Characterization of Value Added Proteins and Lipids from Microalgae

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ABSTRACT

Microalgae have been so far identified as the major producers of organic matter through their photosynthetic activities. In the present work, *Nannochloris sp*. and *Amphora sp*., two marine microalgae, have been investigated for proteins and lipids production. Protein fraction was quantified using Bicinchoninic acid (BCA) assay. Protein content in *Nannochloris sp*. was 16.69 ±4.07 % of dry mass and in *Amphora sp*. it was 39.89 ±2.09 % of dry mass. Enzyme assays were conducted spectrophotometrically. *Nannochloris sp*. had malate dehydrogenase, peroxidase and catalase activities. *Amphora sp*. exhibited malate dehydrogenase, catalase and cytochrome C oxidase activities. These enzymes have several valuable applications in some metabolic pathways and as antioxidant nutrition additives. Besides, lipid extraction was conducted using methanol/ chloroform solvent extraction. Crude lipid extract was analyzed using gas chromatography-mass spectrometry. Lipid contents were 8.14 ±3.67 % in *Nannochloris sp*. and 10.48 ±1.26% on dry basis in *Amphora sp*., respectively. *Nannochloris sp*. fatty acids were composed of C16:0 and C18:0 that are valuable for biodiesel production, and ω-3 C18:3, ω-6 C18:2, ω-6 C16:2 having great nutritional values. In *Amphora sp*., the fatty acids consisted of C14:0, C16:0 and C16:1 shown to be valuable for biodiesel production and ω-3 C22:6 having high nutritional values. Furthermore, a single step conversion of microalgal oil to fatty acid methyl esters was carried out starting directly from lyophilized microalgae. This promising process, in situ transesterification, led to better yields of methyl esters as compared to conventional lipid extraction followed by separate transesterification.

DEDICATION

I dedicate this work to my

- Darling father **Hbib** and mother **Wassila** for their unlimited support, confidence and affection. I strongly owe my success to their help, assistance and encouragements. They were always the nearest to me although far distances between us.
- Lovely grandmother **Ftima** for her advices and care.
- Sweetheart sister **Imen** and her husband **Anouir** and my Brother **Oussema** for their help, confidence and understanding.
- Honey young sister **Dhikra**, May God bring her all the success and happiness in her life.
- My extended family members.

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ABBREVIATION LIST

DHA: Docosahexeanoic acid (ω-3 C22:6)

DNA: Deoxyribonucleic acid

EDTA: Ethylene diamine tetra acetic acid

EPA: Ecosapentaenoic acid (ω-3 C20:5)

FAMEs: Fatty acid methyl esters

GC-MS: Gas Chromatography- Mass Spectrometry

MW: Molecular weight

NADH: Nicotinamide adenine dinucleotide

PUFAs: Polyunsaturated fatty acids

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

U: Unit of enzyme activity (1 micromole of product (or substrate) produced (consumed) per minute.

kDa: kilo Daltons, unit of molecular weight of proteins.

w (or wt): weight

mg: milligram

µg: microgram

g: gram

v: volume

ml: milliliter

µl: microliter

min: minute

s: second

rpm: rotation per minute

×g: centrifuge speed unit

ha: hectar

eg: example

Chapter 1

Introduction and Objectives

Nowadays, there is an increasing quest in the world for the exploration and exploitation of potential microalgae for various potential industrial applications from nutraceutical to biodiesel feedstock (Dayananda et al., 2010). In fact, microalgae possess all the advantages of higher photosynthetic efficiency, higher biomass production and faster growth compared to other energy crops, and they are also easy to handle and do not need much more than light, CO_2 and water for cultivation (Chiu et al., 2009; Miao and Wu, 2006). The high protein content of various microalgal species is one of the main reasons to consider them as an unconventional source of protein (Soletto et al., 2005) which can be valued in many fields such as in food, feed, cosmetic and pharmaceutical (Matsunaga et al., 2005; Kang et al., 2011). Actually, many metabolic studies have confirmed the capacities of microalgae as a novel source of protein: the average quality of most of the algae examined is equal or even superior to that of other conventional high-quality plant proteins (Becker, 2004).

Furthermore, microalgae are potential biofuel feedstocks as they can produce more oil per unit area of land, compared to terrestrial oilseed crops (Widjaja et al., 2009). Indeed, microalgal biofuels possess enormous potential and offer a breakthrough solution to both energy security and global warming concerns (Cheng et al., 2011; Lee et al., 2010). The bioregenerative methods using photosynthesis by microalgal cells have been recently shown to reduce the atmospheric CO_2 which results in a safe and reliable living environment (Chiu et al., 2009). However, it is commonly acknowledged that the profitability of microalgal biofuels is extremely challenging, highly variable, and subject to dynamic and speculative commodity markets (Cheng et al., 2011).

This project emphasizes on the quantification and characterization of protein and lipid contents extracted from two strains of microalgae *Nannochloris sp.* and *Amphora sp.* The goal is to determine some value-added co-products from the identified proteins and lipids in both microalgal strains and their possible fields of applications.

Chapter 2

Literature Review

2.1. Microalgae

2.1.1. Introduction of microalgae

Microalgae are photosynthetic microorganisms with simple growing requirements (light, sugars, CO_2, N, P, and K) that can produce lipids, proteins and carbohydrates in large amounts over short periods of time. These products can be processed into both biofuels and valuable co-products (Brennan and Owende, 2010). Eukaryotic algal groups represent at least five distinct evolutionary lineages, some of which include protists traditionally recognized as fungi and protozoa (Metting, 1996).

The number of algal species has been estimated at between one and ten million, most of which are microalgae. They are ubiquitous in marine, freshwater and terrestrial habitats. The different habitat results in a broad biochemical diversity among microalgal species. The implied biochemical diversity is the basis for many biotechnological and industrial applications (Metting, 1996). Indeed, microalgae varied in their contents of protein (6-52%), carbohydrate (5- 23%), and lipid (7-23%) depending on the type of strain and its relevant culture conditions (Kang et al., 2011). Dozens of microalgae species are produced commercially for single-cell proteins, polysaccharides, healthy food materials such as polyunsaturated fatty acids, and vitamins in the pharmaceutical and the food industries (Kang et al., 2011). Furthermore, algae are considered as good sources of natural antioxidants and bioactive compounds that have potential medicinal applications. The following species, *Spirulina platensis*, *Dunaliella salina*, and *Botryococcus braunii* are important sources of antioxidants for human consumption (Kang et al., 2011).

The advantages of utilization of microalgae are their ability to convert CO_2 to useful materials through photosynthesis and their ability to grow in natural environments under inorganic conditions. Indeed, they are mainly cultivated using seawater, CO_2, and sunlight (Matsunaga et al., 2005).

2.1.2. Biodiversity and Classification

Marine microalgae, the largest primary biomass, have been attracting attention as resources for new metabolites and biotechnologically useful genes. The diversified marine environment harbors a large variety of microalgae (Matsunaga et al., 2005). Here are the common classes of microalgae along with their basic characteristics and examples of commercial applications.

Cyanobacteria

Cyanobacteria are oxygenic photosynthetic prokaryotic microalgae that show large diversity in their morphology, physiology, ecology and biochemistry. This phylum is distributed widely in salt and fresh water (Matsunaga et al., 2005). Many commercial applications have been proposed for marine cyanobacteria, although no marine strain presently is commercially supplied. Potential commercial uses of cyanobacteria typically fall into three categories: bioactive chemical compounds (Volkman, 2003), polysaccharides (Otero and Vincenzini, 2003) and evaluation of new genes for recombinant expression (Matsunaga et al., 2005). Biotechnologically, the most important Cyanobacteria are *Spirulina (Arthrospira) platensis*, *Nostoc commune* and *Aphanizomenon flos-aquae* (Pulz and Gross, 2004).

Rhodophyta

The rhodophytes, or red algae, contain chlorophyll *a*, carotenoids, and phycobiliproteins. They are unicellular, or composed of simple or complex filamentous aggregates. About 600 genera and 5500 species have been recognized, most of them (98%) are marine macroalgal species. Red macroalgae have an important economic value (Wikfors and Ohno, 2001). For instance, the rhodophyte *Porphyra* and a few other species are cultured for human consumption. The production of red algal polysaccharides such as agar, agarose, and carrageenans is also an important industry. These compounds are widely used for laboratory cell culture media, nucleic acid purification, and food processing. Moreover, red microalgae and their polysaccharides were found to be good candidates for the development of antiviral drugs (Huleihel, 2002).

Chlorophyta

Cells of chlorophytes are green due to chlorophyll *a* and *b*, the same predominant photosynthetic pigments as those of land plants. These chlorophyta produce starch in the chloroplast as a storage product of photosynthesis. The Chlorophyta consist of five classes, the *Prasinophyceae, the Ulvophyceae, the Chlorophyceae, the Trebouxiophyceae, and the Charophyceae* (Matsunaga et al., 2005). A marine species of the *Chlorophyceae*, *Dunaliella*, has been cultivated commercially for food supplements and β-carotene production (Apt and Behrens, 1999). Furthermore, the microalgal biomass of some marine *Tetraselmis* and *Pyramimonas*strains in the *Prasinophyceae* family also are used for fish food additives (Brown, 2002). Recently, anti-inflammatory and immune suppressive properties were discovered in the extracted polysaccharides of another marine species, *Chlorella stigmatophora* (Guzman et al., 2003). *Nannochloris sp.*, one of the presently studied microalgae strains, belongs to the *Chlorophyceae* class of the Chlorophyta phylum.

Cryptophyta

Cryptophytes are a small group of mostly unicellular biflagellate microalgae with a broad collection of pigments, including chlorophylls a and c2 and phycobilins, and a single chloroplast with a thylakoid structure intermediate between the red algae and other plants (Metting, 1996). Starch is stored as distinct granules in Cryptophytes and some species also store oils. Indeed, a few strains such as *Rhodomonas minuta* and *Cryptomonas sp.* have been used for aquaculture feeds since they contain significant amounts of polyunsaturated fatty acids (PUFAs) (Brown, 2002).

Heterokontophyta

The phylum Heterokontophyta is the most diverse algal group with huge commercial and biotechnological potentials (Wikfors and Ohno, 2001). They range in size from microscopic unicells to giant kelp averaging several meters. Heterokontophyte microalgae are widely used as feed in mariculture and aquaculture. The genera *Navicula*, *Nitzschia, Cocconeis*, and *Amphora* also are used to feed juvenile abalone. The biotechnological potential of diatoms, which is a class of heterokontophyte microalgae phylum including the microalgal strain *Amphora sp.*, studied in this project, is mainly the production of polyunsaturated fatty acids (PUFAs). Indeed, diatoms were found to have a high content of

ω3 Eicosapentaenoic acid (EPA) 20:5 in *Phaeodactylum tricornutum* and *Nitzschia laevis*were. Hetrokontophytes also are being tested for the production of Docosahexaenoic acid (DHA) from *Schizochytrium, Thraustochytrium* and *Ulkenia* (Matsunaga et al., 2005).

Dinophyta

The Dinophyta include the dinoflagellates, most of which are unicellular, with two dissimilar flagella. Organisms in this phylum have remarkable morphological diversity and are characterized by cell coverings composed of cellulose. Recently, significant biological activities have been attributed to the polysaccharide from *Gymnodinium sp.*, a species of dinophytes (Umemura et al., 2003). These polysaccharides have potent anticancer activity mediated by the inhibition of topoisomerase I and II (Umemura et al., 2003). In addition, optimal growth conditions for *Gyrodinium impudicum* were reported to produce a sulfated polysaccharide that showed antiviral activity against encephalomyocarditis virus (Matsunaga et al., 2005).

Haptophyta

The phylum Haptophyta (haptophytes or prymnesiophytes) is a group of unicellular flagellates characterized by the presence of a haptonema between two smooth flagella. The cells of haptophytes are brownish or yellowish-green containing chlorophylls *a* and *c*1/*c*2 and carotenoids such as *β*-carotene, fucoxanthin, diadinoxanthin, and diatoxanthin. The cells are commonly covered with scales made mainly by carbohydrates or calcium bicarbonate. Microalgal biomass of haptophytes is commonly used as live feed in aquaculture (Matsunaga et al., 2005).

Euglenophyta

Euglenophytes are unicellular organisms with two pantonematic flagella arising from the bottom of a flask-shaped invagination called a "gullet." The chloroplast originating from green algae contains chlorophylls *a* and *b* and carotenoids such as diadinoxanthin, neoxanthin and *β*-carotene. Euglenoids do not have cell walls, but rather possess a proteinaceous pellicle, internal to the cell membrane (Metting, 1996). Although Euglenoids generally are harmless, toxin production has been demonstrated in some freshwater *Euglena sp.* (Triemer et al., 2003). However, *Euglena gracilis Z* is one of the few microorganisms that simultaneously

produce antioxidant vitamins such as β-carotene and vitamins C and E (Matsunaga et al., 2005).

2.1.3. Microalgae growth systems

Large-scale culture systems have been classified as closed and open systems, with the greatest interest given to the light supply (Matsunaga et al., 2005). Closed systems allow better control of growth conditions, while open systems largely depend on external factors and have contact with the open air (Grobbelaar, 1981). However, open systems are often simpler to construct and operate. An intermediate solution between closed and open systems was also developed in order to outweigh the relevant disadvantages of each process, this is hybrid culture systems. Another totally different solution for phytoplankton culture is immobilization, where the cells are trapped in a solid medium (Robinson, 1997).

2.1.3.1. Open Culture Systems

Ponds are open to the environment serving mainly for the production of animal feed ingredients and for wastewater treatment. Generally, the maintenance of single species (unialgal) cultures in open ponds relies on the competitive ability of the microalgae of interest. For instance, *Spirulina* naturally predominates in highly alkaline water, as does *Dunaliella* in saline culture (Matsunaga et al., 2005). Hence, these strains can be well cultured in open ponds due to their tolerance to extreme conditions (high pH and high salinity).

Open culture systems can be categorized into natural waters (lakes, lagoons, and ponds) and artificial ponds or containers. Generally, shallow raceway ponds and circular ponds with a rotating arm mixing the cultures are the two main artificial types used (Richmond, 1990). The raceway pond is set in a meandering configuration with paddle wheel mixers that exert low shearing forces (Figure 2.1). For wastewater treatment, facultative ponds and high rate algal ponds (HRAP) are most commonly used. A facultative pond is usually deeper than one meter, has algae growing in the surface water layers and is anoxic near the bottom (Larsdotter, 2006).

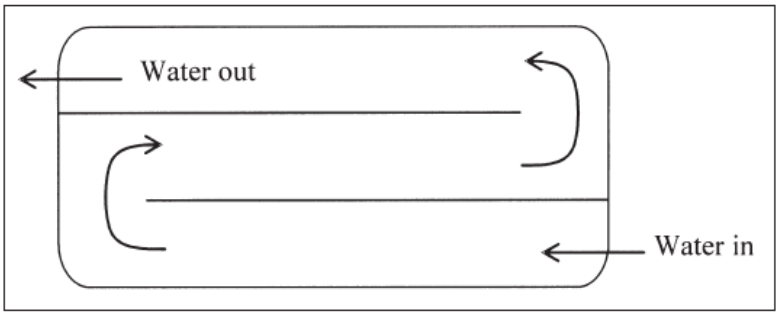

Figure 2.1. Schematic of a raceway pond (Larsdotter, 2006).

The open culture systems are the simplest technology of algal cultivation that has the several advantages. These include the ease of operation, the low construction cost and the easy diffusion of the oxygen resulting from the photosynthesis process to the atmosphere (Lebeau and Robert, 2003). Open cultivation systems result in important production of food sources in aquaculture. However these systems present some drawbacks including the requirement of large surface areas (high land cost), the frequent contamination by different algal species and other organisms, the vulnerability to weather conditions (eg. the rain dilutes the salinity, causing contamination) and the lower productivity of algal biomass as compared to closed cultivation systems (Matsunaga et al., 2005).

2.1.3.2. Photobioreactor (Closed Culture Systems)

Closed systems have been expected to overcome the disadvantages of open culture systems, and several types of photobioreactors have been devised. Closed photobioreactors can be grouped into two major classes: covered raceways and tubular reactors (Richmond, 1990). Airlift is the most common circulating method allowing CO_2 and O_2 to be exchanged between the liquid medium and air as well as providing a mechanism for mixing (Eriksen, 2008). Centrifugal, rotary positive displacement and peristaltic pumps are other techniques of agitation within the photobioreactors. The main advantages of airlift systems are their low shear and relative simplicity of construction. The helical tubular reactor consists of a vertical tower coiled up within a lone tube, increasing the land use efficiency (Matsunaga et al., 2005).

Closed photobioreactors usually have better light penetrating characteristics than open ponds which make it possible to sustain high biomass and productivity with less retention time than is possible in ponds (Borowitzka, 1988). They also facilitate the maintenance of monoalgal cultures by protecting them from contamination, reduce harvesting costs and land

costs (reduced overall volume of algal culture) (Matsunaga et al., 2005). Additionally, cultures in the tubular reactor could be warmed faster than in the open raceway up to 35–37 ^{o}C, the optimal range for growth (Richmond et al., 1993).

However, since they are more technically complicated, often need expert personnel and require more energy than open systems; the operating cost is higher (Larsdotter, 2006). Besides, several criteria regulating algal productivity such as light utilization efficiency, homogeneous mixing (turbulence), low shear environment, temperature control, and efficient gas transfer are critical and significantly affected by location and may limit productivity in certain designs (Borowitzka, 1996). A problem in the closed system is photooxidative damage to the cells caused by accumulation of dissolved oxygen produced by photosynthesis during the light period (Matsunaga et al., 2005).

2.1.3.3. Hybrid culture systems

The hybrid two-stage cultivation is a combination of the distinctive growth stages in photobioreactors and in open ponds. The first stage provides the control of culture factors in order to ensure high biomass productivity with less contamination risk. The second production stage aims to apply certain stress on the algal cells in order to stimulate target molecules production (lipids) (Rodolfi et al., 2008; Huntley and Redalje, 2007). However, the transfer of the culture from photobioreactors to the open pond presents a natural stress for the culture.

2.1.3.4. Immobilized culture systems

In an immobilization system, microalgae are fixed on a solid support that can be, for instance, alginate or synthetic polymers. This process doesn't prevent substances in the water from diffusing to the cells (Robinson, 1997). The algae-medium mixture is often shaped as beads, but can even cover screens or surfaces (Borowitzka, 1988). Researches on immobilized algae have been carried out on living and dead cells. The living cells are studied mainly for nutrient uptake purposes, while dead cells are primarily explored for metal adsorption (Robinson, 1997). Interestingly, the immobilized microalgae harvesting process is easier and more efficient than in other culture systems. Indeed, this system is deemed suitable for a wide range of applications such as wastewater treatment (uptake of metals and nutrients) with the nutrient uptake rates shown to be similar for free and immobilized cells (Matsunaga et al.,

2005). However this cultivation system is still in the research stage at lab-scale, with limited knowledge of how such methods would work in larger scale (Larsdotter, 2006).

