.19

P/S = polyunsaturated/saturated; ω3/ω6 = Σ of the Omega 3 series/Σ of the Omega 3 series; H/H = Σ hypercholesterolemic/Σ hypocholesterolemic; AI = atherogenicity index; and TI = thrombogenicity index.

An additional approach to the nutritional evaluation of lipid profiles is the calculation of an index based on functional effects of fatty acids, *e.g.* the ratio hypocholesterolemic fatty acids/hypercholesterolemic fatty acids (H/H) index, which is based on current knowledge of the effects of individual fatty acids on cholesterol metabolism (SANTOS-SILVA et al., 2002; SIMAT et al., 2015). Nutritionally, higher H/H values are considered more beneficial for human health, because a higher H/H ratio is directly proportional to a high PUFA content. Fatty acids from microalgae that are highly polyunsaturated are thought to have beneficial effects on cholesterol. The highest H/H value (2.04) was found in *C. vulgaris*, followed by *P. cruentum* species (H/H = 1.90). These results are in excellent agreement with H/H values for marine fish such as sardine and mackerel (H/H = 2.46) reported by Fernandes et al. (2014). In addition, Testi et al. (2006) reported H/H values for fish fillets of sea bass and rainbow trout of 2.18 to 2.40.

Two other indexes are used to evaluate the potential for stimulating platelet aggregation, the atherogenicity index (AI) and thrombogenicity index (TI) according to Turan et al. (2007). Lower AI and TI values indicate a greater potential to protect against coronary artery disease. In our study, AI values ranged between from 0.42 to 1.70, with *C. vulgaris* (0.42) and *P. cruentum* (0.49) showing the lowest values (Table 3.3). It is noteworthy that these two species also have the highest PUFA content (~60%). Simat et al. (2015) reported values of 0.59 to 0.92 for the omnivorous fish bogue (*Boops boops* Linnaeus). The lowest thrombogenicity index (TI) value of 0.19 was observed in the marine *P. tricornutum*, which is comparable to TI values for the marine fish sardine of 0.20 reported by Fernandes et al. (2014).

With regard to the ω3/ω6 ratio, typical Western diets have ω3/ω6 ratios that are profoundly skewed toward omega-6, which is believed to promote or cause several diseases (ISSFAL, 2004). This is mainly due to the disproportionately greater consumption of ω-6 rich vegetable oils (*e.g.*, sunflower, peanut, corn) in comparison with the intake of ω-3 rich food sources, such as seafood, nuts, etc. Our data indicate that ω3/ω6 ratios of the algae species decreased in the order of *N. oculata* (6.88) > *P. tricornutum* (6.10) > *C. vulgaris* (2.10) > *P. cruentum* (0.15) > *S. platensis* (0.12) > *N. gaditana* (0.08), (Table 2.3). These results are in accordance with findings of other authors, for example Batista et al. (2013), who reported higher ω3/ω6 ratios in marine algae species than freshwater algae species, suggesting that the marine species studied here

(*i.e. N. oculata* and *P. tricornutum*) could be categorized as beneficial to human health consumption.

3.4 CONCLUSIONS

This study determined the biochemical compositions of the six microalgae strains. Algal biomass from these species contain on average 40 g protein, 18 g carbohydrate, 12 g fiber and 10 g lipid per 100 g of biomass DM. The species *C. vulgaris* and *S. platensis* are rich in ALA (2.8 g/100 g) and GLA (1.9 g/100g), respectively. The marine algae *P. tricornutum* and *N. oculata* contain 42% and 37% PUFA, respectively, with a favorable ω3/ω6 ratio of around 6.5, and are rich in EPA and DHA acids. The alga *P. cruentum* contains high PUFA-ω6 levels, due its high concentration of AA (3.7 g/100 g). Taken together, the results show that microalgae are excellent candidates as sources of high protein (*Spirulina*), high carbohydrate/low fiber (*Chlorella*) and high LC-PUFA-ω3 (*N. oculata* and *P. tricornutum*) contents with a high nutritional value, similar to fish oil.

Chapter 4

POLYUNSATURATED ω3 AND ω6 FATTY ACIDS OF BIOMASS EXTRACTED FROM THREE MARINE MICROALGAE WITH SUBCRITICAL *n*-BUTANE: A NOVEL AND COMPARATIVE STUDY

This chapter is a reproduction of the research paper submitted for Algal Research, only the graphical abstract, abstract, highlights, and acknowledgements were omitted. The chemical characterization of the microalgae biomass used in this work are described in the previous chapter.

4.1. INTRODUCTION

Microalgae have emerged as a potential source of rich-lipids, because of their capacity to synthesize a group of essential fatty acids, the polyunsaturated fatty acids (PUFAs), such as omega-3 (ω3) and omega-6 (ω6) (BATISTA et al., 2013; MATOS et al., 2016; RYCHEBOSCH et al., 2014). Linoleic acid (LA, 18:2-ω6) and α-linolenic acid (ALA, 18:3-ω3) are precursors for the long-chain PUFA (LC-PUFA ≥C20) with high market values, such as eicosapentaenoic acid (EPA, C20:5-ω3), docosahexaenoic acid (DHA, C:22:6-ω3), and arachidonic acid (ARA, C20:4-ω6). These PUFAs can be commercialized for pharmaceutical and therapeutic applications (KHOZIN-GOLDBERG et al., 2011; KOLLER et al., 2014). In addition, an adequate dietary intake of these PUFAs is vital for healthy nutrition and brain function, reducing the occurrence of cardiovascular disease and risk of cardiac death and mental illness (BATISTA et al., 2013; KHOZIN-GOLDBERG et al., 2011; RAPOSO & MORAIS, 2015).

Despite the routine use of lab-scale extraction protocols to determine microalgal lipid content, the variables affecting lipid extraction from microalgal cells are not well understood, making scale-up for commercial products from microalgae difficult (ARAUJO et al., 2013; HALIM et al., 2011; SOARES et al., 2014). There are several extraction methods found in the literature that could be used to extract intracellular lipids carrying LC-PUFAs from microalgae (HALIM et al., 2012; HERRERO et al., 2006; HERRERO & IBAÑEZ, 2015). Though organic solvents have been widely applied to the extraction of microalgal lipids for biodiesel production, such use for pharmaceutical

and food application may lead to contamination of the final product in the form of residues. Furthermore, a large amount of solvent is usually required and its recovery generally involves high temperatures that can affect thermo-labile compounds, for example PUFAs (HALIM et al., 2012; CHENG et al., 2011; MENDES et al., 2003).

For the extraction of high value products (HVPs) such as PUFAs from microalgal biomass, supercritical fluid extraction (SFE) has emerged as a green technology (HERRERO & IBAÑEZ, 2015; MICHALAK et al., 2015; MILLAO & UQUICHE, 2016). SFE is free from extraction solvent at the end of the process, and therefore does not require energy for solvent removal. Carbon dioxide is the most common solvent used for SFE, mainly due to its advantageous physicochemical properties near the critical point and its chemical inertness (HALIM et al., 2011; YEN et al., 2015). In addition to carbon dioxide, other compressed solvents can be used, including ethane, propane, *n*-butane, and dimethyl ether (CAPELETTO et al., 2016; CATCHPOLE et al., 2009; FREITAS et al., 2008; GOTO et al., 2015). The use of these new substances as compressed fluids to replace carbon dioxide usage differs from the work in the subcritical region, as compressed liquids (at least one order of magnitude below the critical point) afford the significant advantage of operating at moderate pressures, which lowers initial investment (CAPEX) and reduces maintenance costs and operational risks (OPEX) (LIPKIN et al., 1942; NOVELLO et al., 2015; OLDS et al., 1944).

Despite the advantages of using, at subcritical compressed conditions, substances which are normally gaseous at room conditions, few examples have been reported in the literature. Catchpole et al. (2009) briefly reviewed the state-of-the-art in the extraction and fractionation of selected specialty lipids using near critical fluids, reporting that propane and dimethyl ether can be used without co-solvents to extract polar lipids (polar lipids are phospho- and glycolipids, while non-polar lipids comprises triacylglycerols) and other complex lipids from a range of raw materials, including dairy powders, eggs, biomass from fermentation, and wet marine biomass. Goto et al. (2015) demonstrated the connection between supercritical CO_2 (SC-CO_2) and liquefied dimethyl ether (DME) as clean extraction processes for bioactive compounds (lipids and carotenoids) from wet algae. The same authors attested that subcritical DME is unique and suitable for extraction of oily substances from high water-content biomaterials. Using *n*-butane as a subcritical solvent, Novello et al. (2015) found that

palmitic (C16:0) and oleic (C18:1) fatty acids were the major components observed in *n*-butane extracts of *Carapa guianesis* seeds. Moreover, Capeletto et al. (2016) achieved high extraction yields and higher antioxidant activity using subcritical *n*-butane compared to SC-CO_2 from *Campomanesia xanthocarpa* seeds.

The investigation of new extraction solvents is an opportunity for the development of new extraction technologies to enable commercial success of HVPs, especially PUFAs from microalgae biomass. In this context, the present study aims to compare the effects of subcritical *n*-butane extraction with two well established methods (Soxhlet, and SC-CO_2) on lipid extraction yields, with a focus on the fatty acids composition of the extracts obtained. For this purpose, three marine microalgae strains (*Phaeodactylum tricornutum*, *Nannochloropsis oculata*, and *Porphyridium cruentum*) were selected for this study because of their high and diverse contents of PUFAs ω3 and ω6, i.e., EPA, DHA, and ARA (MATOS et al., 2016; RYCHEBOSCH et al., 2014; KOLLER et al., 2014).

4.2 MATERIALS AND METHODS

4.2.1 Microalgae strains and biomass production

Three marine microalgae strains (*P. tricornutum*, *N. oculata*, and *P. cruentum*) were used in this study. The algal species were obtained from our internal culture collection at Laboratório de Cultivo de Algas (LCA/UFSC) and cultures were grown in four fiberglass tanks and an acrylic cylinder that we custom designed and built. After starter cultures had grown in 5 L Erlenmeyer flasks, mass cultures of *P. tricornutum* and *N. oculata* were scaled-up in 500 L capacity open tanks (1.60 m diameter and 2.75 m length) containing Conway media (LOURENÇO, 2006) with silicate supply for the diatom *P. tricornutum*. The tanks covered by a transparent roof were constantly aerated (40 L min^{-1}) and the cultures submitted to natural conditions: 22 °C ± 4, irradiance 250 µmol m^{-2} s^{-1} ± 200, pH 8 ± 1, salinity being kept constant at 3.5 % by filtered seawater supply. Cool-white fluorescent lamps were placed over the tanks providing a light intensity of 100 µmol m^{-2} s^{-1} during the night. *P. cruentum* mass culture was scaled-up in a 180 L capacity acrylic cylinder (0.5 m inner diameter and 1.0 length) in Conway media. The surface of the cylinder was continuously exposed to cool white lamps (100 µmol m^{-2} s^{-1}) and controlled culture conditions kept at 22 °C ± 1, pH 7,5 ± 0.5, salinity 3.5 %.

During the cultivation of the three microalgae, nutrients were added to the broth each two days (0.5 mL L^{-1} of Conway main solution) to prevent nutrient limitation. As soon as the cultures reached the stationary growth phase, the entire culture broth was harvested by continuous centrifugation (GEA Westfalia Separator SSD 6-06-007, Germany) at 12,000 rpm and concentrated to a biomass slurry, which was washed (0.5 M ammonium formate) to remove the salt content (ZHU & LEE, 1997) and centrifuged again (Janetzki SD600, Germany) at 3,500 rpm for 35 min. Finally, the wet biomass was spread on tray in thin layer and dried in a dehydrator (45 °C ± 5, 40 h).

4.2.2 Lipids extraction

4.2.2.1 Organic solvent

Organic solvent extraction was performed through Soxhlet apparatus using petroleum ether. The Soxhlet method (AOAC 963.15) is a conventional standard method (AOCS, 1998) used to extract natural products from organic matrices and was adopted to help compare the results of SC-CO_2 and subcritical *n*-butane methods regarding the fatty acids composition of the extracts. Total lipids were extracted from dry biomass continuously during 6 h, after acid digestion with 4.0 N HCl, followed by concentration in a rotary evaporator, dried in an oven (105 °C, 1 h) and weighed.

