Heterotrophic growth of microalgae for the production of lipids

Tracy Ghidossi

Bachelor of Science in Life Technologies, Major in Biotechnology

A dissertation submitted in fulfillment of the

requirements for the award of

Doctor of Philosophy (PhD)

to the

Dublin City University

Faculty of Health and Science, School of Biotechnology

Supervisor: Dr. Rosaleen Devery

Co-supervisor: Prof. Ian W. Marison

External Supervisor: Dr. Darragh Gaffney

June 2018

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed:
(Tracy Ghidossi)

Student ID: 14103095
8th of June 2018

“O frati,” dissi, “che per cento milia
perigli siete giunti a l’occidente,
a questa tanto picciola vigilia

d’i nostri sensi ch’ del rimanente
non vogliate negar l’esperenza,
di retro al sol, del mondo sanza gente.

Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza”.

Dante Alighieri (1265-1321)
La Divina Commedia - L’inferno (C. XXVI, 112-120)

Contents

List of Figures

List of Tables

List of Abbreviations

ALA	α-linoleic acid
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumine
C	Carbon
C/N ratio	Carbon to Nitrogen ratio
DCW	Dry Cell Weight
DHA	Docosahexaenoic Acid
DI water	Deminaralized water
DMSO	Dimethyl sulfoxide
DO	Dissolved Oxygen
DOE	Design of Experiments
DSP	Downstream processing
EPA	Eicosapentaenoic acid
FAME	Fatty Acid Methyl Esters
FDA	Food and Drugs Administration
FID	Flame Ionization Detector
GC	Gas Chromatography
HCD	High Cell Densities
HPH	High Pressure Homogenizer
HPLC	High Performance Liquid Chromatography
LC PUFA	Long Chain Polyunsaturated Fatty Acid
MSG	Monosodium glutamate
N	Nitrogen
NAD	Nicotinamide Adenine Dinucleotide
NIR	Near-infrared
OD	Optical Density
PCB	Polychlorinated Biphenyl
PUFA	Polyunsaturated Fatty Acids
STR	Stirred Tank Reactor
TAG	Triacylglycerides
USP	Upstream processing
YE	Yeast Extract

List of publications and presentations associated with this work

Publications

- Ghidossi T., Marison I., Devery R., Gaffney D., and Forde C.; Characterization and Optimization of a Fermentation Process for the Production of High Cell Densities and Lipids Using Heterotrophic Cultivation of *Chlorella protothecoides*, Industrial Biotechnology, October 2017, 13(5): 253-259, https://doi.org/10.1089/ind.2017.0007

- Ghidossi T., Marison I., Devery R., Gaffney D.; Methods for the disruption of wet microalgal biomass, Algal Research Journal (submitted)

Presentations

- Research Day of School of Biotechnology, Dublin City University, Dublin; Characterisation of the cell wall structure of *Chlorella protothecoides* for the enzymatic extraction of high value biomolecules; January (2015)

- AlgaEurope Conference, Berlin; Characterization and optimization of a fermentation process for the production of high cell densities and lipids using heterotrophic cultivation of *Chlorella protothecoides*; December (2017), (poster presentation)

Abstract

In a world where the demand for natural resources exceeds the supply, microalgae can be seen as a sustainable bio-factory, finding applications in different fields of industry. This thesis focused on the development of a bioprocess to explore the potential of two microalgal strain: *Chlorella protothecoides* and *Schizochytrium* sp. The objective was to improve the total lipid productivity; to develop a cost effective and more sustainable methods to disrupt the cell wall and for the extraction of lipids, and to improve the sustainability of heterotrophic cultivations by using alternative carbon substrates. *Chlorella* and *Schizochytrium* were cultivated heterotrophically in a 5L bioreactor, a two-stage fed-batch process was developed obtaining a final dry biomass of 255 g/L and 90 g/L, respectively. A high carbon to nitrogen ratio triggered the accumulation of 58% of the dry cell weight *Chlorella* as lipids (max. productivity 16.7 g/L/day), rising to 70% for *Schizochytrium* (max. productivity 9-10 g/L/day). Although the main disadvantage of heterotrophic cultivation is the cost of substrate, *Chlorella* showed ability to grow on alternative carbon sources such as glycerol, acetate and molasses, thereby improving the flexibility and the sustainability of the cultivation. The production of the omega-3 fatty acids was studied in *Schizochytrium* sp., achieving a maximum concentration of 4.6 g/L DHA. More than 99% of cell disruption was obtained by developing a standard method using vortexing a microalgal suspension with glass microbeads. Due to the resistant structure of the *Chlorella* cell wall, the potential for enzymatic hydrolysis was explored and the combination of lysozyme and sulfatase showed a 7-fold improvement in lipid extraction. As a conclusion, the results of this research led to increased insights into the process parameters that determine microalgal productivity with respect to biomass an lipid production and methods to efficiently extract the intracellular lipids.

Acknowledgement

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Ian Marison, for the continuous support of my PhD, for his patience, motivation and knowledge. His guidance helped me in many times of my research, I learned how to think in a broader way and I will always be grateful for the opportunity he gave me. My gratitude also goes towards Dr. Rosaleen Devery, I really appreciate the time she took to help me. I must also thank Mary Rafter for her kindness and help in all administrative matters. The project was funded by the Irish Research Council whom I would like to thank for awarding me the PhD scholarship on the Irish Research Council Employment Based Programme and Monaghan Bioscience for being my industrial partner. In particular, I would like to thank my external supervisor Dr. Darragh Gaffney for his support and advices especially at the beginning of this PhD, Ciaran Forde for his great support and also all the nice people who helped make my stay in Monaghan enjoyable. Special thanks go to my colleagues of Biofactory Competence Center, Dr. Tanja Buch and Olivier Bremen. Further, I would like to thank the HES-SO Fribourg for allowing me to use their GC-FID, in particular to Dr. Jean-Pascal Bourgeois for his help in advices in analytical chemistry and Samuel Roth and Laurence Scheurer for their technical help.

On a more personal level, I would like to thank all the new friends I met during this experience. Thank you Aurélie for your initial support and encouragement in taking the choice to start this PhD. Un grazie speciale va ai Terpeni, sono stata davvero fortunata a conoscervi e di poter associare questi anni a Friborgo, non solo a lunghe giornate in laboratorio ma anche a tanti bei momenti e soprattutto alla nostre famose cene. In particolare, vorrei ringraziare Jessica, sei diventata una grande amica e un grande supporto, grazie per aver condiviso le nostre innumerevoli ansie! Grazie anche agli amici rimasti in Ticino e gli amici conosciuti a Sion, ho capito che l'amicizia è anche stare lontani senza che nulla cambi. Enfin, un grand merci à mes colocs, Manu et Jérôme, c'était bien de rentrer à la maison et de vous avoir souvent là.

Alla mia famiglia va il grazie più importante, mi avete sempre mostrato una fiducia cieca, priva di incertezze. Mi avete sempre incoraggiata a sognare in grande e a proteggere i miei sogni. Miscy, sei il mio punto fermo, la mia piccola grande sorella, la donna che mi sarà per sempre accanto. Mamma, mi hai trasmesso il rispetto e l'amore verso la bellezza della natura. Grazie per avermi fatto capire che la gentilezza e la bontà non solo aprono cuori ma anche porte. Papi, pur avendo raggiunto inimmaginabili traguardi professionali, sei stato per noi il migliore dei papà. Sei per me fonte di ispirazione e motivazione, mi hai sempre insegnato che le cose si

conquistano con coraggio e umiltà. Grazie, per gli inestimabili *cinque minuti*. Tutto quello che avete fatto per me non l'ho mai dato per scontato e ve ne sarò per sempre grata.

Finally you Pascal, we met unexpectedly and since the beginning of this thesis you soon became one of the biggest support for me. You bring balance, happiness and a bit too many apéros in my life. You always believed in me more than I did. I feel very, very lucky to have you, grazie amore mio.

Chapter 1

Introduction

1.1 General overview

1.1.1 Food is the new oil

The demand for natural resources from society currently exceeds supply. Some of the most developed countries consume much more than the ecosystems are capable of producing. According to the Living Planet Report of 2012, before 2050 we would need the equivalent of 2.9 planets if natural resources are exploited at such rate [5]. In October 2011, the world population reached over 7 billion and a recent United Nations study estimates that the next milestone of 8 billion people would be expected in 2024, followed by 9.7 billion in 2050 [6].

Overpopulation brings many different problems. Over 80% of the energy that the population uses every day comes from fossil fuels: petroleum, coal and natural gas [7]. Fossil fuels are recognized as an unsustainable source of energy because of the scarcity and contribution to environmental degradation [8]. The increase of oil prices in 2007 affected the cost of fertilizer and food production overall. Food prices were incredibly high and despite the application of economic strategies to stabilize the situation, the prices remained too high for the poor sections of the world populations [9]. The demand and consequent production of food became one of the new main concerns. As a solution to increase food production, an intensive and mechanized agriculture has been developed. The consequences are serious: large amounts of fertilizer, pesticides and herbicides are used, the fertility of the soil is dangerously compromised, deforestation is increasing rapidly, the consumption of water resources is abused and greenhouse gases are produced [10]. Carbon dioxide and other greenhouse gases are normally

associated with pollution caused by the combustion of fossil fuels used for human activities such as transport, heat and electricity generation. However, a report of Worldwatch Institute of 2009 estimated that livestock contributes 51% of the green house gas generation every year. The emissions are due principally to high amounts of exhaled CO_2, the production of methane from animals and the destruction of natural forests, which are replaced with degraded grassland [11]. Crop production for feed and the expansion of farms and pastures for livestock systems are the main forces behind deforestation, leading to significant environmental damage and the extinction of many animals and plant species every year [12]. The demand for freshwater has increased rapidly to maintain an adequate food supply for the population. A study of Mekonnen et al. showed that 15'415 L water were needed to produce 1 kg pork meat compared to 1'644 L to produce cereals, 726 L for fruit and 322 L for vegetables [13]. The results vary strongly depending the production region and system however such values gives an idea about the water footprint of different foods. The marine biodiversity is also endangered because of destructive fishing techniques. The rate of depletion of the world fisheries is faster than ever, with a fish supply increasing faster than the world population, 3.2% against 1.6%. The over-exploitation of fisheries has high risks of stock depletion and collapse driving towards a possible defaunation of the oceans [14] [15] [16]. In the book "Full Planet, Empty Plates", Lester Brown predicts that the world food situation is deteriorating, affirming that "Food is the new oil and land is the new gold." [17]. It is expected that the global demand for food will increase by 70 percent, to feed a population of about 9.7 billion in 2050 [6]. As time goes by it will become more and more difficult to restore the current condition of the natural ecosystem and solve the food scarcity problems. It is incredible how something as simple as the food choices we make every day can be related to this destruction. To prevent a food breakdown it is necessary to stabilize the food system aiming to find solutions to sustainably feed society. There is ongoing research for alternative, sustainable and economic fuels and complete food sources. As an example, "The Protein Challenge 2040" is the first global coalition which is investigating affordable, healthy and environmental friendly sources of protein [18]. New innovations and technologies are required to ensure security for people and to safeguard the natural resources [19].

1.1.2 Biggest changes start small

Microalgae, one of the world's oldest organism, might become a great contribution to solve some of the current challenges, from environmental degradation to find sustainable solutions to feed the world [19]. Microalgae are generally regarded as something unwanted and difficult to get rid of. However, they are an incredible and complete resource with a great potential. Microalgae are fascinating unicellular microorganisms so small that they cannot be seen with the naked eye, they come in many different shapes, colors and properties. Microalgae are one of the earliest forms of life in earth, evolutionary biologists estimated that their evolution started about 2.7 billion of years ago [20]. They are currently spread everywhere on our planet: from frozen lands in Scandinavia to the deserts of the Sahara. They are typically found in oceans and fresh water streams but also on land. The biodiversity of microalgae is vast, it is estimated that several million species exist - compared with only 250'000 species of higher plants - and only 40'000 have already been described and studied [21]. There is much more to discover.

The general advantages of microalgae are the following: (1) they can be grown anywhere so that there is no need for arable land; (2) weather conditions and geography are not an obstacle for the cultivation; (3) they require less water than terrestrial crops; (4) they have a relatively rapid growth; (5) under certain conditions the oil content can be very high, up to 50% or more of the dry weight; (6) they do not require herbicides and pesticides; (7) the biochemical composition has a high nutritional value, that can also be modulated by varying the growth conditions;(8) they are free from allergens (9) under phototrophic cultivation conditions they efficiently use CO_2 [19] [22] [23] [24].

1.2 Microalgal classification

All living organisms can be broadly divided into two groups: prokaryotes and eukaryotes, which are distinguished by the relative complexity of their cells [25]. In general, eukaryotic cells are highly organized and contain membrane-bound organelles, such as the nucleus, while prokaryotic cells do not. Bacteria and archaea are prokaryotes, while all other living organisms protists, plants, animals and fungi are eukaryotes. In the kingdom called Protista, are found any eukaryote (unicellular or multicellular) that is not classified as a fungus, plant or animal. There are three major groups: protozoa (animal-like), protophyta (plant-like) and molds

(fungus-like) [25]. All plant-like protists are autotrophs and they are called algae. Algae are a heterogeneous group of microorganisms which range from the microscopic blue-green algae to large, complex seaweeds, measuring up to a few meters. Based on growth size, algae are classified into two different categories, namely micro- and macroalgae [26]. Eukaryotic microalgae are classified into nine divisions: Glaucophyta, Rhodophyta, Heterokontophyta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta and Chlorophyta [27]. On the basis of their nutritional strategies, algae are into classified four groups: obligate heterotrophs, obligate photoautotrophs, facultative mixotrophs and obligate mixotrophs [27]. As example,the microalgal genus *Schizochytrium* is part of the Thraustochytriaceae family which is included in the Heterokonta phylum [28]. The classification of six genera in the Thraustochytriaceae are hard to distinguish using only a single feature [29], therefore it was based on the cell morphology of various stages during the life cycle. Some of these are the formation of large clusters of cells, the globose sporangia with or without proliferous bodies and the zoospore release caused by partial dissolution of the cell wall of the sporangia [30] [29]. On the other side, *Chlorella* is a genus of single-celled green algae belonging to the division Chlorophyta and more specifically to the Chlorellaceae family [20] [31] [32].

1.3 Cultivation approaches

1.3.1 Nutritional strategies in bioprocesses

In batch cultures, the fermentation starts by inoculating a cell suspension in a certain volume of fresh medium. The volume in the bioreactor remains constant and the biomass increases according to the microbial growth curve (Figure 1.1) while the substrate is consumed. The advantages are several: ease of operation and maintenance, low installation costs, use fresh seed train each production run, lower mutation and contamination risk, easier to integrate with downstream processing (DSP) and easier to validate. The disadvantages are the low productivity, the higher labor costs, the risk of substrate repression of the cell growth, the relatively low final cell densities and the accumulation of toxic products [33]. In fed-batch cultures, the fermentation starts as a batch culture but when the growth is exponential and the substrate is almost depleted, concentrated fresh medium i.e. feed, is introduced in the vessel. Different types of feed exist: linear, exponential, step-wise, etc. Compared to batch cultures, the volume is not constant, the system is called semi-open. Fed-batch strategies are the most important

application when substrate is toxic or overflow metabolism occurs. Other advantages are the less down-time compared to batch cultures, the production rate is extended, the growth and the production phases may be separated and there is the possibility of feeding metabolic precursor. The disadvantages are the higher installation costs compared to batch, the requirement of considerable knowledge about cell growth physiology, kinetics etc. in order to develop fed-batch control strategy and eventually the requirement of sophisticated biosensors and computer automation to control feed rate and maximize productivity [33]. In Figure 1.1 are schematized the two different nutritional strategies. For the fed-batch strategy, an example of stepwise feed is represented.