2.1.4. Microalgae Applications

2.1.4.1. Nutrition

Marine microalgae have been mainly produced and sold as healthy food, due to their high contents of protein, lipid, vitamins, and other nutrient supplements (Kang et al., 2011). In the same context, experiments conducted by Witt et al. (1981) on the marine green microalgae *Nannochloris sp.* aimed to gain information on the physiological, ecological and general suitability of this species as a food item in order to create the best possible growth conditions for mass culture. Furthermore, Kang et al. (2011) have shown in their recent study that the antioxidant enzymatic hydrolysates from *Navicula incerta* efficiently quenched different free radicals: 1,1-diphenyl-2-pycryl-hydrazyl (DPPH), hydroxyl, and superoxide. These results suggest that this enzymatic hydrolysate acts as a good candidate as antioxidant reagents and could be used as a potential functional food ingredient.

Eicosapentaenoic acid (EPA: C20:5) and docosahexaenoic acid (DHA: C22:6) from microalgae are available commercially from at least two companies who market them as additives to various products, including infant formulas (Hodgson, 1996). Nutritional research has focused on *Spirulina* as a source of protein based on standard measures of utilization, including protein content, digestibility, and biological value. As a source of useable protein, *Spirulina* is comparable to meat and dairy products, superior to plant materials, but inferior to poultry and fish (Metting, 1996). Moreover, because of increased awareness of the possible health benefits of including ω-3 fatty acids in the diet, the manufacture of microalgal fatty acids is the subject of many ongoing research and development (Hoeksema et al., 1989).

2.1.4.2. Pharmaceutical component production

As one of the most potent ω-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) has attracted increasing attention due to its important biological properties in improving memory and learning, supporting brain development especially in young children, and its great benefits to people with a history of heart disease (Tang et al., 2011). Marine microalgae, considered as an abundant source of lipids and polyunsaturated fatty acids, are considered as an ideal source of DHA. In the study of Tang et al. (2011), DHA (C22:6) was

identified among the major fatty acids in the lipids extracted from of *Schizochytrium limacinum* using supercritical CO_2 extraction, a commonly used methodology in achieving the extraction of microalgal lipids with high yield and DHA with high purity.

In addition, much effort has been expended on search for new compounds of therapeutic potential and microalgae of all classes possessing components with antibacterial, antifungal, and anticancer properties (Kang et al., 2011). Marine blue-green algae have been the target of a more recent Natural Cancer Institute (Oxford, England) screening program aimed at identifying anticancer and antiviral (anti-HIV) activity, and a number of compounds have been identified with potential for drug development, such as dibromoaplysiatoxin from *Lyngbya majuscula*. Microalgae, including cyanobacteria and colorless (apochlorotic) variants of diatoms, may also be potential sources of antiviral sulfolipids (eg sulfoquinosovyl diglyceride) (Gustafson et al., 1989). Microalgae have been investigated for the production of vitamins and vitamin precursors, including L-ascorbic acid (vitamin C), riboflavin, and α-, β-, and γ-tocopherol for food and cosmetic formulations and mariculture (Brown and Farmer, 1994).

2.1.4.3. Aquaculture feed

Mass culture of microalgae as feed for molluscs (clams and oysters), crustaceans (shrimp), and fish is an important component of the mariculture industry (Benemann, 1992; DePauw and Persoone, 1988). Microalgal feeds are particularly valuable for seafood species with fastidious dietary requirements that cannot be met by formulation with traditional agricultural commodity products, such as corn, soybeans, and fish and food processing byproducts. In these cases, microalgae commonly provide essential amino acids, fatty acids or other unidentified growth factor requirements or are used to provide carotenoids for coloration of the final product. In nearly all cases, the microalgae are produced at the aquaculture facility, frequently by simple fertilization of incoming seawater, and fed directly to the animals as dilute, living cultures (Benemann, 1992).

2.1.4.4. CO_2 mitigation

As a consequence of global warming many technologies are being developed for greenhouse gas mitigation. Biological CO_2 mitigation through microalgae has attracted much attention as a strategic alternative. Indeed, the utilization of industrial gases is becoming a

reality in microalgae cultures, reducing the cost of the cultivation media. The study carried out by Sydney et al. (2010) aimed mainly to quantify the carbon dioxide assimilation rates of four widely used microalgae *Dunaliella tertiolecta* SAD-13.86, *Chlorella vulgaris* LEB-104, *Spirulina platensis* LEB-52 and *Botryococcus braunii* SAG-30.81. Practically, the cultivation vessels of these strains were coupled with sensors in order to measure carbon dioxide in the inlet and outlet gases.

2.1.4.5. Bioplastic production

Bioplastics are a feasible alternative to fossil-based plastics and can easily be biodegraded. Poly-(R)-3-hydroxybutyrate (PHB) is an aliphatic polyester with thermoplastic properties, which is naturally produced by certain bacteria as storage compound and is 100% biodegradable. PHB is synthesized from acetyl-CoA by the action of three enzymes: a ketothiolase, an acetoacetyl-CoA reductase and a PHB synthase. Hempel et al. (2011) recently reported on introducing the bacterial PHB pathway (genes of the three enzymes) into the diatom *Phaeodactylum tricornutum*. Gas chromatography analyses of *P. tricornutum* phaA/phaB/phaC-transfectants transferred to nitrate- containing medium for 7 days revealed that such cells indeed accumulate PHB to levels of up to 10.6% of algal dry weight. Interestingly, in comparison to efforts on PHB synthesis in the cytosol of plants, PHB expression levels in *P. tricornutum* are about 100-fold higher (Hempel et al., 2011).

2.1.4.6. Biofuel production

Microalgae can provide several different types of renewable biofuels. These include hydrogen (through biophotolysis), methane (through anaerobic digestion), ethanol (through alcohol fermentation using microalgae as a feedstock), triglycerides (through extraction of lipids), methyl ester fuels (through transesterification of lipids), and liquid hydrocarbons (from *Botryococcus braunii*) (Matsunaga et al., 2005; Spolaore et al., 2006). Moreover, microalgae produce more oil per unit area of land compared to terrestrial oilseed crops (Widjaja et al., 2009). Technology for producing and using biodiesel has been known for more than 50 years (Demirbas, 2005; Meher et al., 2006). In the United States, biodiesel was produced mainly from soybeans. Other sources of commercial biodiesel include canola oil, animal fat, palm oil, corn oil, waste cooking oil (Kulkarni and Dalai, 2006), and jatropha oil (Barnwal and Sharma, 2005). The typically used process for commercial production of biodiesel is summarized in Figure 2.2.

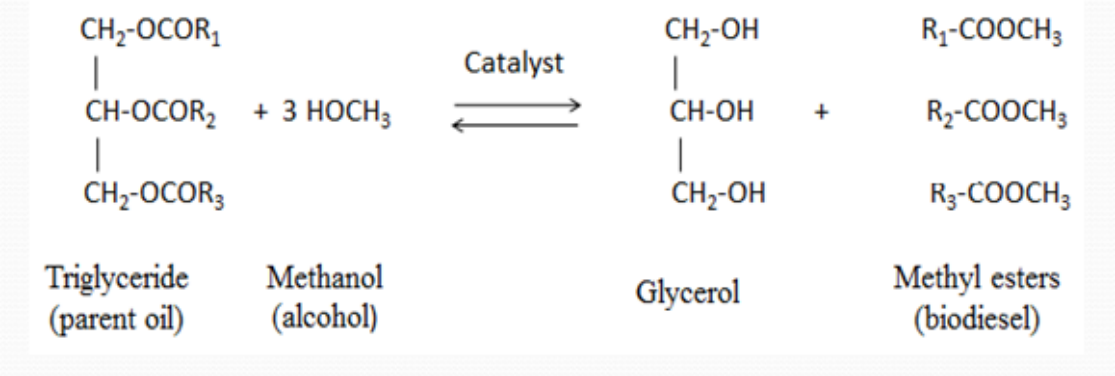

Figure 2.2. Schematic of the transesterification reaction. R1–3 are hydrocarbon groups (Chisti, 2007).

In making biodiesel, triglycerides are reacted with methanol in a reaction known as transesterification or alcoholysis. Transesterification produces methyl esters of fatty acids that are biodiesel, and glycerol (Chisti, 2007). New sources of biodiesel are being investigated as biodiesel contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel (Chisti, 2007). Microalgae have been suggested as very good candidates for biodiesel production as they are very efficient biomass capable of taking a waste form of carbon (CO_2) and converting it into a high-density liquid form of energy (Widjaja et al., 2009). The process suggested by Miao and Wu (2006), which combined bioengineering and transesterification, could be a feasible and effective method for the production of high quality biodiesel from heterotrophic microalgal oil. This study shows that biodiesel from heterotrophic microalgal oil could be a competitive alternative to conventional diesel fuel.

2.1.4.7. Waste water treatment

Microalgae are principal or otherwise important biological components of various systems for treating municipal and industrial effluents. Oxygen from photosynthesis is the primary microalgal contribution to treatment of municipal wastewaters in open ponds or impoundments. In both configurations, microalgal communities are commonly dominated by green microalgae (*Ankistrodesmus, Chlorella, Scenedesmus)* (Metting, 1996).

2.1.4.8. Biofertilizers

Microalgae are employed in agriculture as biofertilizers and soil conditioners (Metting, 1996). Filamentous species of *Anabaena, Nostoc, Aulosira, Tolypothrix*and *Scytonema* are common in China, India and elsewhere in Asia, where they can provide 20 or more kg N $ha^{-1}year^{-1}$, sufficient for one third or more of the requirements of traditional rice cultivars (Roger and Kulasooriya, 1980).

2.2. Microalgal proteins

2.2.1. Protein content

As one of the major constituents of the algae biomass proteins (up to 50% [w/w]) are expected to play an important role in algae biorefinery (Schwenzfeier et al., 2011). A number of microalgal species, for example, *Botryococcus braunii*, *Chlorella sp., Dunaliella primolectal, Nannochloris sp., Neochloris oleoabundans, and Parietochloris sp.* have been shown to have high content of protein in microalgae cells under appropriate cultivation conditions (Kang et al., 2011).

Table 2.1 reveals the protein concentrations relevant to some species belonging to Chlorophyta and Heterokontophyta phylum that include the presently studied microalgae *Nannochloris sp.* and *Amphora sp*., respectively. As shown in Table 2.1, protein content varies among the same phylum upon a large margin of concentrations. This variation is also obvious even for microalgal species of the same class such as *Dunaliella salina* and *Dunaliella bioculata*. These differences are tightly related to species biochemical structures and compositions under different culture, harvesting and treatment conditions.

Table 2.1. Protein content of marine microalgae, as percentage of dry matter.

Microalgae Strain	Protein Content (% dry mass)
Chlorophyta	
D.tertiolecta [a]	11.0
U.fasciata [a]	7.55
C.fastigiata [a]	7.52
Nannochloris *oculata* [b]	23
Dunaliella bioculata [b]	49
Dunaliella salina [b]	57
Clorella vulgaris [b]	51- 58
Tetraselmis maculate [b]	52
Heterokontophyta	
C.minima [a]	10.0
D.menstrualis [a]	7.01
P.gymnospora [a]	11.9
Proschkinia sp. [c]	8.41
Amphora sp. [c]	18.5
Navicula incerta [c]	13.31
Nitzschia sp. [c]	13.58

(*a*) Barbarino and Louren¸co (2005). (*b*) Becker (2006). (*c*) De Viçose et al. (2012).

2.2.2. Cell disruption and protein extraction methods from microalgae

The choice of cell disruption method and device is important to increase the extraction efficiency. Various methods, such as microwaves, sonication, potter homogenization and bead beating have already been used for cell disruption. Effectively, Murphy et al. (2000) reported that sonication was efficient in producing cell-free protein extracts containing active peroxidase, malate dehydrogenase and catalase enzymes. Besides, there are many studies that focus on the extraction techniques of the proteinaceous fraction from microalgal cells. Indeed, the protein isolation method is another key step that influences the rest of the processing work, the quantity and the quality of protein yield recovered in the end. For instance, the proteins from *Tetraselmis sp.* algae were extracted through cell disintegration by bead milling

(Schwenzfeier et al., 2011). The resulting extract was centrifuged then purified via ion exchange chromatography using the absorbent diethyl amino ethyl (DEAE). In this study, the authors highlighted the effect of the bead milling in the enhancement of protein extraction; indeed protein contents increased from 36 % to 64% of the algae biomass after using bead milling.

Another case study aiming to isolate protein extract from *Haematococcus pluvialis* strain prior to 2 dimensional electrophoresis (2-DE) analysis was carried out by Wang et al. (2003). Cell disruption was done by one passage through a pre-cooled French Press Cell. The lysate was centrifuged at 3,000×g for 10 min to pellet unbroken cells and cell debris. The supernatant was dialyzed against a sucrose solution (85% w/v) at 4 ºC for 2 hr. Then the dialysate containing all soluble proteins was utilized for further analyses. These researches focused on the key parameters influencing the efficiency of protein solubilization and recovery from a heterogeneous extract. Indeed, precipitation of protein with trichloroacetic acid (TCA) /acetone showed good resolution as compared to non-precipitated samples and acetone-precipitated samples (Wang et al., 2003).

In addition, further researches carried out by Barbarino and Louren¸co (2005) established a new protein extraction approach applicable for both macroalgae and microalgae. This approach consisted of steeping 50 mg of freeze-dried samples ground manually with pestle and mortar in 4 ml of ultra-pure water for 12 hours, grinding these samples completely using a Potter homogenizer, centrifuging the homogenates at 4°C, 15000×g for 20 minutes and finally precipitating the proteinaceous fraction from the recovered supernatant using a TCA (25%) to homogenate ratio of 2.5. In fact, this approach was deduced from a comparative work between different combinations of three parameters, namely the volume of ultra-pure water utilized (1 ml or 4 ml), the incubation time (6 hours or 12 hours) and the presence or absence of the grinding step. Thus, in all cases, the Potter homogenizer grinding produced higher concentrations of protein yields as compared to non-ground samples. This can be returned to the branched and hard thallus forms of the studied species. In the study by Murphy et al. (2000), the microalgal cells, suspended in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer and cooled in an ice-bath, were disrupted by sonication for up to 7 min (30 s bursts, 1 min cooling between bursts). The sonicated mix was centrifuged at 20,000×g for 30 min, and the supernatant was used as the cell-free extract. Barbarino and Louren¸co (2005) reported that the efficiency of protein extraction from microalgae seems to be

influenced generally by two main factors: the chemical composition of the species and its morphological and structural characteristics.

2.2.3. Protein quantification methods

Determination of protein content of algae can provide important information on the chemical characteristics of algal biomass. The methods most commonly used to quantify protein are: (1) the alkaline copper method (Lowry et al., 1951); (2) the Coomassie Brilliant Blue dye method (Bradford, 1976); or (3) determination of crude protein (N × 6.25) (Barbarino and Louren¸co, 2005). In the study by Schwenzfeier et al. (2011), protein content from the commercially available microalgae *Tetraselmis sp*., was calculated as the sum of amino acid residue weights analyzed according to the method of Moore and Stein (1963).

Barbarino and Louren¸co (2005) utilized Lowry assay, Bradford assay and the sum of amino acid residues method in order to quantify and analyze the protein concentration of three microalgal strains. The values for the total amino acid residues were calculated by summing up the amino acid masses retrieved after acid hydrolysis. They noticed that the protein values obtained using Lowry assay, with BSA as a standard protein, are closer to those obtained by summing up the mass of amino acid residues. In this study, the Bradford assay gives lower protein content since the dye, Coomassie Brilliant Blue-G250, binds only to basic and aromatic amino acid residues that are present in low concentration in the studied strains. The fact that Bradford's method would generate lower protein values for a large number of organisms compared to Lowry's method is also suggested by other authors (Kaehler and Kennish, 1996).

Murphy et al. (2000) also applied the Lowry method for the quantification of the peroxidase enzyme extracted from three different microalgal species; *Porphyridium purpureum, Phaeodactylum tricornutum* and *Dunaliella tertiolecta.* It is important to note that several substances may interfere with both the Lowry and Bradford methods, such as phenol and phenolases (Mattoo et al., 1987), glucosamine and detergents (Peterson, 1979) and flavonoids (Compton and Jones, 1985) among many others (Peterson, 1979; Stoscheck, 1990). These substances could affect analyses by either increasing the absorbance (overestimating values), or decreasing the measurements by inhibiting the action of specific reagents. These limitations are overcome by the trichloroacetic acid (TCA) precipitation step, which allows the recovery of protein in the pellet (Murphy et al., 2000).

2.2.4. Characterization methods of microalgal proteins

The protein isolate extracted in the study of Schwenzfeier et al. (2011) was identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were analyzed based on proteomics data of different microalgal species. Thus the main proteins were deemed to correspond to photosynthesis enzymes or other essential activities for survival and growth within microalgal cells. This experiment showed that bands with the highest intensity represented proteins with a molecular size of about 50, 40, 25 and 15 kDa under reducing conditions. A distinct band with a molecular mass of about 50 kDa was identified by immunoblot analysis as the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) enzyme. The SDS–PAGE results imply that the majority of these polypeptide chains are smaller than 50 kDa.

In another case of study, protein amino acids profile was determined using an automatic analyzer (Kang et al., 2011). This analysis revealed that the microalgae *N. incerta* was mainly composed of lysine, arginine, aspartic acid and glutamic acid that together represent 51.3% of the total amino acid. Amino acid profile can also be investigated using ion-exchange chromatography with an automatic integrator (Barbarino and Louren¸co, 2005). The comparison of the amino acid composition showed that aspartic acid, glutamic acid and arginine are different among all the studied algal strains (15 species). On the other hand, some other residues such as glycine and leucine had comparable contents. The study of Murphy et al. (2000), aiming for peroxidase identification and quantification, used a spectrometric method to determine the activity of this enzyme in the presence of urea-hydrogen peroxide coupled with one of many possible factors such as ascorbic acid (290nm), iodide (350nm) and glutathione/NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) (340nm). The molecular weight of the recovered peroxidase was found to be equal to 36 kDa through Sephadex G-75 gel filtration and the protein concentration was identified using the method of Lowry et al. (1951).

2.3 Microalgal lipids

2.3.1. Microalgal lipid content

The total oil and fat content of microalgae ranges from 1% to 70% of the dry weight and tends to be inversely proportional to the rate of growth with greater accumulations during stationary phase. The percent of total lipid as neutral lipid, glycolipid, and phospholipid also

varies widely among and within groups of microalgae (Borowitzka, 1988). Microalgae was reported to produce up to 58,700 Liter/hectare (L/ha) of oil (for strains having 30% oil on biomass weight basis), which is much higher than the oil yield recovered from energy crops such as corn, soybean and canola producing 172 L/ha, 446 L/ha and 1190 L/ha, respectively (Chisti, 2007). Interestingly, certain microalgal species have been shown to synthesize polyunsaturated fatty acids (PUFAs) such as lignoceric acid (C24:0) in the premnesiophyte *lsochrysis,* ω-3 linolenic acid (C18:3) in the golden-brown alga *Ochromonas,* and arachidonic acid (C20:4) in the red microalga *Porphyridium* (Chen et al., 1990). These PUFAs possess high nutritional quality for humans as well as for animals (Spolaore et al., 2006).

2.3.2. Extraction and quantification of microalgal lipids

Schwenzfeier et al. (2011) carried out the lipid quantification gravimetrically after exhaustive extraction of freeze dried samples by Soxhlet extraction approach using chloroform/methanol (2:1 v/v) as a solvent. In the study of Chiu et al. (2009), a sample of lyophilized biomass (30 mg) was mixed with chloroform/methanol solution (1:2, v/v) and sonicated for 1 hour. After precipitation of the mixture, chloroform and 1% NaCl solution were then added to give a ratio of methanol, chloroform, and water of 2:2:1, respectively. The mixture was centrifuged at 1000×g for 10 min and the chloroform phase was removed under vacuum in a rotary evaporator and the remainder was weighed as the lipid fraction.