4.2.2.2 Supercritical CO_2 (SC-CO_2)

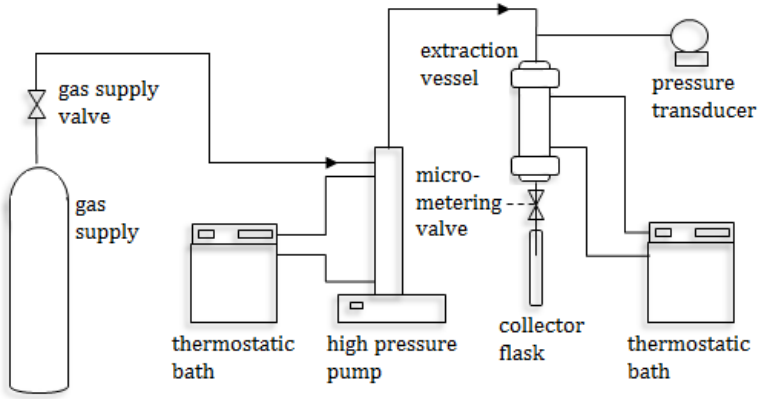
The SC-CO_2 extraction procedure was performed at 300 bar, 40 °C, 9 mL min^{-1} for 2 h. The experimental conditions were taken from the literature (HALIM et al., 2012; MICHALAK et al., 2015; YEN et al., 2015). Briefly, the extraction procedure consisted of filling a 100 cm^3 jacketed extraction vessel amounts of 20 g (± 0.5) of microalgal biomass (average particle size 5 mm) placing glass beads and cotton at both ends to form a fixed bed before closing the vessel. The cylinder gas supply (White Martins, CO_2 99.9% purity) fed the fluid directly into the extraction line to the high pressure pump (Thermo Separation Products, Model 3200P/F, USA). The temperature inside the pump was kept constant by the thermostatic bath (0 °C) to guarantee liquid CO_2. The high pressure pump directed the pressurized fluid through the extraction line to the jacketed extractor, with internal temperature adjusted by the second thermostatic bath which kept the temperature condition (40 °C) constant. The extraction pressure was monitored with an analogical

manometer. After the depressurization the solute was collected in an amber flask with a rapid separation of the fluid which goes to a gaseous state at room temperature.

4.2.2.3 Subcritical *n*-butane

For subcritical *n*-butane, the extraction procedure was basically the same as described for SC-CO_2, however different control conditions were adopted as well as the components needed to build the extraction unit due to a lower pressure in the system. The extraction unit (Fig. 4.1) consisted of a gas supply (White Martins, C_4H_{10} 99.5% purity), two thermostatic baths, a high pressure pump (ISCO 260D), an extraction vessel (130 cm^3 internal volume), an absolute pressure transducer (Smar LD301) equipped with a portable programmer (Smar HT 201) and a collector flask.

Figure 4.1 Schematic diagram of the experimental extraction unit.



Source: Author.

The same amount of microalgal biomass, used for SC-CO_2 extractions (i.e. 20 g) was added into the extraction vessel. In the case of *n*-butane a 5 °C temperature was enough to ensure fluid liquid inside the pump kept by the thermostatic bath. When the pump is operating at constant pressure (experimental condition), the micrometering valve is adjusted and the flow rate established.

The extraction time was determined according to the kinetics assay. To obtain the overall extraction curve (OEC), the extract samples were collected at pre-established time intervals. The mass of extract was

weighed, and the collector flask was reconnected to the equipment. This procedure was performed until no significant further mass was extracted. In order to work in the subcritical region, these assays were carried out using *n*-butane at 15 bar, 40 °C and solvent flow rate of 3 mL min^{-1}. Taking the OEC into account, subcritical *n*-butane extraction time of 2 h was chosen in order to recover all the extractable material.

4.2.3 Fatty acids analysis

The fatty acid composition of the extracts was determined after conversion of the fatty acids to their corresponding methyl esters. The fatty acid methyl esters were characterized on a gas chromatograph, model GC-2014 (Shimadzu, Kyoto, Japan), equipped with split-injection port, flame-ionization detector and 105 m-long Restek capillary column (ID = 0.25 mm) coated with 0.25 μm of 10% cyanopropylphenyl and 90% biscyanopropylsiloxane. Injector and detector temperatures were both 260°C. The oven temperature was initially set at 140°C for 5 min, programmed to increase at 2.5°C min^{-1}, and held at 260ºC for 30 min. The injection volume was 1μL, and the split ratio was 10:1 (AOAC, 2005). Nitrogen was used as the carrier gas (flow rate was 2.2 mL min^{-1}) at a constant pressure of 130.3 kpa. Fatty acid methyl esters were identified by comparing the retention times of the peaks with the respective fatty acids standard (Sigma, St. Louis, USA). The proportions of the individual acids were calculated by the ratio of their peak area and expressed as mg g^{-1} of extract.

4.2.4 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Software STATISTICA version 7.0 from (Statsoft Inc., USA), in order to detect significant differences between values of extraction yields between the extraction methods used. The significant differences ($p<0.05$) were analyzed by Tukey test.

4.3. RESULTS AND DISCUSSION

4.3.1 Lipids extraction

The experimental results obtained by the three different methods tested (Soxhlet, SC-CO_2, and subcritical *n*-butane) for lipid extraction are shown in Table 4.1. The highest lipid extraction yields were achieved by the Soxhlet method (range from 53.2 to 172.5 mg g^{-1} of dry

weight (DW)) followed by subcritical *n*-butane (range from 2.4 to 17.2 mg g^{-1} DW), and SC-CO_2 (range from 4.5 to 14.7 mg g^{-1} DW). A statistical difference was observed between lipid contents among the methods (p<0.05). The soxhlet method extracted ~10-fold more lipids than the other two methods which were statistically similar. A slightly difference was observed between the lipid extraction yields for SC-CO_2 and subcritical *n*-butane. SC-CO_2 extracted more lipids for *P. tricornutum* (14.7 mg g^{-1} DW) and for *P. cruentum* (4.5 mg g^{-1} DW) while *n*-butane extracted more lipid for *N. oculata* (17.2 mg g^{-1} DW).

Table 4.1 Lipid extraction yields from the three marine microalgae biomass (average of two determinations). Data expressed in mg g^{-1} DW.

Extraction method	*P. tricornutum*	*N. oculata*	*P. cruentum*
Soxhlet	149.7 ± 0.6^{a}	172.5 ± 1.0^{a}	53.2 ± 0.4^{a}
SC-CO_2	14.7 ± 0.8^{b}	15.6 ± 1.2^{b}	4.5 ± 0.5^{b}
Subcritical *n*-butane	12.8 ± 0.6^{b}	17.2 ± 1.0^{b}	2.4 ± 0.2^{b}

Values in the same column with different superscript letters are significantly different ($p < 0.05$).

Despite the slight difference observed in the lipid extraction yield between SC-CO_2 and *n*-butane, the fact that the flow rate during the extraction process for the subcritical *n*-butane (3 mL min^{-1}) was 3-fold less than the flow rate adopted in the SC-CO_2 extraction (9 mL min^{-1}) means that subcritical *n*-butane was more efficient for lipid extraction since less solvent was expended in the process compared to SC-CO_2. In this case, working at the same fluid flow rate, the use of subcritical *n*-butane would allow a reduction of extraction time, while avoiding the extraction of co-products (e.g., pigments) (CAPELETTO et al., 2016; NOVELLO et al., 2015).

At the temperature investigated (40 °C), subcritical *n*-butane is not a supercritical fluid and it utilizes a hydrostatic mechanism, working as a mechanical press or piston fluid that increases the system pressure, favorably changing the extraction of lipophilic compounds (CAPELETTO et al., 2016). Such characteristic (solvent density) may be advantageous in order to save energy and reduce operational pressure expenditures during lipid extraction from algae.

In spite of its high lipid extraction yields, the Soxhlet apparatus suffers from high energy requirements due the continuous solvent

distillation, making it useful primarily for lab-scale procedures (HALIM et al., 2012). Nevertheless, the use of organic solvents at large scale is not a preferable process for food application, since solvent residues may remain as a contaminant in the final product and possibly lead to degradation of thermo-labile compounds (CHENG et al., 2011; MICHALAK et al., 2015).

3.3.2 Comparison between the extraction methods on the fatty acids composition

Fatty acids composition of the microalgae biomass extracts was analyzed for the three extraction methods used. The main fatty acids were identified, as well as the proportion of total saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) ω3 and ω6 fatty acids. Fourteen fatty acids, ranging from of C12:0 to C22:6, were identified and quantified as mg g^{-1} of the total fatty acid content of the samples (Table 4.2).

Table 4.2 Fatty acids composition of the algae biomass extracts using the three extraction methods. Data is an average of two analyses in mg g^{-1}.

	Phaeodactylum tricornutum			*Nannochloropsis oculata*			*Porphyridium cruentum*		
	Extraction Method								
Fatty acids	Soxleth (AOAC)[1]	Supercritical CO_2	Subcritical *n*-butane	Soxleth (AOAC)[1]	Supercritical CO_2	Subcritical *n*-butane	Soxleth (AOAC)[1]	Supercritical CO_2	Subcritical *n*-butane
C12:0	8	-	6	11	18	8	4	176	2
C14:0	56	78	64	61	86	65	2	41	5
C16:0	**136**	**190**	**155**	**194**	**163**	**198**	**295**	**524**	**416**
C18:0	2	86	3	3	5	5	8	28	24
Others SFA	36	33	47	23	72	32	32	55	32
Σ SFA	238	**387**	275	292	**344**	308	341	**824**	479
C15:1	28	88	105	9	37	13	15	10	27
C16:1	**156**	**189**	**164**	**225**	**230**	**216**	14	19	12
C18:1	**130**	37	**116**	29	13	24	21	**52**	22
Others MUFA	35	22	21	73	12	64	2	-	-
Σ MUFA	349	336	**406**	**336**	292	317	52	**81**	61
C18:2-ω6 (LA)	24	23	22	29	23	35	**105**	50	96
C18:3-ω6 (GLA)	3	4	3	5	4	4	2	-	3
C20:3-ω6 (DGLA)	12	2	8	12	-	4	13	-	11

C20:4-ω6 (ARA)	9	7	7	-	-	-	**386**	45	**267**
Σ PUFA-ω6	48	36	**40**	46	27	**43**	**506**	95	**377**
C18:3-ω3 (ALA)	9	7	7	-	4	-	-	-	-
C20:5-ω3 (EPA)	**289**	**161**	**206**	**311**	**255**	**313**	2	-	8
C22:6-ω3 (DHA)	**8**	**4**	**5**	-	-	-	-	-	-
Σ PUFA-ω3	306	172	**218**	311	259	**313**	2	0	**8**
ω3/ω6	6.38	4.78	5.45	6.76	9.59	7.27	<0.1	<0.1	<0.1
EPA+DHA	297	165	211	311	255	313	2	0	8
Unknow	59	69	61	15	78	19	99	0	75
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000

[1]Method used AOAC 963.15 (Official methods and recommended practices for the American Oil Chemists' Society. 1995).
Numbers in bold indicate contents of the major fatty acid in the microalgal extracts.

4.3.2.1 Saturated fatty acids

Palmitic acid (C16:0) was the most abundant SFA extracted for the three extraction methods tested, comprising more than 50% of the total SFA content in all the microalgae species. The SC-CO_2 method showed the highest extraction of SFAs (range from 344 to 824 mg g^{-1}), followed by *n*-butane (range from 275 to 476 mg g^{-1}) and Soxhlet (range from 238 to 341 mg g^{-1}) extraction of total fatty acids. In particular, total SFA extraction by SC-CO_2 was more effective in the microalga *P. cruentum* (824 mg g^{-1}), corresponding to 2.4 and 1.7-fold compared to Soxhlet (341 mg g^{-1}) and *n*-butane (479 mg g^{-1}) extractions, respectively.