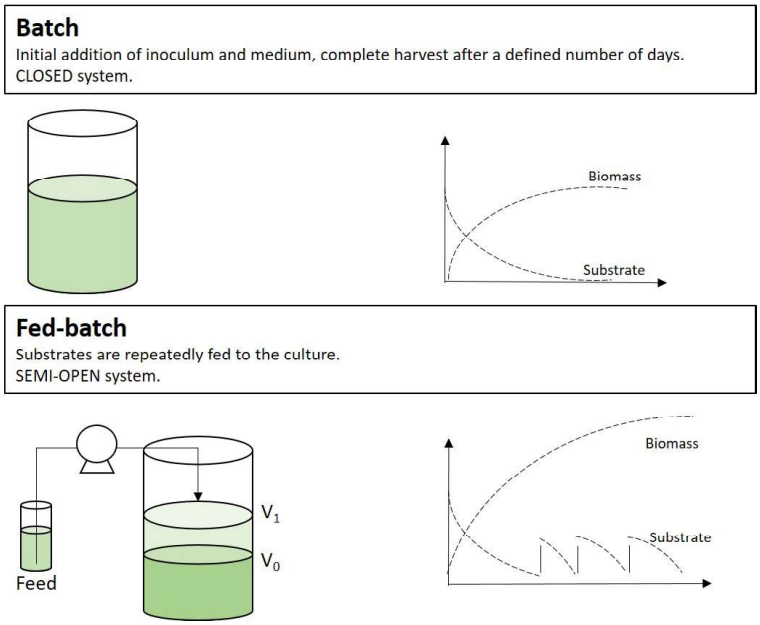


Figure 1.1: *Schematic representation of a batch and a fed-batch cultivation strategy.*

1.3.2 Phototrophic growth

Most microalgae grow photoautotrophically, obtaining the necessary energy to carry out metabolic processes exclusively from light sources through the photosythesis. The energy is then stored in carbohydrate molecules, which are synthesized from atmospheric CO_2. Phototrophic cultivation of microalgae is carried out in photobioreactors or in large-scale open pond systems, as represented in Figure 1.2, where the natural environmental conditions of microalgae are mimicked. Despite being cheap and relatively easy to clean and to maintain; the risk of contamination from bacteria, protozoa or other microalgae is very high [22] [34]. For these reasons, algal biomass and bio-products cannot be sold in the food or pharmaceutical market [35].

Figure 1.2: *Open pond raceways*

Figure 1.3: *Photobioreactor at AlgaeParc, Wageningen*

Several parameters cannot be controlled, the temperature fluctuates within a diurnal/seasonal cycle and the local weather conditions have a big impact on the process and reproducibility [8]. Non-potable water is suitable for growth of many algal species however, when microalgae are grown in open ponds the water requirements may increase due to evaporation [24]. In general, the microalgal biomass concentration remains low, mostly because of the limitation in the illumination area. The light does not penetrate more than a few centimeters through a dense algal culture therefore, scale-up is based on surface area rather than volume.

Photobioreactors are closed systems that, unlike the open pond, avoid contamination and permit single-species culture of microalgae. On the other hand, when installed indoors, they require artificial illumination which makes the process more expensive because of the high-energy inputs and the cost of the sophisticated construction is relatively high [20] [36]. When the photobioreactors are used outdoors, the process depends on the environmental conditions and the maintenance of optimal temperatures could be difficult, depending on the location and on the cultivation purpose [37]. As an example, a non-cooled closed system can imply culture tem-

peratures above 60°C, making temperature control indispensable to cultivate microalgae [38]. The cost of cooling and heating has a considerable impact on the final price of algal products. The most important factor limiting algae productivity in phototrophic cultivation is light. The design of the photobioreactors imposes limitations such as self-shading effects therefore, high cell densities ($>$ 20 g/L) are difficult to achieve. Light cannot diffuse through highly concentrated fermentation broths [22] [35]. Not at least, the technical difficulty in sterilizing the photobioreactors has impeded the application for algal culture for specific end-products such as high value pharmaceutical products [22] [39].

1.3.3 Heterotrophic growth

A feasible alternative to open pond and photobioreactors is heterotrophic cultivation. Organic substrates are used both as energy and as carbon sources [35] [22]. Although most known microalgal strains grow phototrophically, some microalgae are also able to grow in the absence of light and the advantages of this cultivation method are multiple. First of all, algal growth is independent of the light and the biomass is fed with organic carbonic sources. This supports high cell density cultures and higher productivity, at an improved growth rate. Lipid accumulation is also enhanced [40]. Conventional bioreactors can be used, avoiding to set-up or buy specific photobioreactors. No expertise with autotrophic growth and light controls are required [41]. The operations are relatively simple as is the daily maintenance. The microalgal cultivation process becomes similar to well-established procedures developed for fungal and bacterial strains. Microalgae are capable of modifying their metabolism according to varying culture conditions consequently, it is possible to modify, control and maximize the production of target compounds [42]. It has been observed that biomass, fatty acid profile and DHA content of cells vary largely depending on the physiological conditions and nutrient composition of the medium. Results could be improved adding to the medium components that have a biochemical effect [43].

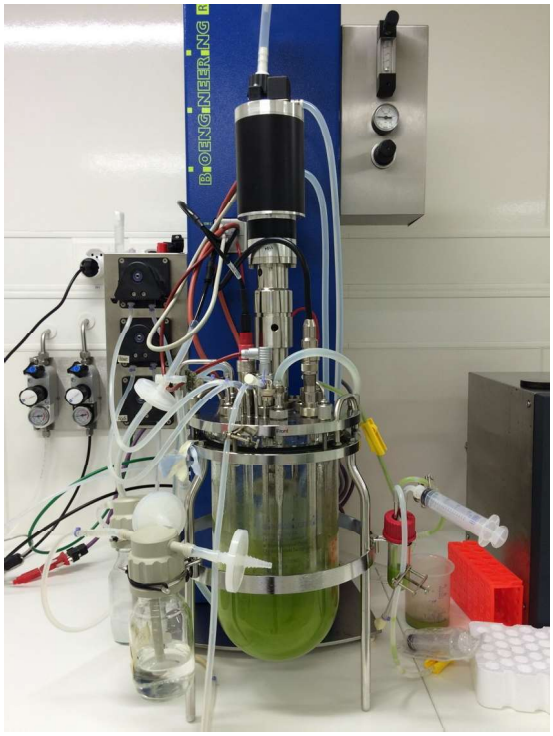


Figure 1.4: *In-house heterotrophic cultivation of* Chlorella protothecoides

Heterotrophic cultivation has been judged as the best option to grow microalgae through this project (Figure 1.4). However, this type of cultivation is not appropriate for all types of microalgae and the number of current commercially important processes where experience is gained, is limited [35]. The main disadvantages of heterotrophic culture, compared to phototrophic, is that the simplicity of the concept of microalgae as cellular factories producing liquid fuel from pure sunlight and CO_2 is lost [44]. The use of organic carbon sources adds an additional cost, along with the environmental impact of its own production.

1.3.4 Mixotrophic growth

Phototrophic and heterotrophic cultivation are two common methods to cultivate microalgae, however some microalgae have the ability to grow mixotrophically. In this growth regime, both the organic and inorganic carbon sources are used for cellular growth in the presence of light as the energy source [45] [46] [47] [48] [49]. An an example, high inoculum concentration of heterotrophic algal seed can be used as an effective method for contamination control in phototrophic systems [50].

1.3.5 Choice of microalgal strain

The next step was to select the most suitable microalgal strain that not only satisfies the requisites of heterotrophic cultivation, but also that has the potential to deliver successful results accordingly to the principal objectives of this project, the production of lipids.

To be suitable for heterotrophic culture, microalgae need to have different pre-requisites:(1) Facility of cell division and active metabolism in the absence of light; (2) Ability to grow in culture media with carbon sources in a well-defined mineral medium; (3) Ability to adapt rapidly to the medium conditions; (4) Capacity to resist mechanical and chemical stress in bioreactors, (5) non-toxicity [51] and (6) ideally they should be unicellular microorganism because they are easier to cultivate compared to filamentous strains, due to the great number of factors influencing the rheological properties of mycelial fermentations [43] [52]. The strains presented below satisfy all the requirements for heterotrophic cultivation.

1.3.6 The reference strain: *Chlorella protothecoides*

For this study a model microalga will be considered: *Chlorella* sp.. The strain used for this research, *Chlorella protothecoides* (ATCC 30411), was found in a freshwater ecosystem in Holland and it is represented in Figure 1.5(a). *Chlorella* is a genus of spherical unicellular green microalgae, the size of which varies between 2 to 10 μm. *Chlorella* reproduces asexually by auto-sporulation. Inside the cell wall of the mother cell, several daughter cells are formed, already completed with their own cell wall. After maturation the mother cell wall ruptures and the daughter cells are liberated [53]. *Chlorella* was cultivated for the first time in the laboratory of Otto Warburg in 1919 and after years of research, it became clear that its nutritional value was higher than plants, showing a high accumulation of lipids under specific conditions [54]. Its annual production exceeds 2,000 tonnes, mostly used for dietary supplements and nutraceuticals, with a minor share destined to the cosmetic market and aquaculture [55] [56] [57] [20] [58] [59]. *Chlorella* is one of the strains dominating the market because of its high protein content, high lipid content and the whole nutritive value. It is one of the few strains employed for human consumption; a toxicology study with high FDA standards has been conducted revealing that the cellular material of this strain is safe and it can be sold as a food supplement, adding to its economic viability [60]. It is also easy to grown both auto-

Table 1.1: *Overview of microalgal strain cultivations focused on the production of DHA*

Species	Carbon source	μmax [h^{-1}]	DCW [g/l]	Tot. lipid [%]	DHA [%]	DHA [g/L]	Prod$_{DHA}$ [mg $l^{-1}h^{-1}$]	Ref.
Crypthecodiunium cohnii	Glucose	0.089	2.73	-	56.8	-	-	[63]
Crypthecodinium cohnii	Acetic acid	0.053	61	51	32	19	48	[64]
Schizochytrium limacinum SR21	Glucose	-	48.1	77.5	35.6	13.3	138	[65]
Schizochytrium LU310	Glucose	0.04	88.6	-	-	24.7	241.5	[66]
Schizochytrium limacinum SR21	Glycerol	-	61.8	65.2	72.6	20.3	123	[67]
Schizochytrium sp. HX-308	Glucose	-	-	-	60	-	-	[68]
S. mangrovei	Glucose/Fructose	-	27.9	-	20.9	5.8	80.6	[69]
Thraustochytrium sp.	Glucose	-	24.7	53.5	31.4	4.6	95.8	[70]
Thraustochytrium sp.	Glucose	-	24	57.8	40.1	5.6	117	[71]
Thraustochytrium roseum 28210	Starch	-	6.1 - 17.1	-	48.3 - 58.2	-	-	[72]
Thraustochytrium aureum	Glucose/Fructose	-	1.1 5.0	-	41-75	-	-	[28]

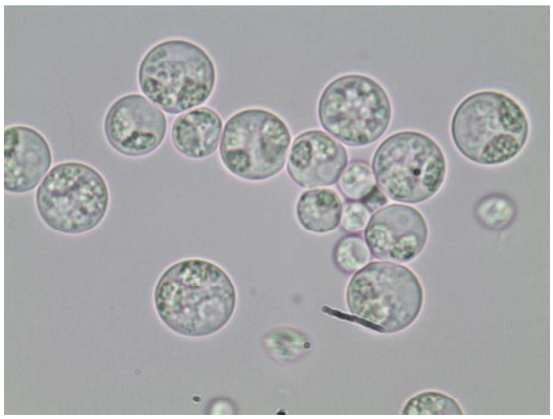
trophically and heterotrophically, it can achieve high biomass and it has a high rate of lipid productivity, even using alternative carbon sources [61]. In heterotrophic conditions, *Chlorella protothecoides* reached four times the lipid level observed in photoautotrophic cultures with a higher growth rate [62].

1.3.7 An alternative strain: *Schizochytrium*

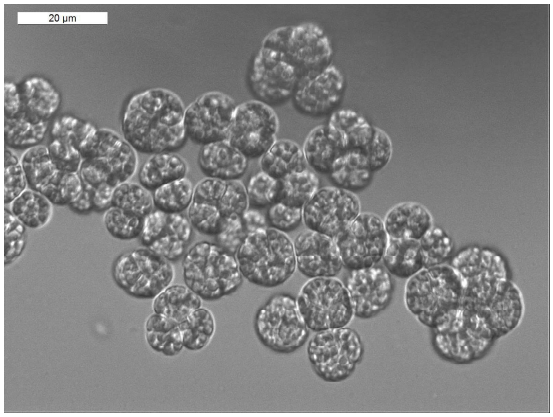
Table 1.1 shows different heterotrophic algal strains which are currently used for the production of omega 3 fatty acids. *Schizochytrium* is a spherical unicellular microorganism that belongs to the family of Thraustochytriaceae [73]. It was selected as principal strain because of several advantages specific to Thraustochytrids. They are classified as oleaginous microorganisms due to the high production and accumulation of omega-3 fatty acids. The potential for the extraction of novel and valuable bio-active products is attractive for the medical and food industries [28]. In Table 1.1, *Schizochytrium* is the strain with the highest DHA production reaching up to 24 g/L, which is the reason why it is considered a promising and commercially viable alternative DHA feedstock [66].

In general, Thraustochytrids are widely distributed in marine and estuarine environments. In particular, the strain used for this project, *Schizochytrium* sp. (ATCC 20888) was isolated

from estuarine water in California. Cells of *Schizochytrium* are thin-walled, spherical and pale yellow. Continuous binary cell divisions release zoospores which form large colonies [29] [30]. In culture media containing high chloride concentrations, cells tend to aggregate forming clumps bigger than 150 microns [74]. *Schizochytrium* has also the characteristics, over other strains, to grow at faster rate, to have higher tolerance to shear stress, high glucose concentrations and high salinity levels [43].



(a) *Chlorella protothecoides*



(b) *Schizochytrium* sp.

Figure 1.5: *Optical microscopy images of the two microalgal strains. The magnification was 1000x with immersion oil.*

The taxonomic structure of Thraustochytriaceae has been the subject of many discussion, especially the genus level classification. Yokoyama and Honda reorganized the genus *Schizochytrium* and named two new genus: *Auranthyochytrium* and *Oblongichytrium* [29]. Classification was based mainly on the cell morphology, pigments, fatty acid profile and molecular analysis of the components of the small eukaryotic ribosomal subunit i.e. 18S rRNA, which is useful to reconstruct the evolutionary history of organisms and to decide the classification [29] [75]. Generally, *Schizochytrium* strains are used naturally, meaning that they are not modified genetically

as for example in the industrial process of Martek, Biosciences (USA) [76]. They can accumulate large amounts of triacylglycerol, up to 50%-80% of dry biomass, with a high proportion of long chain polyunsaturated fatty acids (PUFA). They are well known for a high production of omega-3 fatty acids and in particular of docosahexaenoic acid (DHA), which in certain cases can account for 20-40 % of total lipids. Some strains can also produce docosapentaenoic acid (DPA), arachidonic acids (AA) and eicosapentaenoic acid (EPA) [70] [77] [75]. *Schizochytrium* sp. ATCC 20888 possesses an enzyme called the PUFA synthase complex which appears to be unique for Thraustochytrids; normally only marine bacteria have the gene that encodes for such an enzyme complex. It has been proposed by Hauvermale et al. that in the past, *Schizochytrium* obtained this gene from bacteria by lateral gene transfer [78]. In 2003, the FDA reported that *Schizochytrium* ATCC 20888 was not pathogenic: chemical and biological analysis of the strain confirmed the absence of common algal toxins. Therefore, DHA microalgal oil was generally recognised as safe (GRAS) and from 2012, Martek Bioscience Corporation began to market microalgal oil rich in DHA produced from *Schizochytrium* sp. in baby food and other food supplements. Also the production of carotenoids, such as astaxanthin, zeaxanthin, canthaxanthin, echinenone, and beta-carotene from Thraustochytrids has received attention from the industry [70] [79].