Another case of study done by Yang et al. (2010) dealt with microalgal lipid extraction as follows; for each sample, an amount of cells were weighed and mixed with chloroform solution containing C19:0 fatty acid as an internal standard. The lipids were exhaustively extracted using chloroform/methanol (2:1, v/v). The extracts were dried by rotary evaporator (40°C), re-dissolved in pure chloroform, and then dried again using a nitrogen stream. Dayananda et al. (2010) also extracted and quantified the lipid fraction from *Botryococcus sp.* using chloroform/methanol (1:2) and the common gravimetric method, respectively. Results showed that *Botryococcus sp.* lipid content was equal to 14% on dry weight basis, which allowed authors to admit its relevance for prospective application in biodiesel production. The study by Widjaja et al. (2009) showed that the use of hexane as the solvent in the extraction of lipids from microalgae resulted in poor yield and that the extraction using chloroform/methanol mixture was therefore later employed in further essays.

Prabakaran and Ravindran, (2011) revealed that the sonication of microalgal cells had a good influence on the lipid extraction. Indeed, higher lipid content was observed in all

isolates when using the sonication method as compared to other tested cell disruption methods, including microwaving, osmotic chock, bead beating and autoclaving. The microwave and osmotic shock methods were also efficient among the methods tried while the autoclaving method recorded the lowest efficiency of lipid extraction. This study concluded that the sonication method would be the most applicable for large-scale lipid extraction from microalgae. A similar work carried out by Lee et al. (2010) with *Botryococcus sp.*, *Chlorella vulgaris* and *Scenedesmus sp.* showed the highest lipid yield using microwave oven method. Additionally, the fatty acid profile of three microalgae strains studied by Prabakaran and Ravindran (2011) determined using GC analysis revealed that oleic acid (C18:1) was higher in *Nostoc sp.* and *Tolypothrix sp.* while linoleic acid (C18:2) was found higher in *Chlorella sp.* It is commonly known that as the oleic acid content increases the oxidative stability for longer storage is enhanced. So among the tested microalgal species, *Nostoc sp.* and *Tolypothrix sp.* were the most suitable for the production of good quality biodiesel.

2.3.3. Transesterification analysis of microalgal lipids

In the study of Yang et al. (2010), a lipid transesterification reaction was carried out using methanol and HCl as an acid catalyst. The resulting fatty acid methyl esters (FAME) samples were then analyzed using gas chromatography (GC). The fatty acids contained in each sample were quantified according to their peak area relative to the C19:0 fatty acid internal standard, and expressed as a percentage of total fatty acid content. The process of biodiesel production from microalgae indicated that 90% of the process energy is consumed by oil extraction, indicating that any improvement in lipid extraction will have a significant impact on the economics of the process (Lardon et al., 2009). Moreover, an alternative method of transesterification, termed as in situ or direct transesterification, was suggested by Lepage and Roy (1984) to extract and convert microalgal triglycerides into biodiesel in a single step. This helped to avoid the loss of large quantities of extraction solvents and their phase separation costs, as compared with the conventional method (Vicente et al., 2009; Dufreche et al., 2007). In situ transesterification was also adopted by two recent cases of study by Wahlen et al. (2011) and Ehimen et al. (2010). These new researches investigated the optimal reaction conditions of time, temperature, catalyst concentration, methanol concentration and volume, stirring and moisture content of biomass for maximal biodiesel production from algal biomass.

The study by Wahlen et al. (2011) revealed that, in addition to simplifying the production process, direct transesterification resulted in improved yields of FAME when compared to the conventional extraction followed by oil to biodiesel conversion. In addition, conducting the in situ transesterification reaction using different types of alcohols resulted in approximately equal amounts of FAME. As a result, the lowest cost alcohol (methanol) was selected for the optimization of the in situ transesterification reaction. Then, sulfuric acid was chosen as catalyst for in situ transesterification because acid-catalyzed reactions have been shown to be effective at converting both triacylglycerols (TAG) and free fatty acids (FFA) into FAME.

The results of research conducted by Ehimen et al. (2010) indicated an improvement of the microalgae oil conversion to FAME with increasing temperature and increasing alcohol volume. In addition, biomass drying was shown to play an important role. In fact, the increase in moisture content of the biomass results in significant reductions of the equilibrium of FAME conversion yields. Finally, Biodiesel yields were also shown to improve significantly with process stirring (Ehimen et al., 2010).

2.3.4. Fatty acids composition in microalgae

In microalgae culture, when nutrients become limited, cell division stops and carbon is then stored as lipids and carbohydrates to be used under more favorable conditions (Flynn et al., 1992). Then, the harvest of microalgae strains is recommended at the beginning of the stationary phase when they have a higher cell density and a higher enrichment of ω-3 polyunsaturated fatty acids and docosahexaenoic acid (C22:6) (Roncarati et al., 2004). Obviously, there are huge variations in the fatty acid composition between different microalgae. These variations depend on species, culture techniques and physical factors such as light intensity, pH, salinity, temperature and CO_2 supply (Roncarati et al., 2004). Table 2.2 and Table 2.3 reveal the variability of the fatty acid profiles of a couple of microalgal strains belonging to the Heterokontophyta phylum (diatoms class) and to the Chlorophyta phylum (Eustigmatophycea and Prymnesiophycea classes), respectively.

Table 2.2. Fatty acid composition (% of total fatty acids) of four diatoms (De Viçose et al., 2012).

Heterokontophyta (Diatoms)				
Fatty acids	*Amphora sp.*	*Navicula incerta*	*Nitzschia sp.*	*Proschkinia sp.*
Saturated fatty acids	32.53	31.52	26.08	28.63
C14:0	7.37	5.83	10.00	2.15
C16:0	20.54	18.06	10.74	21.85
Monounsaturated fatty acids	31.99	41.65	31.44	34.83
ω-7 C16:1	25.31	32.14	24.72	29.40
ω-9 C18:1	3.87	4.27	3.85	1.64
Polyunsaturated fatty acids	34.66	33.01	48.30	37.28
ω-3 Polyunsaturated fatty acids	23.30	26.35	33.50	26.15
ω-3 C20:5	16.46	21.8	18.87	19.52
ω-3 C22:6	1.64	0.07	2.34	3.81

As shown in Table 2.2, the fatty acid profiles of the diatoms were variable but they remain typical of the diatom family. The fatty acid profiles of the diatoms tested were characteristic of most diatoms, presenting high proportions of 16:0, 16:1 and Ecosapentaenoic acid (EPA, C20:5) (De Viçose et al., 2012).

Table 2.3. Fatty acid composition (% of total lipids) of four Chlorophyta microalgae strains, analyzed during the stationary phase (Roncarati et al., 2004).

Chlorophyta phylum				
Fatty acids	*Nannochloropsis sp.*	*Nannochloropsis oculata*	*Nannochloris atomus*	*Isochrysis sp.*
Saturated fatty acids				
C14:0	6.03	8.38	**0.72**	8.05
C16:0	32.88	26.08	24.62	12.25
C18:0	0.67	0.98	0.98	0.85
Monounsaturated fatty acids				
C16:1	27.04	27.94	**2.01**	**4.59**
C18:1	17.38	12.99	10.18	22.65
Polyunsaturated fatty acids				
ω-6 C18:2	2.66	4.15	**16.94**	5.11
ω-6 C18:3	0.05	0.15	**28.70**	8.87
ω-3 C20:5	9.03	12.50	**0.14**	**0.57**
ω-3 C22:6	0.00	0.03	0.00	12.40

Differences of fatty acid profiles (highlighted in Table 2.3) are related to the taxonomic group and they do exist even within the same algal strain, mainly in relation to the culture phase and the exposure to different concentrations of carbon dioxide (Roncarati et al., 2004).

2.4. Microalgal to biofuel conversion technologies

To date, there are various sophisticated technologies employed for mass production and processing of photoautotrophic microalgae, the annual world production of all species is estimated to be 10,000 tones/year (Richmond, 2004).

The conversion technologies of microalgal biomass to biofuel can be divided into two basic categories including thermochemical and biochemical conversion. The desired form of energy, the economic aspects and the project specificity are among the primary factors that influence the choice of the conversion process (Brennan and Owende, 2010). Figure 2.3 summarizes almost all conversion technologies of microalgal biomass to different forms of biofuels.

2.4.1. Thermochemical conversion

Thermochemical conversion is to thermally decompose the organic components in biomass to yield fuel products. This includes direct combustion, gasification, thermochemical liquefaction and pyrolysis (Tsukahara and Sawayama, 2005).

2.4.1.1. Gasification

Gasification is based on a partial oxidation of biomass into a combustible gas mixture under high temperatures (800–1000 ºC) (Clark and Deswarte, 2008). Gasification characteristics of microalgal biomass have been investigated by several researchers. Hirano et al. (1998) partially oxidized *Spirulina* at temperature ranging from 850 to 1000 ºC, and determined the gas composition required to generate theoretical yield of methanol of 64 % (0.64 g methanol from 1 g of biomass). Minowa and Sawayama (1999) also conducted a gasification assays on the microalgae *C. vulgaris* in using a novel system with nitrogen cycling to obtain methane-rich fuel with all the nitrogen component of the microalgae converted into fertilizer ammonia.

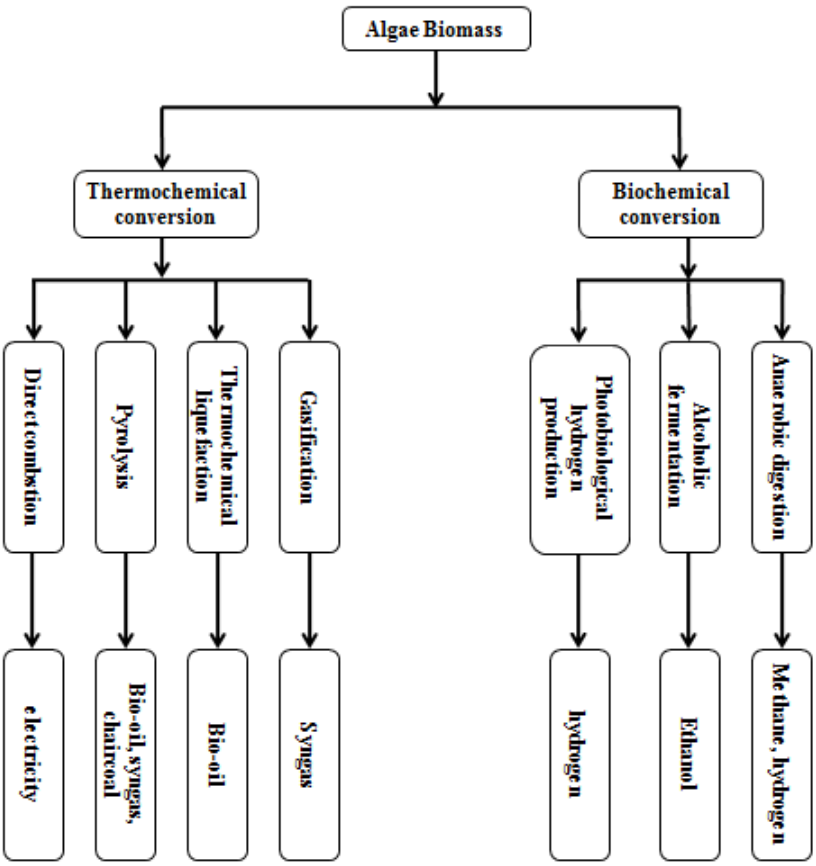

Figure 2.3. Potential algal biomass conversion processes (adapted from Tsukahara and Sawayama, 2005).

2.4.1.2. Thermochemical liquefaction

Thermochemical liquefaction is the process of conversion of wet algal biomass material into liquid fuel. It is a low-temperature (300–350 ºC) and high pressure (5–20 MPa) process aided by a catalyst in the presence of hydrogen to yield bio-oil (Goyal et al., 2008). Dote et al. (1994) successfully applied thermochemical liquefaction at 300 ºC on *B. braunii* and reached a maximum oil yield equal to 64% dry weight basis. The resulting higher heating value (HHV) was important (45.9 MJ kg-1) and the energy balance of the process was positive. In a similar study, an oil yield of 42% dry weight was obtained from *Dunaliella tertiolecta* microalgae giving a HHV of 34.9 $MJ.kg^{-1}$ and a positive energy balance (Minowa et al., 1995).

2.4.1.3. Pyrolysis

Pyrolysis is the conversion of biomass to bio-oil, syngas and charcoal at medium to high temperatures (350–700 ºC) in the absence of air (Goyal et al., 2008). Flash pyrolysis, at moderate temperature (500 ºC), short hot vapor residence time (about 1s), is considered as a promising technology producing biomass derived liquid fuels that could replace the fossil-fuels. This is because of the high biomass-to-liquid conversion ratio (95.5%) that can be achieved (Demirbas, 2006). Demirbas (2006) experimented with *Chlorella prothothecoides*, and achieved a HHV of 39.7 MJ kg^{-1}, at temperatures ranging from 502 to 552 ºC. Furthermore, this latter author indicated that bio-oils from microalgae are of a higher quality than those extracted from lignocellulosic materials.

2.4.1.4. Direct combustion

Direct combustion process is based on burning the biomass in the presence of air to convert the stored chemical energy in biomass into hot gases (Goyal et al., 2008), usually in a furnace, boiler, or steam turbine at temperatures above 800 ºC. Direct combustion is only feasible for biomass with moisture content less than 50% of dry weight. Combustion of biomass for heat, power, and steam ranges from very small scale utilities up to large-scale industrial processes in the range of 100–300MW (Mc Kendry, 2002). Researches on the feasibility of the use of algal biomass in direct combustion in literature is still limited, but a life cycle assessment (LCA) of coal-algae co-firing (Kadam, 2002) suggested that this fuel could lead to lower greenhouse gas (GHG) emissions and air pollution.

2.4.2. Biochemical conversion

The biochemical conversion processes of biomass into other fuels include anaerobic digestion, alcoholic fermentation and photobiological hydrogen production (USDOE, 2002).

2.4.2.1. Anaerobic digestion

Anaerobic digestion is the conversion of organic wastes into a biogas, primarily methane (CH_4) and carbon dioxide, with traces of other gases such as hydrogen sulphide. Anaerobic digestion process is appropriate for high moisture content (80–90% moisture) in organic wastes (Mc Kendry, 2002) and can be useful for wet algal biomass. The anaerobic digestion process is done in three sequential steps of hydrolysis, fermentation and methanogenesis. In hydrolysis the biomass macro molecules are broken down into soluble

sugars. Then, fermentative bacteria convert these sugars into alcohols, acetic acid, volatile fatty acids (VFAs), and a gas containing H_2 and CO_2. This complex is later metabolized into CH_4 (60–70%) and CO_2 (30–40%) by methanogens (Cantrell et al., 2008). It has been estimated that the energy resulting from algal anaerobic digestion is comparable to that obtained from the extraction of cell lipids, with the possibility of recycling the nutrient rich waste product as a new algal growth medium (Olguı´n, 2000 ; Phang et al., 2000).

2.4.2.2. Alcoholic fermentation

Alcoholic fermentation is the conversion of biomass materials containing sugars, starch or cellulose into ethanol (Mc Kendry, 2002). The biomass is pretreated to produce starch that is converted to sugars mixed with water and yeast and kept warm in large fermenters. A purification process (distillation) is required to separate the diluted alcohol product (10–15% ethanol) from water and other impurities in the medium. The concentrated ethanol is later condensed into liquid form, which can be used as a supplement or substitute for gasoline in cars. The solid waste from the process can be used for cattle-feed or for gasification. This helps offset feedstock costs which typically represents up to 55–80% of the final alcohol selling price. Microalgae such as *C. vulgaris* are a good source of ethanol due to the high starch content (37 % dry weight), and for which up to 65% ethanol conversion efficiency has been reported (Hirano et al., 1997).

2.4.2.3. Photobiological hydrogen production

Hydrogen (H_2) is a naturally occurring molecule, which is a clean and efficient energy carrier. Microalgae possess the necessary genetic, metabolic and enzymatic characteristics to photoproduce H_2 gas, under anaerobic conditions. In fact, there are two fundamental approaches for photosynthetic H_2 production from water. The first H_2 production process is a two stage photosynthesis process where photosynthetic oxygen production and H_2gas generation are separated from each other (Ghirardi et al., 2000). The second approach involves the simultaneous production of photosynthetic oxygen and H_2 gas. Melis and Happe (2001) found that using the two-stage photosynthesis process, the theoretical maximum yield of hydrogen produced by green algae could be about 198 kg H_2 ha^{-1} per day.

2.4.2.4. Algal biomass-to-biodiesel

Biodiesel is a mixture of monoalkyl esters of long chain fatty acids derived from a renewable lipid feedstock such as algal oil. After the extraction processes, the resulting algal oil can be converted into biodiesel through a process called transesterification. Algal biodiesel has similar physical and chemical properties to petroleum diesel and compares favorably with the international biodiesel standard for vehicles (EN14214). Algal biodiesel has several advantages over petroleum diesel in that it is derived from biomass and therefore it is renewable and biodegradable. Biodiesel is also non-toxic and contains reduced levels of particulates, carbon monoxide, soot, hydrocarbons and SO_x. It must be noted that compared to first generation biodiesel, algal biodiesel is more suitable for use in the aviation industry where low freezing points and high energy densities are key criteria. Another major advantage of algal biodiesel is the decrease of CO_2 emissions of up to 78% compared to emissions from petroleum diesel (Sheehan et al., 1998).

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Chapter 3

Material and Methods

3.1. Material

3.1.1. Strains

Nannochloris sp. (Chlorophyta phylum, *Chlorophyceae* class) and *Amphora sp.* (Heterokontophyta phylum, diatom class) are two marine microalgae collected from Sfax coast, Tunisia. Specimens were previously isolated through serial dilutions method and identified morphologically and genetically, through an advanced molecular analysis of the microalgal 18S ribosomal DNA, by the research group of the Tunisian Collection of Microorganisms laboratory (CTM) in the Center of Biotechnology of Sfax (CBS).

3.1.2. Culture medium

The following medium compositions are F/2 Provasoli medium with some modifications by the research team of the CTM Laboratory for improved growth of the microalgal strains.

Table 3.1. *Nannochloris sp.* medium composition.

Components	Quantity per liter of cultural medium
$NaNO_3$ solution	75 mg
NaH_2PO_4 solution	2 mg
Vitamin B	0.06 mg
Na_2EDTA	0.075 mg
$ZnSO_4$ (7 H_2O)	0.0165 mg
$CoSO_4$ (6 H_2O)	0.0075 mg
$MnCl_2$ (4 H_2O)	0.135 mg
Na_2MoO_4 (4H_2O)	0.00473 mg

Table 3.2. *Amphora sp.* medium composition.

Components	Quantity per liter of cultural medium
$NaNO_3$ solution	100 mg
NaH_2PO_4 solution	10 mg
Na_2SiO_3 solution	80 mg
Vitamin B1	0.035 mg

In addition, the culture medium consisted of sea water brought from the littoral near the Center of Biotechnology of Sfax.

3.1.3. Reagents and buffers

3.1.3.1. Protein analysis

Protein assay reagents

Pierce Bicinchoninic acid (BCA) protein assay kit and bovine serum albumin (BSA) standard (2 mg/ml) was purchased from Pierce (Rockford, IL, USA).