Due to the non-polar property of carbon dioxide molecules, SC-CO_2 is considered a suitable solvent for the extraction of lipids. However, it has been reported that SC-CO_2 is selective for neutral lipids (non-polar lipids) such as triacylglycerols (TAGs), but did not solubilize phospho- and glycolipids (polar lipids). Moreover, the lipids obtained by SC-CO_2 extraction are mainly composed of TAGs (CRAMPON et al., 2013; MOUAHID et al., 2013). Considering these findings, our results suggest that in the case of SC-CO_2 selectivity for neutral lipids (TAGs), these are composed more for saturated than polyunsaturated fatty acids in the studied algae. The lowest solvent polarity (dielectric constant) of SC-CO_2 also suggest that this solvent is more suitable for the extraction of neutral lipids compared to the other methods, once the lower is the solvent polarity more neutral lipids can supposedly be extracted. The dielectric constant increases in order of carbon dioxide (1.6) < *n*-butane (1.8) < petroleum ether (2.0) (NOVELLO et al., 2015; MULLER & HUBSCH, 2000).

4.3.2.2 Monounsaturated fatty acids

Among the MUFA, palmitoleic acid (C16:1) is the main fatty acid extracted from *P. tricornutum* (189 mg g^{-1}) and *N. oculata* (230 mg g^{-1}), when SC-CO_2 was used. Oleic acid (C18:1) was also observed in the microalga *P. tricornutum* with significant extraction yields (more than 100 mg g^{-1}) using Soxhlet and *n*-butane methods. C18:1 was the main MUFA present in the microalga *P. cruentum* with 52 mg g^{-1} achieved using the SC-CO_2 method. With respect to total MUFA extraction (i.e. Σ MUFA), similar extraction yields were observed in the composition of the extracts between the methods for the three studied microalgae (Table 4.2). According to literature findings the range of C16:1 and C18:1 content for *P. tricornutum* (155-225 mg g^{-1} and 13-67 mg g^{-1},

respectively) (MATOS et al. 2016; FELLER et al., 2014; MAADANE et al., 2015; MEDINA et al., 1998), for *N. oculata* (224-235 mg g^{-1} and 28-78 mg g^{-1}, respectively) (MATOS et al., 2016; CAMPOS et al., 2010), and for *P. cruentum* (14-85 mg g^{-1} and 20-65 mg g^{-1}, respectively) (MATOS et al., 2016; MEDINA et al., 1998; OH et al., 2009) are in agreement with our work except for C18:1 of *P. tricornutum* (range 37-130 mg g^{-1}). However, the fatty acid composition can vary depending of the culture media and environmental conditions (HARUN et al., 2010; MATOS et al., 2015).

4.3.2.3 Polyunsaturated ω3 and ω6 fatty acids

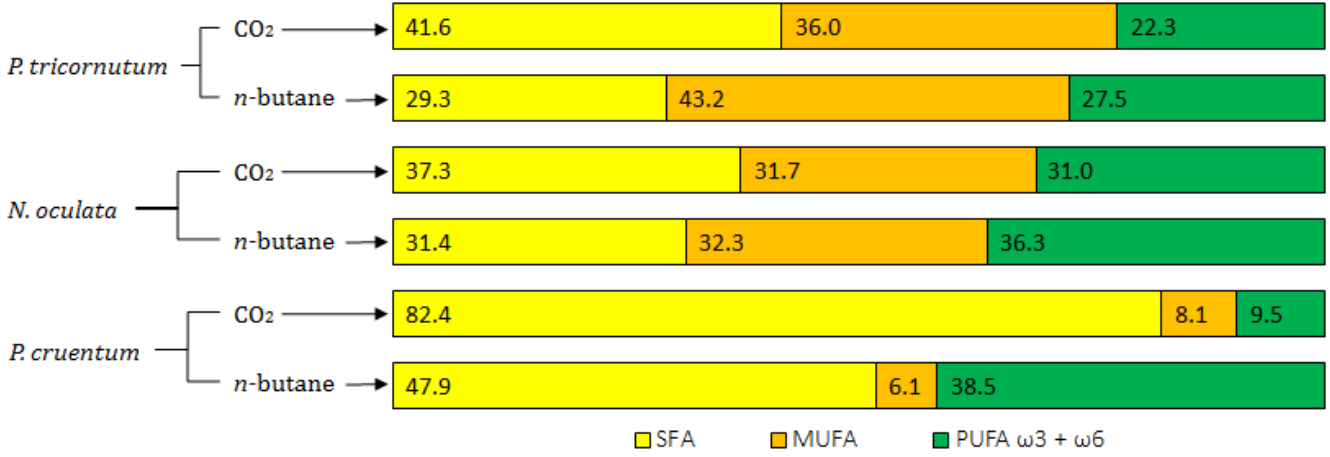
With regards to PUFA-ω6, low concentrations were noted (< 50 mg g^{-1}) in *P. tricornutum* and *N. oculata*. The PUFA-ω6 contents were very similar for these two species using the three extraction methods (average of 40 mg g^{-1}). On the other hand, high amounts of linoleic acid (LA, C18:2-ω6) and ARA were extracted (105 mg g^{-1} and 386 mg g^{-1}, respectively) by using the Soxhlet method for *P. cruentum*. ARA and LA were also extracted at high concentrations using *n*-butane (267 mg g^{-1} and 96 mg g^{-1}, respectively) from the red alga, indicating that subcritical *n*-butane is a powerful method for extraction of these two fatty acids from *P. cruentum.*

The microalga *P. cruentum* is well recognized as a rich source of ARA. Medina et al. (1998) reported production of 360 mg g^{-1} and that under optimal growth conditions it could reach 417 mg g^{-1}. Because LA is a precursor of ARA in the biosynthesis of LC-PUFA-ω6 group, its presence was also expected.

With respect to PUFA-ω3, a high concentration of eicosapentaenoic acid (EPA, C20:5-ω3) occurs in *P. tricornutum* and *N. oculata,* with more than 93% of their overall PUFA-ω3 content being EPA. The *N. oculata* extract provided the highest content of EPA (313 mg g^{-1}) when the *n*-butane method was used. Low concentrations of α-linolenic acid (ALA, C18:3-ω3) in *P. tricornutum* using the three extraction methods was noted (range from 7 to 9 mg g^{-1}). In addition, docosahexaenoic acid (DHA, C22:6-ω3) was only observed in *P. tricornutum* in low concentrations (range from 4 to 8 mg g^{-1}).

Figure 4.2 shows the proportion of SFA, MUFA, and PUFA (ω3 + ω6) in the overall fatty acid composition of the three microalgae biomass extracts, comparing the *n*-butane extraction method with SC-CO_2.

Figure 4.2 Fatty acid content of the biomass extracts. Comparison between SC-CO_2 and subcritical *n*-butane extraction methods. Data shown normalized to 100 percent.



Source: Author.

As can be seen in Figure 4.2, the proportion of SFA extracted was higher in all microalgae species studied when SC-CO_2 was used (values ranged from 37.3 to 82.4%), *P. cruentum* showed the highest values for SFA (82.4%). In the case of MUFA, SC-CO_2 and *n*-butane methods showed slight extraction differences between the species used (*P. tricornutum* 7.2%, *N. oculata* 0.6%, and *P. cruentum* 2% of the total fatty acids). It is noted that low amounts of MUFA (6.1-8.1%) are seen in *P. cruentum* biomass composition, independent of the method used. As revealed in Fig. 4.2, the extraction of PUFA ω3 and ω6 by subcritical *n*-butane extraction method was shown to be the best option, because it extracted more PUFAs ω3 and ω6 (*P. tricornutum* 27.5%, *N. oculata* 36.3%, and *P. cruentum* 38.5%) in comparison to SC-CO_2 (*P. tricornutum* 22.3%, *N. oculata* 31.0%, and *P. cruentum* 9.5%) of the total fatty acids. The solubility of PUFAs in *n*-butane was higher for all the species tested, with significant values (27.5-38.5%). For *P. cruentum*, *n*-butane showed greater ARA extraction (26.7%) than using SC-CO_2 (4.5%).

It is important to note that fatty acids in algae can be found in the polar lipid fraction, being part of the structure of cell walls and cell membranes, and also in the non-polar lipid fraction which comprises triacylglycerols (TAGs) used as cell energy storage (KHOZIN-GOLDBERG et al., 2011; HALIM et al., 2012. As an example of the occurrence of PUFAs in these two different classes of lipids, Medina et al. (1998) reported the fractionation of the lipid extract in polar and non-polar fractions of *P. tricornutum*, finding greater proportions of PUFAs (76.8% of total lipids) in phospho- and glycolipids (polar lipids) and 23.2% in the form of TAGs. Ryckebosch et al. (2014) found that EPA was highest in glycolipids for *N. oculata* (68.3% of total lipids), and in TAGs for *P. tricornutum* (31.7%). The authors also found that DHA was highest in phospholipids (3.9% of total lipids) for *P. tricornutum*. Thus, we conclude that EPA and DHA are not incorporated in a specific lipid class, although preferential incorporation in polar lipids class was expected, since EPA and DHA are structurally important fatty acids to cell membranes.

Since the extraction process is based on the solubility of compounds in the solvent fraction used, it is difficult to conclude whether *n*-butane is better to extract polar or non-polar lipids since the presence of PUFAs constituting TAGs, phospho- and glycolipids may depend on the microalgae species and also the culture condition

adopted. Thus the lipid class content should be determined by fractionation (e.g., silica solid phase extraction) to identify the affinity of polar lipids in *n*-butane (RYCHEBOSCH et al, 2014; HALIM et al., 2012. Nevertheless, independent of which class of lipids PUFAs were present, subcritical *n*-butane was shown to be a good and alternative method for the extraction of these HVPs for the three marine microalgae studied.

There is a general agreement that PUFA-ω3, particularly EPA and DHA, are associated with important health benefits. According to the literature, an intake of 250 mg EPA + DHA/day has shown to give primary prevention against cardiovascular disease and this is also the intake recommended by WHO and EU (RYCHEBOSCH et al, 2014; KOLLER et al., 2014). This nutrition recommendation strengthens the suitability of *n*-butane in the extraction of lipids from the studied algae. The extracts obtained by subcritical *n*-butane provided higher EPA + DHA values (Table 4.2) for *P. tricornutum* and *N. oculata* (211 mg g^{-1} and 313 mg g^{-1} respectively) than supercritical CO_2 (165 mg g^{-1} and 255 mg g^{-1}, respectively). For *N. oculata*, extracts obtained by *n*-butane showed highest content of EPA + DHA (313 mg g^{-1}) compared to Soxhlet method (EPA + DHA = 311 mg g^{-1}). In addition, our data indicate higher ω3/ω6 ratios (Table 4.2) from *P. tricornutum* and *N. oculata* extracts obtained by the three extraction methods used (*P. tricornutum* ~5.53 and *N. oculata* ~ 7.87). The ω3/ω6 ratio recommended for nutrition human health is ≤1:5. These results suggest the use of the studied microalgae biomass for nutritional proposes (MATOS et al., 2016; KHOZIN-GOLDBERG et al., 2011).

4.4 CONCLUSION

P. tricornutum, *N. oculata*, and *P. cruentum* biomass extracts provided an appreciable fatty acid composition. Subcritical *n*-butane extracted more PUFA ω3 and ω6 than supercritical CO_2 in all microalgae species studied. This fact might be attributed to the higher solubility of PUFAs in the *n*-butane than in carbon dioxide. Compared to the Soxhlet method, subcritical *n*-butane showed better results for the extraction of EPA from *N. oculata* and *P. tricornutum*. Despite this, the Soxhlet method achieved the highest extraction yields, though this method is not applicable at large scale mainly due to high energy requirements. Based on the results obtained in this work, *n*-butane was shown to be a powerful solvent for use in the extraction of lipid from microalgal biomass when the target compounds are PUFAs. In addition,

the properties of the subcritical *n*-butane method are suitable for saving energy and reducing operational pressure expenditure for the extraction of high value lipids from microalgae.

Chapter 5

ANTIOXIDANT ACTIVITY OF THREE MARINE MICROALGAE EXTRACTS OBTAINED BY SUPERCRITICAL CO_2 AND SUBCRITICAL *n*-BUTANE: CORRELATION WITH ITS TOTAL CAROTENOID CONTENT

5.1 INTRODUCTION

The previous chapter described the extraction of lipid fraction from three marine microalgae biomass regarding to the fatty acids composition of the extracts. However, there are several other compounds present in the microalgae lipid fraction that are also extracted. For example, natural antioxidants, such as phenolic compounds, tocopherols, vitamin C, carotenoids, among others (GOIRIS et al., 2015; RODRIGUEZ-GARCIA & GUIL-GUERRERO, 2008). The focus of this present work was to evaluate the antioxidant activity of the biomass extracts obtained by supercritical CO_2 (SC-CO_2) and subcritical *n*-butane for their *in vitro* antioxidant activity using DPPH free radical scavenging assay. Since carotenoids are lipophilic compounds and have been known as important antioxidants for human health (PALIWAL et al., 2016; AHMED et al., 2014), total carotenoids were also determinate in order to evaluate the correlation between antioxidant activity and carotenoid content of the extracts.