1.3.8 Nutritional requirements for medium selection

Chlorella and *Schizochytrium* belong to different classifications: the first derives from a division of freshwater and terrestrial green algae of the family Plantae (Viridiplantae) and the latter is a Thraustochytrid, a type of marine algae classified as Protist [80] [81]. The nutritional requirements of the two species are different. *Schizochytrium* has a broad salinity tolerance and requires high concentrations of Na^+ ions for substrate transport and other metabolic activities. Sodium ions have a positive effect on particular enzymes, stimulating growth and improving respiration and substrate uptake [82]. Sodium and potassium are also important for osmotic regulation of *Chlorella* in a process called sodium/potassium pump, however it was observed that its adaptation to high salinity was characterized by the accumulation of osmolytes, which are compounds with the function to maintain the cell volume counteracting osmosis, such as carbohydrates [83]. In general, the essential nutritional needs for microalgae include macro-nutrients such as carbon, hydrogen, nitrogen, phosphorus, sulfur, magnesium and potassium. To begin, it was decided to select a medium with glucose as the main carbon source

because it is the most commonly used carbon source for heterotrophic cultures of microalgae and many other microbial species. It possesses higher energy content per mole compared to many other substrates (glucose produces 2.8 kJ/mol of energy compared to 0.8 kJ/mol for acetate) [84]. Furthermore, glucose is reported to promote physiological changes in *Chlorella vulgaris*, which strongly affect the metabolic pathways of carbon assimilation, size of the cells, volume densities of storage materials, such as starch and lipids, protein, chlorophyll, RNA, and vitamin contents [35]. Yeast extract (YE) is frequently used as organic nitrogen source. It was reported to be the best nitrogen source for biomass production of *Chlorella protothecoides* among different organic and inorganic sources such as urea and glycine [62]. After carbon, hydrogen and oxygen, nitrogen is the most important element, accounting about 10% of the dry cell weight [85]. Nitrogen has particular importance in the accumulation of lipids, the carbon to nitrogen ratio is a key factor in the optimization of the cultivation medium [45]. Microalgae require vitamins of the B group as growth factors which are also contained in yeast extract. It is especially rich in thiamine (Vit B1) which is a co-factor for several essential carboxylase enzymes, including acetyl coenzyme A (CoA) carboxylase, which is involved in fatty acid synthesis [86]. Fatty acid synthesis is regulated by the enzyme acetyl coenzyme A carboxylase (ACCase). The carboxylation during the elongation of the fatty acid chain is dependent on biotin. This co-factor is consequently very important and is found also in yeast extract [87]. Other important nutrients for cell growth are phosphorus, magnesium, sulfate and calcium [88] [57]. In some microalage, iron deficiency is one of the factors limiting algal biomass productivity in batch culture conditions. Studies showed that adding $FeCl_3$ to the medium prolonged the period of the exponential growth phase of *Chlorella vulgaris* and increased the final cell density [89]. Elements such as boron, zinc, copper, manganese and cobalt are important, although they are required in smaller amounts. Most of these elements serve either a structural or functional role in the cells, i.e co-factors for enzymes [90]. All these points were taken care during the selection of the cultivation medium of the two microalgal strains.

1.4 Lipid metabolism in microalgae

Lipids are a broad group of biological molecules that are insoluble in aqueous solution and soluble in organic solvents. Lipids are (1) involved in the formation of cell membranes; (2) they are present in complex structures such as cholesterol and other biologically active molecules;

(3) they act as energy storage molecules. Below are listed three groups of lipids.

- **Fatty acids** are long-chains of hydrocarbon molecules containing a carboxylic acid moiety at one end. A fatty acid is saturated when no double bounds exists between carbons whereas unsaturated fatty acids contains double bonds (C=C). Monounsaturated fatty acids have one C=C bond, and polyunsaturated have more than one C=C bond. Saturated lipid tends to be solid at room temperature and unsaturated fatty acids are fluids.

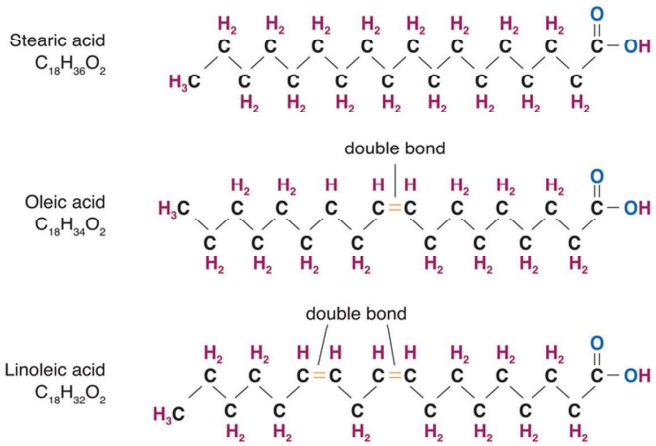


Figure 1.6: *Structure of different types fatty acids*

- **Triglycerides** are formed by the combination of glycerol with three fatty acid molecules through ester bonds.

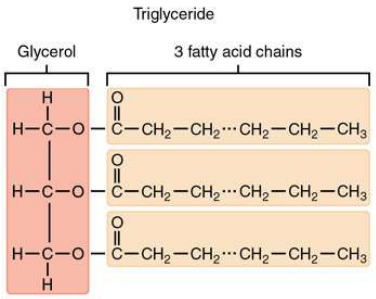


Figure 1.7: *Structure of a triglyceride*

- **Other main groups of lipids** involve phospholipids, sphingolipids, sterol lipids such as hormones and cholesterol, phenols such as carotenoids, saccharolipids and polyketides.

Lipids produced by microalgae are grouped into two main classes (1) storage lipids (neutral lip-

ids) and (2) structural lipids (polar lipids). Storage lipids are mainly in the form of triglycerides (TAGs). Research suggest that TAGs are predominantyl constitued of saturated and monounsaturared fatty acids, whereas long-chain polyunsaturated fatty acids are found in structural lipids. Phospholipids and glycolipids are present in the cell membranes [91] [92] [93] and particular strains of microalgae, such as *Schizochytrium* are capable of accumulating significant amounts of long-chain polyunsaturated fatty acids (lcPUFA) in their membrane [94].

1.4.1 Fatty acid metabolism

The mechanisms involved in the fatty acid biosynthetic pathways in microalgae have not been extensively studied and most information has been theoretically deduced by homology with bacterial and plant systems [95]. The synthesis of fatty acids take place in the chloroplast and begins with the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACCase) to form malonyl-CoA [96]. There is evidence that ACCase activity is a primary determinant of the rate of fatty acid synthesis. Therefore, the over-expression of ACCase has become an enzymatic target to increase algal lipid yields [95] [97]. The growing fatty acids are esterified to acyl-carrier protein (ACP), this can be seen as a flexible arm, which transfers the growing fatty acid chain to each of the enzymatic centres of the fatty acid synthase complex. ACP is an apoprotein, it needs an additional component called a prosthethic group to show full biological activity and become a holoprotein. The prosthetic group is phosphopantetheine, derived from coenzyme A, and is tightly bound to the serine residue. In 1971, Sirevag and Levine used *Chlorella reinhardtii* mutants to study algal fatty acid biosynthesis and concluded that FAS was ACP-dependent [96] [95] [97]. The enzyme malonyl-CoA:ACP transacylase transfers a malonyl group from malonyl-CoA to homo-ACP forming malonyl-ACP [97]. β-Ketoacyl-ACP-synthase III (KASIII) condenses acetyl-CoA with malonyl-ACP to form a -ketone, the acetyl group is again extended with a 2-carbon unit. In *de novo* fatty acid synthesis (FAS), the cyclic condensation reactions extend the acyl group to a middle chain length fatty acid. The process occurs seven times to form C16 (palmitoyl-ACP). The end products of this synthesis are usually the saturated fatty acids palmitate and stearate (C18) with the latter predominating (in most plants by 2-3 times that of palmitate) [95]. Generally, after the synthesis, stearic acid is desaturated to oleic acid by stearoyl-CoA desaturases [1]. Lipid catabolism comprises two main steps: the lipolysis and the β-oxidation. The first step occurs in cell membrane or lipids

droplets, lipases catalyzes the hydrolysis of triglycerides into glycerol and free fatty acids. Afterwards, the degradation of fatty acids to acetyl-CoA, which occurs in peroxisomes through the -oxidation pathway in green microalgae, and can sometimes occur in mitochondria in some other algal species [98] [99].

1.4.2 Mechanism of enhanced lipid accumulation

Under particular conditions, microalgae can accumulate high amounts of lipids [70] [28] [100]. In some microalgae, the lipid content increases significantly after being subjected to stressful conditions, $20-50\%$ by weight of dry biomass and sometimes exceed 80% [101]. Such stress conditions are very different compared to the optimal conditions for the normal development and growth of microalgae and they can cause considerable changes at all functional levels of the organism. Stress factors are classified into two groups: nutrimental and physical [102]. The nutrimental factors are considered as manipulation of culture media composition (carbon source, nitrogen, phosphorus, iron and silicate deficiency), while physical are described as manipulation of operation conditions and external factors that affect the microalgae growth (high light intensities, temperature, pH, salinity, dissolved oxygen and electromagnetic fields) [103] [102] [22].

The increase in total lipid content in the cells can be regarded as a storage of energy. In fact, the carbon uptake continues even when essential substances are limited and it is accumulated inside cells as long chains of carbon. Storage lipids, primary triglycerides, are a compact and efficient cellular store of carbon and energy, they are relatively stable and they can be packed into lipid vesicles. Oxidation of lipids generates more energy than carbohydrates, providing an excellent reserve for biomass production once nitrogen becomes available. Production of lipids consists in a dense, highly reduced storage compound that allows the cells to survive [102] [103].

Nitrogen limitation is the most frequently reported method of enhancing lipid content [66] [35] [87] [101] [104]. It has a reliable and strong influence on lipid content in many species [102]. Nitrogen is a very important element for metabolism, as a main constituent of protein and genetic material. After carbon, oxygen and hydrogen, nitrogen is the most abundant element in microalgae. Therefore, when cells divide they require a supply of nitrogen [103]. Under nitrogen limiting conditions, the ability of cells to synthesize molecules necessary for cell

growth such as proteins, nucleic acids and chlorophyll, is compromised. Consequently, the growth rate becomes dependent on the intracellular nitrogen concentration [105] [106] [102]. However, the genes and the enzymes inducing the metabolic switch to triacylglycerol accumulation in microalgae are poorly known [107]. It was reported by Morales et al. that under nitrogen deprivation, *Chlamydomonas reinhardtii* showed triglyceride accumulation and up-regulation of a gene encoding an enzyme with an unusual structure: glycerol-3-phosphate dehydrogenase (GPD). This enzyme catalyze the formation of glycerol-3-P (G3P) which is the backbone of TAGs and membrane lipids. It appears that TAG accumulation is influenced by affecting G3P levels and that the glycerol is used as an intermediate for TAG synthesis in *C. reinhardtii* [108] [109]. The factors leading *Chlorella* to accumulate lipids are not only related to the higher production of lipid-synthesizing enzymes but also to the cessation of the synthesis of other enzymes associated with cell growth and proliferation [35]. As a result, the starvation mechanism has a contradictory nature [101]. The accumulation of lipids under unfavorable conditions unfortunately limits biomass production and overall lipid productivity, which is the total amount of lipid formed per unit culture volume and time [84]. A strategy aiming to increase the lipid accumulation without reducing the cell growth needs to be developed.

1.4.3 Accumulation of lipids in alternative single-cell microorganisms

Microalgae are not the only microorganisms with the capacity to accumulate lipids. Different cellular and biochemical mechanisms are involved in neutral and polymeric lipid accumulation; four main single-cell microbial groups exists: yeasts, microalgae, bacteria and archaea [27]. Generally, in oleaginous yeast, TAG and/or sterol esters are the primary neutral lipids accumulated inside the cell to a level corresponding to more than 20% of their biomass [110] [111]. As an example, *Yarrowia lipolytica* has been engineered to improve lipid production, achieving more than 50% of the biomass. Other known oleaginous yeasts are *Candida, Cryptococcus, Rhodotorula, Rhizopus, Trichosporon and Yarrowia* [110]. In specific types of bacteria and archaea, polyhydroxyalkanoates (PHA) are mainly accumulated and only to a lesser extent TAG are stored [27]. PHA are bio-degradable, environmentally friendly and biocompatible thermoplastics, the main PHA producers are the bacterial strains *Bacillus, Alcaligenes, Pseudomonas, Azotobacter, Ralstonia, Cupriavidus necator,* etc. [112]. As a similarity to microalgae, such type of lipids are accumulated when bacterial growth is limited by depletion of nitrogen,

phosphorous or oxygen and an excess amount of a carbon source is still present [112]. The microbial fungus *Mortierella alpina* has been known to produce polyunsaturated fatty acids such as gamma-linoleic acid and arachidonic acid under normal growth medium conditions, whereas under stress condition, this fungus produces omega-3 fatty acids in the mycelial biomass [113] [114].

1.4.4 Omega-3 fatty acids biosynthesis

Omega-3 fatty acids belong to the family of polyunsaturated fatty acids (PUFAs). The molecule have a double bond at the third carbon atom from the end of the chain. The methyl end is called omega and the other end finishes with a carboxylic acid. Synthesis of omega-3 fatty acids occurs via the elongation of the fatty acid chains with elongases or the insertion of additional cis-double bonds with desaturases, in the endoplasmic reticulum [1] [115]. Figure 1.8 shows the PUFA synthetic pathways to 22-carbon fatty acids from oleic acid in eukaryotic systems via diverse fatty acid desaturases.

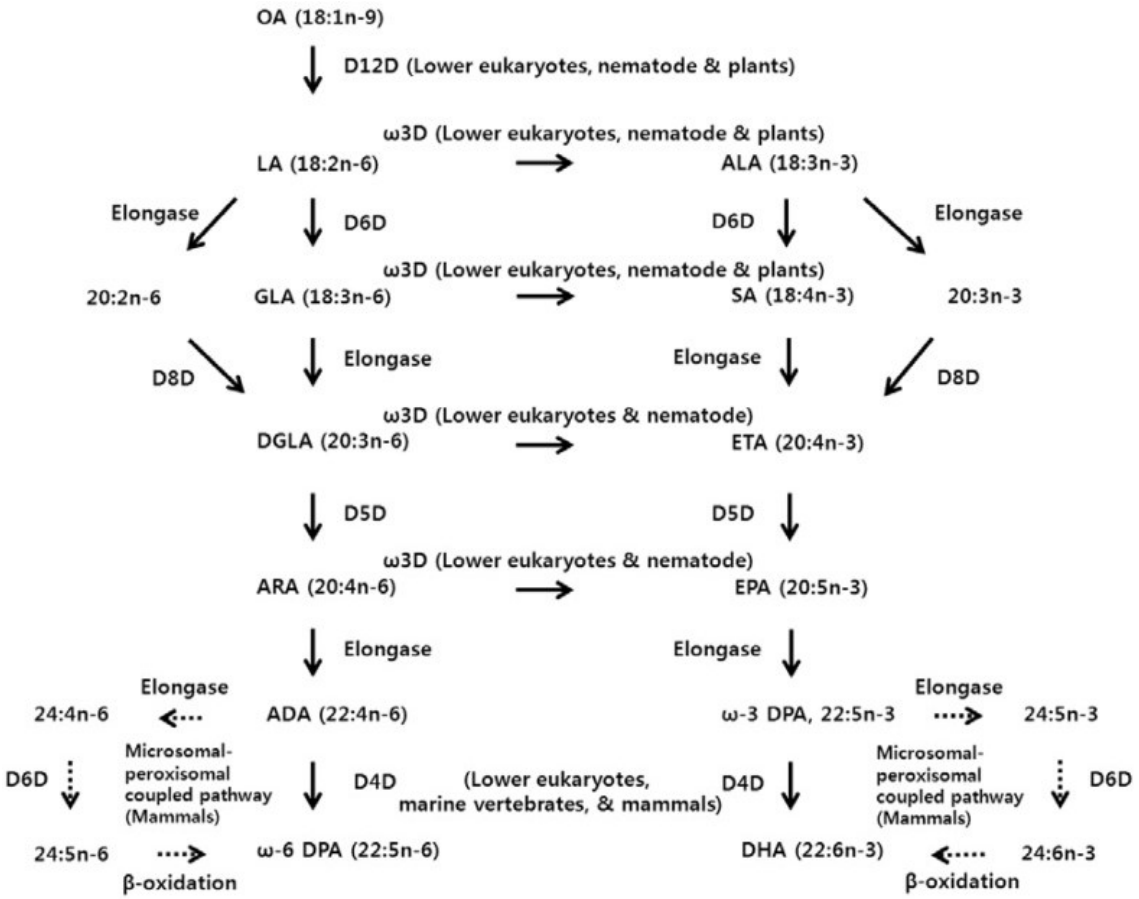


Figure 1.8: *PUFA synthetic pathway to 22-carbon fatty acids from oleic acid in eukaryotic systems. On the left side are represented the n-6 pathways and on the right side the n-3 pathways [1].*