SDS-PAGE reagents

All sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) products including NuPAGE LDS (lithium dodecyl sulfate) sample buffer, NuPAGE reducing agent and 2-(N-morpholino)ethanesulfonic acid (MES) (1×) SDS running buffer (50 ml 20× NuPAGE MES- SDS Running Buffer, 950 ml deionized water) were purchased from Invitrogen (Carlsbad, CA, USA).

Malate dehydrogenase assay reagents

0.006 M oxaloacetic acid and 0.00375 M NADH were freshly prepared in 0.1 M phosphate buffer, pH 7.4.

Peroxidase assay reagents

For peroxidase assay in the presence of 4-aminoantipyrine (4-AAP), 0.05 mM urea-H_2O_2 was prepared in 0.2 potassium phosphate buffer (pH 7.0) and 0.0025 M 4-AAP was prepared in 0.17 M phenol solution. For peroxidase assay in the presence of O-dianisidine, 0.75 mM of urea-H_2O_2 and 0.5 mM of O-dianisidine were prepared in 0.2 potassium phosphate buffer (pH 6.0). Substrates were prepared freshly before peroxidase enzyme assay.

Catalase assay reagents

0.059 M hydrogen peroxide (30%) was prepared in 0.05 M potassium phosphate (pH 7.0).

Cytochrome C oxidase assay reagents

100 mM potassium phosphate buffer (pH 7.0) (**Reagent A**). 10 mM potassium phosphate buffer (pH 7.0) (2 liters were prepared in deionized water using Reagent A and adjusted to pH 7.0, **Reagent B**). 1.0 % (w/v) cytochrome C reduced solution (100 mg of cytochrome C was dissolved in 8 ml of Reagent B. The cytochrome C was reduced by adding 5 mg of L-ascorbic acid, sodium salt. The excess of ascorbate was removed by dialyzing against Reagent B for 24 hours at 0 - 4 ° C with three changes of buffer. The dialysate was then removed and brought up to a final volume of 10 ml with Reagent B, **Reagent C**). 100 mM potassium ferricyanide solution (**Reagent D**). 250 mM sucrose solution was prepared with 1% (v/v) tween 80 (**Reagent E**). Cytochrome oxidase C enzyme solution (Immediately before use, a solution containing the crude extract was prepared in cold Reagent E, **Reagent F**).

3.1.3.2. Lipid analysis

1 mM ethylene diamine tetraacetic acid (EDTA) (Sigma, St. Louis, MO, USA) was dissolved in 0.15 M acetic acid solution. Other substrates used for lipid analysis include methanol, chloroform, 0.8% (v/v) KCl solution, hexane, 5% (v/v) sulfuric acid in methanol, 2% (v/v) sulfuric acid in methanol, 0.2% (w/v) C17:0 triacyl glycerol (TAG) standard.

3.2. Methods

3.2.1. Microalgae culture

The strains were primarily cultured in 1 liter Erlenmeyer scale under continuous illumination. The source of carbon was introduced by bubbling CO_2 gas, through a fine membrane filter (cutoff = 0.22 µm), into the culture medium. The scale up of the cultures was performed in two photobioreactors containing 8 liters of culture medium in the case of *Amphora sp.* and 10 liters in the case of *Nannochloris sp.* These cultures were maintained for 15 days. At this step, the beginning of the stationary growth phase was reached for both microalgal strains.

3.2.2. Biomass harvesting

The culture media were concentrated using microfiltration columns. The retentate (nearly 1 liter volume) contained concentrated microalgae. The cells were recovered by centrifugation at 9110×g (Fisher Scientific, Waltham, MA, USA) for 10 minutes. Pellets belonging to the same microalgal strain were pooled together using a minimum sterile water volume and then maintained at -80 ºC overnight. The lyophilization cycle was carried out the next day at -80 °C. The lyophilized samples were recovered in sterile plastic pots and conserved at -20 °C until further assays.

3.2.3. Dry mass determination

Dry mass was determined based on lyophilized and wet microalgal biomass. A mass of lyophilized microalgae equal to 50 mg was put on Watman paper (10 cm× 6 cm) and kept in the oven at 105 °C. The change of mass was followed until a constant weight was obtained. For wet biomass analysis, a quantity of biomass was harvested from the culture by centrifugation in conical tubes. The pellet was then gathered, weighed and kept in the oven at 105 ºC until a constant weight was obtained. Two replicates per each strain were utilized to determine the microalgal dry mass.

3.2.4. Cell disruption and protein aqueous extraction

The protein fraction was extracted from microalgae in aqueous phase (water) with reference to the best conditions investigated by Barbarino and Lourenço (2005) with some modifications. In this study, 50 mg of lyophilized microalgae were added to 4 ml of potassium

phosphate buffer (20 mM, pH 7.4) and 400 µl of phenylmethylsulfonyl fluoride (PMSF: endogenous proteases inhibitor) solution (1M). The mixture was kept at 4 °C for 15 hours and then sonicated in order to promote cell disruption. This experiment was conducted according to the method by Murphyet al. (2000) using disruption up to 7 min (30 s bursts, 1 min cooling between bursts), with a Sonic Dismenbrator (Fisher Scientific, Waltham, MA, USA) fixed at 30 % of amplitude. The resulting mixture was centrifuged at 15,000×g for 20 min, and the supernatant was used as the cell-free extract. 1 ml of 0.1 M NaOH was added to the pellet and kept for 1 hour at room temperature with shaking occasionally. This was later centrifuged at 15,000×g for 20 min at room temperature; the supernatant was collected and combined with the first one. This crude extract was used for protein quantification and further analyses.

3.2.5. Protein quantification

Protein quantification was done using the Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). This kit is a detergent-compatible formulation based on BCA for the colorimetric detection and quantitation of total proteins. The Bovine serum albumin (BSA) was used as the standard protein in this experiment. A series of dilutions of known concentration were prepared from the BSA protein and assayed alongside the unknown(s) before the concentration of each unknown was determined based on the standard curve. All assays were carried out in 96-well flat bottom Greiner microtiter plates (USA Scientific, Ocala, FL, USA) and performed in duplicates. For the protein assay, 25 µL of sample or standard was added to each empty well followed by addition of 200 µL of prepared BCA reagent as specified by the manufacturer. The samples were allowed to incubate at 37 ºC temperature for 30 minutes. Absorbance measurements were read at 562 nm on a synergy microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

3.2.6. SDS-PAGE

Different crude extract pretreatments were carried out in order to determine the best contrast of the Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile. Indeed, these pretreatments tend to eliminate the impurities that interfere with the protein fraction and to concentrate the protein sample before its application to SDS-PAGE. To do so, diafiltration, dialysis plus ultrafiltration, dialysis plus freeze drying and unique freeze drying the crude extract were investigated before SDS-PAGE. The SDS-PAGE was applied under reducing conditions using NuPAGE Novex Bis-Tris Mini gel (Stacking gel: 4%,

Separating gel: 12%) and *XCell SureLock* Mini Cell that were purchased from Invitrogen company (Carlsbad, CA, USA). 5 µL of sample was mixed with 2.5 µL NuPAGE LDS sample buffer, 1 µL NuPAGE reducing agent and 1.5 µL deionized water. The samples were vortexed and heated at 70 ºC for 10 min in a water bath. The gels were run for 35 minutes at 200 V. The concentration of protein applied per well was equal to 10 µg. The NuPAGE MES (2-(N-morpholino) ethanesulfonic acid) SDS (1×) solution was used as a running buffer during electrophoresis. After running, the gels were washed with deionized water and stained with Coomassie blue SafeStain for 1 hour. The gels were scanned with a Bio-Rad ChemiDoc XRS imager (Bio-Rad, Hercules, CA, USA) and analyzed using Quantity One Software.

3.2.6.1. Diafiltration

The crude extract was diafiltred in small centrifugal filters with 10 KDa as a cutoff (Millipore, Bedford, MA, USA). The centrifugation was run at 15,790×g for 5 minutes. Then the concentrate was washed with 20 mM potassium phosphate buffer, (pH 7.4). Finally, a reverse centrifugation was carried out at 2,430×g for 3 minutes, to recuperate the concentrate in new empty centrifugal tubes. This retentate is later used to carry out SDS-PAGE analysis.

3.2.6.2. Dialysis plus ultrafiltration

The dialysis of the crude extract was done in dialysis cassette (Pierce, Rockford, IL, USA) against 20 mM potassium phosphate buffer, (pH 7.4). The buffer was replaced twice in the first 4 hours and then kept overnight under agitation. Dialyzed samples are then recovered via a syringe needle and concentrated using ultrafiltration. Practically, samples were ultrafiltred at 15,790×g for 5 minutes, in centrifugal membrane filters.

3.2.6.3. Dialysis plus freeze drying

The dialysis was run as described before, then the resulting volume was concentrated using the freeze drying machine.

3.2.6.4. Freeze drying

The crude extract is concentrated directly using freeze drying method in Eppendorf tubes containing 500 µl each for 2 hours.

3.2.7. Enzyme assays

Some enzyme assays were carried out with microalgal protein aqueous fraction to identify the presence of certain enzymes.

3.2.7.1. Malate dehydrogenase

2.6 ml of 0.1 M phosphate buffer was introduced into a cuvette. 0.2 ml of 0.00375 M NADH and 0.1 ml of 0.006 M oxaloacetate were added to the cuvette. The mixture was incubated in the spectrophotometer for 3-4 minutes to achieve temperature equilibration. 0.1 ml of sample (crude extract) was added to the cuvette and the decrease of absorbance at 340 nm (A_{340}) was recorded for 3-5 minutes. The variation of A_{340} (ΔA_{340}) per minute was calculated from the initial linear portion of the curve. A unit of enzyme activity (U) represents one micromole of substrate consumed per minute. The formula used to calculate the dehydrogenase activity (Worthington, 1993) is shown below.

$$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{\frac{6.22 \times \text{mg of enzyme}}{\text{ml of reaction mixture}}} \qquad (1)$$

3.2.7.2. Peroxidase

1.4 ml of 0.0025 phenol/aminoantipyrine solution and 1.5 ml of 0.0017 M hydrogen peroxide were introduced into a cuvette. The mixture was incubated in the spectrophotometer at 25°C for 3-4 minutes to achieve temperature equilibration. 0.1 ml of sample (crude extract) was added and the increase of A_{510} was then recorded for 4-5 minutes. ΔA_{510}/minute was calculated from linear portion of the curve. A unit of enzyme activity (U) represents one micromole of product synthesized per minute. The peroxidase activity was calculated using the following formula (Worthington, 1993).

$$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{510}}{\text{min}}}{\frac{6.58 \times \text{mg of enzyme}}{\text{ml of reaction mixture}}} \qquad (2)$$

3.2.7.3. Catalase

1.9 ml of reagent grade water and 1.0 ml of 0.059 M hydrogen peroxide were introduced into a cuvette. The mixture was incubated in the spectrophotometer for 4-5 minutes to achieve temperature equilibration. 0.1 ml of sample (protein crude extract) was

then introduced and the decrease in absorbance at 240 nm was recorded for 2-3 minutes. ΔA_{240}/min was calculated from the initial linear portion of the curve. The catalase activity was calculated using the following formula (Worthington, 1993).

$$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{240}}{\text{min}} \times 1000}{\frac{43.6 \times \text{mg of enzyme}}{\text{ml of reaction mixture}}} \qquad (3)$$

3.2.7.4. Cytochrome C oxidase

Reagents shown in Table 3.3 were detailed in the Material part in the paragraph of cytochrome C oxidase assay reagents.

Table 3.3. Composition of reagents mixture for cytochrome C oxidase assay.

Reagents	Test	Blank
Reagent B	2.7 ml	2.7 ml
Reagent C (cytochrome C, Reduced)	0.2 ml	0.2 ml
Reagent D (Potassium Ferricyanide)	-	0.1 ml
Mix by inversion and equilibrate to 37 °C. Then add:		
Reagent F (Enzyme solution)	0.1 ml	-

The blend, introduced into a cuvette, was mixed by inversion immediately and the decrease in A_{550nm} was monitored for 10 minutes, recording the A_{550} nm values at one minute intervals. The formula used to calculate $\Delta A_{550\ nm}$ (ΔE) is shown below.

$$\Delta E = E_t - E_f \qquad (4)$$

E_t = the A_{550} nm value at the time points 0 through 10 minutes for the test.

E_f = the A_{550} nm value at the 10 minutes time point for the blank.

Ln (ΔE) was plotted versus time and the slope (M) was determined. The activity was calculated using Equation 5. The cytochrome C oxidase activity was calculated using the following formula (Wharton and Tzagoloff, 1967).

$$\frac{\text{Units}}{\text{mg solid}} = \frac{(\text{M})}{\frac{\text{mg solid}}{\text{RM}}} \quad (5)$$

M = Slope of Ln (ΔE) versus time, RM = Reaction mixture volume, mg solid = quantity of total protein introduced in the mixture.

3.2.8. Lipids extraction and quantification

3.2.8.1. Lipids extraction starting from wet biomass

The microalgal culture was harvested as previously described. The pellet was weighed and used as the starting wet biomass material. This biomass was dried at 105 ºC for 1 hour. In fact, the drying step has been shown to enhance the lipid extraction process and lipids to FAMEs conversion yields (Ehimen et al., 2010). The extraction of lipids from microalgal strains was carried out based on the Bligh and Dyer, (1959) protocol with some modifications; the dried sample was blended with 1 ml of 1 mM EDTA in 0.15 M acetic acid solution and pulverized in a mortar to promote cell disruption. The resulting mix was transferred into glass tubes and supplemented with 3 ml of methanol/chloroform (2:1) and vortexed for 10 minutes. 1 ml of chloroform and 0.8 ml of 0.8% KCl were then added to the solution. The reaction mix was slightly vortexed and centrifuged for 2 minutes at 3,640×g under 4 ºC. The lower phase was taken aside (extracted) in another glass tube previously weighed. 1 ml of hexane was added to the remaining phase (upper phase) to extract the rest of lipids. A second centrifugation was then done in the same operating conditions, and the supernatant was added to the first lipid extract. The solvent fraction was evaporated under nitrogen flow and the remaining lipid content was determined gravimetrically as compared to the empty weighed tube and expressed in terms of the starting wet mass and relevant dry mass.

3.2.8.2. Lipids extraction starting from lyophilized biomass

The same method of Bligh and Dyer (1959) applied in the previous section was applied on the lyophilized microalgal biomass in order to compare the profile of lipids extracted from different starting biomass. The elimination of moisture was ensured in this

section by evaporation under low temperature (lyophilization). Practically, 50 mg of lyophilized biomass was used for the lipid extraction process, without the drying step, and the fatty content was then assessed based on the gravimetric method.

3.2.9. Lipids transesterification

Prior to gas chromatography-mass spectrometry (GC-MS), the extracted lipid fraction was transesterified in the presence of methanol to produce fatty acid methyl esters (FAME) that can be analyzed by GC-MS (Agilent Technologies). To do so, 1 ml of 5% sulfuric acid (H_2SO_4) in methanol was added to the lipid extract. H_2SO_4 acts as a catalyst for the transesterification process since it shortens the reaction time and enhances the specificity. Indeed Ehimen et al. (2010) mentioned that acidic catalysts foster better FAME yields from microalgal lipids than alkaline catalysts (e.g. NaOH), under the same reaction conditions. Furthermore, sulfuric acid was chosen as a catalyst in the studies of Wahlen et al. (2011) because acid-catalyzed reactions are effective at converting both triacylglycerols (TAG) and free fatty acids (FFA) into FAMEs. This mainly because microalgal lipids have high free fatty acid content which leads to saponification reactions if mixed with alkaline catalysts (Ehimen et al., 2010). Moreover, 5 µg of the internal standard C17:0 was introduced into the reaction mixture (1 ml). The transesterification step consists of the incubation of the mixture of lipids, methanol and catalyst at 80 ºC, in a hot water bath, for 90 minutes. Later, 1.5 ml of hexane was added to dissolve the FAME fraction. The upper phase was extracted and another 1.5 ml of hexane was added to the lower phase in order to extract the remaining esters. The resulting FAME fraction was analyzed by GC-MS for fatty acids composition.

3.2.10. In situ transesterification

Recent studies have emphasized on the production of biodiesel from plant and microalgal oils in a single step using the in situ transesterification process. Actually, it is highly valuable to be able to extract and convert triglycerides in microalgae directly into biodiesel, bypassing the use of large quantities of organic solvents (Ehimen et al., 2010).

Hence, in situ transesterification was conducted in this study using lyophilized samples aiming to compare the lipid compositions and FAMEs yields between the conventional transesterification and the direct transesterification processes. To do so, 50 mg of lyophilized biomass was incubated with 2 ml of methanol containing 2 % H_2SO_4, with reference to the best operating conditions conducted by Wahlen et al. (2011). The mix was

vortexed for 10 minutes to increase the homogeneity and dissolution of the microalgal cells in methanol. The in situ transesterification was carried out at 80 ºC, in a hot water bath, for 90 minutes. C17:0 fatty acid was also introduced in this assay as an internal standard. Then, the resulting FAMEs fraction, extracted using hexane, was analyzed by GC-MS for fatty acids yield and content.

3.2.11. GC-MS conditions

The GC-MS analysis of FAMEs was performed on Agilent 6890-N gas chromatography instrument coupled with an Agilent MS-5975-B inert mass selective detector (MSD) and an Agilent autosampler 7683-B injector (Agilent Technologies, Little Fall, NY, USA). A non-polar capillary column HP-5MS (5% phenyl methylsiloxane) with dimension of 30 m × 0.25 mm and 0.25 µm film thickness (Agilent Technologies, Palo Alto, CA, USA) was used for the separation of fatty acid methyl esters. The injection volume was 1 µl. The initial oven temperature of 100 ºC was raised to 190 ºC at the rate of 20 ºC/min, kept at 190 ºC for 15 min, raised again from 190 ºC to 280 ºC at the rate of 4 ºC/min and hold at 280 ºC for 5 min. The splitless mode was used for the front inlet, and helium was used as a carrier gas with a constant flow rate of 1 ml/min and an average velocity equal to 37 cm/s. The MSD transfer line heater temperature was 280 ºC and the temperature of the injector was 250 ºC. Characterization and identification of FAMEs was performed in the full scan mode. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 50–550 m/z. Data acquisition and processing were performed with the ChemStation 6890 Scale Mode software. GC-MS chromatogram obtained was compared with Wiley7n library which provides reliable information about the identification of fatty acid present in the samples. The match quality of fatty acids was only retained for values equal or higher than 90% of similarity to the authentic data base standards.

The oven heating cycle previously described was not efficient for the analysis of FAMEs extracted from *Nannochloris sp*. wet mass. Therefore, a new heating cycle had been processed in this case. In fact, the oven temperature was initiated at 160 ºC, then raised to 220 ºC at the rate of 2 ºC/min, kept at 220 ºC for 10 min, increased again to 300 ºC at the rate of 10 ºC/min and finally hold at 300 ºC for 10 minutes. The other GC-MS conditions were kept similar as advanced above.