The extracts analyzed in this work were obtained according to the previous chapter, and the same extraction conditions (SC-CO_2: 300 bar, 40 °C, 9 mL min^{-1}, and 2 h; subcritical *n*-butane: 15 bar, 40 °C, 3 mL min^{-1}, and 2 h) were adopted.

5.2 MATERIALS AND METHODS

5.2.1 Total carotenoid content quantification by UV-Vis spectrum of extracts

Determination of carotenoids content of the microalgae extracts was carried out spectrophotometrically according to Lichtenthaler and Buschmann (2001). Aliquots of the extracts were prepared at concentration of around 2.5 mg mL^{-1} in methanol, and manually homogenized. The samples were then subjected to scanning profile using UV-Vis spectrophotometer (U-1800, Hitachi), for a spectral window of 200-800 nm for obtaining the image. Because the absorption maxima of extracted pigments strongly depend on the type of solvent,

the pigment content (chlorophyll a, chlorophyll b and carotenoids) was calculated using the Lichtenthaler equations which are used for methanol dilutions and given in µg mL^{-1}. Therefore, absorbance of samples was measured at 470, 652 and 665 nm in triplicate, methanol was used as blank.

(1) $Chl_a = 16.72 \times A_{665} - 9.16 \times A_{652}$

(2) $Chl_b = 34.09 \times A_{652} - 15.28 \times A_{665}$

(3) $Carotenoids_{total} = (1000 \times A_{470} - 1.63 \times Chl_a - 104.96 \times Chl_b) / 221$

5.2.2 DPPH radical scavenging assay

The antioxidant activity of the microalgae extracts was evaluated using 2,2-diphenyl-1-picrilidrazin (DPPH) radical scavenger method as described by Mensor et al. (2001). The DPPH method has been developed for measuring the antioxidant activity as the ability to scavenge free radicals generated in aqueous and lipophilic phases (ANTOLOVICH et al., 2002). DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. In presence of antioxidant compounds the DPPH is reduced producing a non-colored solution. The reducing ability of antioxidants towards DPPH can be evaluated by monitoring the absorbance decrease at 515-528 nm. The DPPH assay is technically simple, rapid, needs only a UV-Vis spectrophotometer and is a widespread antioxidant screening method.

For the procedure, each extract obtained from SC-CO_2 and subcritical *n*-butane were mixed with a 0.3 mM DPPH ethanol solution, to give final concentrations of 5, 10, 25, 50, 125, 250 and 500 µg extract mL^{-1} DPPH solutions. After 30 min at room temperature and protected from light, the absorbance values were measured at 517 nm in spectrophotometer (UV-VIS, Techcomp Ltda., Kowloon, Hong Kong). The higher the antioxidant activity of the tested sample, more stable becomes the radical DPPH, causing discoloration of the solution (DPPH and extract) and decreasing the absorbance. Thus, the inhibition percentage of the tested samples on the DPPH radical can be calculated by converting the absorbance as a percentage of the antioxidant activity (AA%), according to:

$$AA\% = 100 - \left[\frac{(Abs_{AMOSTRA} - Abs_{BRANCO}) \times 100}{Abs_{CONTROLE}} \right]$$

This activity was also expressed as the effective concentration at 50% (EC_{50}), i.e., the concentration of the solution required to give a 50% decrease in the absorbance of the test solution compared to a blank solution and expressed in µg of extract mL^{-1} DPPH. The EC_{50} values were calculated from the linear regression of the AA% curves obtained for all extract concentrations. The AA% and EC_{50} for all extracts were obtained considering the mean value of triplicate assays.

5.2.3 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Software STATISTICA version 7.0 from (Statsoft Inc., USA), in order to detect significant differences between values of antioxidant activity (AA% and EC_{50}) and total carotenoid contents. The significant differences ($p<0.05$) were analyzed by Tukey test.

5.3 RESULTS AND DISCUSSION

5.3.1 Total carotenoid content

Carotenoids are an important and well-known class of lipophilic antioxidants, and microalgae are an excellent source of these compounds. These pigments play an important role in quenching reactive oxygen species (ROS) generated during photosynthesis, especially singlet oxygen, which are known as important antioxidants for human health (AHMED et al., 2014; GOIRIS et al., 2012). In order to investigate the effectiveness of supercritical CO_2 and subcritical *n*-butane methods in the extraction of carotenoids of the microalgae studied, total carotenoids content of the extracts in mg g^{-1} extract weight (EW) for both methods were determined. As shown in Table 5.1, *n*-butane extract of *P. cruentum* displayed the highest total carotenoid content (5.2 ± 0.70 mg g^{-1} EW), followed by SC-CO_2 extract of *N. oculata* (4.4 ± 0.90 mg g^{-1} EW), and *n*-butane extract of *N. oculata* (3.1 ± 0.30 mg g^{-1} EW) whereas lowest carotenoids contents were found in *n*-butane extract of *P. tricornutum*, SC-CO_2 extract of *P. cruentum*, and SC-CO_2 of *P. tricornutum* (Table 5.1).

Table 5.1 Total carotenoids content of supercritical CO_2 and subcritical *n*-butane extracts of the microalgal biomass calculated with the Lichtenthaler equations (average of three determinations). Data expressed in mg g^{-1} extract weight (EW).

Microalgae strain	Supercritical CO_2	Subcritical *n*-butane
P. tricornutum	2.6 ± 0.24^{abc}	1.3 ± 0.20^{a}
N. oculata	4.4 ± 0.90^{bc}	3.1 ± 0.30^{abc}
P. cruentum	2.0 ± 0.19^{ab}	5.2 ± 0.70^{c}

Values with different superscript letters are significantly different ($p < 0.05$).

Comparing the methods, SC-CO_2 showed better results in the extraction of carotenoids for *P. tricornutum* and *N. oculata*, extracting 2.0 and 1.4-fold more carotenoids than subcritical *n*-butane, respectively. *P. cruentum*, *n*-butane extracted 2.6-fold more carotenoids than SC-CO_2, and significant differences were observed ($p<0.05$).

These results are not far from findings of Liau et al. (2010), which reported carotenoid content of *N. oculata* 7.61 mg g^{-1} EW using supercritical CO_2 extraction at 350 bar, 323K and 16.7 wt% of ethanol. The authors demonstrated that the addition ratio of co-solvents (ethanol or dichloromethane) is important factor on the increase of extraction efficiency of carotenoids. These results can be attributed to the fact that carotenoids are naturally lipophilic and more polar than triglycerides, which explains the highest solubility of this compounds in the extraction solvents (CO_2 + co-solvent) than only in CO_2.

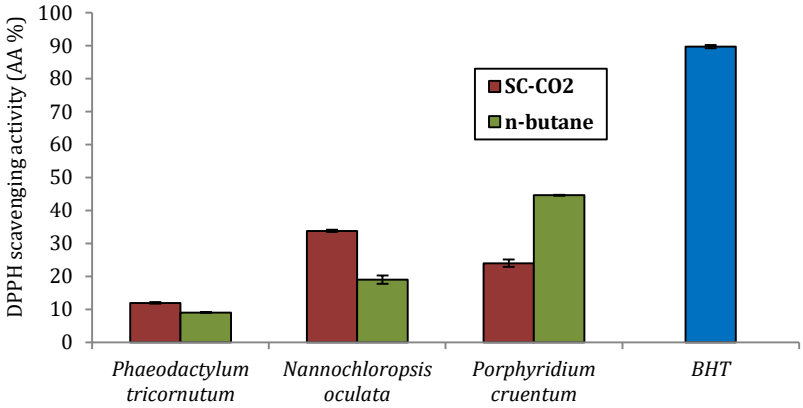
In another study, Millao and Uquiche (2016), have found a content of carotenoid of ranging from 4.02 to 6.23 g kg^{-1} oil from *Nannochloropsis gaditana* biomass using supercritical CO_2 method. The authors have shown a positive linear effect in the temperature range, i.e. when the temperature increased from 50 to 64 °C, the carotenoid content in microalgal extracts increased 1.45 and 1.35 times at 914 and 956 kg m^{-3}, respectively. They also found a positive effect of CO_2 density. The carotenoid content increased 1.24 and 1.19 times when CO_2 density increased from 914 to 956 kg m^{-3} at 36 and 64 °C, respectively. According to Mendes et al. (1999), the solubility of β-carotene in supercritical CO_2 increased with a change of pressure from 12 to 30 MPa (40-60 °C), while from 20 up to 30 MPa, its solubility increased with the temperature (40-60 °C). In another work, de la Fuente et al. (2006) reported that the solubility of astaxanthin in supercritical CO_2 increased with the increases of temperature (40-60 °C) and pressure (10-38 MPa). Considering these literature findings, the content of carotenoid

in the obtained extract may be favored, to a certain level, by the increase in solute vapor pressure at higher temperatures, and enhanced of solubility by higher densities.

5.3.1 DPPH radical scavenging activity

DPPH radical scavenging activities in percent of antioxidant activity (AA%) of different extracts of three marine microalgae are presented in Figure 5.1. All microalgae biomass extracts tested possess the ability of scavenging DPPH at various degrees. For example, *P. cruentum* extract showed the highest scavenging activity (44.63%) using *n*-butane methods, followed by SC-CO_2 extract of *N. oculata* (33.85%). For these microalgae extracts, statistical difference among the methods (p<0.05) were noted. The scavenging effect on the DPPH radical decreased in the order of *n*-butane extract of *P. cruentum* (44.63%) > SC-CO_2 extract of *N. oculata* (33.85%) > SC-CO_2 extract of *P. cruentum* (24.04%) > *n*-butane extract of *N. oculata* (19.05%). Among the microalgae studied, *P. tricornutum* extracts showed the smallest DPPH scavenging activities, i.e. the ability to scavenging DPPH was 12.01% and 9.06% using SC-CO_2 and subcritical *n*-butane, respectively, which were statistically similar (p<0.05).

Figure 5.1 Antioxidant activity of three microalgae extracts for the two methods tested performed by DPPH radical scavenger assay. Data is an average of three determinations.



Source: Author.

In this study, *P. cruentum* extract was found to be the most potent scavenger (44.63%) when subcritical *n*-butane was used. The scavenging activity detected in *P. cruentum* might be attributed to the wide range of antioxidant compounds found in this species, e.g. tocopherols, vitamin K, phycoeritrin, sulfated polysaccharides and a large amount of carotenoids (ARAD & LEVY-ONTMAN, 2010). Compared to the scavenging effect of the standard butylated hydroxytoluene (BHT) used as positive control (89,7%) (BENELLI et al., 2010), *n*-butane extract of *P. cruentum* presented half of its scavenging ability. Currently BHT as well as butylated hydroxyanisole (BHA) are widely used as antioxidants to prolong the shelf life of foodstuffs. Since these synthetic antioxidants are suspected carcinogens and there is a search to replace these products with natural antioxidants, our results suggest that *n*-butane extract of *P. cruentum* could be an alternative and potential source of antioxidant for food and other applications, such as cosmetics or pharmaceutics. In this case, antioxidant compounds can be increased in microalgae by manipulating the appropriate cultivation conditions (GOIRIS et al., 2012; SPALAORE et al., 2006).

In respect to synthetic antioxidants, Rodriguez-Garcia and Guil-Guerrero (2008) determined the antioxidant activity of ethanolic extracts of *Phaeodactylum tricornutum*, *Porphyridium cruentum*, and *Chlorella vulgaris* by using the β-carotene bleaching assay, indicating antioxidant activity of *C. vulgaris* and *P. cruentum* extracts higher than BHT and BHA. In another study Hemalatha et al. (2013) found highest DPPH scavenging activity in methanol extracts than in acetone and hexane extracts of *Chlorella marina* and *Dunaliella salina* (23.08% and 17.66%, respectively). In addition, BHT scavenging activity was 15.25%; lower than the microalgal extracts. Despite these authors have found higher antioxidant activities in microalgal extracts compared to synthetic solvents (BHT and BHA), faster and selective extraction methods, e.g. supercritical fluids, are indicated to recover food-grade antioxidant compounds which are food-compatible and environment friendly solvents/methods (GUEDES et al., 2013).