With a few exceptions, that are going to be illustrated later, both higher and lower eukaryotes share a common pathway leading to the production of EPA, differing slightly depending on the species. As an example, EPA biosynthesis in the diatom *P. tricornutum* involves different elongation and desaturation steps combining the n-6 and n-3 pathways. Arachidonic acid (ARA 20:4*n*-6) is converted to EPA by a omega-3 desaturase [96], as represented in Figure 1.8. However, differences are found in the mode of converting EPA into DHA [115]. Microalgae species included in the eukaryotic kingdom Chromista, possess a specific $\delta 5$ elongase and $\delta 4$ desaturase for converting EPA into DHA [96] [1]. This pathway is simpler than the one found in fish and other animals. Chromista do not produce DHA via the presence of the so-called Sprecher pathway. In such a process DHA (22:6n-3) is produced from EPA (20:5n-3) through two successive elongations to 24:6n-3 and then converted by a $\delta 6$ desaturase followed by a peroxisomal chain shortening reaction (Figure 1.8) [116]. Mammals, including humans, cannot synthesize omega fatty acids *de novo* because they lack $\delta 12$ and $\delta 15$ omega-3 desaturase and they must consume them in the diet or in other nutritional supplements. Plants generally cannot synthesize PUFAs containing more than 20-carbons, except a few species that produce gamma-linolenic acid (GLA) and stearidonic acid (SDA) [1].

1.4.4.1 PUFA synthase

Recent studies discovered that *Schizochytrium* sp., other eukarotic thraustochytrids and some marine microbes, posses an alternative anaerobic pathway for n-3 LC-PUFA synthesis [117]. Previously, it was assumed that all LC-PUFA were produced by variations of the same basic pathway which involves a series of elongase and desaturase reactions to generate PUFAs from pre-existing, short-chain fatty acids [78]. This alternative enzymatic system was first described in marine bacteria producing EPA, such as *Shewanella pneumatophori* [73]. It was shown that LC-PUFA were synthesized *de novo* from malonyl-CoA by subunits of an enzyme complex called PUFA synthases [117] [96]. PUFA synthases do not have a requirement for molecular oxygen for insertion of the carbon-carbon double bonds and the synthesis mechanism is sometimes referred as the "anaerobic pathway" [118]. Some domains of such an enzymatic complex show homology to those found in FAS systems and polyketide synthase (PKS) systems [78] [118]. Depending on the specific PUFA synthase, the main product can be a single PUFA such as DHA, EPA, or ARA, or a mix of PUFAs such as DHA and DPA n-6 or DHA and EPA [118]. Genes similar to the one found in *Shewanella pneumatophori* have been iden-

tified in a number of deep-sea bacteria such as *Photobacterium profundum*, *Moritella marina*, *Colwellia psychrerythraea* and Thraustochytrid marine protists including *Schizochytrium* and *Thraustochytrium* [73]. Hauvermale et al. confirmed that the DHA and DPA present in the oil of *Schizochytrium* (ATCC 20888) are the products of a single PUFA synthase enzyme [78]. In general, PUFA synthases characterized in marine bacteria appear to produce predominantly a single product: either EPA or DHA [78].

1.4.5 Genetic manipulation and engineering of microalgae

Due mainly to the high cultivation cost of microalgae and low biomass productivity, genetic modifications of the available microalgal strains are demanded for further improvement in the development of microalgae-based biorefinery [119]. However, the identification and subsequent targeted genetic manipulation of key enzymes to improve algal lipid production are difficult and this area has received little attention and is still in its infancy [120] [121]. One of the more successful attempts is the overexpression of glycerol-3-phosphate dehydrogenase (G3PDH), an enzyme involved in supplying glycerol-3-phosphate (G3P) required for TAG formation [108]. Appropriate genetic engineering could also help overcome the inability of obligate photoautotrophs to grow in presence of organic sources. This is often the main obstacle preventing the efficient heterotrophic production of microalgal metabolites [51]. As an example, Zaslavskaia et al. demonstrated the feasibility of introducing a gene encoding a glucose transporter [122]. While genetically optimized microalgae may appear to offer great potential, there are also several disadvantages [123]. Genetically engineered microalgae grown in open ponds have a potential risk to spread in the environment and their advantage to grow could results in devastating effects on the existing ecosystem [108]. Furthermore, when microalgal products are sold as food supplements, nutraceuticals or baby food, genetic modification could influence negatively the choice of costumers, which are likely to be part of a segment more attentive to health and environmental impacts.

1.5 Application areas of microalgal biotechnology

Despite the fact that microalgae have been known for centuries, commercial large-scale production found applications in different fields of industry only a few decades ago. New techniques and products were developed even though the complete potential in biotechnology is currently

underexploited and further research is needed [51]. The industrial interest is mainly due to the biodiversity, the versatility and flexibility and the opportunity to modify the formation and production of the targeted natural compounds [51] [120].

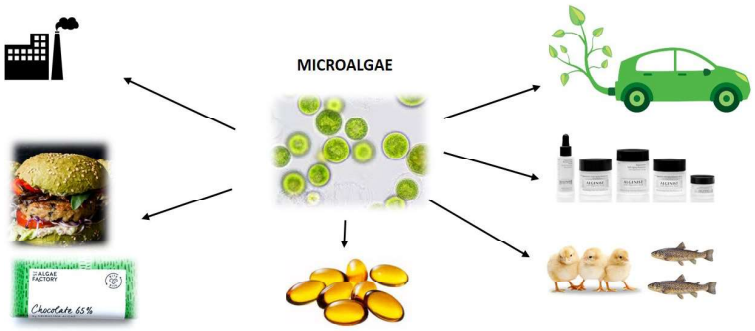


Figure 1.9: *Industrial application of microalgae*

Microalgal biomass is one of the predominant products in microalgal biotechnology and can be sold as feed for animal nutrition and in aquaculture, but also for human health in the health food market [120]. Microalgae contain highly valuable compounds such as essential amino acids and fatty acids, antioxidants, vitamins, iron and other essential trace minerals [124]. Therefore, they have been used to fortify conventional food, increasing the nutritional or health benefit [125]. There is also potential of bioactive molecules from microalgae such as antioxidants, to be used as primary active ingredients in cosmetic products and in pharmaceutical industry [59] [38] [126]. Microalgae produce a large variety of compounds to help their adaptation and survival in different environmental conditions [114]. This research focuses particularly on another surprising capacity: some microalgal strains can accumulate lipids up to 30-70% of their dry weight [127]. Microalgal lipids constitute a promising alternative source for the production of biodiesel but also for humans, animal feed and personal-care products.

1.5.1 Microalgae as a source of biodiesel

The global demand for energy is continuously growing and alternative fuels are necessary for environmental sustainability and fuel security. The most promising solutions are biofuels, which are liquid or gaseous fuels that are predominantly produced from biomass. The first generation of biofuels was generated from plant oils, sugar beet, starch, etc.; however, this technique was criticized due to the negative effect on the biomass prices of the global food market. The second generation was produced from non-edible sources such as wood, organic

waste, food crop waste and from the process of different biomass [128]. Ideally, a biofuel must provide a net energy gain over the energy sources used to produce it, have environmental benefits, be economically competitive, and be producible in large quantities without reducing food supplies [128]. In 1978, the United States Department of Energy funded a program called the Aquatic Species Program, to produce renewable fuels focusing on biodiesel from algae. After two decades the funding for the program was discontinued, even though promising advances were made in the engineering of microalgal production systems [129]. Reasonable productivities and the high accumulation of oil make microalgae a very promising sustainable resource. After the extraction processes, the algal oil can be converted into fuel through a process called trans-esterification. Triglycerides react with alcohol in the presence of a catalyst producing mono-esters, better known as biodiesel [130]. Further research and efforts are still necessary before the process would become cheap enough to compete with fossil fuels. Numerous problems must be overcome before algae will be an economical, sustainable alternative to offset petroleum. Currently some companies can already produce thousands of gallons of oil but not at a commercially economic value, therefore work was shifted toward the production of high valuable biomolecules present in microalage.

To make microalgal products more attractive, a solution would be to take advantage of all the various components of the biomass maximizing the profitability and also preserving the environment [131]. The biorefinery or algorefinery concept is inspired by the petroleum refinery concept where the aim is to minimize the initial costs and energy requirements by improving the product yield [114] [132]. All valuable components from the microalgal biomass must be valorised and used in order to become competitive [133].

1.5.2 Microalgae as a source of high valuable molecules

Algal biomass is used as feed for animals and in aquaculture because of the positive effect on animal physiology, boosting their immune system [134]. In aquaculture, microalgae are used to nourish molluscs, shrimps and fish. Microalgae are the natural food source of such animals, providing high nutritional values. It is important to not forget that microalgae are at the bottom of the food chain in aquatic ecosystems: fish are rich in omega-3 fatty acids because of their consumption [114]. Artificial diets in aquaculture lack natural sources of pigments that give organisms such as salmon and trout their characteristic coloration. Thus, carotenoid pigments

like astaxanthin must be supplied with the diet. Another example is the greening of oysters using the diatom *Haslea ostrearia*, after this process the product market value increases by 40%. The annual worldwide aquaculture market for this pigment is estimated at US$ 257 million with an average price of US$ 2500/kg. Astaxanthin is used also in human health because of the potent antioxidant activity which seems to be beneficial in cardiovascular, immune, inflammatory and neurodegenerative diseases [59] [135]. Carotenoids are a broad class of pigments produced naturally from plants, bacteria and also microalgae. Beside astaxanthin, the most known and commercialized is β-carotene, which is used in food supplements as vitamin A precursor. Lutein is sold to prevent eye diseases including age-related macular degeneration, cataracts, and retinitis pigmentosa but also for colon cancer, breast cancer, diabetes and heart disease [59] [136]. Chlorophyll is extracted from microalgae and used as an additive in pharmaceutical, cosmetic products but also as a natural food coloring agent. Additionally, it has antioxidant and anti-mutagen properties [137]. In skin care products such as creams, mask scrubs, serum beauty oils and also in sun protection and hair care products, it is possible to find microalgae due to the high content of fatty acids and antioxidants that seems to prevent dryness, skin problems and aging effect [59].

1.5.3 Food industry and nutraceuticals

As already mentioned, one of the new challenges of our society is to provide optimal nutrition efficiently and economically, eliminating food scarcity and restoring a healthy ecosystem [138]. Microalage are one of the most promising sources of innovative ingredients in food and pharmacological companies. Popular lifestyle considers microalgae, in particular *Chlorella* and *Spirulina* as a "superfood" but the high nutritional value of microalgae was known already in ancient civilizations such as the Aztecs in Mexico and other tribes in Africa [21]. Microalgae were used to survive during the Chinese famine [59] and since thousand of years Chinese herbalists use microalgae to treat vitamin deficiency diseases [90]. Algae have a rich composition and a high nutritional value: they are made up of protein, carbohydrates, healthy fats and other micro-nutrient such as vitamins, minerals and antioxidant, constituting a complete nutritional product for the human consumption. Nowadays, they are marketed in different forms such as tablets, capsules and liquids. They can also be found in pasta, snack food, candy bars, gums and beverages [59]. Their composition enhance the nutritional content of conventional food preparations, affecting positively the health of the consumer. Algae are

used more and more as replacements of different fundamental ingredients in the food industry. Eggs, for example, are added in processed food to provide texture, thickness, mouth-feel and retain moisture. However, because of egg shortages - due to the consequences of avian influenza outbreak [139] [140] - price increases and dietary requirement of the population, many manufacturers decided to stop or reduce their use, developing egg-replacements. The leading company in the microalgal food technology Solazyme, introduced a microalgal high lipid powder that provides exactly the same results as eggs but with the advantage of adding more nutritional value, reducing the calories, saturated fats and cholesterol. The protein content in microalgae is high and with a complete aminoacid pattern, the quality was shown to be equal or even higher than other high quality plant proteins [138]. The founder of Solazyme, Rob Rhinehart affirmed "The algae production of food ingredients is leagues more sustainable than using traditional agriculture. I really think thats the future. It needs to happen because our burden on the environment is unsustainable." [138]. Nutraceuticals include any food or food products that provide nutrition and pharmaceutical-health benefits such as prevention or treatment of diseases [126]. Microalage found a good place in this sector, being sold as dietary supplements because of the high number of essential bioactive compounds such as vitamins (vitamin A, vitamin of the B groups, vitamin C, vitamin E, biotin, folic acid and panthothenic acid), minerals (iodine, magnesium, potassium, calcium, and magnesium and iron), antioxydants, phenolic compounds, sterols, long chain polyunsaturated fatty acids (omega-3 and omega-6), essential amino acids and polysaccharides with different activities i.e. antitumor, anticoagulant, antiviral, etc. [125] [126] [124] [141] [114]. Algal material must be analyzed to prove their harmlessness. Most of the microalgal products derived from well known strain such as *Chlorella*, *Spirulina*, etc. commercialized on the market were approved from FDA and considered Generally Recognized as Safe (GRAS) [60].

1.5.3.1 Omega-3 fatty acids

Three types of omega-3 fatty acids are important in human physiology: one with a short carbon chain (18 carbons): α-linoleic acid (ALA) and two with long chain ($>$ 20 carbons): eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

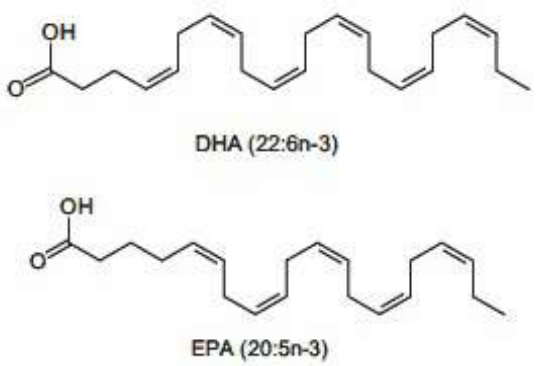


Figure 1.10: *Structure of EPA and DHA omega-3 fatty acids*

Omega-3 are essential fatty acids, which means that the body cannot synthesize them and they need to be included with the diet. The alpha-linoleic acid (ALA) is the only omega-3 fatty acid found in terrestrial lands and it can be obtained by consuming walnuts, flaxseed, echium and vegetable oils such as canola, soy and perilla [141] [142]. ALA (18:3n-3) has a short carbon chain and theoretically our metabolism could convert it into longer carbon chain of fatty acids such as DHA and EPA but in reality the conversion is difficult and limited by various factors [142] [143] [144].