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Chapter4

Results and Discussions

4.1. Dry mass determination

In this project, microalgae were tested under two forms including wet mass and lyophilized mass. Therefore, dry mass was determined starting from both microalgal mass natures to ensure the extrapolation of relevant results on dry basis, independently of the feedstock pretreatment method. The dry mass of *Nannochloris sp.* microalgae was equal to 86.2 ± 3.8% (w/w) of lyophilized mass, after 48 hours of drying at 105 °C. Its dry content based on wet mass was equal to 28.3 ±2.3% (w/w) determined after incubation 24 hours at 105 ºC. Under the same respective conditions as for *Nannochloris sp.*, the dry mass of *Amphora sp.* microalgae was equal to 78.6 ±3.6% (w/w) of lyophilized mass and 10.7 ±0.9% (w/w) of wet mass. These values are comparable with the range of dry mass values based on wet mass determined in the study by Schwenzfeier et al. (2011). The dry weight starting from the wet mass, where the water quantity is high, is lower than that based on lyophilized biomass. Indeed, starting from the same mass of microalgae, the dry matter is higher considering the lyophilized mass as compared to wet mass. However, it seems that water evaporation from lyophilized mass took longer time to evaporate as compared to that of wet mass. This can be explained by the difficulty of evaporation of structural water (binding water) of the relatively dry cells in the lyophilized microalgae and the ease of water evaporation from freshly recovered cells out from the aqueous culture. This analysis is based on the relative cell wall resistance to water elimination under heat.

4.2. Protein quantification

Total protein concentration was measured with spectrophotometric method using BCA assay and bovine serum albumin (BSA) as a standard protein. Each concentration of BSA dilutions was tested in duplicate. Absorbance at 562 nm of each BSA dilution was recorded and shown in Table 4.1.

Table 4.1. Diluted BSA standards and their absorbance at 562 nm.

A 562 (Replicate1)	A 562 (Replicate2)	A562 nm (mean value)	BSA concentration (µg/ml)
0.176	0.17	0.173	0
0.233	0.258	0.2455	25
0.392	0.389	0.3905	125
0.596	0.59	0.593	250
0.987	0.96	0.9735	500
1.642	1.643	1.6425	1000

The standard curve of the BSA concentrations versus their absorbance at 562 nm (A_{562}) is shown in Figure 4.1. This curve allows the determination of the microalgal protein concentrations.

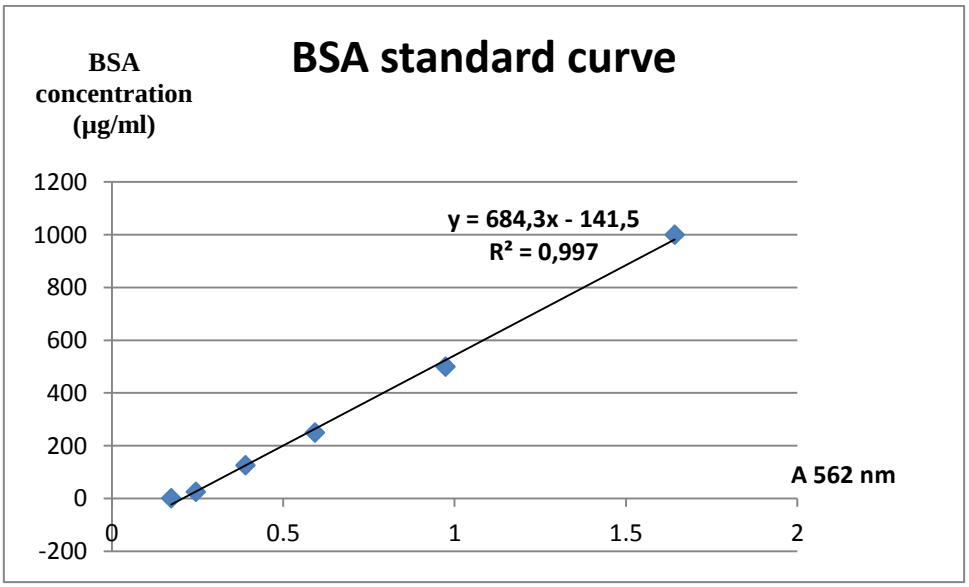


Figure 4.1. BSA protein standard curve.

For *Nannochloris sp.*, the protein content was 16.69 ±4.07% (w/w) of dry mass (14.36 ±3.44% (w/w) of lyophilized microalgae). This strain has comparable protein content with some other Chlorophyta species reported by Barbarino and Louren¸co (2005). Another species of the same class, *Nannochloris oculata*, was shown to have a higher protein content of 23% on dry basis (Becker, 2006).The protein content of *Amphora sp.* was 39.89 ±2.09% (w/w) of dry mass (31.29 ±0.79% (w/w) of lyophilized microalgae). *Amphora sp.* has a high protein quantity (39.89 ± 2.09% of dry mass), similar to that of *Porphyridium cruentum* (28-39 % dry basis) (Kim and Kang, 2011), which is considered as one of the commercially

valuable strains. *Amphora sp.* presents a protein content much higher than that reported by De Viçose et al. (2012) for the same species (18.5 % Dry weight). The variability of protein contents between species of the same class can be attributed primarily to differences in culture, harvesting and pretreatment conditions of these species.

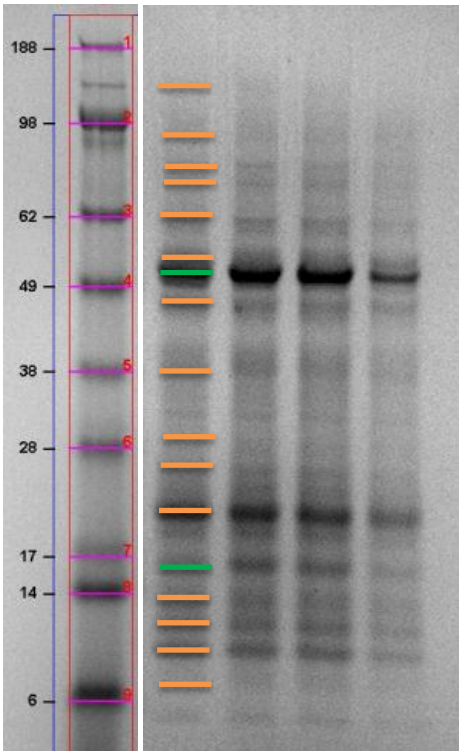
4.3. Protein characterization

4.3.1. SDS-PAGE

SDS-PAGE analyses were conducted to investigate the intrinsic protein profiles of the studied microalgal strains *Nannochloris sp.* and *Amphora sp.* An SDS-PAGE profile, characterized by the number of protein bands and their molecular weight, can be useful for the determination of the nature of some proteins by conducting a comparative study with SDS-PAGE profiles of other microalgal strains.

4.3.1.1. Nannochloris sp

The SDS-PAGE profile obtained from *Nannochloris sp.* protein crude extract is shown in Figure 4.2. This figure shows the effect of different pretreatments applied to the crude extract prior to running the gel electrophoresis. These pretreatments were applied to select better profiles with less contrast background.



M A B C D

Figure 4.2. SDS-PAGE profile of *Nannochloris sp.* proteins stained with Coomassie blue SafeStain**:** 10 µg of protein per well: M: molecular weight marker (kDa), A: diafiltred crude extract, B: dialyzed+ ultrafiltred sample, C: dialyzed+ freeze dried sample, D: freeze dried crude extract.

The different optimized pretreatment methods gave similar high resolutions in this case. Indeed, all lanes showed clear protein SDS-PAGE profile. Interestingly, a protein SDS-PAGE profile presents an intrinsic "footprint", characteristic to each microalgal species although some similarities always exist among the same phylum and genera. This specific profile is useful in further proteomic studies of different microalgal species (Wang et al., 2003). Furthermore, a clear SDS-PAGE profile is highly required for further protein characterization using sequencing starting directly from the gel bands, for instance in the case of Edman sequencing (Smith, 2001).

In addition, only concentrating the protein crude extract from *Nannochloris sp.* using freeze dryer (Lane D) led to clear SDS-PAGE profile. This proves that the current protein extraction method efficiently resulted in low interfering molecules with the protein fraction. SDS-PAGE gel was analyzed using Bio-Rad densitometer software and the results are shown in Table 4.2.

Table 4.2. Molecular weight identification of *Nannochloris sp.* protein bands using Bio-Rad densitometer.

Band Number	MW (kDa)
1	114.6
2	95.4
3	87.5
4	72.6
5	69.6
6	55.7
7	52.6
8	47.6
9	34.4
10	29.9
11	27.4
12	23.6
15	15.0
16	12.9
17	10.7
18	9.6
19	8.4

Previous studies of microalgal SDS-PAGE profiles showed that bands with the highest intensity, as revealed in the present study, represent proteins with a molecular size of about 50, 40, 25 and 15 kDa under reducing conditions. Schwenzfeier et al. (2011) also carried out an SDS-PAGE analysis on the commercial microalgae *Tetraselmis sp.* and identified a distinct band with a molecular mass of about 50 kDa and 15 kDa as the respective large and small subunits of Rubisco using immunoblot method. These bands are highlighted in green in Figure 4.2.

4.3.1.2. Amphora sp

The result of SDS-PAGE analysis of the protein extract from *Amphora sp.* is shown in Figure 4.3.

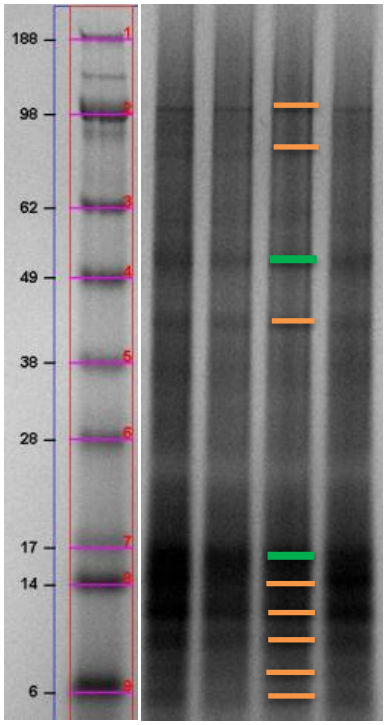


Figure 4.3. SDS-PAGE gel of *Amphora sp.* proteins stained with Coomassie blue SafeStain: 10 µg per well of protein: M: molecular weight marker (kDa), A: diafiltred crude extract, B: dialyzed+ ultrafiltred sample, C: dialyzed+ freeze dried sample, D: freeze dried crude extract.

Dialyzed and freeze dried sample (Lane C) gave slightly better results and was then used to identify protein bands. However, it is obvious that the crude protein extract from *Amphora sp.* contains a lot of impurities, which resulted in a low contrast resolution after running SDS-PAGE analysis. Alternative pretreatments of *Amphora sp.* protein extract might be investigated in order to increase the purity of recovered proteinaceous fraction. This can include protein precipitation using Trichloroacetic acid (TCA) in the ratio 2.5:1 (TCA: protein crude extract), as suggested by Barbarino and Louren¸co (2005). Indeed, these authors reported that 25% TCA precipitation resulted in a good protein recovery after microalgal cell disruption and aqueous extraction.

In the study by Wang et al. (2003), a number of key chemical reagents were proposed for the precipitation of protein prior to two-dimensional electrophoresis (2-DE) from the green microalgae *Haematococcus pluvialis*. These reagents include acetone, TCA, urea, thiourea, dithiothreitol, and tributyl phosphine. The research aimed to increase the number and staining intensity of protein bands while minimizing the smearing on the second dimensional SDS-gel. Results showed that the combination of all mentioned reagents removed efficiently the photosynthetic pigments along with other interfering substances from the protein fraction, which enhanced the resolution of the 2-DE SDS gel (Wang et al., 2003). Besides, protein SDS-PAGE profile from *Amphora sp.* can be further improved by manipulating the protein samples loading capacity per SDS-PAGE gel wells. This approach was considered by Wang et al. (2003) as an effective purification strategy for selectively investigating proteins of different abundances and characteristics.

Table 4.3. Molecular weight identification of *Amphora sp.* protein bands using Bio-Rad densitometer.

Band Number	MW (kDa)
1	120.7
3	71.1
4	56.4
5	42.2
6	13.2
7	11.4
8	9.6
9	8.3
10	7.1
11	6.3

Starting from SDS-PAGE analysis, protein composition can be identified using the sequencing of each protein band separately starting from SDS-PAGE gel. Besides, proteomic data can help to characterize some of the common protein spots among species within the same phylum. The fact that different microalgal strains are characterized by high similarities in their ultrastructural and biochemical characteristics was proved by Matsunaga et al. (2005) mainly within Heterokontophyta phylum that includes the currently studied *Amphora sp.* diatom strain.

According to previous reports (Contreras et al., 2008; Wang et al., 2003; Schwenzfeier et al., 2011), most proteins in the extract of microalgae are enzymes involved in photosynthesis or other essential activities for survival and growth. These enzymes (e.g.

Rubisco) can consist of multiple polypeptide chains with polypeptide chains smaller than 50 KDa, which is also noticed from SDS-PAGE profiles in Figure 4.2 and Figure 4.3 for *Nannochloris sp.* and *Amphora sp.,* respectively. The next part of the microalgal protein characterization consists of the investigation of the presence of some enzymes having significant value as metabolic, nutritional or pharmacological additives.

4.3.2. Enzyme assays

4.3.2.1. Malate dehydrogenase assay

Malate dehydrogenase (MDH) is an enzyme that reversibly catalyzes the oxidation of malate to oxaloacetate. This reaction is part of many metabolic pathways, including the citric acid cycle. Malate dehydrogenase is found in all eukaryotic cells as two isozymes: mitochondrial (m-MDH) and cytoplasmic (soluble, s-MDH) (Worthington, 1993). MDH activity has a key role in the citric acid cycle, also known as the tricarboxylic acid cycle (TCA cycle) and the Krebs cycle, that generates energy through the oxidization of acetate derived from carbohydrates, fats and proteins into carbon dioxide. The citric acid cycle begins with the transfer of a two-carbon acetyl group from acetyl-CoA to the four-carbon acceptor compound (oxaloacetate) to form a six-carbon compound (citric acid). MDH is the enzyme ensuring the production of oxaloacetate from malate by the reduction of NAD^+ to NADH. The NADH generated by the TCA cycle is fed into the respiration pathway, oxidative phosphorylation. The net result of these two closely linked pathways is the oxidation of nutrients to produce usable energy in the form of ATP (Wolfe and Jahoor, 1990).

The molecular weight of the MDH is equal to 70 kDa containing two subunits of 35 kDa each. Besides, this enzyme is characterized by an isoelectric point (pI) equal to 6.14 for cytoplasmic malate dehydrogenase (Teague and Henney, 1976). The MDH enzyme is of interest to clinicians as its activity in serum and cerebral/spinal fluid is of diagnostic significance (Sharpe et al., 1973).

In this study, MDH activity was detected at 340nm wavelength, in the presence of oxaloacetate and NADH as substrates. The decrease in absorbance at this wavelength (A_{340}), during the first 4 to 5 minutes, revealed the presence of MDH activity (Worthington, 1993). As indicated in Figures 4.4 and 4.5, malate dehydrogenase activity was detected in both microalgal strains *Amphora sp.* and *Nannochloris sp.* The variation of A_{340} per minute (ΔA_{340})

was calculated from the initial linear region of the curve and the MDH activity was determined according to specific calculation formula. MDH activities per mg of total protein extracts are shown in Table 4.4.

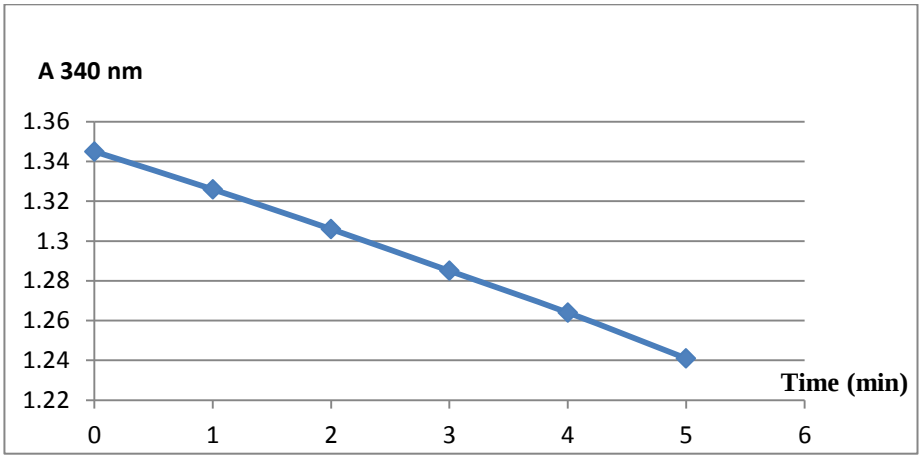


Figure 4.4. Plot of $A_{340\ nm}$ as function of time for malate dehydrogenase activity determination in *Amphora sp.*

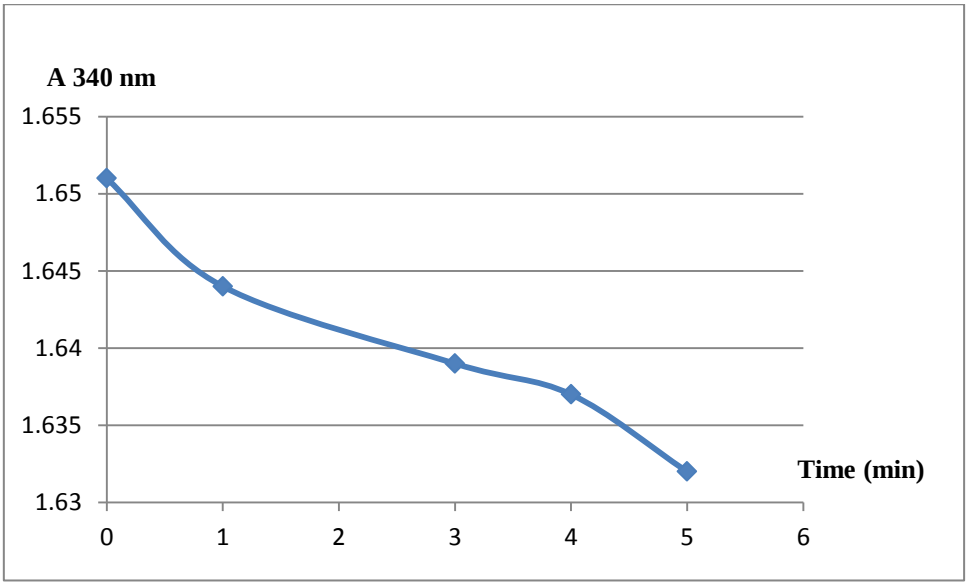


Figure 4.5. Plot of $A_{340\ nm}$ as function of time for malate dehydrogenase activity determination in *Nannochloris sp.*

Table 4.4. Malate dehydrogenase activity in *Amphora sp.* and *Nannochloris sp.*

Microalgal strain	***Amphora sp.***	***Nannochloris sp.***
Malate dehydrogenase activity **(Units/mg of total protein)**	**0.207** ±0.017	**0.002774** ±0.00006

The identification of malate dehydrogenase activity in both microalgal strains is important to assess the effectiveness of the cell disruption by sonication treatment in providing cell-free protein fraction containing active enzymes (Murphy et al., 2000). It is obvious that MDH activity in *Amphora sp.* is much higher than that in *Nannochloris sp.* which allows considering the first strain for prospective purification of malate dehydrogenase. MDH activity in *Amphora sp.* is quite comparable with MDH activity in heterotrophic algae *Chlorogenium elongatum* (0.5 micromole/min/ml of gradient fraction) reported by Stabenau and Beevers, (1974) after cell disruption and fractionation of the protein homogenates on sucrose gradients. Enzymes distribution can be determined on a linear sucrose gradient as a good analytical method for the recognition of the cytosol and mitochondrial MDH activities that would be recovered at different densities after centrifugation of the cell free homogenates on a sucrose density gradient (Stabenau, 1974).