Using supercritical extraction Millao and Uquiche (2016) studied the effects of temperature and fluid density of CO_2 on the content of carotenoids and tocopherols on the lipid fraction from *Nannochloropsis gaditana*. The authors observed that when the content of carotenoids increased from 4 to 6 g kg^{-1} oil, the antioxidant activity increased 1.4-fold for β-carotene bleaching assay, 1.5-fold for TROLOX equivalent

antioxidant capacity (TEAC) and 1.3-fold for Ferric-reducing antioxidant power (FRAP) assay. When the tocopherol content increased from 2 to 4 g kg^{-1} oil, the antioxidant activity increased 1.3-fold for β-carotene bleaching assay, 1.5-fold for TEAC, and 1.3-fold for FRAP. Thus, they conclude that the antioxidant activity of the extracts depends on the amount of carotenoids and tocopherols present in the lipid fraction. The highest values of antioxidant activity and carotenoids + tocopherol content were achieved at 64 °C and 956 kg m^3.

Apart from the above work, there is a lack of information in the literature regarding the antioxidant activity of microalgal extracts obtained by supercritical CO_2 and other compressed fluids. Although the knowledge of an isolated compound present in an organism is not always a good indicator of its antioxidant activity, but the interaction between them (term synergism) (RODRIGUEZ-GARCIA & GUIL-GUERRERO, 2008), the use of solvent-free methods applied to microalgal biomass found in the literature are focused on the optimization of the extraction process targeting specific compounds, e.g. polyunsaturated fatty acids (PUFAs) and carotenoids (MICHALAK et al., 2015; GOTO et al., 2015; LIAU et al., 2010; MENDES et al., 2003; MILLAO & UQUICHE, 2016). This fact is attributed to the selective characteristic of these methods, controlled by temperature and pressure conditions. For this reason, the literature is scarce to search for comparative information on the antioxidant activity of microalgal extracts for the tested methods.

In order to compare the results, another issue is the wide number of methods for the determination of antioxidant activity found in the literature, e.g. ORAC, TRAP, FRAP, TEAC, β-carotene bleaching assay, DPPH, among others (ANTOLOVICH et al., 2002; KARADAG et al., 2009). These wide range of alternatives for the determination of antioxidant activity allow studies to indicate that microalgae are rich source of natural antioxidants, however, the comparison of the results is difficult due to the differences in assay formats, experimental conditions, and data presentation strategies.

Regarding to the effective concentration at 50% (EC_{50}), the lower value means of EC_{50} the higher powerful antioxidant capacity. The highest radical scavenging activity was obtained with subcritical *n*-butane extract of *P. cruentum*, exhibiting the lowest EC_{50} value (577.53 μg mL^{-1}), which were 2.2-fold higher than BHT (261 μg mL^{-1}). SC-CO_2 extract of *N. oculata* presented EC_{50} value of 743.55 μg mL^{-1}, statistically similar to subcritical *n*-butane extract of *P. cruentum* (Table

5.1). The data expressed as EC_{50} showed statistical difference ($p<0.05$) between the EC_{50} values according to Table 5.2.

Table 5.2 DPPH radical scavenger activities of the three marine microalgae biomass extracts expressed as effective concentration at 50% (EC_{50}). Data is an average of three determinations.

Microalgae strain	Extraction method	EC_{50} (μg mL^{-1})
P. cruentum	*n*-butane	577.53 ± 2.63^{b}
N. oculata	SC-CO_2	743.55 ± 4.00^{bc}
P. cruentum	SC-CO2	962.13 ± 34.71^{c}
N. oculata	*n*-butane	1320.95 ± 63.08^{d}
P. tricornutum	SC-CO_2	2294.57 ± 53.84^{e}
P. tricornutum	*n*-butane	3486.13 ± 34.00^{f}
BHT	---	261.00 ± 12^{a}

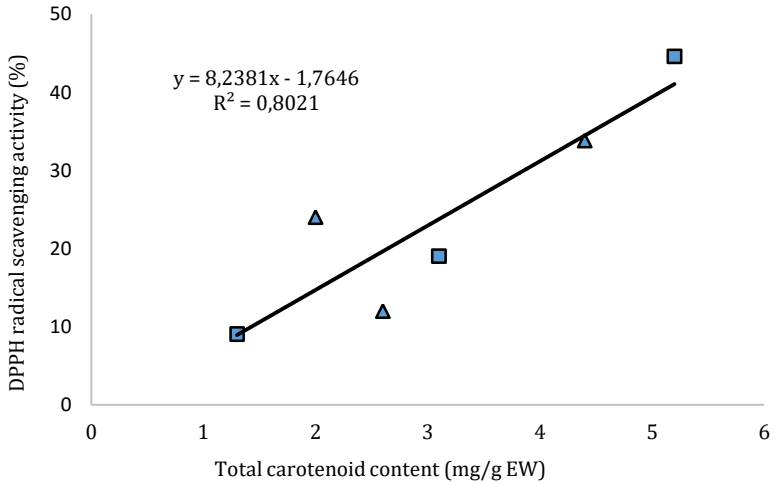
Values in the same column with different superscript letters are significantly different ($p < 0.05$).

Our results are in disagreement with Maadane et al. (2015), which determined the DPPH radical scavenging activity described by Fukumoto and Mazza (2000). The authors reported EC_{50} values of nine microalgae ethanolic extracts ranging from 247 to 464 μg mL^{-1} and EC_{50} value of 6.2 μg mL^{-1} for BHT. These results are ambiguous since EC_{50} value of BHT presented at least 40-fold stronger antioxidant activity than the microalgae extracts and they concluded that the microalgae extracts obtained are potential new source of natural antioxidants. Although macroalgae have received much attention as a potential source of natural antioxidants (DUAN et al., 2006), there has been very limited information on antioxidant capacity of microalgae (AHMED et al., 2014).

5.3.3 Correlation between antioxidant activity and carotenoid content

The correlation between the antioxidant activity and total carotenoid content of the supercritical CO_2 and *n*-butane extracts of the three microalgae strains was determined (Figure 5.2). When the data were plotted, a correlation coefficient of $R^2 = 0.8021$ was determined. These results indicate that carotenoid compounds might be the major contributor to the antioxidant capacities of these microalgae.

Figure 5.2 Correlation between the antioxidant activity and carotenoids content of the SC-CO_2 and subcritical *n*-butane extracts of the three microalgae biomass. Triangle represents extraction with *n*-butane and square represents extraction using supercritical CO_2.



Source: Author.

There are few studies on the relationship between carotenoids, phenolic compounds, and tocopherols in the antioxidant activity of microalgae and results are contradictory. Based on the R^2 value of the multiple regression for FRAP (0.549), TEAC (0.510), and AIOLA (0.310), Goiris et al. (2012) suggest that, besides carotenoids and phenolics, also other components contributed to the antioxidant activities measured in 32 microalgal biomass extracted with organic solvents and water. The authors found highest carotenoid content in the ethanol/water extracts (ranging from 0 to 7.8 mg g^{-1} biomass); highest phenolic content in the hot water fraction and low in the hexane fractions, attesting the typically more polar and hydrophilic nature of these compounds. These findings are in agreement with Hajimahmoodi et al. (2010), who found the highest phenolic content in the hot water fraction of 12 microalgae strains when using hexane, ethyl acetate, and water as solvents.

Hajimahmoodi et al. (2010) also evaluated the antioxidant activity according to FRAP and DPPH-HPLC assays. These authors reported a positive correlation between antioxidant capacity and phenolic content only in FRAP assay and not in DPPH-HPLC method. In another work,

Millao and Uquiche (2016) developed a model correlating total content of carotenoids and tocopherols with the antioxidant activity determined by TEAC, β-carotene bleaching assay and FRAP, showing good correlation (R^2>0.84) for the methods tested, except for FRAP.

Besides DPPH scavenging assay is a simple, practice and wide used method to estimate the antioxidant activity of a sample, more than one method need to be tested, to take into account different antioxidant mechanisms (GOIRIS et al., 2012; KARADAG et al., 2009). Since the assays principles are different, to attest the results more than one method to determine the antioxidant capacity must be tested. Thereby, correlations can be performed to estimate which compound might contributed for the antioxidant activity of a sample, thus the methods could be compared, attesting the results.

As carotenoids have been divided into carotenes and xanthophylls based on their chemical structure, xanthophylls have oxygenated functional groups making them more polar than carotenes, which are hydrocarbons (AHMED et al., 2014). This feature gives a more polar nature of phenolic compounds compared to some carotenoids, i.e. carotenes (GOIRIS et al., 2012). Our correlation achievements suggest that the antioxidant activity found in the microalgae extracts, could be caused by more carotenoids than phenolic compounds since CO_2 and *n*-butane are considered less polar solvents. Several compounds can be responsible for the antioxidant activity of our species. For example, *P. tricornutum* has been identified as a rich source of fucoxanthin (ten times more than macroalgae) (KIM et al., 2012). The Eustigamatophyceae *N. oculata* sp. are able to synthesize a high range of carotenoids and the major is zeaxanthin, this genus also produces tocopherols (MILLAO & UQUICHE, 2016). Cells of *P. cruentum* have a red color, provided by the accessory pigment phycoeritrin and it is well-known recognized as a producer of sulfated polysaccharide (exopolysaccharide), among other important metabolites such as tocopherols, vitamin K and β-carotene (ARAD & LEVY-ONTMAN, 2010; FUENTES et al., 2000; WANG et al., 2007).

To sum up, this work gives the first description of total carotenoids content coupled with antioxidant activity, presented in three microalgae biomass using SC-CO_2 and subcritical *n*-butane methods. In order to improve the extraction of antioxidant compounds by subcritical *n*-butane, further studies and analysis are required, for example to optimize the temperature and pressure values that could extract the maximum carotenoids content in microalgae.

Since, in SC-CO_2 extractions the raise of these conditions provides an increase in the content of antioxidant compounds, we hypothesized that it might work for subcritical *n*-butane due to some particularities in the extraction processes. Also, most of pigments are high influenced by temperature and pressure at certain level (MILLAO & UQUICHE, 2016; MENDES et al., 1999; de la FUENTE et al., 2006). Another approach to increase the extraction of more polar antioxidant compounds is the use of co-solvents, but therefore organic solvents are not indicated for food-grade and pharmaceutical products (GUEDES et al., 2013; HERRERO & IBÁÑEZ, 2015).

5.4 CONCLUSION

The three microalgae biomasses used in this study presented antioxidant activity for the DPPH radical scavenger assay. Subcritical *n*-butane extract of *P. cruentum* showed the highest scavenger activity (44.63%), suggesting that it could be an alternative source of natural antioxidant, which can be used for preserving foodstuffs and replacing synthetic antioxidants. Comparing the two methods, supercritical CO_2 showed better results in the extraction of carotenoids for *P. tricornutum* and *N. oculata*, whereas for *P. cruentum*, *n*-butane extracted 2.6-fold more total carotenoids than CO_2. Based on the results, *n*-butane was shown to be a good method for the extraction of antioxidant compounds and carotenoids mainly from *P. cruentum*. For further studies, we suggest performing experiments with different temperature and pressure conditions, in order to improve the extraction of antioxidant compounds from microalgae by subcritical *n*-butane. This could lead to energy saving and reduced operational pressure expenditure.

Chapter 6

APPLICATION OF THE PROTOZOAN *TETRAHYMENA* AS A HARVESTING METHOD FOR MICROALGAL CULTURE AT PILOT SCALE

The work presented in this chapter was developed under the supervision of Ian Ross and Ben Hankamer from the Institute for Molecular Bioscience (IMB) at The University of Queensland, Australia. It was supported by National Council for Science and Technology Development (CNPq/Brazil), who provided a sandwich scholarship (process number 205269/2014-1) by the program Science without Borders (CsF) to Rafael Feller and IMB who funded all the materials and structure needed for the study.

John Roles, Juliane Wolf and Evan Stephens also provided technical support for the development of this work.