DHA (22:6n-3) is the major PUFA in neuronal cell membranes forming approximately 3% of the dry weight of the brain. It has a role in membrane fluidity, it regulates important enzymatic reactions in the cells, such as the rhodopsin response, it is involved in signal transduction and ion channels and it controls apoptosis of neural cells [145]. Studies showed that a correct intake of DHA may prevent disease such as cognitive decline, Alzheimer and depression [94] [115] [146] [147]. Photoreceptor cells present in the retina are composed of 60% DHA, a lack of this fatty acid can lead to age-related macular degeneration (AMD), retinitis pigmentosa (RP) and other retinal degenerative diseases [136] [125]. EPA (20:5n-3) is a precursor of prostaglandin-3, an anti-inflammatory hormone that inhibits excessive and uncontrolled inflammation reactions of the body [144]. This omega-3 fatty acid is contained in the cell membrane increasing their fluidity [148]. DHA and EPA fatty acids are both essential for proper fetal development and the intake is highly recommended during pregnancy [142].

Along with omega-3 fatty acids, omega-6 fatty acids play a crucial role in human health promoting the inflammatory response in the immune system. Omega-6 are good for the body but only in the right quantity as they are very reactive, forming free radicals, because of the numer-

ous double bonds in the molecular structure. The oxidation of double bonds causes inflammation disorders in the body, leading to rheumatoid arthritis, heart disease and cancer [143] [144]. The ratio between omega-6 to omega-3 has to be balanced to provide beneficial effect for the health, meaning a ratio around 3:1. The current Western diet is characterized by an over consumption of refined sugars, salt, saturated fat, refined grains, processed meat and oils. This type of diet is very rich in omega-6 (i.e they are added in process food) and pro-inflammatory components, resulting in a very unbalanced ratio of about 20:1. Especially in the less-developed countries, many people do not receive an adequate intake of calories and micronutrients, which are vital for optimal immune function. As a consequence, chronic illnesses and health problems such as obesity, cardiovascular disease, diabetes, osteoporosis and cancer are increasing [149].

The beneficial effects of omega-3 fatty acids can be obtained by eating fish several times a week or by daily intake of concentrated fish oil. All fish contain some omega-3s, however quantities vary among species and according to factors such as the environment and diet. Fish oil can be processed and encapsulated as a dietary supplement however, there are several drawbacks including bio-accumulation of fat-soluble vitamins and toxins i.e heavy metals, PCBs, dioxins, high levels of saturated and omega-6 fatty acids, fish odour and regurgitation effects, unpleasant taste, poor oxidative stability, it is not sustainable because of intensive fishing and it is not recommendable for vegetarians and vegans [22]. Fish itself obtain omega-3 fatty acids by eating microalgae, these microorganisms are at the bottom of the food chain [150]. It is clear that the production of omega 3 from microalgae has numerous advantages. Their potential, their importance and their contribution for the environment and for the human health were highlighted.

1.6 Downstream processing in microalgal processes

Bioprocesses use living cells such bacteria, yeast, mammalian cells, algae or enzymes to obtain desired products. Usually they consist of a fermentation step i.e upstream processing (USP) followed by the downstream processing (DSP) where the product is separated for recovery and purification. DSP is an expensive and critical step in most biotechnological process but in the particular case of microalgae the extraction of intracellular products, in this case omega-3 fatty acids, is even more challenging because of various reasons. (1) The cell wall of certain microal-

gae can be very thick, structurally robust forming a protective barrier essential for survival in an aquatic environment [58]. (2) The composition of the cell wall is poorly understood and (3) it is variable between different species and there are also inter-species variations that depends on the cultivation methods, growth phase, existence of stress factor, etc. It is difficult to predict or develop a universal method or even only make predictions and comparison with the existing literature [151]. (4) Not least, the cells are small (10 μm) and (5) the moisture content might interfere with solvent extractions [152].

1.6.1 The microalgal cell wall

The composition of the microalgal cell wall depends not only on the microalgal species but also on cultivation conditions and biomass quality [153] [154]. Some microalgal cells are easier to break compared to others, because of the different composition of the cell wall [152]. This aspects has a big influence on the choice of the more adapted microalgal strains in a bioprocess. For many microalgae, the complex structure of the cell wall and its complete composition is not clearly defined and several theories exist [155] [80]. In some microalgal strains, such as *Nannochloropsis* sp., *Chlorella* sp., *Chlorococcum* sp., *Neochloris oleoabundans*, *Scenedesmus* sp., *Haematococcus pluvialis*, *Pavlova* sp., *Dunaliella* sp, etc., is reported the existence of rigid and thick cell wall with complex structure which requires disruption prior to extraction [58] [32] [153] [132] [152] [127]. The resistant cell wall constitutes a barrier necessary for survival in aquatic environments [58], which unfortunately might affect certain processes in algal biotechnology. In such situation, cell disruption is an integral part of the downstream operations that facilitates the release of intracellular products essential for lipid extraction but involves higher costs.

The cell wall of *Chlorella* sp. have rigid wall components embedded in a polymeric matrix. The rigid components are predominantly amino sugars such as chitin (poly-β-,4-D-N-acetylglucosamine) whereas the matrix contains variable quantities of neutral and charged polysaccharides such as uronic acids, rhamnose, arabinose, fucose, xylose, mannose, galactose and glucose [58] [156]. Loos and Meindl showed that the isolated cell wall of *Chlorella fusca* was composed of about 80% carbohydrate, 7% protein and the remaining was 13% was not identified. A high mannose content was reported - accounting for most of the carbohydrates - followed by glucose, rhamnose and fucose and as minor component also glucuronic acid. An

X-ray diffraction diagram of the walls showed no reflections characteristic of cellulose. The cell walls from young cells contained about 51% carbohydrate, 12% protein, and 37% unidentified material. Mannose and glucose were also the main sugars; their absolute amounts per wall increased 6-7 fold during cell growth. According to the authors, these data and other published cell wall analyses show a wide variability in cell wall composition of the members of the genus *Chlorella* [157] [158] [159]. According to other studies, cellulose is reported to be the main structural component of the cell wall of most microalgal species, but differing views and theory exists [156] [133] [80]. For certain microalgal species, including *Chlorella*, there is an outer and dense trilaminar layer (TLS) of algaenan, an insoluble and non-hydrolysable aliphatic polymer responsible for the strong resistance of microalgae. It is formed from the polymerization of high molecular weight lipids and a network of long hydrocarbon chains, however its structure has not been fully characterized [160]. The cell wall also contains protein (glycoproteins) along with phenolics and complexed cations [161]. The composition and the thickness of the cell wall varies during the growth phases. During the autosporangium stage - inside the mother cell wall - it is more fragile forming only a 2 nm unilaminar layer. After maturation, it gradually increases up to 17-21 nm. However, even in the mature stage, the cell wall can change influenced by the environmental conditions [53].

Little is known about the particular compostion of *Schizochytrium* cell wall. According to the research of Darley et al. [162], in a dry weight basis the cell walls contain 21-36% carbohydrate and 30-43% protein. The principal sugar (>95%) of *Schizochytrium* cell wall was l-galactose. Also Margulis et al. reported that in some Thraustochytrid walls, a high-sulfate galactan was detected [163]. Fan et al. reported in Thraustochytrid the presence of L-galactose as the primary monomer of the cell walls [164]. Ultrastructurally the cell walls consisted of a multilaminated structure that is presumed to be composed of Golgi body-derived scales [162] [29].

1.6.2 Current disruption and extraction methods

The traditional lysis methods that have been tested on microalgae are outlined below. Table 1.2 gives a general overview of the different methods and it is separated into two classes: mechanical methods and non-mechanical methods.

Dounce homogenizer and Potter-Elvehjem are manual pestels; the number of strokes, the grinding speed and the operator way of working determines the success of the breakage, therefore

Table 1.2: *Overview of the traditional methods to lyse the cell wall*

Method	Tools	Description
Homogenization [165]	Waring Blender Polytron Douce Homogenizer French Press Bead Mill Mortar and pestle	Cells are sheared forcing them through a narrow space
Sonication [166]	Ultrasonication	High frequency sound waves forms microscopic vapor bubbles that implode causing cavities in the cell wall
Freeze-thaw [34]	Freezer Liquid nitrogen	Repeated cycles of freezing and thawing disrupt cells through ice crystal formation
High temperatures [152]	Autoclave Microwave	High temperatures and pressures break the cell wal
Chemical permeabilization [100]	Organic solvents Detergents, surfactants Antibiotics	Permeabilization of outer wall barriers
Acid/alkali treatment [152]	Acid Base	Solubilization of cell membranes
Osmotic shock [167]	Salts General solutes	Rapid change in the solute concentration around a cell, causing the movement of water across the membrane and its breakage

their reproducibility is very low. Blenders such as the Polytron, relies on the use of rotating blades to grind and homogenize the samples. They have been used already to treat microalgal cells, however they are more indicated to disperse plant and animal tissue or grain and seed [165].

The principle of high pressure homogenizer (HPH) is relatively straightforward, a fluidic product i.e the microalgal cell suspension, is forced at high pressures (between 150-400 MPas) through a narrow space called a homogenizing nozzle. The pressure differential combined with the shear forces caused by the impact of the flow induces the rupture of the cell wall [168]. Samarsinghe et al. showed that HPH was an effective technique to rupture *N. oculata* cell walls [151]. Althought HPH is one of the most preferred industrial-scale methods for microalgal breakage, the main disadvantages are the limited use due to low cell density suspensions, it is not suitable for sensitive compounds and the equipment constitutes a costly investment [152].

Bead mills are instruments for ultra-fine processing of solids. Originally, they were designed for size reduction of pigments, chemicals, pharmaceutical mineral particles and food. However, they have been shown to be a useful technique for the disintegration of microbial cells and also of microalgae [169]. A bead mill is a type of grinder composed of a cylindrical device

in which beads are rapidly agitated by means of discs inside an enclosed chamber, resulting in a high-shear environment. The suspended cells are also disrupted by collision with the beads. Dyno Mill is probably one of the most well-known bead mills on the market, offering a size reduction from 500 microns to 0.30 microns [170]. A degree of cell disintegration higher than 95% has been obtained using a concentration of *Chlorella* of 110 g/L, glass beads of 0.42-0.58 mm and with the speed of agitator discs around 10-14 m/s [169]. Postma et al. showed that over 97% of cells were disintegrated at different biomass concentrations (24-145 g/kg) under different agitator speed (6-12 m/s), using 1 mm ZrO_2beads and with a filling percentage of 65% [171]. The advantage of this technique is that is very simple, effective and there is the possibility to treat high cell densities suspensions. However, beside the expensive initial investment, the energy consumption is also considerable [152].

Microwaves interact with water, the movement between inter- and intra-molecules forms frictional forces that generate intense local heating. The heat and the pressure cause damage to the surface of the cells, which is visible under scanning electron microscopy. Some studies proved that this technique appears to be a very effective and fast method for disrupting certain microalgal strains [172]. The advantage is that it is rapid, economical and it does not require de-watering of algal biomass [173]. On the other hand, the formation of free radicals, the temperature increase and the chemical conversions might cause damage to the lipids [152] [174].

Ultrasonication involves pulsed, high frequency sound waves to agitate and lyse cells, bacteria, spores and tissues. The sound waves are delivered using an apparatus with a vibrating probe that is immersed in the liquid cell suspension. Mechanical energy from the probe causes the formation of microscopic vapor bubbles that when imploding cause shock waves through the sample creating cavities in the cell wall [166]. Sonication processes generate lower temperatures compared to microwave and autoclave treatment however, in the micro-regions near the implosion of the bubbles, temperature and pressure might also reach high values [152]. The micro-bubbles can form highly reactive free radicals that can react with lipids [166]. Lee et al. found that ultrasonic disruption was not effective and the study of MC Millan et al. proved that ultrasonication had poor disruptive rates compared to other methods [174] [172]. Safi et al. showed that ultrasonication did not cause any change for *H. pluvialis* however. it showed an effect on the cell wall of *N. oculata* and *C. vulgaris* [20].

Non-mechanical methods aim to cause the permeabilization of the cell wall and cell membrane by creating canals. Osmotic shock treatments can be categorized as a mechanical permeabilization which involve the use of high substrate i.e salts concentration, in the aqueous medium around the cell which changes the osmotic pressure balance between the interior and exterior of the cell. As a result the rapid movement of water can damage the algal cell wall. Lee and al. tested a 10% NaCl solution for 48 hours on different algal strains, the results were lower than the disruption rate obtained with other methods and it required a longer treatment time [174]. On the other hand, Rakesh et al. recorded the highest lipid extraction with osmotic shock (15% NaCl) treatment for *Botryococcus* sp. [167]. The method has the advantage to be simple, easy and not expensive.

Chemical treatments have been employed to extract intracellular components by permeabilizing the cell wall barriers by creating canals, using organic solvents such as toluene, ether, phenylethyl alcohol, DMSO, benzene, methanol, chloroform Theories regarding the need to previously pre-treat the cell wall by non-mechanical method are contradictory. On one hand, in some research such as the one of Ranjan et al., it is suspected that a disruption method is not necessary. According to their hypothesis, lipids seems to diffuse across the cell wall when the biomass is suspended in a solvent with higher selectivity and solubility for lipids [175]. On the other hand, other studies affirm that the presence of water in the wet biomass forms a film preventing the solvent from reaching the lipid, preventing efficient lipid extraction. Thus, the cell wall needs to be mechanically disrupted to liberate the lipids and allow them to come into contact with the solvent [100].

To sum up, it is not possible to define from previous literature studies which is the best method to extract lipids from microalgae. Published results are contradictory between different studies because of the differences between microalgal species. Due to differences in cell wall structure, not all microalgae respond the same to pretreatment. Cell disruption methods depend on microalgae species, cultivation conditions and biomass quality in term of concentration, dried/wet status and stage of growth [153] [154]. The efficiency of cell disruption, and the consequent lipid extraction, differs according to the species and cultivation method. A standard effective method applicable to all microalgal strains does not exist and further research is needed to

optimize the extraction process of the current algal strains used in this research.

1.6.3 Enzymatic treatment - A solution?

In order to develop a completely profitable and eco-friendly process, the extraction method must be sustainable, avoiding (or reducing) the use of toxic solvents and high-energy inputs. The best solution may be the enzymatic lysis of the cell wall, prior to lipid extraction. Enzymes are globular proteins which linked to specific substrates can act as biocatalyst in chemical reactions. The amino acid chain is folded into a precise shape giving the protein its function, the substrate binding site is called active site and the amino acid residues that form it are arranged to interact specifically with the substrate. Enzymatic treatments have the advantage that they can be carried out under mild and very specific conditions, under low energy consumption and the high selectivity to substrate allows to minimize side products [176] [97]. In the field of industrial biotechnology, enzymes play an important role in cell wall lysis. Proteins or other valuable molecules manufactured by microorganism are sometimes intracellular and they cannot be easily secreted by the cell. Lytic enzymes are used for the selective release of recombinant proteins or other valuable products. An enzyme can selectively degrade a specific chemical linkage, binding to specific molecules in the cell membrane or cell wall to hydrolyse specific bonds (Figure 1.11), whereas mechanical methods destroy almost every particle existing in the solution [177] [2].

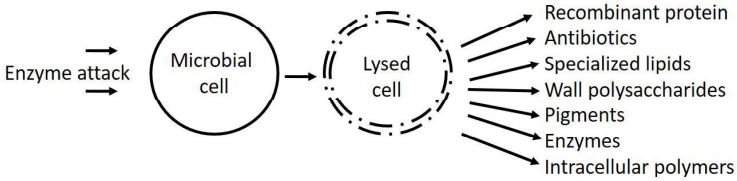


Figure 1.11: *Example the enzymatic lysis of a microbial cell [2]*

Although only few studies investigated the effects of enzymatic treatments on microalgae, results were encouraging [58] [152] [178] [179] [180] [181]. As an example, Fu et al. observed an improvement on lipid extraction from 32% to 56% after enzymatic hydrolysis of the cell wall [182]. The drawbacks of the use of enzymes could be the longer process times (compared to mechanical disruption) and their relatively high cost. The immobilization of enzymes could lower the needed amount and reduce the downstream process costs, since separation of the en-

zymes from the products would be avoided [152]. In Table 1.3 is represented a list of enzymes commonly used to harvest products from microalgae.