Under anaerobic conditions, activities of the enzymes belonging to the citric acid cycle (eg. malate dehydrogenase) and to the respiration pathway (eg. cytochrome C oxidase) were shown to be low and mitochondria were reported to be absent or very few in number (Chapman and Bartley, 1968). This fact can be also induced heterotrophically by cell growth on high concentration of glucose. As *Nannochloris sp.* (10 liters culture volume) was cultured under lower aeration conditions as compared to *Amphora sp.* (8 liters culture volume in a similar reactor total volume), possible removal of mitochondrion organelles is expected to be more pronounced in *Nannochloris sp.* than in *Amphora sp*. Less mitochondria result in less MDH activity and NADH yield.

In addition, the activity of certain enzymes varies when assayed at different growth phases. Indeed, the specific activity of enzymes was shown to decline by 50% for every cell division. Such a rate of activity loss was named the dilution rate. Namely, malate dehydrogenase was shown to decrease during yeast cells growth at the dilution rate (Chapman and Bartley, 1968). This can be another possible reason for the actual low MDH activities knowing that the present studied microalgae were harvested at the end of the exponential growth phase. Then, further MDH assays can be carried out at different microalgal growth steps to characterize the expression profile of this enzyme as function of growth time. Moreover, low MDH activities can be due to enzymatic repression by oxaloacetate (substrate) (Lemaire et al., 2005). Indeed, yeast mitochondrial MDH was inhibited by concentration of oxaloacetate above 0.3 mM and 2.5 mM oxaloacetate was 66% of that with 0.25 mM oxaloacetate in the aerobic yeast extract (Chapman and Bartley, 1968). Hence, optimum

substrate concentration can be further determined by testing different oxaloacetate contents with the protein crude extracts from *Amphora sp*. and *Nannochloris sp*.

4.3.2.2. Peroxidase assay

Peroxidase is a hemoprotein catalyzing the oxidation by hydrogen peroxide of a number of substrates such as ascorbate, ferrocyanide, cytochrome C and the leuco form of many dyes (Worthington, 1993). Peroxidase has been found well suited for the preparation of enzyme conjugated antibodies, due in part to its ability to yield chromogenic products, and in part to its relatively good stability. Furthermore, Sternberger et al. (1970) and Moriarty et al. (1973) discovered another interesting application of the soluble peroxidase-antiperoxidase techniques in the fields of immunohistochemistry and immunoassay. The use of the highly specific, sensitive and very stable horseradish peroxidase with a chromogenic donor has been proven very useful for assay systems producing hydrogen peroxide, for example in the determination of glucose or galactose by their respective oxidases and in the determination of certain L-amino acids in conjunction with L-amino acid oxidase (Malmnstadt and Hadjiioannou, 1963).

Peroxidase is a monomer estimated to have a molecular weight (MW) of 40 kDa (Worthington, 1993) and a pI of 4.7 (Overbaugh and Fall, 1985). Murphy et al. (2000) identified a peroxidase enzyme in the cell-free extract of *Porphyridium purpureum* microalgae with a molecular weight of 36 kDa. Here, peroxidase activity was detected using spectrophotometric method at 510 nm wavelength. The presence of peroxidase activity was taken into account by the increase of A_{510}, during the first 4 to 5 minutes (Worthington, 1993). Peroxidase activity was detected in *Nannochloris sp.* strain in the presence of urea peroxide and 4-aminoantipyrine (AAP) (Figures 4.6) and in the presence of urea peroxide and O-dianisidine (Figures 4.7) as substrates. No peroxidase activity was detected in *Amphora sp.* strain. ΔA_{510} per minute was calculated from the initial linear part of the curve and the peroxidase activity was determined according to its specific calculation formula. Peroxidase activities are indicated in Table 4.5.

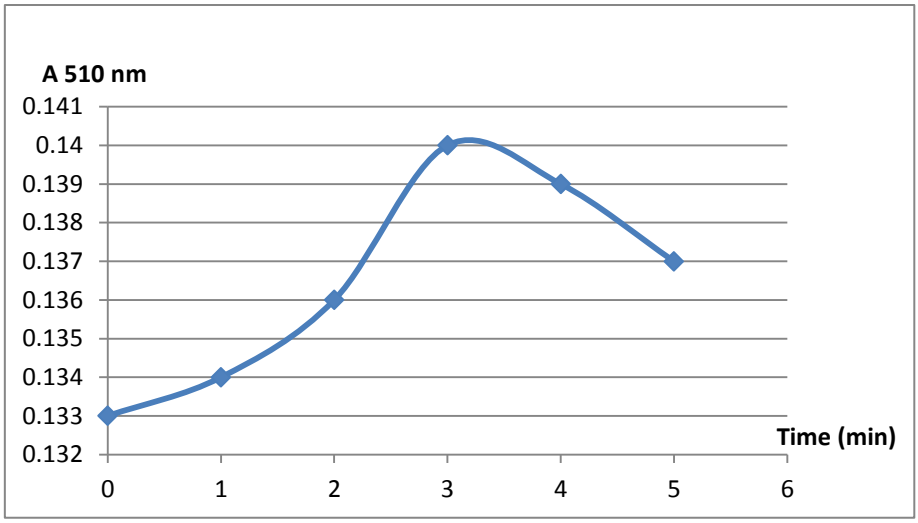


Figure 4.6. Plot of $A_{510\ nm}$ as function of time for the determination of *Nannochloris sp.* peroxidase activity in the presence of urea peroxide and 4-aminoantipyrine (AAP).

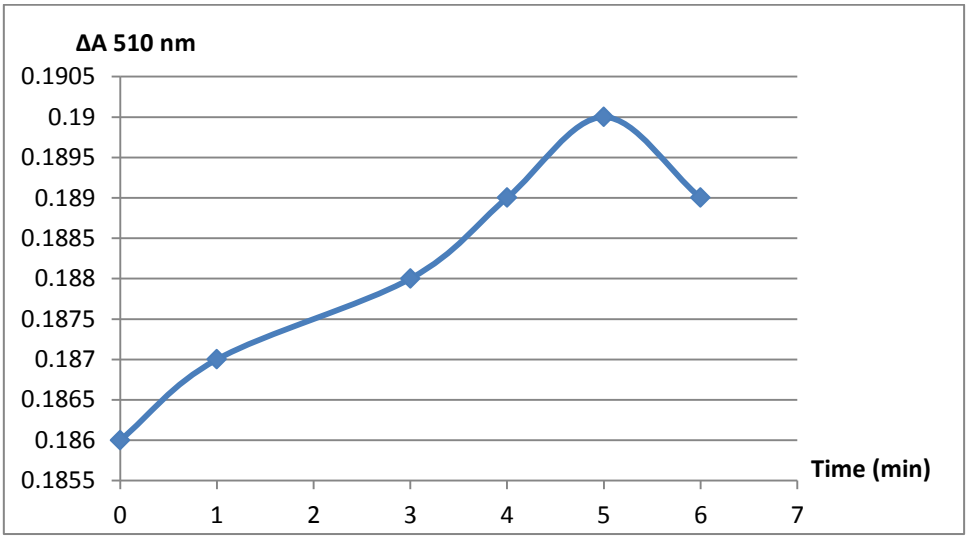


Figure 4.7. Plot of $A_{510\ nm}$ as function of time for the determination of *Nannochloris sp.* peroxidase activity in the presence of urea peroxide and O-dianisidine.

Table 4.5. Peroxidase activity in *Nannochloris sp.* and *Amphora sp.*

Microalgal strain	***Amphora sp.***	***Nannochloris sp.***
Peroxidase activity in presence of urea peroxide plus 4-aminoantipyrine (AAP) **(Units/mg of total protein)**	-	**0.004624** ±0.0015
Peroxidase activity in presence of urea peroxide plus O-dianisidine **(Units/mg of total protein)**	-	**0.001783** ±0.00044

Peroxidase activity was also detected in cell-free extracts of three species of the marine microalgae, *Porphyridium purpureum*, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, in the study of Murphy et al. (2000). These three strains respectively yielded 1.1, 0.32 and 0.07 Units/mg of total protein in the presence of O-dianisidine as a substrate. Peroxidase activity, 0.045 Units/mg of total protein, was also reported by Overbaugh and Fall (1985) in *Euglena gracilis* algae extract in the presence of glutathione as substrate. Peroxidase activity in *Nannochloris sp.* seems low compared to the values reported in literature. However, the peroxidase activity was, interestingly, identified in this species although its cultivation in non-inducing conditions. This result encourages further investigation of the expression and isolation of this antioxidant enzyme under specific culture conditions. This includes the addition of oxidative stressors to the culture medium of *Nannochloris sp.* (Buckova et al., 2010) to enhance its production of peroxidase.

Actually, green microalgae biomass is well expected to represent an innovative proteinaceous bioresource for developing enzymatic protein hydrolysate suitable for pharmacological nutrition (Kim and Kang, 2011). Hence, the green microalgae *Nannochloris sp.* would be a good candidate as food ingredient regarding the benefits of the peroxidase antioxidant activity. Interestingly, antioxidants are important industrial additives mainly used to avoid the deterioration of oxidizable products, such as food, drugs, and cosmetics. Many of these studies have been performed with compounds extracted and purified from terrestrial plants, but researchers are also focusing on the antioxidative properties of bioactive molecules from marine organisms (Kim and Kang, 2011).

4.3.2.3. Catalase assay

Catalase is an enzyme responsible for the degradation of hydrogen peroxide that is primarily derived from mitochondria after the reduction of oxygen to water (Radi et al., 1991). It represents a key antioxidant defense mechanism present in nearly all animal cells. Recently, catalase has been investigated as a possible agent to support intracellular drug delivery (Siwale et al., 2009). Catalase has also been incorporated into an assay for cholesterol quantification (Robinet et al., 2010) and a biosensor for alcohol determination (Hnaien et al., 2010).

Catalase is a tetrameric enzyme (4 subunits) with a MW of 240 kDa and a pI 5.4. Each subunit (60 KDa) contains a heme group and NADPH in its active center (Scibior and Czeczot, 2006). Catalase activity was revealed using spectrophotometric method at 240 nm wavelength (Worthington, 1993), in the presence of hydrogen peroxide as a substrate. The decrease of A_{240}, during the first 2 to 3 minutes, is used to calculate the catalase activity. As indicated in Figures 4.8 and 4.9, catalase activity was identified in *Amphora sp.* and *Nannochloris sp.*, respectively. Catalase activities per mg of total protein are summarized in Table 4.6 for both microalgal strains.

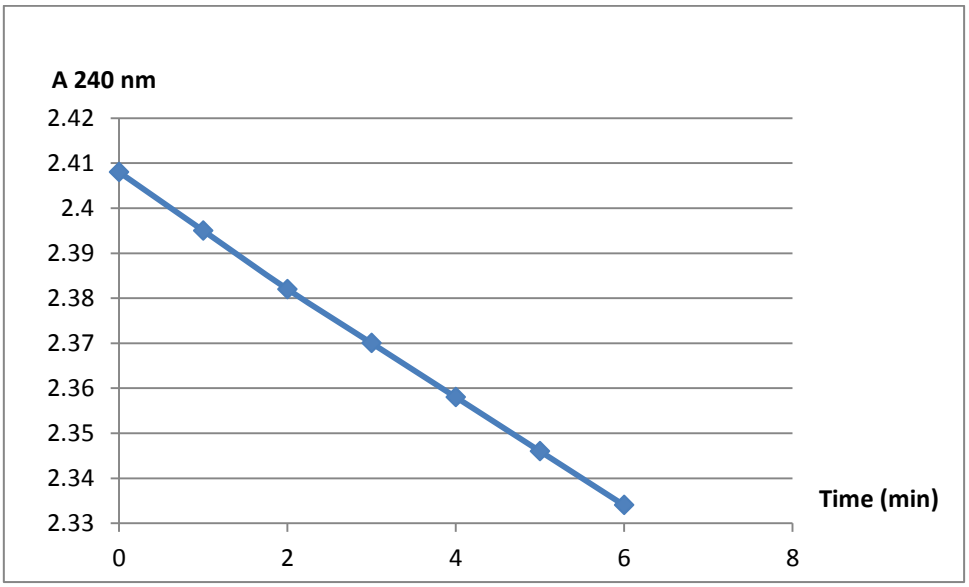


Figure 4.8. Plot of $A_{240\,nm}$ versus time for catalase activity determination in *Amphora sp.*

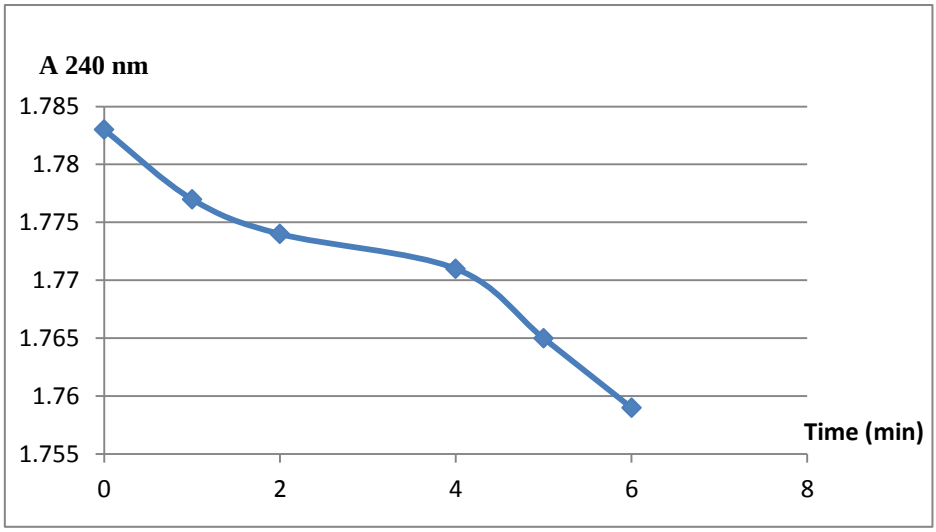


Figure 4.9. Plot of $A_{240\ nm}$ as function of time for catalase activity determination in *Nannochloris sp.*

Table 4.6. Catalase activity in *Amphora sp.* and in *Nannochloris sp.*

Microalgal strain	***Amphora sp.***	***Nannochloris sp.***
catalase activity **(Units/mg of total protein)**	**1. 077** ±0.145	**1.072** ±0.153

Catalase activities were identified in *Nannochloris sp.* and *Amphora sp.* but the values seem lower than that of *Chlorella pyrenoidosa* algae (11 Units/mg of total protein) studied by Frederick et al. (1973) using the same spectrophotometric enzyme assay. However, the present catalase activities are still comparable with catalase activity from bacterial *Comamonas sp.* isolates (Buckova et al., 2010) and the catalase activity identified in heterotrophic *Chlorogenium elongatum* algae (Stabenau and Beevers, 1974). Actually, quantitative electron microscopic immunocytochemistry and electrophoretic analysis followed by Western blotting of microalgal mitochondria and peroxisomes can be other alternative methods to identify, measure and localize the currently studied enzymes, instead of biochemical analysis (spectrophotometric enzyme assays) (Radi et al., 1991).

Further investigation of catalase, as well as peroxidase, activities as a function of microalgal growth steps would be important since these enzyme activities were shown to vary significantly from the beginning and middle of the exponential phase until the entry of the stationary phase, as previously reported by Buckova et al. (2010). These authors highlighted also the effect of the nature and concentration of the oxidative stressor (H_2O_2, o- or p-

phenylenediamine (o-PDA and p-PDA)) on the increase of catalase and peroxidase expression. Interestingly, constitutively expressed catalase was found to represent the major pathway for the resistance of *Comamonas terrigena* cells to toxic peroxides (Buckova et al., 2010). Hence, exposing microalgal cells to various oxidative stressors may result in higher catalases (catalase and peroxidase) activities.

Because the dietary intake of antioxidant-rich food is of great interest for the prevention of oxidative stress and age-dependent diseases (Kim and Kang, 2011), the extraction of catalase from *Nannochloris sp.* and *Amphora sp.* can be of high importance in the enhancement of the value added nutrients for humans. Actually, catalases (catalase and peroxidase) are involved as one of the mechanisms used to protect cells against the damage caused by reactive oxygen species (ROS) to cellular components, including nucleic acids, lipids and proteins (Buckova et al., 2010). Furthermore, catalases are not only essential for the protection against oxidative stress, but with peroxidation they can also participate in the metabolic changes of ubiquitous contaminants in soil and water such as phenolic compounds (Buckova et al., 2010).

Besides, further nutritional and toxicological evaluations of both microalgal strains are required to confirm their antioxidant activities and to ensure their suitability as valuable feed supplement (Becker, 2006). Indeed, as spectrophotometric enzyme assays still have a limited precision (King, 1963); then detailed nutritional tests are later recommended to consider *Nannochloris sp.* and *Amphora sp.* as harmless food additives.

4.3.2.4. Cytochrome C oxidase assay

Cytochrome C oxidase is the final protein complex in the electron transport chain, respiratory chain, of the inner mitochondrial membrane. It couples electron transfer from cytochrome C to O_2, terminal electron acceptor, with the movement of protons across the inner membrane, creating an electrochemical gradient that is utilized to synthesize the adenosine triphosphate (ATP) (Michel et al., 1998). The number of cytochrome C oxidase subunits varies between 3 and 5 in bacteria and up to 13 in mammalian mitochondria (Ostermeier et al., 1997). In all eukaryotes there are three large subunits, termed I, II, and III, whose genes are located in the mitochondrial genome (Michel et al., 1998). However, the comprehensive analysis of plant cytochrome C oxidase and that of other plant mitochondrial enzymes has been greatly hindered by the difficulty of obtaining sufficient quantities of

starting material. The presence of cytochrome C oxidase activity was revealed by the decrease of A_{550}, during the first 10 minutes, in the presence of cytochrome C as a substrate. $\Delta E = E_t - E_f$ was calculated with E_t as the A_{550} nm value at the time points 0 through 10 minutes for the test and E_f as the A_{550} nm value at the 10 minute time point for the blank. The slope (M) of the plot Ln (ΔE) versus time is used for the calculation of cytochrome C oxidase activity (Figure 4.10).