6.1 INTRODUCTION

One of the main costs associated to microalgae biomass production is related to the harvesting process, as it usually accounts for about 20–30% of total operating outlay. The main challenge is the separation of small cells (2-20 µm) from a very dilute culture (less than 1 g L^{-1}) (BARROS et al., 2015; UDUMAN et al., 2010). Several authors have suggested that low-cost harvesting of microalgae can be achieved by means of a two-stage harvesting process in which the biomass is pre-concentrated by flocculation prior to final dewatering using a mechanical method such as centrifugation or filtration. The key requirement for the pre-concentration step is to minimize the total volume that needs to be treated in the final dewatering step (GRIMA et al., 2003; BENEMANN & OSWALD, 1996; MOHEIMANI et al., 2015). Apart from energy balance, effective harvesting systems must be able to process large volumes, be applicable to a wide variety of microalgal strains and achieve high biomass concentrations (VANDAMME et al., 2013).

Flocculation enables gravity settling or flotation and has long been attractive as a low energy dewatering technique. The method involves the destabilization of the microalgal cells in suspension by reducing or neutralising the cell surface charge (coagulation), for the following process - the aggregation of the destabilised microalgae cells (flocculation) (HOWE et al., 2012). Flocculation may be initiated

through the use of inorganic coagulants, organic coagulants (often polymers) or by using electrofloculation, and bioflocculation procedures (BOROWITZKA & MOHEIMANI, 2013). Despite this alternatives studies have shown a big gap between technical and economic viability for microalgae harvesting technique at larger scale (WAN et al., 2015).

Currently, biological approaches are emerging techniques that can lead to an effective harvesting method for microalgae cultures. One process can be characterized by the presence of extracellular polymeric substances (EPS) in the medium that is believed to induce the aggregation of the algae cells to form flocs (VANDAMME et al., 2013). Bacterial and fungal biomass has been used as a source of EPS for bioflocculation (LEE et al., 2009; PARK et al., 2015; PRAJAPATI et al., 2014) but typically require substantial flocculation times. Recently, Jakob et al. (2016) demonstrated that the protozoan *Tetrahymena sp.* offers a controllable, rapid, low-energy and potentially cost-effective harvesting technology. The method triggers exocytosis in starved *Tetrahymena* cells that then initiates bioflocculation in algal cultures. They suspect that the extracellular polymer produced by the protozoan *Tetrahymena* is released into the medium and then acts to trap algal cells (*Chlorella sorokiniana*).

Based on this study the present work aims to demonstrate the technical feasibility of a biological flocculation using *Tetrahymena* as a harvesting method for the pre-concentration of microalgal cultures at pilot scale. The use of *Tetrahymena* for cost-effective harvesting of algae culture is an attractive alternative, however to date *Tetrahymena* cells have not been exploited for industrial production and use. An explanation for this underutilization is that protozoa are more difficult to culture on a large scale than bacteria or fungi and only a few fermentation strategies yielding biomass comparable to bacteria and yeast cultures have been developed for *Tetrahymena* (CAILLIERET-ETHUIN et al., 1998; KIY & TIEDTKE, 1992; de CONINCK et al., 2000).

Experiments at laboratory scale proved that the protocol is likely to be applicable to greater volumes and has significant potential for industrial scale application. Other algal species could also be effectively captured indicating that the observed aggregation is not dependent on the actual algal species utilized, suggesting that *Tetrahymena spp*. could be used as an economical and controllable bioflocculant. However, it is not known whether the *Tetrahymena* based method can compete with other flocculation techniques and whether it is a viable technology for

industrial scale algal bioflocculation. The main objective of this work is to conduct the necessary experimentation to investigate the viability to use the protozoan *Tetrahymena* as a pre-concentrating technology for the harvesting of microalgal cultures at pilot scale.

6.2 MATERIALS AND METHODS

6.2.1 Scaling up *Tetrahymena* growth

In the literature (CAILLIERET-ETHUIN et al., 1998; KIY & TIEDTKE, 1992; KIY & TIEDTKE, 1992a) low-cost skimmed milk based-media has been described to achieve the highest cell densities and growth rate compared to other media based on e.g. proteose peptone based-media. In order to evaluate the growth of our particular species in skimmed milk based-media, cells were first grown in 150 mL flasks (25 mL of skimmed milk based-media plus 1 mL inoculum ~1 x 10^6 cells/mL) swirling (150 rpm) in a shaker (Bioline orbital shaker Model BL8136) at room temperature.

In order to obtain the growth curve cells were counted in a Neubauer chamber through a microscope (Olympus BX40F4) achieving a maximal cell concentration of ~3.5 x 10^6 after 50 h. The media chosen was composed of 2% skimmed milk powder (Woolworths Ltd), 1% D-glucose, 0.5% yeast extract (KIY & TIEDTKE, 1992) autoclaved separately in stock solutions (10% skimmed milk, 10% D-glucose, 5% yeast extract) at 121 ^{0}C for 15 min. To achieve the number of cells needed for each flocculation experiment, first 30 mL of *Tetrahymena* culture (~1 x 10^6 cells/mL) from Neff media was used as inoculum into 300 mL of skimmed milk media amounting to 2.4 L, split into 8 flasks (2 L each).

6.2.2 Cells starvation

The second step for using *Tetrahymena* as a harvesting method is to ‘’starve’’ the cells. Cells under starvation are more sensitive and release much more EPS in presence of a stimulator than replete cells. The use of stimulators (trigger compounds) are chiefly limited to starved cells in low ionic-strength medium (e.g. Dryl’s solution) (TURKEWITZ et al., 1999). To starve the cells a protocol described by Turkewitz et al. (1999) with some modifications was used. The procedure was originally developed to optimize capsule formation as a test of exocytic competence. After 24 h growing in skimmed milk media (1.5-2.0 x 10^6

cells/mL) 2.4 L of culture was centrifuged (Beckman Coulter J-26 XPI) at 600 *g* for 4 min in a 500 mL buckets. The supernatant was discarded and the concentrate cells poured in 2.4 L of starvation buffer (0.15 mM sodium citrate, 0.1 mM NaH_2PO_4, 0.1 mM Na_2HPO_4, 0.1 mM $MgCl_2$ and 0.5 mM $CaCl_2$) split in the same 8 flasks. Cells were starved overnight (20-24 h) in this buffer, swirling (150 rpm) at room temperature. The effectiveness of starvation was judged by two physical characteristics which accompany exocytosis – the cells become thin and elongated, and they swim very rapidly compared to their normal rate in a complete medium.

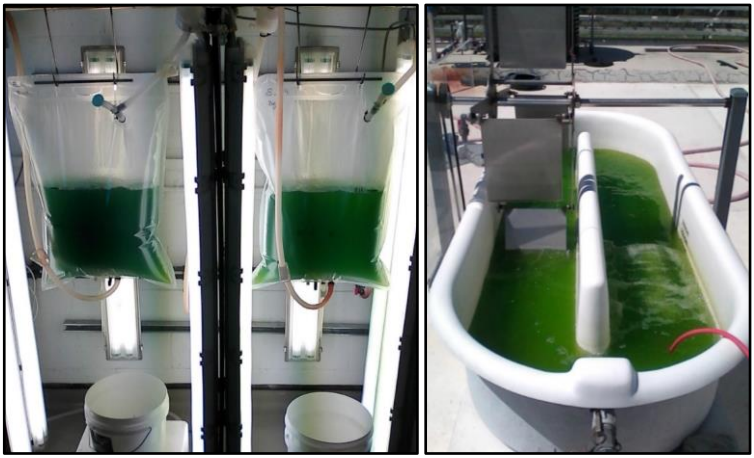
6.2.3 Cultivation of microalga strain used as model for the bioflocculation assays

The local wild algal strain *Chlorella sorokiniana* (8-C4) used as a model organism for the flocculation experiments was obtained from the internal culture collection at the Institute for Molecular Bioscience (IMB) and was previously isolated from freshwater habitat in Queensland, Brisbane. The microalga cultivation as well as the flocculation experiments were conducted at the Solar Biofuels Research Centre (SBRC). To inoculate the pond, the inoculation volume (~30 L) was established from a stock culture into 10 mL TP medium (Tris-Phosphate medium) which is then scaled up to approximately 3 L volumes to a culture density of OD_{750} = ~2. The culture was subsequently transferred into gamma sterilized hanging bag culturing systems (Pure Biomass, USA) growing for two weeks to a scale up volume of up to 30 L, targeting an OD_{750} of up to 2 (constant irradiance at 310 *mE* m^2 s^{-1}, temperature 25 ^{0}C, aeration ~0.5 L min^{-1} sterile air). The pH was monitored and regulated every two days to pH 7 by adding filter sterilized NaOH (5M).

The nutrient media, for the alga growth, used in the pond was previously optimized by Wolf et al. (2015) and reformulated based on elemental equivalence for the use of agricultural grade nutrients as would be necessary in commercial scale systems. For the pond inoculation all chemicals, except calcium nitrate, were separately pre-dissolved in RO (reverse osmosis) water prior to addition into larger volumes. Prior to the algae inoculation, the media was mixed in the pond in approximately 3/4 of the final volume. After approximately 5 minutes of mixing and dissolving, the pH of the medium was adjusted with NaOH (5M) until a stable value of ~ pH 7 was achieved then

calcium nitrate was added. The EDTA chelated iron sulphate mixture which had been previously neutralised was added last. The pond volume was topped up to its final amount (250 L) after inoculation with the algae broth (Figure 6.1).

Figure 6.1 8-C4 microalgae cultures. Hanging bags used to inoculate the pond (left) and open pond culture used for the flocculation experiments (right).



Source: Author.

The pond (volume capacity up to 500 L) provided continuous mixing of the algae cultivation solution performed mechanically by paddle wheel system (drive motor generally set at 50 Hz corresponding to a rotational speed of ~5 rpm) and micro-bubble diffuser (bubble size 100-500 micron). The water was supplied to compensate for evaporation, assisted by a fill height mark, while the pH was controlled manually by adding NaOH (5 M). For the culture density determination absorbance (λ= 750nm) of 1 mL alga culture were measured (PG Instruments T60 UV-Visible Spectrophotometer– Software: UV Win) in duplicates each two day using 1 cm path length plastic cuvettes (10 x 4 x 45 mm polystyrene cuvettes - Sarstedt, Germany).

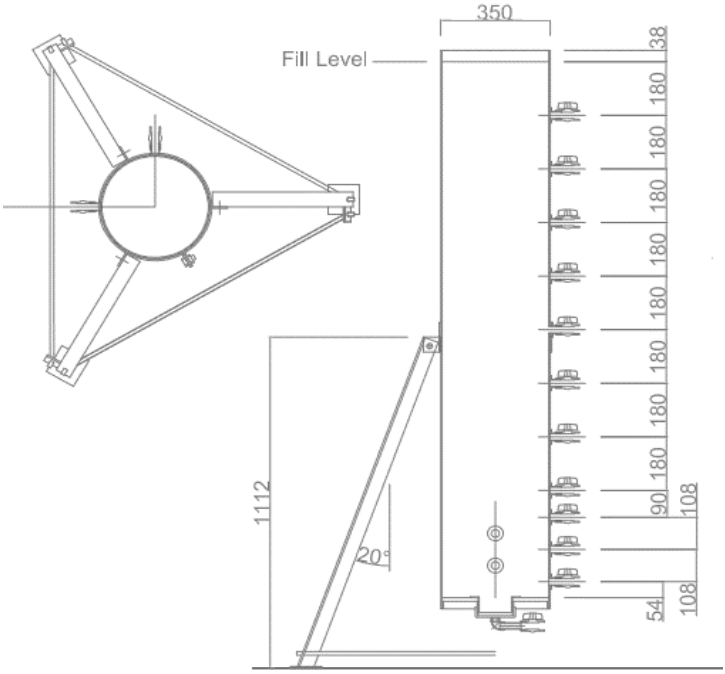
6.2.4 Flocculation assays

The key parameters governing the performance of any separation technique are separation efficiency, cut ratio and time. All of these factors govern the size and cost of full scale separation operations. In order to find out a separation profile in pilot scale (200 L) an acrylic

cylinder was designed (Figure 6.2) and flocculation assays in batch were conducted. The hight of the column was set at 2 m (approximately 50% of the depth of a full scale separator), a dimension reasonable to extrapolate the results. A 0.4 m diameter was selected to be sufficient to minimize side wall effects on the settling rate.