Table 1.3: *Enzymes commonly used to harvest products from microalgae.*

Enzyme	**Microalgal strain**	**Final product**	**Reference**
Cellulase	*Chlorococcum* sp.	Ethanol	[156]
Cellulase	*Chlorella* sp.	Reducing sugars	[182]
Alpha-amylase Amyloglucosidase	*Chlamydomonas reinhardtii*	Ethanol	[183]
Cellulase Chitinase beta-glucosidase Laminarase ... Lysozyme Lyticase Sulphatase Trypsin	*Chlorella* sp.	-	[58]
Lysozyme Cellulase	*Chlorella* sp., *Ankistrodesmus braunii*, *Pseudochlorococcum* sp., *Tetraselmis* sp., *Nannochloropsis* sp., *Scenedesmus* sp., *Chlamydomonas* sp.	Proteins, pigments	[178]
Lysozyme	*Scenedesmus* sp.	Biodiesel	[100]
Cellulase Lysozyme Snailase	*Chlorella vulgaris*	Total lipids	[184]

1.7 Problem identification

Presently, microalgal lipids are mainly sold in niche markets but different factors - such as the increased social and political awareness for sustainability, the instability of fossil fuel prices, the pressure on agriculture crops for non-food applications, the growth in population and the limited availability of arable land - are pushing the industry to look for alternative and more sustainable feedstocks to supply the commodity markets [185]. The cultivation of microalgae

for lipids is transitioning to commercial-scale systems however, it is a technology that so far is not ready and its potential in biotechnology is underexploited [24] [59] [186] [55] [133] [35]. Several bottlenecks remain to be overcome to make the large-scale production of microalgal lipids commercially viable [187].

- **Productivity**

  The industrial production of microalgal lipids is not yet economically viable, mainly because of the low productivity. The production costs of microalgal oil need to be drastically decreased to become economically viable [185] [133]. The effectiveness of microalgae production depends on lipid productivity, which is a combined effect of biomass productivity and lipid content [100]. High-cell-density cultivations with a good lipid accumulation are essential to increase the productivity of bio-products [188]. In general, the ultimate objective for any bioprocess is to achieve the highest product concentration in the shortest time [51].

- **Downstream processing**

  Besides the low productivity, another problem of microalgal cultivation is the high operational cost of the disruption of microalgal cell walls to liberate the enclosed products, such as lipids [189]. Due to the robust structure of microalgal cell walls, many pretreatment processes required are usually energy intensive increasing the operational costs and resulting in excessive heat generation, which may affect the properties of sensitive compounds [184]. Other traditional methods involve the high consumption of organic solvents, which are not sustainable and not ideal for the safety of large-scale operations [152] [151]. Furthermore, the recovery of microalgal lipids has been studied mainly from dried microalgae however, biomass dewatering is a time consuming, energy intensive process which increases further the DSP cost [100] [190] [191].

- **Cost of the substrates**

  Heterotrophic growth of microalgae is superior to phototrophic cultivation, due to the potential for high final product concentration however, the cost of the raw materials is the major concern from a commercial point of view, especially when pure glucose is used as carbon source [41]. Efforts are needed to reduce the cost of heterotrophic algal

cultivation and make it economically viable for low-value commodities [42]. A zero-waste strategy could be applied to a microalgal biorefinery by using by-products or waste materials as substrates, in order to increase the sustainability of the process [35]. The use of cheap organic carbon sources such as molasses or glycerol can be considered as an alternative. Nevertheles, it was reported that biomass and lipid production of microalgae is also greatly affected by different carbon sources and concentration [192]. Therefore, it is necessary to investigate and select a suitable medium that maintains a rapid growth and a high lipid content.

1.8 Objectives

This project was developed in collaboration with an industrial partner Monaghan Bioscience (Ireland), which aim was to explore the potential of commercial exploitation of microalgae as a sustainable source of oil, biomass and other high-valuable products such as omega-3 fatty acids. One of the main challenges was to collect and adapt scientific studies described by the literature into the development of a small-scale industrial bioprocess. Being a relatively new technology, different bottlenecks needs to be overcome to reach the full potential of microalgae as cell-factories. To summarize, the main aims of this chapter are as follow:

- **Increase of lipid productivity**

  Microalgal cells accumulate high concentrations of lipids only when cultivated under particular unfavourable conditions, such as in a nitrogen deficient medium. However, this is a contradictory mechanism: when cells are in stressed conditions, the proliferation is not optimal resulting in an overall low productivity. Here, the aim was to develop a two-stage heterotrophic cultivation process where in the first step, the medium is carbon-limited allowing to achieve high cell densities and afterwards, under a nitrogen-limited condition, it is expected to stimulate the lipid accumulation. For this research, *Chlorella protothecoides* was chosen as reference strain because it is one of the most understood microalgal species in current research and industry applications whereas *Schizochytrium* because it is well known for its high accumulation of a particular category of lipids, omega-3 fatty acids.

- **Explore potential alternatives in downstream processing**

  Typically, downstream processing of industrial biotechnological products accounts for 20-40% of the total production costs, while for microalgal biorefinery the costs are substantially higher (50-60%) [190]. The principal objective was to develop and investigate the potential of an extraction method for algal lipids, which is more sustainable, cost effective and that could be applied on larger scale. The strategy was to develop two different disruption methods. First a standard method, applicable in lab-scale, relatively quick, based on classic methodologies already described in the literature, which then was compared to an alternative disruption method, based on enzymatic cell wall hydrolysis, that could be applied at larger scale.

- **Improve sustainability of the cultivation process**

  Another objective was to increase the sustainability and the costs of the heterotrophic two-stage fed-batch cultivation process by using alternative carbon sources. The potential of molasses, glycerol and acetate - which are waste products of other industries - were evaluated based on the effect on the cell growth and lipid accumulation.

- **Investigate the production of specific high-valuable molecules**

  It is anticipated that varying the culturing conditions can alter lipid production as well as the chemical composition of microalgae. The objective was to improve the accumulation of omega-3 fatty acids, in particular of DHA and EPA, by testing different conditions.

1.9 Thesis outline

The present thesis includes seven chapters, which are represented in the schematic flowchart process in Figure 1.12. The structure of each chapter is discussed in more detail in the following sections.

- **Chapter 2**

  One of the major bottlenecks in microalgal bio-refineries is the disruption of microalgal cell walls, to liberate the enclosed lipids. In this Chapter, the first objective was to develop and optimize a standard method to disrupt cell walls of two microalgal strains, *Chlorella protothecoides* and *Schizochytrium* sp., to enable efficient lysis in order to allow intracellular lipid determination. In the second part, the potential for enzymatic hydrolysis of wet biomass was explored.

- **Chapter 3**

  This chapter is based on the characterization and the optimization of a fermentation process for the production of high cell densities and lipids using the heterotrophic cultivation of *Chlorella protothecoides*. First of all, the effect of the carbon to nitrogen (C/N) ratio on growth and lipid accumulation was studied. The final objective was to develop a two-stage fed-batch cultivation process for high lipid productivity. The expectation was that with a low C/N ratio the medium will be carbon-limited, promoting cell proliferation and consequently the achievement of high cell densities whereas with a higher C/N ratio, under nitrogen limitation, lipid would be accumulated.

- **Chapter 4**

  The process developed for *Chlorella protothecoides* in Chapter 3 was applied to a second microalgal strain, *Schizochytrium* sp. The expectation was to achieve a high lipid productivity under nitrogen limitation in a two-stage fed-batch cultivation. The potential of both microalgal strains was compared and evaluated.

- **Chapter 5**

  The high cost of the fermentation substrates causes restrictions in the production of heterotrophic microalgae. In this study, the potential of alternative carbon sources to pure glucose was evaluated. The objective was to grow *Chlorella protothecoides* with different carbon sources such as glycerol, molasses, acetate and to define the effect on cell

growth and lipid accumulation.

- **Chapter 6**

  Marine Thraustochytrid and in particular *Schizochytrium* strains are reported to contain a high proportion of omega-3 polyunsaturated fatty acids in their lipid composition. In particular, docosahexaenoic acid (DHA) has received particular attention for its health benefits. This chapter investigated the potential of the production of high valuable bio-products and the ability of modifying the conditions of cultivation with the biotechnology techniques to enhance the production of particular products.

- **Chapter 7**

  This last chapter summarizes the conclusions from Chapters 2 - 6 and gives an outline of further applications of the present findings. An overall conclusion of the work carried is drawn.

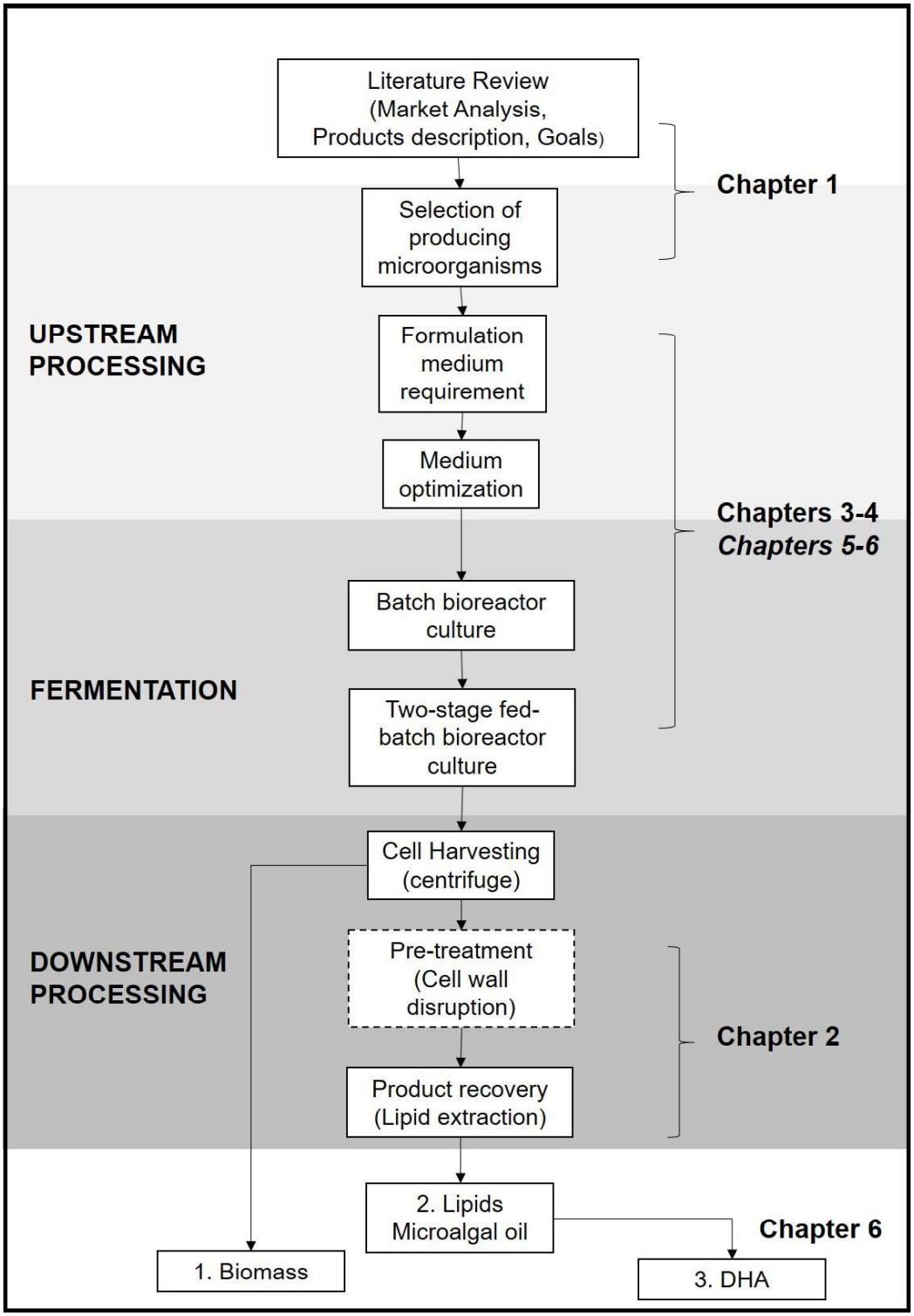


Figure 1.12: *Flowchart process plan*

Chapter 2

Development of methods for the disruption of wet microalgal biomass and for the extraction of lipids

2.1 Introduction

2.1.1 Background and problem

Downstream processing (DSP) is a critical component of biotechnological processes that involves the different stages occurring after the completion of a fermentation or bioconversion process, which recover and purify biosynthetic products. Figure 2.1 represents a general flowchart of downstream processing, it can be observed that supplementary steps are required when the desired product is intracellular [193] [3].

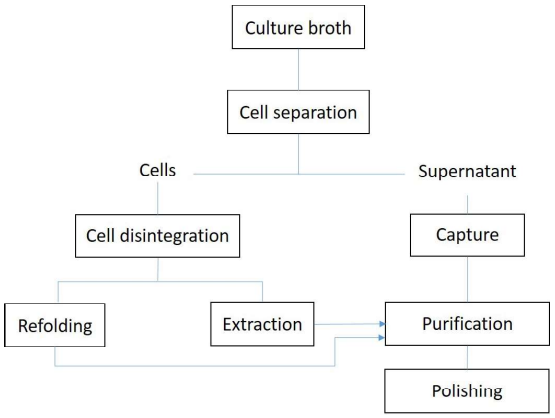


Figure 2.1: *General set of unit operations in downstream processing [3]*

One of the major bottlenecks in microalgal bio-refineries is the disruption of microalgal cell walls, to liberate the enclosed lipids [191]. An efficient cell disruption method is needed to maximize the product recovery and to make algal bio-products more economically attractive. Due to the robust structure of algal cell walls, many pretreatment processes require a high consumption of organic solvents or high energy loads [100]. The ideal extraction process should not negatively affect sensitive compounds such as omega-3 fatty acids, which are highly susceptible to oxidation and other chemical changes by exposure to air, light, or heat. Also in the case of biodiesel production, thermal degradation of triglycerides yields several compounds, many of which cannot be converted to biodiesel [191].

The recovery of microalgal lipids has been studied mainly from dried microalgae [194] [64] [195] [147] [196] [100]. However, biomass de-watering is a time consuming, energy intensive process which involves high costs [196]. According to Taher et al., drying is responsible for 89% of the required energy input [100]. Processes which avoid the drying step, by extracting lipids directly from the wet and concentrated biomass, require development. New methods, which are ideally environmentally friendly, need to be established to ensure low operating costs, high product recovery and high quality of the recovered lipids. This is primordial for the development of efficient microalgae bio-refineries [58].

2.1.2 Strategy and scope

This chapter describes the development and the adaptation of specific methods for the disruption of the cell wall of *Chlorella* and *Schizochytrium* and the extraction of total lipids. The efficiency of the disruption method is largely dependent on the microalgal species because of inter- and intra-species variations [152]. The metabolism and cell wall characteristics are often affected by the culture conditions such as medium composition, temperature, pH and pressure but also on the biomass conditions in term of concentration, dried/wet status and stage of growth. Therefore, it is difficult to predict which method will be the optimum for each process [135] [191]. This chapter focuses on the following three objectives:

- Develop a standard method to disrupt the microalgal cell wall
- Define a method to extract total lipids
- Define an alternative and sustainable method for lipid extraction

2.1.2.1 Disruptions of cell walls

The literature reports that in some microalgae, cell walls are extremely resistant [58]. The aim of this part of the work was to develop a standard method which needs to be applicable at lab-scale, relatively quick, inexpensive, provide reproducible disruption of the cell wall, which should result in a minimum of 80% cell lysis and it has to operate under relatively mild conditions. The quality of polyunsaturated fatty acids, which might be unstable and highly susceptible to oxidation and other chemical changes should be preserved [146]. A general standard method applicable to every microalgal strain does not exist and research regarding the optimal disruption are frequently in contradiction between each other [34].