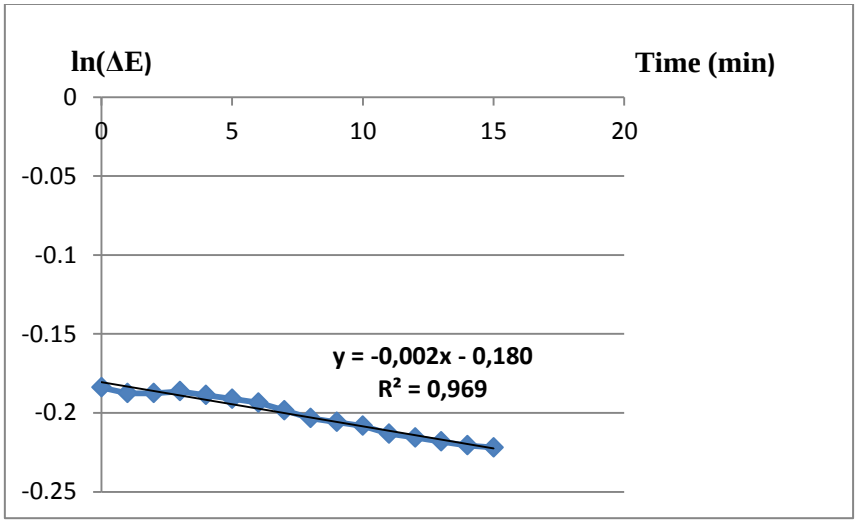


Figure 4.10. Plot of Ln (ΔE) versus time for the determination of cytochrome C oxidase activity in *Amphora sp.*

Table 4.7. Cytochrome C oxidase activity in *Amphora sp.* and *Nannochloris sp.*

Microalgal strain	***Amphora sp.***	***Nannochloris sp.***
Cytochrome C oxidase activity **(Units/mg of total protein)**	**0.0173** ±0.0066	-

The study by Stabenau and Beevers, (1974) also revealed relatively similar cytochrome C oxidase activity (10 nanomole/min/ml of gradient fraction) in the heterotrophic *Chlorogenium elongatum* algae. Cytochrome C oxidase activity was detected in *Amphora sp.* protein extract and not recognized in *Nannochloris sp.* although its crucial role in the respiration pathway. Many assumptions can be made in order to explain the absence of cytochrome C activity in *Nannochloris sp.* This includes the possibility of having an alternative NADH oxidase which oxidizes the NADH in the cytosol rather in the mitochondrial matrix and passes these electrons to the ubiquinone pool (Rasmusson et al., 2004). The alternative NADH and ubiquinone oxidases can be produced under external

culture stresses (eg. cold, reactive oxygen species) and have low ATP yields than the full electron transport pathway (Vanlerberghe and McIntosh, 1997).

Secondly, the negligible cytochrome C activity in *Nannochloris sp.* can be explained by the low MDH activity determined in the same strain, as the TCA cycle and the respiration pathway are linked by the NADH gradient resulting from the MDH activity. Indeed, a close correlation between the activity of cytochrome c oxidase (respiration pathway) and the rate of pyruvate decarboxylation (Glycolysis and TCA cycle) was reported by Van Hinsbergh et al. (1980). Hence, the low MDH activity in *Nannochloris sp.* does decelerate the enzyme activities of the electron transport chain including cytochrome C oxidase activity.

Besides, the study by Xue et al. (1996) on the interactions between photosynthesis and respiration in the green alga *Chlamydomonas reinhardtii* showed a decrease in the rate of respiratory carbon flow during photosynthesis, and a simultaneous increase in respiratory O_2 consumption during that may be mediated by the export of photogenerated reductant from the chloroplast. Indeed, CO_2 exchange data were consistent with a photosynthesis-dependent decrease in the rate of respiratory carbon flow and a decrease in the rate of CO_2 evolution from the TCA cycle, in favor of the rate of O_2 consumption (Xue et al., 1996). This fact can be also behind the decrease of malate dehydrogenase and cytochrome C oxidase activities during photosynthesis carried out by *Nannochloris sp.* and *Amphora sp.* exposed to continuous illumination during their culture.

Furthermore, some studies reported that concentrations of ferricyanide exceeding 5 mM led to the inhibition of succinate dehydrogenase (King, 1963). Then, actual used ferricyanide concentration (100 mM) can be also considered for the inhibition of cytochrome C oxidase in *Nannochloris sp*. This fact can be taken into account by the optimization of the adequate concentration of ferricyanide for better cytochrome C activity. As the protein quantity, the enzyme quantity, is lower in *Nannochloris sp.* the cytochrome C oxidase inhibition process by high ferricyanide concentration may be more meaningful in this strain as compared to *Amphora sp*. The pattern of disappearance of cytochrome C oxidase was also shown to vary more than that of any other enzyme tested by Chapman and Bartley (1968).

Actually, some other enzyme assays were tested using the protein crude extracts of *Nannochloris sp.* and *Amphora sp.*, but no activity was detected from these assays. These enzyme assays include carbonic anhydrase that was identified in the marine microalgae *Tetraselmis gracilis* (*Chlorophyta*) in the study by Masini et al. (2003). Regarding the

importance of the antioxidative activities reported in microalgae, acyl co-A oxidase (antioxidant enzyme) was also tested in *Nannochloris sp.* and *Amphora sp.* protein crude extracts. However, results showed the absence of acyl co-A oxidase activity in both strains. Furthermore, since microalgae cells had been shown to accumulate high amounts of lipids (Widjaja et al., 2009), lipase activity assay was investigated, but results didn't reveal lipase activities from *Nannochloris sp.* and *Amphora sp.* protein crude extracts.

4.4. Lipid content in microalgae

4.4.1. Lipid content based on wet microalgal mass

Starting from wet microalgal mass harvested by centrifugation and dried 1 hour at 105 ºC, *Nannochloris sp.* lipid fraction was determined to be 8.14 ±3.67% on dry basis. The calculation was done with reference to the gravimetric method for lipid content determination (Bligh and Dyer, 1959). Lipid content in *Nannochloris sp.* is still comparable to the values, ranging from 8.9 % and 16.7% of dry mass, reported by Dunstan et al. (1992) for ten species of microalgae belonging to the Chlorophyta phylum.

For *Amphora sp.* the lipid quantity was equal to 10.48 ±1.26% on dry basis. This value is in the same range of the lipid contents reported by Sydney et al. (2010) for *Chlorella vulgaris* (9.95 ±2.1%), *Spirulina platensis* (11 ±2.2%), and *Dunaliella tertiolecta* (11.44 ±1.8%) that were shown to accumulate high contents of lipids. *Amphora sp.* presented higher lipid content as compared to the same species (7.11±0.29% on dry basis) studied by De Viçose et al. (2012).

4.4.2. Lipid content based on lyophilized microalgal mass

Starting from lyophilized microalgal biomass, *Nannochloris sp.* lipid fraction was equal to 13.69% on dry basis. This lipid content was comparable to another species of *Chlorella protothecoides,* photoautotrophically grown by Miao and Wu, (2006) and identified with high lipid content equal to 14.57 ±0.16% on dry basis. For *Amphora sp.*, the lipid content was 21.37% on dry basis. It has been reported that microalgal lipid yields were shown to reach up to 50% of total dry weight (Wahlen et al., 2011), but current study reveals lipid contents that are still similar to some published studies on several strains of microalgae. Allard and Templier, (2000) extracted lipids from a variety of freshwater and marine microalgae and reported that lipid contents varied from 1 to 26% on dry basis.

Lipid content was higher in lyophilized microalgae than in wet mass, for both strains *Nannochloris sp.* and *Amphora sp.* This can be attributed to the fact that better lipid yield is generally obtained when the quantity of moisture is low (Ehimen et al., 2010). Similarly, Zhu et al. (2002) extracted fungal lipid of *Mortierella alpina* and made comparison between wet biomass and biomass dried at 80 ºC and found that dry extraction was more effective than wet extraction in terms of lipid yield. Hence, it is worth concluding that biomass pretreatment method does influence the lipid yield. Indeed, the lipid extraction yield is sensitive to many factors relevant to biomass cultivation system, pretreatment approach and extraction method. For instance, lipid content in heterotrophic *Chlorella protothecoides* cells reached as high as 55.20%, which was about four times that in autotrophic cells (14.57%) (Miao and Wu, 2006).

Furthermore, Prabakaran and Ravindran (2011) studied different cell disruption methods applied to three microalgal strains *Chlorella sp.*, *Nostoc sp. and Tolypothrix sp*. These methods include autoclave, bead beating, microwave, osmotic shock and sonication. Data revealed that the different cell treatments resulted in different lipid contents with the highest levels obtained when applying the sonication method. In addition, the study by Cheng et al. (2011) identified different lipid crude extracts from *Pavlova sp.* equal to 17.6 wt%, 13.8 wt% and 44.7 wt% when using toluene/methanol, hexane/methanol, and ethyl acetate/methanol mixed solvents, respectively. All these examples revealed that different lipid yields can be obtained starting from the same microalgal biomass but using different treatment methods.

4.5. Analysis of FAMEs content using GC-MS starting from microalgal wet mass

The FAME yield was obtained from the transesterification of the crude lipid which was then quantified by GC-MS with an internal standard (C17:0). This approach allows the separation of the different fatty acid methyl esters based on their intrinsic hydrophobicity. The elution of the FAME elements is characterized by specific retention time, as indicated in Table 4.8. Different peaks obtained in the output chromatogram, are assigned to precise constituents identified by comparing their retention times and fragmentation patterns with those of authentic standard in the internal data base system (Wiley7n) (Cheng et al., 2011). The area of each peak is proportional to the concentration of the corresponding fatty acids.

Amphora sp.

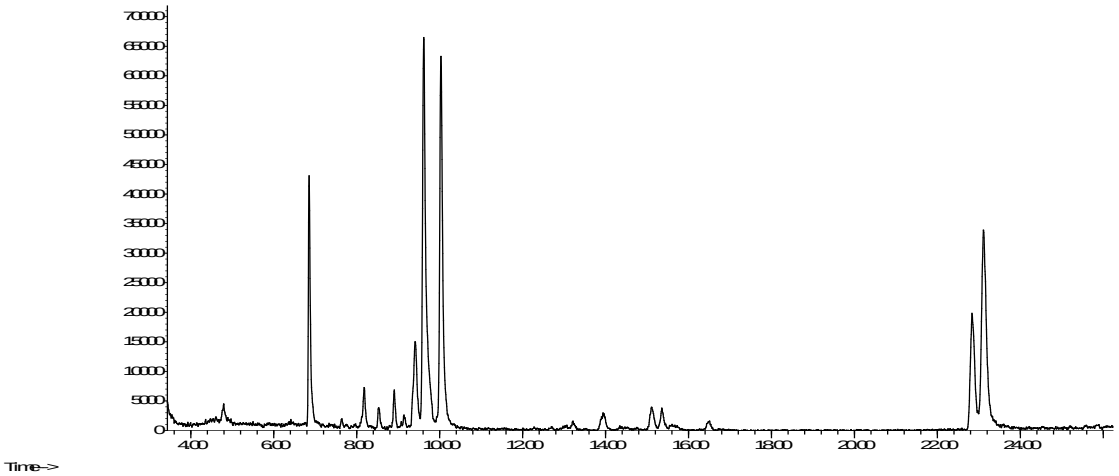


Figure 4.11. GC-MS chromatogram of the transesterified lipids extracted from *Amphora sp.* wet mass.

Table 4.8. Fatty acid components of *Amphora sp.* FAME after GC–MS analysis.

Sample	Retention time (min)	Fatty acid component	Shortened formula
1	6.852	Myristic acid	**C14:0**
2	9.621	Palmitoleic acid	**C16:1 ω-7**
3	10.033	Palmitic acid	**C16:0**
4	23.114	DHA: Docosahexeanoic acid	**C22:6 ω-3**

The fatty acids contained in each sample were quantified according to their peak area relative to the C17:0 fatty acid internal standard (5 µg of C17:0 standard has a peak area percent equal to 0.575, averaged over many assays), and expressed as a percentage of total fatty acids content (Yang et al., 2010). The FAMEs content in *Amphora sp.* was equal to 11.069 ±4.692% of total lipid basis (1.020 ±0.433% of dry mass basis). This value of FAME is lower than the total lipid content extracted from *Amphora sp.* (10.48 ±1.26% dry basis) indicating that a large quantity of impurities was extracted from the microalgal biomass using chloroform/ methanol lipid extraction. The difference between crude lipids and FAME yield was also observed by Cheng et al. (2011), Mulbry et al. (2009) and Johnson and Wen, (2009).

Diatoms are characterized, in general, by a high content of ω-7 C16:1, C16:0 and ω-3 polyunsaturated fatty acids such as C20:5 (Ecosapentaenoic acid, EPA) and C22:6

(Docosahexaenoic acid, DHA) (De Viçose et al., 2012). Figure 4.12 shows that indeed *Amphora sp.* is rich in palmitoleic acid (34.04%), palmitic acid (29.32%), ω-3-docosahexaenoic acid (23.15%), and myristic acid (13.49%). The content of each fatty acid is expressed as a percentage of the mass sum of the total fatty acids that are recognized within the crude lipid with high match quality (more than 90 %), as compared with the authentic Wiley 7n data base standards of the GC-MS machine. The fatty acid composition of *Amphora sp.*is highly comparable to that presented by De Viçose et al. (2012), for the same species (Table 2.2). In fact, *Amphora sp.* was reported to have palmitic acid (20.54%) and myristic acid (7.37%) as the major components of the saturated fatty acids, palmitoleic acid (25.31%) with the higher content among the monounsaturated fatty acids and a total ω-3-polyunsaturated fatty acids of 23.30% where the EPA (C20:5) is the major component (16.46%) (De Viçose et al., 2012).

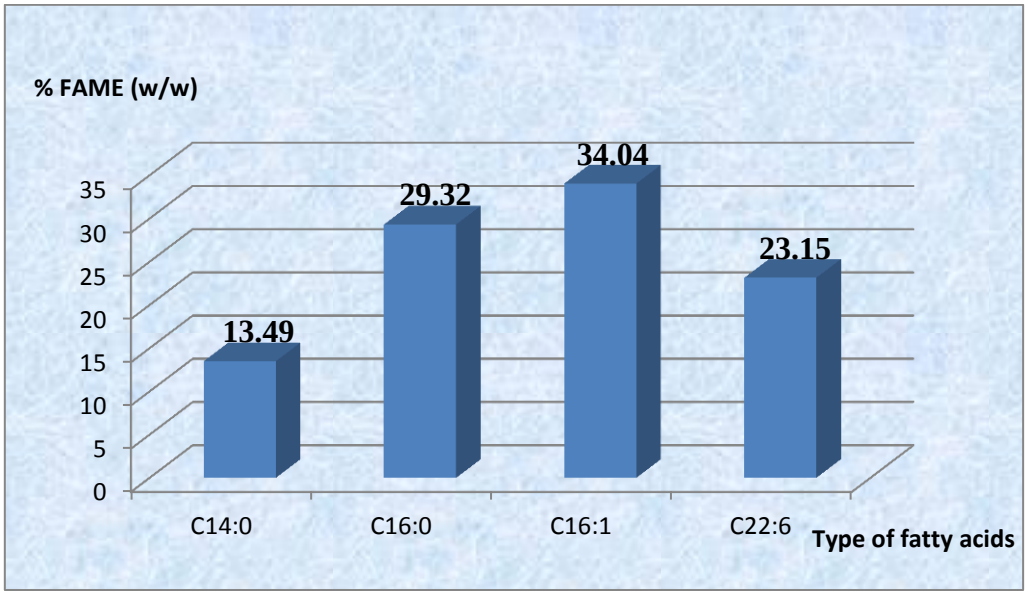


Figure 4.12. Percentage of fatty acids per total FAME extracted from *Amphora sp.*

Amphora sp. has a considerable amounts of saturated (and monounsaturated) fatty acids (C14:0, C16:0, C16:1) and hence it could be a good candidate for biodiesel production. The study by Yu et al. (2009) on two strains *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* belonging to the same phylum as *Amphora sp.* showed that their oil extracts were predominantly composed of triacylglycerols (TAGs) having palmitic (16:0), palmitoleic (16:1), and myristic (14:0) acid substituents. *Amphora sp.* fatty acid profile is also quite similar to that of *Schizochytrium limacinum* studied by Tang et al. (2011). These researchers

intended to increase the crude lipid yield through supercritical CO_2 (SC-CO_2) extraction and to enrich and purify DHA (C22:6) that was equal to 29.7% of total fatty acids. High DHA content among Heterokontophyta phylum (including *Amphora sp.*) was also highlighted by Matsunaga et al. (2005).

DHA (C22:6) as one of the ω-3 PUFA is well-known for its medical advantages for human beings as well as for other vertebrates (Muskiet et al., 2004). Hence, many efforts were focused to find high-quality DHA sources which are sustainable and also free of toxins (Yang et al., 2010). Hence, DHA found in *Amphora sp.* is of great importance and its prospective purification may have valuable applications.

Nannochloris sp.

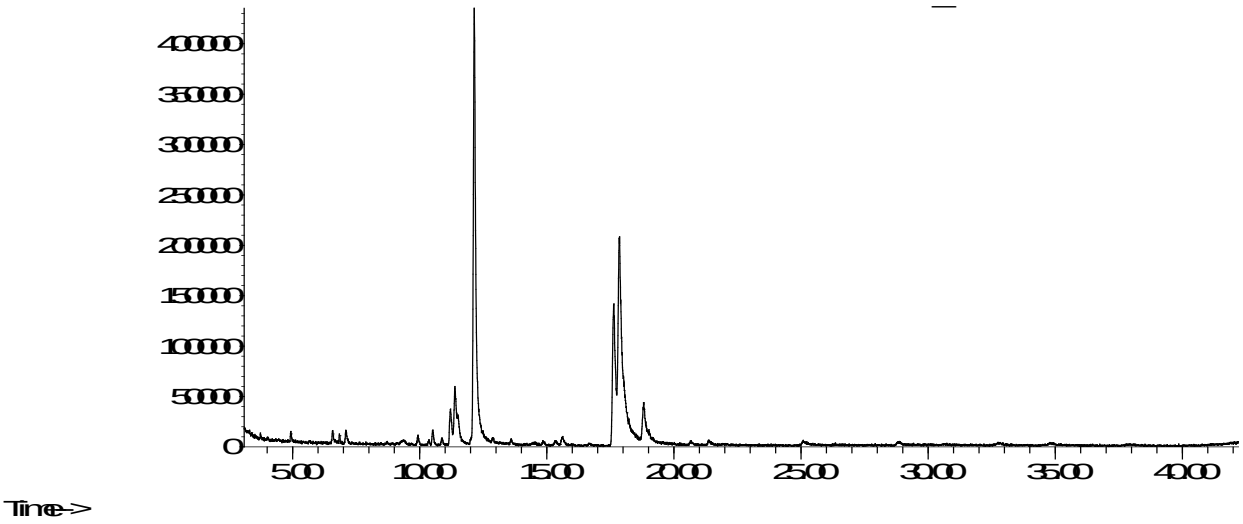


Figure 4.13. GC-MS chromatogram of the FAME extracted from *Nannochloris sp.* wet mass.

Table 4.9. Fatty acid components of *Nannochloris sp.* FAMEs after GC–MS analysis.

Sample	Retention time (min)	Fatty acid component	Shortened formula
1	11.212	Hexadecadienoic acid	**C16:2 ω-6**
2	12.150	Palmitic acid	**C16:0**
3	17.632	Linoleic acid	**C18:2 ω-6**
4	17.855	Linolenic acid	**C18:3 ω-3**
5	18.811	Octadecanoic acid	**C18:0**

The FAME content identified in *Nannochloris sp.* is equal to 24.46 ±1.03% of total lipid basis (0.39 ±0.001% of dry mass basis). Similar to *Amphora sp.*, there is a large difference between FAMEs content and the crude lipid yield. As biodiesel is mainly composed of FAMEs, it is worth using the FAMEs yield, instead of total lipids yield, as a suitable index for comparing the lipid contents of microalgae, and the extraction efficiency for biodiesel applications. This is explained by the large difference that can be found between FAMEs and crude lipid yields extracted from microalgae (Cheng et al., 2011). Moreover, low FAMEs yields can be increased by optimizing many parameters relevant to biomass culture conditions (long depletion in nitrogen), biomass pretreatment (drying temperature; lyophilization, freeze drying), cell disruption method (sonication or bead beating), extraction method (supercritical fluid extraction SFE), extraction factors (solvent type) and transesterification factors (methanol to lipid ratio, catalyst content, reaction temperature, reaction time, in situ transesterification). These perspectives are further discussed in the following chapter.