To obtain the settlement profile of each experiment, the tube is equipped with 13 sampling points with internally projecting inlets to avoid side wall effects, the lower collection points being spaced radially also to reduce cross effects from the sampling. Anticipating that a full scale separator should operate at a cut ratio of around 5% the sample point spacing was also compressed in the lower collection points. The sampling points were separated in standard distances from the bottom: 0, 3, 6, 9, 12, 15, 20, 30, 40, 50, 60, 70, 80, and 90%. The cylinder is also equipped with a flat removable bottom to make easy the collection of flocs.

Figure 6.2 Apparatus designed for the bioflocculation assays. Values in millimeters.



Source: Author.

For the quantification of setting rates, the same procedure as used for the density determination of the algae culture (section 6.2.3) was employed. For all the assays the ratio of *Tetrahymena* to algae cells were kept ~1:250 through previous dilution and cell count via microscopy (Olympus MODEL BX41TF). The algae culture was pumped until the cylinder was full almost to the top (200 L). For the experiments different approaches were designed in order to find out the best way to initiate floc formation according to 4 different methodologies:

- 1° Experiment: the trigger compound (4 L of NaCl 5 M) was added first to the algae broth (final concentration 100 mM) and air mixing was performed through the bottom of the cylinder for 30 seconds. Then *Tetrahymena* cells were added rapidly (1.2 L poured manually in less than 5 s).
- 2° Experiment: *Tetrahymena* cells were added first (1.2 L) and air mixing was performed through the bottom of the cylinder for 30 s. Then the trigger compound (4 L of NaCl 5M) was added to the algae broth (final concentration 100 mM) and 30 s of air mixing performed again.
- 3° Experiment: the trigger compound (4 L of NaCl 5 M) was added first to the algae broth (final concentration 100 mM) and air mixing was performed through the bottom of the cylinder for 30 s. Then *Tetrahymena* cells were added in a slow manner (1.2 L poured manually in approximately 2 min).
- 4° Experiment: the trigger compound was added to the *Tetrahymena* flasks in a final concentration of 100 mM to pre-triggering the cells. Then the *Tetrahymena* broth (1.2 L) was poured manually into the algae broth.

The samples were collected and measured on time: 0, 5, 10, 15, 20, 30, 40, 50 and 60 min. The OD_{750} was then plotted against time, to estimate the rate of settlement. The recovery efficiency was calculated as follows:

$$\text{recovery efficiency (\%)} = \left[1 - \frac{OD_{750\,(t)}}{OD_{750\,(t0)}}\right].100$$

where the $OD_{750}(t)$ is the turbidity of the collected sample at each time and $OD_{750}(t_0)$ the turbidity of the initial culture.

6.3 RESULTS AND DISCUSSION

6.3.1 Issues involving *Tetrahymena* growth at large volumes

Tetrahymena cells were easily grown in a high surface area (300 mL in 2 L Erlenmeyer flasks) allowing cell density between 1.5 and 2.0 x 10^6 cells mL^{-1}. However, to setup many flasks each containing a small amount of media, in order to provide a high surface area, quickly becomes an impractical system. For this reason, an apparatus based on the cells growth requirements was built, in order to grow enough cells for the large scale flocculation assays. A 10 L flask was used as a reactor equipped with an oxygen (99.5%) and air supply to provide the right rate of oxygen dissolution in the media controlled by two flow meters at rates of 0.5 L/min and 5 L/min respectively. An oxygen sensor (Sensorex DO1200 – Teflon membrane) was used to monitor the oxygen saturation. To collect the samples and to provide gas exchange two additional tubes were used across the stopper on the top of the flask. Filters were connected at the gas supply tube and gas exchange tube to avoid contamination. A magnetic bar was also used (150 rpm) to provide better oxygen dissolution and to keep the cells and nutrients in suspension. All the procedures were performed in the laminar flow chamber.

To achieve the number of cells needed for each flocculation experiment, first 400 mL of *Tetrahymena* culture (~1 x 10^6 cells/mL) from Neff media was used as inoculum into 4 L of the skimmed milk media. To prevent foaming the medium was supplied with 0.01% silicone defoaming agent (Antifoam-CYNDAN). According to literature review (KIY & TIEDTKE, 1992; de CONINCK et al., 2000) a certain level of oxygen (range 20-60%) is required for the *Tetrahymena* cultivation. For our species, in the beginning of cultivation the oxygen proportion was increased to 60% and then air supply was kept at 5 mL/min and the oxygen kept at 0.5 L/min in order to provide an oxygen dissolution ~30% during the growth (~24 h).

Despite the use of oxygen together with the air supply into the culture it was not possible to provide enough oxygen for the cells growth. As the cells start to grow at a certain level the oxygen concentration starts to decrease. Although these issues were not reported in the literature, it was considered possible that cellular respiration could be leading to carbon dioxide being dissolved instead the oxygen in the culture medium resulting in both cell death and consequently a pH

decrease. In some cases, bacterial contamination was also detected. These suggest that a better understanding about the gas requirements of the cells and a more sophisticated reactor may be required. In particular, automated control of the oxygen level is required.

The aseptic conditions required for the *Tetrahymena* cells growth for long periods of time (more than 50 hours) is another issue that will lead to increasing operational costs. We found that in our case these conditions are not necessarily needed. Through experiments using autoclaved and non-autoclaved media it was found that a period of 24 h of growth is not enough for the bacteria to affect the *Tetrahymena* culture health, enabling the cells to be starved and be used as bioflocculants. More than 24 h of cultivation could be a risk because bacteria population into the culture media starts appearing to a high degree, endangering the *Tetrahymena* culture.

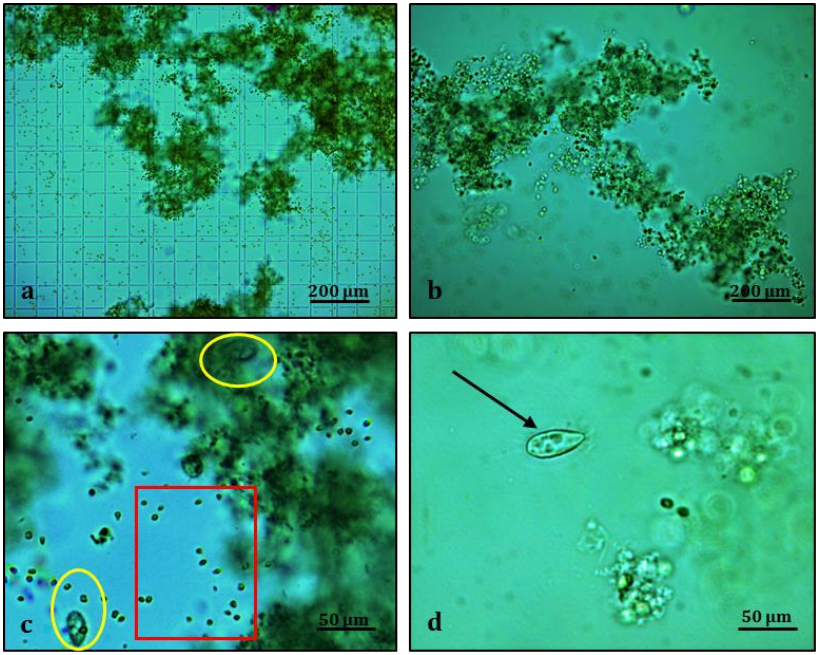
An option to rescue the culture in case of a long period or eventual contamination is the use of antibiotics (CASSIDY-HANDLEY, 2012). Consequently, our particular *Tetrahymena* species was submitted to different concentrations of antibiotics to identify their sensitivity to these agents. This may enable culture rescue in case of an eventual contamination. The antibiotics tested were Ampicillin, Kanamycin, Spectinomycin, and Cefotaxime in concentrations of 50, 100, 200, 400 and 800 μg/mL. Assays were performed in 150 mL flask (25 mL of media) in duplicate in skimmed milk based-media (growth step) and starvation buffer (starvation step). The cell density was kept at ~1 x 10^6 cells/mL for both media tested. The *Tetrahymena* survived all the concentrations tested, enabling the use of these antibiotics suitable to kill bacteria. However, this procedure is not economically viable for routine growth of the cells and is anticipated to be used in case of culture rescue.

To scale up *Tetrahymena* cell growth to a sufficient level to be used as an alternative algae dewatering, not only the culture medium is relevant for a cost-effective process. Oxygen supply, pH control, temperature and contamination are also conditions that must be controlled. Due to these conditions needed for cell growth and subsequent starvation, parameters such as reactor design, automated control, purity of nutrients supply in a system allowed to be used for algae cultures should be considered in a manner which will not increase the cost and energy inputs to high levels.

6.3.2 Flocculation results using *Chlorella sorokiniana* (8-C4) as a model strain

Chlorella sorokiniana (8-C4) culture growth was performed on average for a week reaching OD_{750} ~ 0.5 corresponding to a cell density of ~ 4 x 10^6 cells mL^{-1}. The ratio *Tetrahymena* to microalga cell was set to 1: 250 for each experiment by diluting the culture through pumping RO water into the cylinder. For all four methodologies, the flocs formation occurred, where it was possible to observe, *in loco*, different behaviors of the flocs formed (settelment, flotation and stability in suspension at some point). Although, the possibility to observe the formation of small flocs during the experiments, after the flocculation trials was not possible to check, by naked eye, differences in turbidity of cultures through the cylinder. The separation process was not efficient enough to enable the cells to aggregate forming sufficiently consistent flocs in a short time (60 min) to provide a phase separation visualization. Through Figure 6.3 it is possible to see the flocs formed and many algal cells in suspension.

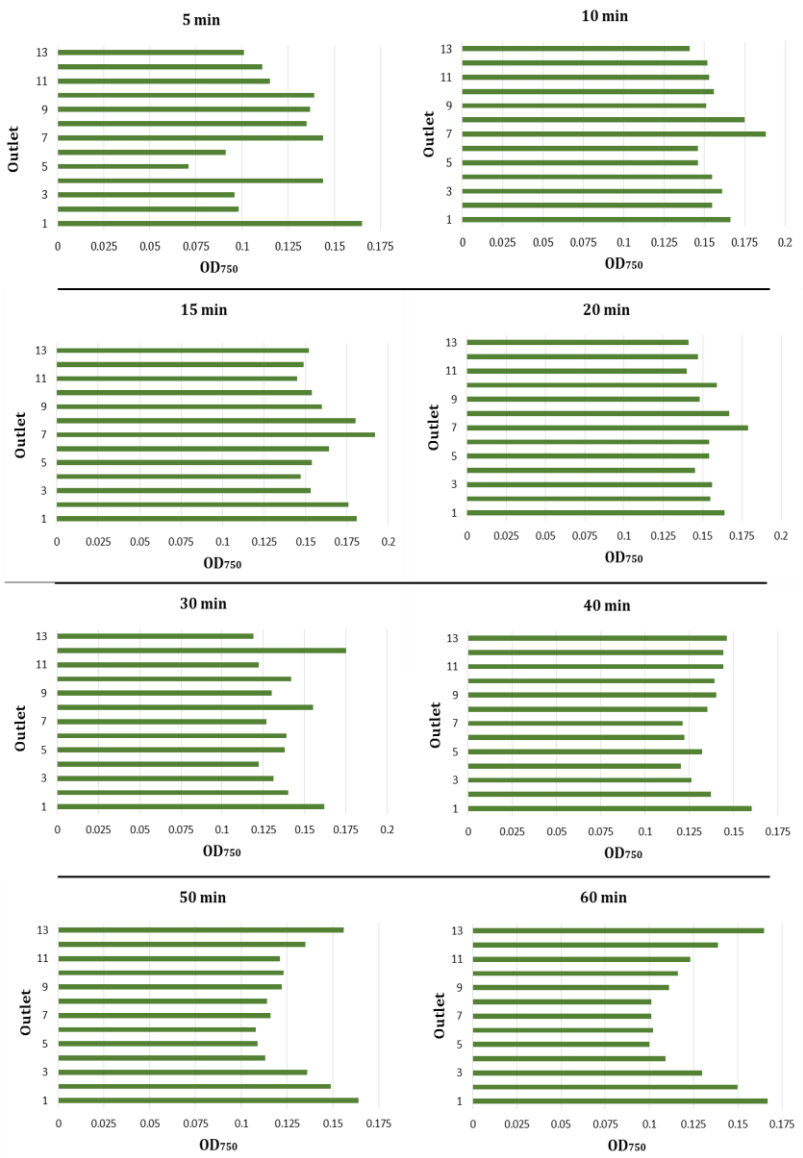
Figure 6.3 Images viewed under a microscope of a sample collected from the 3º experiment. **a** and **b** are flocs formed, which can be seen in the picture **a** many still suspended algae cells. **c** shows, circled in yellow, cells of *Tetrahymena*, and in red, algal cells. In **d**, a *Tetrahymena* cell can be clearly identified amid solution.