2.1.2.2 Total lipid extraction

The efficiency of the extraction of lipids using solvent mixtures depends on the polarity of the solvents used. Indeed, polar solvent release the lipids from protein-lipid complexes of the cell wall, allowing lipids to dissolve in the non-polar solvent. Water and solvent are removed using liquid-liquid separation methods such as evaporation or vacuum distillation. Bio-safety issues are the main disadvantage of solvent extraction. The objective of this part of the work, was to define a standard procedure for lipid extraction from microalgae. The ideal solvent mixture was chosen based on the yield of total lipid extracted in combination with a green chemistry approach: ideally the solvents needed to be bio-compatible and have a low toxicity level.

An efficient, reproducible disruption of cell walls was required to maximize the recovery of intracellular lipids and other bio-products from the biomass. However, Ranjan et al. suggested that disruption methods are not necessary to extract lipids from microalgae. Lipids appear to diffuse across the cell wall when the biomass is suspended in a solvent with higher selectivity and solubility for lipids [175]. The hypothesis of Ranjan et al. needed to be confirmed on the microalgal strains used in this research. It is suspected that the outcome is predominantly dependent on the cell wall thickness/permeability. Therefore, it is planned to compare the lipid yield after solvent extraction with and without previous disruption of the cell wall.

2.1.2.3 Enzymatic hydrolysis

Once standard methods are set-up, the potential of alternative methods, more adapted in view of larger scale application will be explored. It is suspected that enzymatic degradation of the algal cell walls prior to lipid extraction has the potential to facilitate both lipid extraction and post-extraction use of the algal biomass. Weakened cell walls could reduce solvent requirements and energy inputs needed for lipid extraction, by improving accessibility of the cell walls. Furthermore enzymatic pre-treatment can be carried out directly on wet biomass under mild conditions, lowering the energy consumption [178]. The effectiveness of enzymatic treatments will to be evaluated in term of percentage of total lipids extracted from dry cell weight, instead of a product concentration. In fact, the substrate is an undefined molecule present in the cell wall and the product is consequently also more difficult to measure. The hypothesis was that in the algal cell wall, there are defined molecular key-points holding the complex and layered structure [135] [58] [20] [22]. When these points are attacked, cell walls become more easily permeable to solvents - and lipid release - without necessarily involving complete cell wall disruption. It is important to point out, that unlike classic enzyme assays, the measured quantity of product (i.e extractable lipids) may not be directly proportional to the enzyme activity on cell wall. Despite substrates and products are not going to be quantified, they could give interesting insight on the composition of cell walls. The expectation is that that the efficacy of the pre-treatment method is proportional to the quantity of total lipids extracted from the biomass. Ideally, the percentage of lipids extracted from the positive control (standard analytic disruption method) will be the same as the one extracted with enzymes.

Enzymes chose for the study were selected from the existing literature.

- **Lysozyme (E.C. 3.2.1.17)**

  Hydrolysis of (1→4)-beta-linkages between N-acetylmuramic acid and N-acetyl-D- glucosamine residues in a peptidoglycan and between N-acetyl-D- glucosamine residues in chitodextrins.

- **Cellulase (E.C. 3.2.1.4)**

  Endo-hydrolysis of (1→4)-beta-D-glucosidic linkages in cellulose.

- **Sulfatase (E.C. 3.1.6.1)**

  Hydrolysis of sulfuric ester bonds.

2.2 Material and methods

2.2.1 Materials

All chemicals were purchased from either Sigma Aldrich (St. Louis, USA) or Thermo Fisher Scientific (Waltham, USA) if not stated otherwise.

2.2.2 Cell count

The sample was diluted to a suitable concentration between $2.5 \cdot 10^5$ and $2.5 \cdot 10^6$ cells/mL. The glass cover sliding was placed on the top of the Neubauer chamber and the chamber was filled with 10 μl of sample. The liquid should enter by capillary action. The consistency in filling is important, if more liquid than necessary is introduced, the distance between the bottom of the chamber and the glass cover will be higher and the results will be inaccurate. The cells were counted in each square and the concentration determined using equation 2.1. The measurements were repeated in triplicate (n = 3) to check the reproducibility of the results, the standard deviation error (SD) is reported in the experimental results or represented graphically as error bars.

$$Conc\,[cells/mL] = \frac{Avg_{tot\,cells}}{Num_{square}} \frac{1}{V_{square}} dilution \tag{2.1}$$

where:

V_{square} = 0.1 cm x 0.1 cm x 0.01 cm = $1 \cdot 10^{-4}$ mL

Num squares = 4

2.2.3 Bradford assay

An alternative method to evaluate the disintegration of the microalgae for the release of intracellular products i.e. lipids can be the determination of protein. The Bradford method was set up using the standard protein BSA (Bovine Serum Albumin). The method describing the Bradford assay is described by the supplier [197], 50 μL of standards or sample were mixed in a test tube with 1 mL of Bradford reagent at room temperature and incubated for 5 min. The absorbance was read at 595 nm with a spectrophotometer (Unispec2, LLG Labware, Meckenheim, Germany). Each point of the calibration curve was measured in triplicate (n=3) while the measurements of the samples were repeated in duplicate (n = 2) to check the reproducib-

ility of the results, the standard deviation error (SD) is reported in the experimental results or represented graphically as error bars.

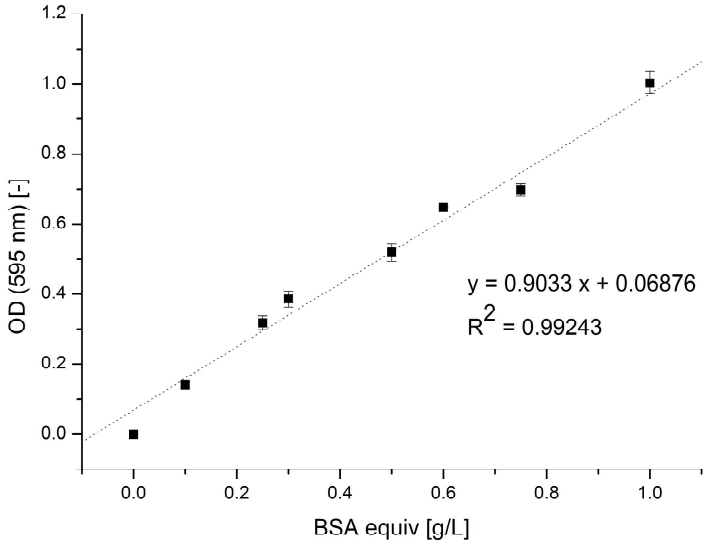


Figure 2.2: *Standard calibration curve of BSA using Bradford assay. The measurements were repeated in triplicate and the standard deviation is represented.*

2.2.4 Solvent extraction of total lipids

In a glass test tube, 1mL of microalgal suspension - of known dry cell weight (DCW) concentration - was regularly vortex-mixed with a mixture of 2 mL methanol and 4 mL dichloromethane. After 2 h of incubation at room temperature, 1 mL methanol and 2 mL dichloromethane were added and centrifuged (FL40R, Thermo Fisher Scientific) at 1,000 rpm. The aqueous upper layer was removed, and the organic layer was filtered through a 1 m glass fibre pre-filter (AP1507500, Millipore, Burlington, USA) in a Buechner funnel in a pre-weighed test tube (W_0). Solvents were evaporated in a water bath at max. 50°C. The remaining lipids were flushed with nitrogen to dry completely. The content of total lipids was determined gravimetrically (W_1) according to Equation 2.5. The method was adapted from various methods described in the literature [34] [92] [198] [174] [173] [199]. The measurements were repeated in duplicate (n = 5) to check the reproducibility of the results, the standard deviation error (SD) is reported in the experimental results or represented graphically as error bars.

$$Lipid\,\% = \frac{W_1 - W_0}{DCW}\,100 \tag{2.2}$$

where:

W_1 = weight of empty tube [g]

W_2 = weight of the tube with the extracted oil [g]

DCW = dry cell weight of microalgal paste [g]

In Figure 2.3 the standard solvent extraction procedure using dichloromethane and methanol is schematically represented.

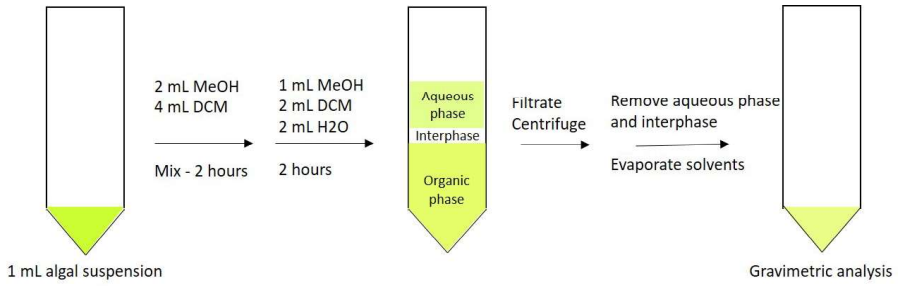


Figure 2.3: *Schematic representation of solvent extraction with dichloromethane and methanol.*

2.2.5 Polytron homogenizer

Polytron handheld homogenizers are generally used to homogenize, emulsify and disperse plant and animal tissues (Figure 2.4). Applications can also include disruption of bacterial cells, therefore it was decided to test it on a suspension of *Chlorella protothecoides* aiming to disrupt cell walls. In the first part, in a 50 mL test tube, 10 mL of *Chlorella protothecoides* cell suspension were treated for 20 min at two different homogenization speeds (i.e 3 and 5). In the second part of the experiment, the cell suspension was incubated in a final concentration of 1M H_2SO_4 for 1h, 2h and 14 h. The treatment with the homogizer lasted 20 min at speed 3. Cells were counted under optical microscopy (Section 2.2.2) and the results are represented as fractional concentration of the un-disrupted cells (Equation 2.3).

$$Fractional\,concentration = C/C_0 \tag{2.3}$$

where:

C = number of counted cells after the treatment

C_0 = number of counted cell at time 0

Figure 2.4: *Graphic representation of Polytron hand-held homogenizer.*

2.2.6 Vortex bead milling technique and Design of Experiment (DoE)

The vortex bead milling technique was developed based on the principle of classic bead mills, which are instruments for ultra-fine processing of solids but also for the disintegration of cells. Cells are disrupted by the collision with the microbeads, which are rapidly agitated inside an enclosed chamber, resulting in a high-shear environment [169] [174] [184]. A 50 mL test tube containing the cell suspension and microbeads was continuously mixed on a vortex (VELP Scientifica, Usmate, Italy) at 40 Hz. Every minute, a sample (200 μL) was taken over a total treatment period of 15 minutes. The samples were centrifuged at 21379 x g (Z126M, Hermle, Gosheim, Germany) for 10 min. The supernatant was analysed by Bradford assay and the released protein were calculated as the ratio of initial cell concentration to the final protein released.

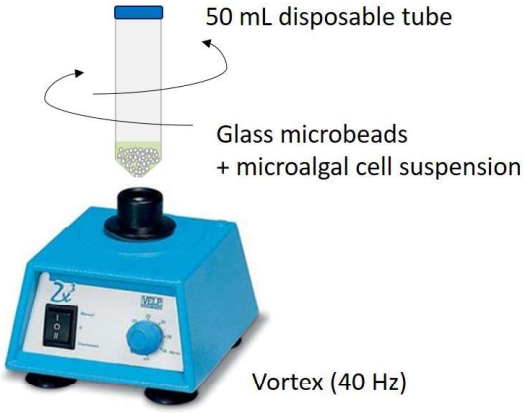


Figure 2.5: *Schematic representation of the vortex bead milling system.*

The efficiency and the repeatability of the cell disruption using the vortex bead milling method was evaluated by carrying out a screening analysis with the software MODDE 9.0 (MKS Umetrics, Malm, Sweden). The objective of the screening analysis was to define which factors had an influence on the process and the optimal range. A two-level full factorial design in three factors (2^3) was constructed. This implied 8 runs and in addition three replicated experiments (center point) were added generating a total of eleven experiments. The repeated testing of the standard condition allowed to determine the replicate error. The experiments were proposed in a randomized order to prevent systematic time trends that might influence the experimental values. Factors considered in the design of experiments were the following: concentration cell suspension (40 g/L, 80 g/L, 120 g/L), diameter of glass microbeads (0.1 mm, 0.5 mm, 1.0 mm) and volume fraction of microbeads (50%, 65%, 80%). (Annexe 2) The experimental design supported the model represented in Equation 2.5 [200].

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \varepsilon \tag{2.4}$$

where:

y = response

$x_{1,2,3}$ = factors

$\beta_{1,2,3}$ = regression coefficients (linear terms)

ε = residual response variation not explained by the model

2.2.7 Enzymatic hydrolysis

The enzymes selected for the study: cellulase (1'000 U/g, density 1.2 g/mL, C2605), sulfatase (10'000 U/g, S9626) and lysozyme (40000 U/mg, L7651) were purchased from Sigma Aldrich (St. Louis, USA). They were chosen based on the positive outcome of the hydrolysis of the cell wall reported by different studies [152] [58] [100] [179] [180] [181]. *Talaromyces* sp. enzymatic cocktail, composed of glucanase (2403 U/mL), laminarase (56 U/ml), xylanase (430 U/ml), β-glucosidase (28 U/ml) and cellobiohydrolase (10 U/mL) was produced in house by Monaghan Bioscience (Monaghan, Ireland) and it was tested in order to evaluate its potential in the treatment of *Chlorella* cell wall. Before the analysis, aliquoted microalgal suspension samples were thawed, centrifuged and re-suspended in 35 mL of the appropriate buffer solution. A certain enzyme concentration and 200 L Pen-Strep (Penicillin Streptomycin, 15140122, Thermo Fisher Scientific, Waltham, USA) were added followed by gentle mixing. Mixture was incubated in an orbital shaker incubator (Model 3500I, VWR, Radnor, USA) at 100 rpm under the appropriate conditions (Table 2.1). Antibiotic was added to avoid bacterial overgrowth during incubation but was shown to have no effect on the enzyme reactions. Samples were taken at several intervals until the end of the experiment and total lipids were extracted. The experiments were divided in three parts, as indicated in Table 2.1 (A, B, C). In the first part, the effect of the different enzymes on Chlorella protothecoides cell wall was tested separately, whereas in the second part lysozyme was combined to cellulase and to sulfatase. Different temperatures and pH conditions were tested in order to identify the optimum conditions for both enzymes. In the last part, the combination of lysozyme and sulfatase was re-tested to define whether the increase of enzyme concentration, keeping the substrate constant, showed a proportional increase in the reaction rate.

Single enzyme kinetics were carried out over 48 hours, according to Table 2.1 (part A), which describes the concentration of enzyme added and the incubation conditions. Tests with combinations of enzymes were carried out according to Table 2.1 (part B), over 24 hours. Total lipids were extracted at the end of each experiment. The last experiment (Table 2.1 - part C) was a kinetic study of a combination of lysozyme and sulfatase over 48 hours. Samples were taken and lipid extraction was performed at different intervals. Negative controls were carried out in both buffer without the addition of enzymes - with an incubation of 48 hours.

The result of the enzyme treatments was evaluated based on extractable lipids, which is the ratio of total lipid composition determined after enzyme hydrolysis and after cell disruption with cell bead vortexing.

$$Extractable\,lipids\,[\%] = \frac{Tot.lipids_{enzym}}{Tot.lipids_{positive\,control}} 100 \tag{2.5}$$

Table 2.1: *Description of enzymatic experiments in term of type of enzyme, enzyme concentration [mg/mL] and [U/mL], pH and temperature of incubation [°C]. For pH between 4.5 and 5.5, acetate buffer (100mM) was used and for pH at 7.0, Tris HCl 10 mM. Cellulase was provided in liquid form with a density of 1.2 g/L. Talaromyces enzyme mixture was also provided in liquid form. Furthermore, due to the complex composition in enzymes, the concentration in U/ml cannot be shown. Part A, B, C of the Table correspond to the different experiments carried out*

	Enzyme	**Concentration** [mg/mL]	**Concentration** [U/mL]	**pH** [-]	**Temp.** [°C]
A	Cellulase	6	6	5	50
	Sulfatase	0.5	5	5	37
	Lysozyme	0.05	2000	7	37
	Talaromyces	22.86 μl/mL	*	4.5	37
B	Lysozyme	0.1	4000	5.5	40
	Cellulase	12	12	7.0	
	Lysozyme	0.1	4000	5.5	37
	Sulfatase	1	10	7.0	
	Lysozyme	0.1	4000	4.5	37
	Talaromyces	22.86 μl/mL	*		50
C	Lysozyme	0.1 (1-fold) - 0.2 (2-fold)	4000 - 8000	5.5	37
	Sulfatase	1.0 (1-fold) - 2.0 (2-fold)	10 - 20		

2.3 Results and discussion

2.3.1 Disruption of the microalgal cell wall

The first step of this study was to define a standard method to disrupt microalgal cell walls in order to enable the quantitative analysis of the intracellular lipids. These development studies used the reference strain *Chlorella protothecoides*, since the cell wall composition was reported

to be more dense and resistant than *Schizochytrium* sp. [162] [169] [201] [184].