Dunstan et al. (1992) showed that major fatty acids in the Chlorophyceae strains were C16:0, C16:1, C16:2, C16:3, C18:2 and C18:3. Indeed, in the present work, *Nannochloris sp.* is also primarily rich in palmitic acid (C16:0, 38.98%), ω-3-linolenic acid (C18:3, 28.32%) and ω-6-linoleic acid (C18:2, 19.82%). This strain has lower amount of ω-6 hexadecadienoic acid (C16:2, 8.19%) and octadecanoic acid (C18:0, 4.69%) (Figure 4.14). The proportion of each fatty acid is expressed as a percentage of the total fatty acids that are recognized in the crude lipid with a high match quality as compared to the library. In the output GC-MS chromatogram, some eluted fragments do not correspond to fatty acids. Indeed, HP-5MS non polar column, used in this study, can be applied for the identification of a variety of compounds, including alkaloids, semivolatiles, drugs, pesticides and halogenated compounds besides FAMEs determination.

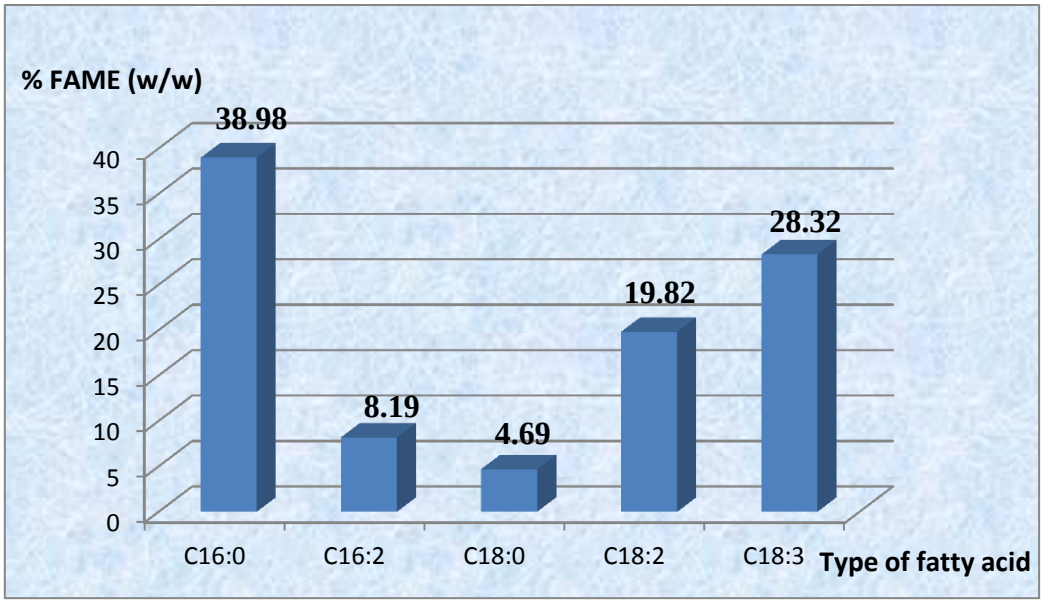


Figure 4.14. Percentage of fatty acids per total FAME extracted from *Nannochloris sp.*

The considerable amounts of palmitic and linoleic acids in *Nannochloris sp.* along with the octadecanoic acid content make this strain suitable for the production of good quality biodiesel. In a previous report (Knothe, 2008), palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids were recognized as the most common fatty acids contained in biodiesel. Indeed, the preference of the advanced fatty acids for biodiesel production ensures its oxidative stability for longer storage and safety (Knothe, 2005).

Furthermore, ω-6 C16:2, C18:2 and ω-3 C18:3 fatty acids can also be valuable in human consumption (Biller and Ross, 2011). Hence, the separation of fatty acids destined for biodiesel production and those valuable in food consumption is possible (Bar et al., 1995), for both strains of microalgae *Nannochloris sp. and Amphora sp.*

4.6. Comparison of lipid profiles in conventional and in situ transesterification experiments using lyophilized mass

Conventional and in situ transesterification were carried out starting from lyophilized microalgae. Conventional approach consists of a lipid extraction step followed by a separate transesterification reaction of the crude lipids, whereas in situ transesterification combines both steps in a single process aiming to the conversion of microalgal oil to FAME directly from cells, avoiding the separate extraction step. The resulting FAME contents extracted using hexane were analyzed by GC-MS machine. The weight of FAME components were

calculated according to the internal standard (C17:0) peak area and concentration correlation. The total FAME content, which is the sum of the different fatty acids' weights, expressed in terms of dry basis is summarized in Table 4.10.

Table 4.10. FAME yields of *Nannochloris sp.* and *Amphora sp.* after conventional and in situ transesterification.

	Conventional transesterification		**In situ transesterification**	
Microalgal strain	***Amphora sp.***	***Nannochloris sp.***	***Amphora sp.***	***Nannochloris sp.***
FAME yield (% of dry mass basis)	8.189	3.527	14.745	3.638

The profile of fatty acids resulting from GC-MS analysis of FAMEs extracts using conventional and direct (in situ) transesterification are shown in Figure 4.15 for *Amphora sp.* and Figure 4.16 for *Nannochloris sp*.

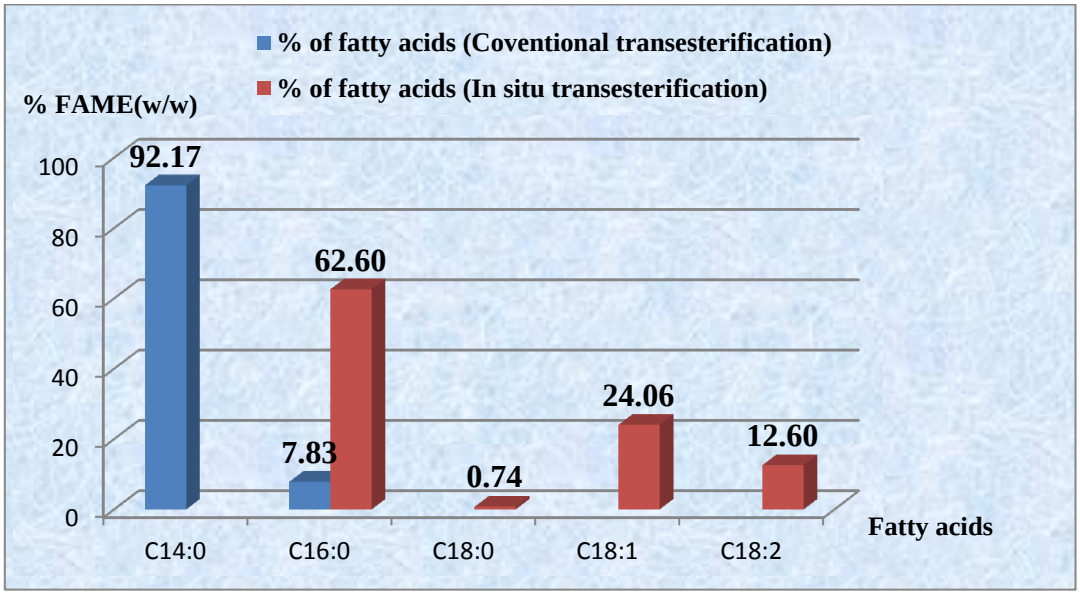


Figure 4.15. Percentage of fatty acids per total FAME extracted from *Amphora sp.*

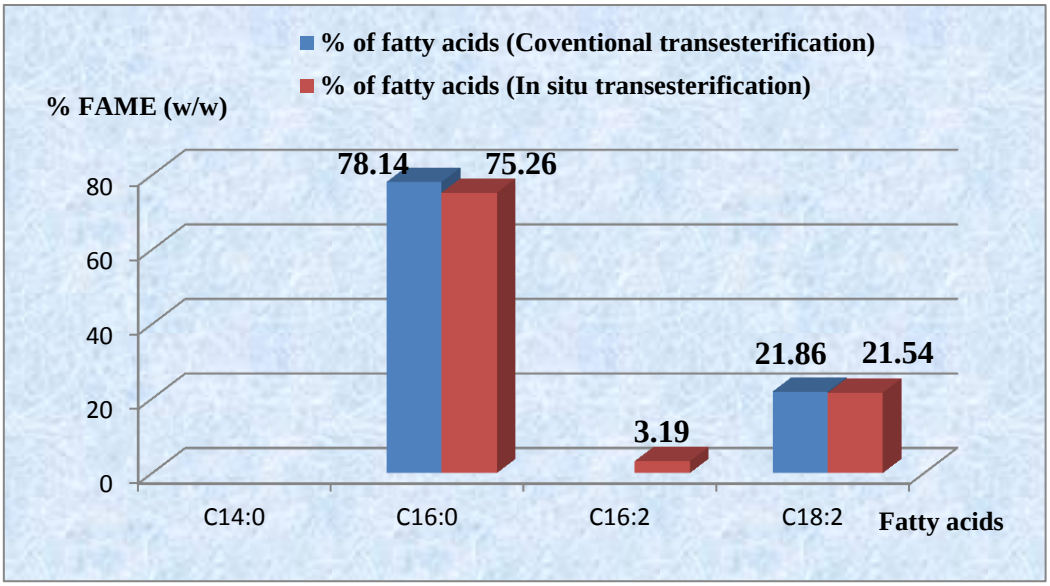


Figure 4.16. Percentage of fatty acids per total FAME extracted from *Nannochloris sp.*

In addition to simplifying the production process, direct transesterification resulted in improved yields of FAMEs when compared to a conventional extraction followed by conversion approach. Indeed, FAMEs contents increased from 8.189% to 14.745% and from 3.527% to 3.638% in *Amphora sp.* and *Nannochloris sp.*, respectively, when using in situ lipid-FAME conversion (Table 4.10). The increase of FAME yield from microalgal lipids due to in situ transesterification as compared to conventional process was also observed by Wahlen et al. (2011) and Lewis et al. (2000). Using the in situ method with sunflower seeds as feedstock, Harrington and D'Arcy-Evans (1985) achieved an increase in biodiesel yields of up to 20% compared to the conventional process. Ehimen et al. (2010) assumed also that direct transesterification may be especially advantageous for use with microalgae, since the extraction of microalgae lipids is usually accomplished via solvent extraction and not with the use of cheaper physical extraction methods (for example, expellers) as utilized for conventional oil crops. The difference between in situ and conventional transesterification reactions is also taken into account when considering the FAME profiles of the two studied microalgae (Figures 4.15 and 4.16). It is obvious that in situ transesterification allows the conversion of higher amounts of fatty acids than in the two-steps reaction; this can be assigned to the efficient simultaneous lipid extraction and FAME production in the direct transesterification process. This was previously described by Wahlen et al. (2011) who showed that direct transesterification approach yielded significantly more biodiesel than would be expected from available triglycerides, indicating the capture of fatty acids from

membrane phospholipids. These results were deduced from wide phylum of microalgae including diatoms, green algae, cyanobacteria, and a wild mixed culture, despite their diverse lipid compositions (Wahlen et al,. 2011).

Interestingly, the FAME yield obtained starting from lyophilized microalgal biomass, even when using conventional transesterification, is higher than that obtained from wet mass. This elucidates the advanced analysis (see 4.4 of this chapter) about lipid yield increase in the presence of less moisture in the feedstock. Ehimen et al. (2010) studied the influence of moisture content on FAME production using in situ transesterification process and they indicated that the increase in the biomass moisture content had a significant negative effect on the equilibrium conversion of oil to biodiesel, with improved FAME yields observed when using completely dried samples compared with moisture containing samples.

4.7. Fields of application of the microalgal proteins and lipids from *Nannochloris sp.* and *Amphora sp.*

Regarding the protein analysis, microalgal proteins from *Nannochloris sp.* and *Amphora sp.* can be applied as food additives, pharmaceutical components and valuable intermediates in metabolic pathways. This fact is feasible regarding the presence of enzymes with interesting metabolic and analytical properties including malate dehydrogenase and cytochrome C oxidase and enzymes with antioxidant activities such as peroxidase and catalase. Hence, these two strains can be new competitive unconventional nutrition sources regarding their high content of protein and their antioxidant activities that can prevent oxidative stress and age depending diseases (Kang et al., 2011). In addition based on the lipid analysis, *Amphora sp.* presents high content of DHA, one of the ω-3 PUFAs, which would allow its application as a healthy food additive. Similarly, *Nannochloris sp.* contains ω-6 C16:2, ω-6 C18:2 and ω-3 C18:3 PUFAs which encourages their integration in food consumption. Indeed, among all the fatty acids in microalgae, some fatty acids of the ω-3 and ω-6 families are of particular interest (Spolaore et al., 2005).

Kang et al. (2011) also revealed that diatoms (including *Amphora sp.*) are the major component of many food webs and that green microalgae (including *Nannochloris sp.*) would represent an innovative proteinaceous bioresource for pharmacological nutrition. Furthermore, these two microalgal strains can be used as cosmetic product supplements because of the fact that protein-rich microalgae promote positive effects on face and skin tissues (Matsunaga et al., 2005). Moreover, nowadays microalgae for human nutrition are

being marketed in different forms such as tablets, capsules and liquids. They can also be incorporated into pastas, snack foods, candy bars or gums, and beverages (Yamaguchi et al., 1997; Liang et al., 2004).

Due to their protein and lipid composition, *Nannochloris sp.* and *Amphora sp.* can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals. The results of the experiments so far conducted by Witt et al. (1981) indicated that *Nannochloris sp.* is a suitable food item for mass cultures. In fact, 30% of the current world algal production is sold for animal feed applications (Becker, 2004). It is also obvious that microalgae should meet some criteria in order to be applied in feed and aquaculture such as ease of culture, non-toxicity, high protein content, high nutritional quality and cell wall digestibility. In addition, highly unsaturated fatty acids such as docosahexaenoic acid (DHA) content are of major importance for human beings as well as for other vertebrates (Spolaore et al., 2006; Muskiet et al., 2004).

The enrichment in polyunsaturated fatty acids is of great interest and can be obtained by urea complexation with microalgae lipids. Urea complexation method was used by Tang et al. (2011) to concentrate polyunsaturated fatty acids (PUFAs) from saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs). Urea surrounded straight-chain SFAs and MUFAs in urea hexagonal crystal structure, leaving PUFAs outside due to their larger molecular volume and steric structure. The urea complexes were then separated from the non-urea complexes (PUFAs) under vacuum filtration. Tang et al. (2011) concluded that urea complexation was an effective tool to enrich and purify DHA from microalgae lipids since DHA purity doubled from 29.7% to 60.4%.

Nowadays biodiesel is getting more interest and new biodiesel resources are being investigated. The properties of a biodiesel fuel, including its ignition quality, combustion heat, cold filter plugging point, oxidative stability, viscosity and lubricity, are determined by the structure of its component fatty esters (Prabakaran and Ravindran, 2011). Because C16:0, C18:0, C18:1, C18:2 are the most common fatty acids contained in biodiesel (Knothe, 2008), *Amphora sp.* and *Nannochloris sp.* lipids can be good candidates for biodiesel production. Interestingly, microalgae were estimated to offer many potential advantages as a non-food feedstock for biodiesel production (Chisti, 2008; Hu et al., 2008).

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Chapter 5

Conclusion and perspectives

Results obtained in this study showed that *Nannochloris sp.* and *Amphora sp.* microalgal strains have high protein contents equal to 16.69 ±4.07% and 39.89 ±2.09% of dry mass, respectively, which makes them comparable with some commercially valuable microalgae (Barbarino and Lourençco, 2005; Kim and Kang, 2011). Based on spectrophotometric enzyme assays, four interesting enzymes were identified in *Nannochloris* and *Amphora sp.* These enzymes include malate dehydrogenase and cytochrome C oxidase that can be applied as intermediates in metabolic pathways and therapeutic ingredients, and peroxidase and catalase enzymes that are highly valuable as natural antioxidants in nutrition. Perspective work on optimum protein yield and composition in *Nannochloris sp.* and *Amphora sp.* includes monitoring the culture conditions (eg. culture medium composition) and identifying the maximum protein yield within microalgal growth phases. Besides, cell disruption and protein extraction methods are key features influencing protein content and analysis, which make them worth investigation.

Lipid characterization using methanol/chloroform (2:1) extraction and GC-MS analysis, starting from microalgal wet biomass, showed that *Nannochloris sp.* and *Amphora sp.* have considerable lipid contents equal to 8.14 ±3.67% and 10.48 ±1.26% on dry basis, respectively. GC-MS profiles indicated that biodiesel contents (FAMEs) were 24.46 ±1.03% and 11.07 ±4.69% of total crude lipids extracted from *Nannochloris sp.* and *Amphora sp.*, respectively. FAMEs composition in *Nannochloris sp.* justifies its possible application in food and feed viewing the presence of polyunsaturated fatty acids ω-3 C18:3 (28.32%), ω-6 C18:2 (19.82%) and ω-6 C16:2 (8.19%) and in biodiesel production because of its contents of C16:0 (38.98%) and C18:0 (4.69%). In *Amphora sp.*, FAMEs are composed of C16:1 (34.04%), C16:0 (29.32%) and C14:0 (13.49%) that are fatty acids basically valuable in biodiesel production and polyunsaturated ω-3 DHA (C22:6) that has crucial health and pharmaceutical applications such as improving memory and brain development.

In situ transesterification is a good alternative in avoiding solvent wastes during the extraction of lipids and allowing better analysis and specific characterization of biodiesel

yields (Haas et al., 2007). In this study, in situ transesterification led, indeed, to better FAME yields, for both microalgae strains, as compared with the conventional lipid extraction followed by a separate transesterification.

Further enhancement of lipid production and extraction during microalgal growth phases, biomass pretreatment, extraction and transesterification processes are still required for better biodiesel yields from microalgal strains, *Nannochloris sp.* and *Amphora sp.* Heterotrophic growth of microalgae may be a good alternative to the autotrophic growth for the production of higher biomass content and metabolites such as lipid, as indicated by Miao and Wu (2006).

In fact, among other possible ways to increase the lipid extraction yield in microalgae is the use of an efficient cell disruption method such as bead beating or sonication. Indeed, because microalgae lipids are within rigid cell walls (Lee et al., 2010), bead beating was the appropriate approach applied by Cheng et al. (2011) in order to disrupt the cells and release the maximum of lipids. In other case of study, sonication was proved to be an effective method to enhance lipid yields from microalgae (Prabakaran and Ravindran, 2011). Moreover, lipid extraction solvent is an influencing factor on the recovered lipid yield. Toluene/ methanol and acetate/ methanol can be alternative mixed solvents to chloroform/ methanol in order to investigate better extraction methods (Cheng et al., 2011). Instead of solvent extraction, supercritical fluid extraction (SFE) is also a promising process for extracting lipids containing labile PUFAs because the extraction method can be conducted at a low temperature (Cheng et al., 2011). Moreover, supercritical fluid extraction offers new opportunities for using CO_2, which is a non-toxic, non-flammable, inexpensive, and environmentally friendly solvent.

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