Source: Author.

The 3º experiment, where the methodology adopted was primarily the addition of the trigger compound to algal broth; mixing the solution with air injection and finally slowly added the *Tetrahymena* cells, was the only experiment possible to obtain a clear concentration profile (Figure 6.4).

Figure 6.4 OD_{750} profiles within the flocculator for the 3º experiment. Charts shown the evolution of the separation process along the cylinder for the different times.



Source: Author.

Observing the OD_{750} data from Figure 6.4, it is clear that over the time OD_{750} values become higher at the extremities and smaller at the vertical center of the cylinder. These OD_{750} profiles demonstrate a concentration gradient along the flocculator, showing that there is a solids (cell) movement inside the cylinder. As can be seen by the profiles, in the course of time occurs the decanting of some flocs and the flotation of others. From 40 minutes, the process begins to assume a defined behavior to a final time of 60 minutes, with higher concentrations at the extremities and smaller in the center. Higher concentration values found in the bottom of the cylinder indicate some formed flocs settling. The occurrence of this phenomenon was already expected, since normally when the flocs become denser than the medium around, it tends to move down. In the case of higher concentrations on the upper part of the cylinder which indicates the flotation of flocs, it was also visualized *in loco*. This flotation was generated by the attachment of flocs to small remaining bubbles of compressed air injection, used as a mixing agent at the beginning of each experiment.

Despite, the fact that the bioflocculation efficiency was not in line with expectations, the approach used in the 3º experiment provided the formation of flocs and a OD_{750} profile along the cylinder. In this experiment the flocs formed were not dense enough to settle, and the air injected for mixing eventually carried some flocs to the top of the flocculator. These observations show that changes must be done on the procedure in order to give the right direction to the flocs and consequently improve the efficiency of the process. For example, mix the system using a paddle to enable the flocs to settle or use a continuous air flow injection during the flocculation process to give direction for the flocs to the top of the flocculator (flotation). Through these findings, it was noted that the *Tetrahymena* cells were not doing a complete exocytosis. This fact could be observed adding the trigger compound (NaCl 5M) to the *Tetrahymena* culture and visualizing through the microscope. The EPS capsule formed was thinner compared to that usually found. Nevertheless, experiments at laboratory scale have found satisfactory flocculation, the extrapolation for a pilot scale did not show similar results.

6.4 CONCLUSIONS

The use of the protozoan *Tetrahymena* as a source of bioflocculation for microalgal cultures is still in the early stages of research, needing more understanding of the mechanisms of the flocs formation and functioning of the metabolism of protozoan cells. More experimentation should be done to optimize the pre-concentration step which is the most promising approach towards lowering microalgal harvesting costs. Thus, it may be possible to achieve the expected efficiency of the process for application on a large scale. Following some concluding remarks about this work.

- Since the cost of skimmed milk powder is approximately 1% of the cost of the proteose peptone together with a high cell density and rapid growth rate, it shows as a good alternative for the growth of *Tetrahymena* cells.
- Considering the conditions necessary for the *Tetrahymena* growth at large scale it is recommended to use a more sophisticated reactor. In particular, with automatic control of oxygen level.
- Flocculation experiments at intermediate scales must be successfully completed before attempting in large volumes (200 L).
- The release of dissolved air in the process may be another option for biomass harvest, since small bubbles induce flotation of the flocs.

Chapter 7

GENERAL DISCUSSION AND CONCLUSION

The main contributions of this thesis are: a screening of the chemical composition of six microalgae species indicating important candidates to enhance the nutritional value of foods (Chapter 3); the use of subcritical *n*-butane as an alternative and powerful method for the extraction of rich-lipids (ω3 and ω6) from microalgae biomass (Chapter 4); the potential use of *n*-butane for the extraction of antioxidant compounds from microalgae, and a significant correlation between total carotenoid content and antioxidant activity of extracts obtained by SC-CO_2 and subcritical *n*-butane (Chapter 5); an initial understanding about of the biological flocculation using the protozoan *Tetrahymena* for microalgal culture harvesting (Chapter 6).

Furthermore, this is the first work to use *n*-butane as a solvent applied to microalgae biomass, comparing the suitability of the method with two other well established extraction methods (Soxhlet and SC-CO_2), focused on fatty acids composition. Also, the antioxidant activities from microalgae biomass extracts obtained by SC-CO_2 and subcritical *n*-butane were compared. Based on these results, it is clear that subcritical *n*-butane is the best choice for the extraction of rich-lipids from the studied algae compared to SC-CO_2, and in some cases, even more than the Soxhlet method. Also, this alternative method has shown to have potential benefits for the extraction of antioxidant compounds from microalgae biomass. In addition, it was the first scale-up of *Tetrahymena* as a harvesting process for microalgal culture from the laboratory to pilot scale.

For the obtainment of microalgae biomass, the harvesting process has shown to be the biggest hurdle in the whole of the downstream processing. Due, to the different cell morphologies, cell sizes, and organic matter (i.e. polysaccharides) present in the medium, finding an optimal centrifugation rate for each species becomes a difficult procedure, and sometimes an addition step must be adopted. In our case, the three marine microalgae (*P. tricornutum*, *N. oculata*, and *P. cruentum*) were harvested in two centrifugation steps: using a continuous centrifuge followed by a batch centrifuge for the obtainment of a biomass slurry around 10-20%, which is suitable for drying. Apart from this issue, current mechanical harvesting processes such as centrifugation are energy intensive and not cost-effective. For this

reason, the flocculation as a step before the mechanical dewatering is desirable, and the search for alternatives such as the case study on bioflocculation using *Tetrahymena* are promising approaches for a cost-effective harvesting of microalgae cultures.

The chemical characterization of microalgae biomass is crucial due to the wide range of existing microalgae species, coupled with the varieties of natural compounds produced by these microorganisms. This approach opens a significant opportunity to isolate and identify new species from natural environments. This allows the possibility of finding suitable species for the production of different products (nutraceuticals, metabolites, vaccines, fuels, etc.). Based on this fact, future isolation and characterization of new microalgae species could be a more interesting approach than the use of known algae species.

Since, subcritical *n*- butane has shown good results in the extraction of rich-lipids from microalgae biomass, this alternative method must be optimized for the extraction of these value compounds. A prospective platform suggested for future works would be to use pressurized *n*-butane at 30, 40, and 50 $^{\circ}$C and pressures of 10, 15, and 20 bar. However, the lipid class (polar and non-polar) content in the extracts should be determined to identify the affinity of these lipids in *n*-butane.

Regarding to antioxidant compounds, subcritical *n*- butane extracts have shown different scavenging capacities according to DPPH method, and also different experimental conditions could be tested in order to improve the extraction of natural antioxidants of microalgal biomass. Due, to the fact that a wide range of methods for the determination of antioxidant capacity are found in the literature, more than one method should be used to give a more accuracy and higher confidence in the results.

In summary, the present work has provided an innovative method for the extraction of rich-lipids and antioxidant compounds from microalgae biomass, suggesting an economic analysis of the whole process. Since, the scaling-up of the bioflocculation process using the protozoan *Tetrahymena* is in early stages of research, more experimentation should be done, thus it may be possible to achieve the expected efficiency improvements for application on a large scale.

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APPENDIX

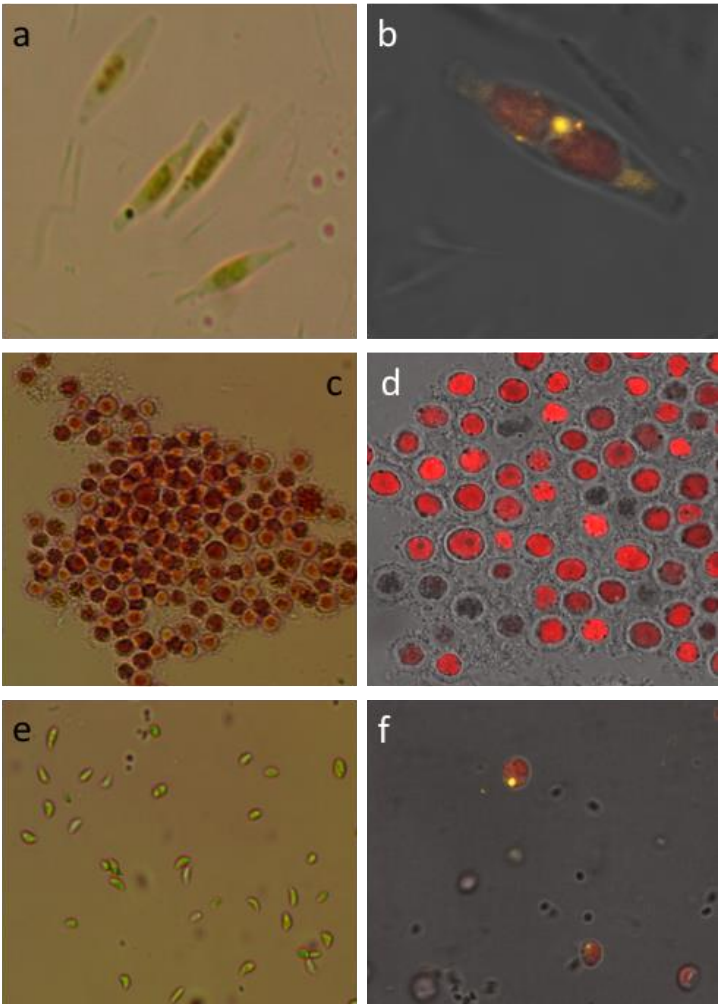

Figure A1 Micrograph images obtained from the studied microalgae. *Phaeodactylum tricornutum* viewed under optical microscope (a**)**, and under scanning confocal microscopy (b**)**, where the yellow intracellular lipid bodies of the algal cells were visualized using Nile Red stain. *Porphyridium cruentum* viewed under optical microscope (c), and under scanning confocal microscopy (d), where red fluorescence appearance from chlorophyll after UV emission. *Nannochloropsis oculata* viewed under optical microscope (e), and under scanning confocal microscopy (f), where the yellow intracellular lipid bodies of the algal cells were visualized using Nile Red stain.

Figure A2 Inoculum preparation. Cultures in initial stage, (a) and scaling up (b).

Figure A3 Cultures before the harvesting process by centrifugation. (a) *Phaeodactylum tricornutum*, (b) *Nannochloropsis oculata*, and (c) *Porphyridium cruentum.*

Figure A4 Centrifuges used to harvest the microalgae biomass. (a) continuous centrifuge (b) batch centrifuge. Obtained biomass. (c) wet paste spread in thin layer, (d) dried biomass.

Figure A5 Dried biomass before the extraction process. From the left to the right, *Phaeodactylum tricornutum, Nannochloropsis oculata*, and *Porphyridium cruentum*.

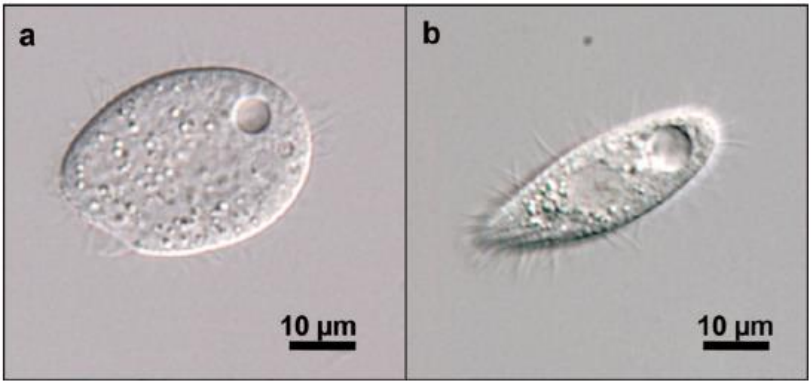

Figure A6 Non-starved (a) and starved (b) *Tetrahymena* cells. Starved *Tetrahymena* are significantly thinner than non-starved cells, and swim more rapidly.