The purpose of this study was to compare and evaluate a range of different treatments on the disruption of *Chlorella* for lipid recovery. Initially, different disruption methods such as repeated freeze-thawing cycles, freeze drying, autoclaving and microwave treatment were initially tested. However, the outcome was not positive, showing results lower than 30% of cell disruption (results not shown). The treatments were probably too soft against the resistance of *Chlorella* cell wall. Use of a Polytron homogenizer gave slightly better results, although the most efficient method was vortex bead milling. The results of both methods are described below.

2.3.1.1 Polytron homogenizer

Polytron handheld homogenizers was tested on a suspension of *Chlorella protothecoides* aiming to disrupt cell walls. Figure 2.6 shows disruption results over 20 min of treatment at two different homogenization speeds (i.e 3 and 5), whereas Figure 2.7 shows results of same experiment (20 min at speed 3) after - 1h, 2h, 14 h - pre-treatments in diluted H_2SO_4. Cells were counted under optical microscopy and the results are represented as fractional concentration of the undisrupted cells (C/C_0) (Equation 2.3).

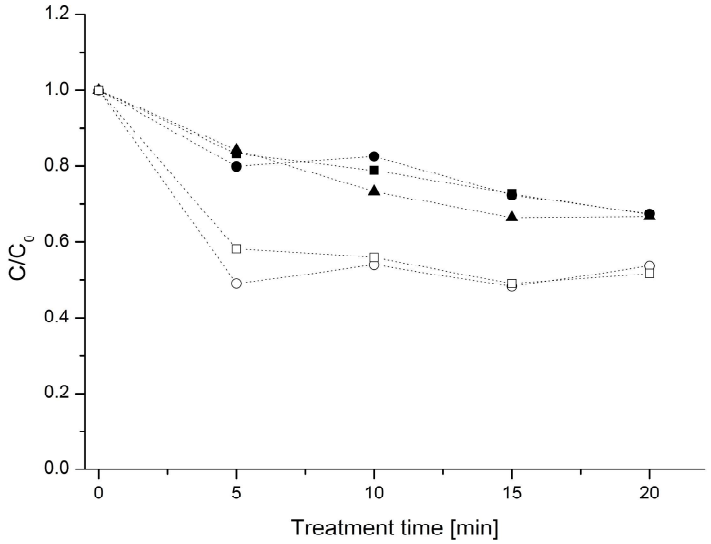


Figure 2.6: *The fractional concentration of the un-disrupted cells is represented as a function of the homogenizing time. The homogenization speed tested was at 3 (black symbols, n=3) and at 5 (white symbols, n=2*

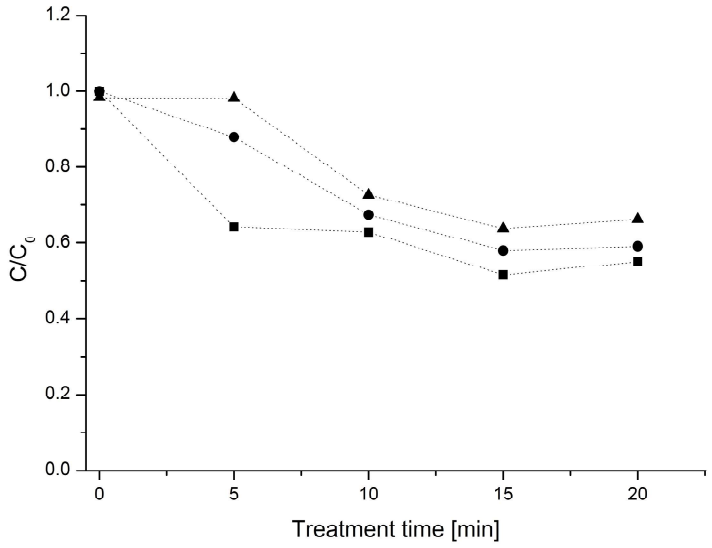


Figure 2.7: *The fractional concentration of the un-disrupted cells is represented as a function of the homogenizing time. The homogenization speed was at 3 and it was tested after different incubation times in H_2SO_4: 1h (▲), 4h (●), 14h (■)*

Figure 2.6 shows that the speed of the Polytron homogenizer had an influence on the microalgal cell disruption. Indeed, using speed 3, the disruption was 40% (SD= 5.7%, n=3) and at a speed of 5 the disruption was slightly higher, 47% (SD= 1.4%, n=2). At higher speeds, the maximum disruption was achieved after 5 min whereas at lower speed after 15-20 min. Acid pre-treatment seemed to have an effect on the cell disruption when compared to results without pre-treatment, at the same homogenization speed of 3. Over 33.75% of algae were disrupted after 1h of acid treatment, 40.94% after 4h and increased slightly to 44.96% after an overnight acid treatment ($>$ 14h) (Figure 2.7). The maximum percentage of disruption obtained with the Polytron homogenizer was less than 50% which is significantly lower than the requested 80%. The production of heat during the treatment of the sample was considerable and the acid pre-treatment is not optimal for the stability of sensitive compounds.

2.3.1.2 Vortex Bead Milling optimization with design of experiments

An inexpensive method, based on the principle of bead milling was developed and tested using a disposable 50 mL tube filled with glass microbeads and agitated at 40 Hz on a bench-top vortex. The disruption of cell wall was measured not only by counting the number of intact cells but also by measuring the release of soluble protein in the medium. The hypothesis was

that released protein could simulate the behavior of lipid molecules [171]. The measure of soluble proteins released in the medium is relatively easier than measuring total lipids and a lower amount of sample is required to perform the analysis. It was hypothesized that the release of soluble protein could be proportional to amount of disrupted cells and consequently also to the release of intracellular lipids.

The efficiency and repeatability of cell disruption using vortex bead milling was evaluated by carrying out a screening analysis with the software MODDE 9.0 (MKS Umetrics, Malm, Sweden). Factors comprised in the design were: concentration of cell suspension, diameter of microbeads and fraction of microbeads in the lysis suspension (%). Efficiency of the methods was based on the percentage cell disruption, by counting intact cells before and after treatment and the release of water soluble protein. Release of protein over time was relatively easy to measure using the Bradford assay and it was used as means to estimate lipid release. After completing the experimental plan, the results of which are reported in Annexe 1, data were analysed using the software based on the model reported in Section 2.2.6 - to define which factors were influencing the response and how. Regression coefficients are represented in Figure 2.8.

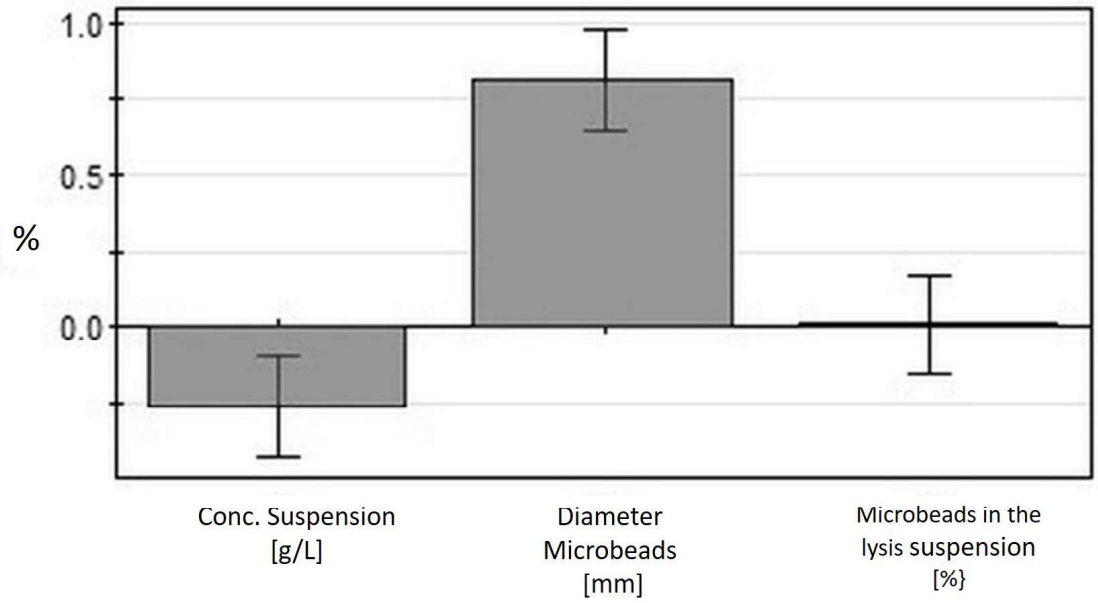


Figure 2.8: *Representation of the regression coefficients of the model: concentration of microalgal suspension (g/L), diameter of microbeads (mm) and percentage of microbeads in the lysis suspension (%). The confidence interval is represented with error bars and it is stated at the 95% of confidence level.*

Graphic representation of regression coefficients allows to distinguish between real effects and

experimental noise. It can be noticed that the strongest influences are due to the microalgal suspension concentration and to the microbead diameter (Figure 2.8). Coefficients were respectively -0.26% and + 0.81%. As an example, when the diameter of the microbeads was increased from the standard condition - 0.5 mm - to its high level of 1 mm, by keeping the other factors fixed at their standard condition, the percentage of cell disruption increased by 0.81%. The microbead volume percentage of the lysis chamber was not statistically significant therefore, it was removed from the model. The re-fitted model is represented in Figure 2.9 as a double response cell disruption and protein release - contour plot.

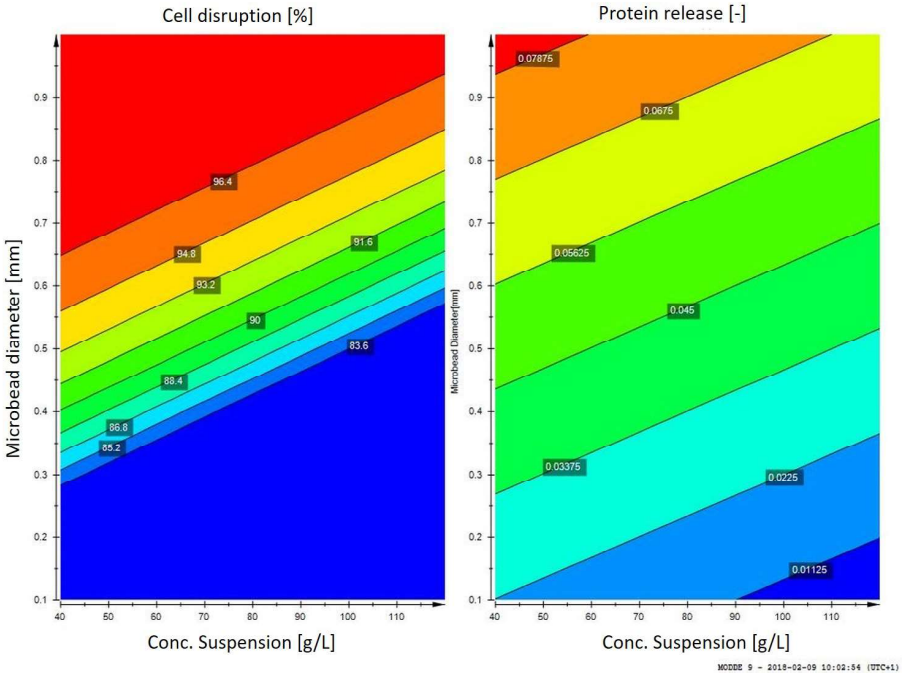


Figure 2.9: *Response contour plots for the cell disruption [%] and for the release of soluble proteins in BSA equivalent [-]. The influence of two responses: bead diameter [mm] and concentration of the cell suspension [g/L] is represented.*

The experimental goal was to increase the percentage cell disruption and consequently the release of protein. The two graphs represented in Figure 2.9 have the same trend, meaning that under the same conditions, the two responses react in the same way, indeed the two aims are not in conflict. The highest response is predicted to be achieved in the top-left corner region of the response contour plot, where the cell disruption is higher than 96.4% (Figure 2.9a), meaning with a low cell suspension concentration (40-60 g/L) and high bead diameter (0.7-1.0 mm). An example of the tested cell wall disruption of *Chlorella protothecoides* is represented in Figure 2.10.

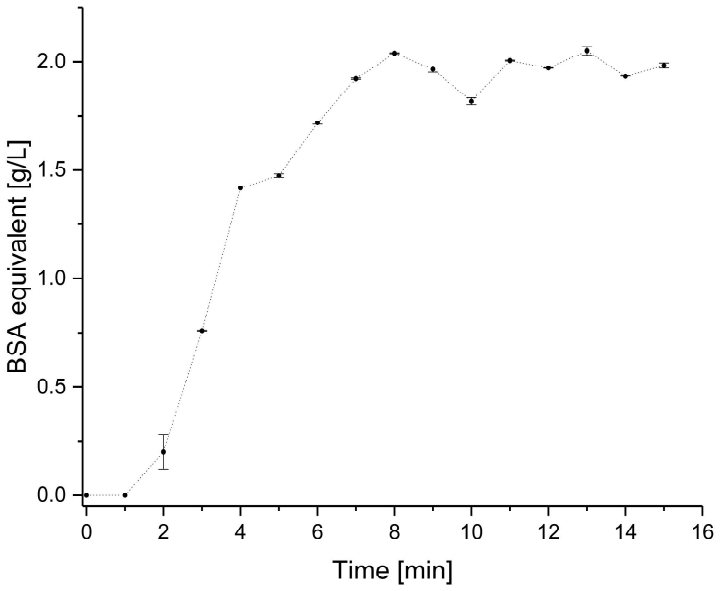


Figure 2.10: *Example of* Chlorella *sp. cell wall disruption by vortex bead milling a 40 g/L suspension using 1 mm diameter glass microbeads. Release of protein is represented as BSA equivalent as a function of the treatment time. Experiments were repeated in duplicate (n=2) and the standard deviation is represented as error bars.*

Figure 2.10 shows the protein release in BSA equivalents as a function of the bead vortexing treatment time with 10 mL *Chlorella protothecoides* suspension (40 g/L DCW) and 37 g of 1 mm diameter glass bead beads, composed of 50% microbeads in the lysis suspension. It can be seen that a plateau was achieved after about 8 min. Intact cells were counted at time 0 min and 15 min showing a cell disruption of 99.1 % (SD= 0.09%, n=3).

2.3.1.3 Validation of the methods on *Schizochytrium* sp.

The optimization of the vortex bead milling technique was carried out on the reference strain *Chlorella protothecoides*, results satisfied the expectations of achieving a disruption of the cell walls higher than 80%. It was necessary to determine whether the optimal conditions for the disruption, determined in Figure 2.9, allowed to obtain similar results with *Schizochytrium*. The cell wall of *Schizochytrium* appeared to be less resistant than the one of *Chlorella* therefore the expectation was to obtain a disruption close to 100%. In Figure 2.11 the lysis was carried out on 10 ml *Schizochytrium* suspension (40 g/L DCW) and 37 g of 1 mm diameter glass bead beads (50% microbeads in the lysis suspension).

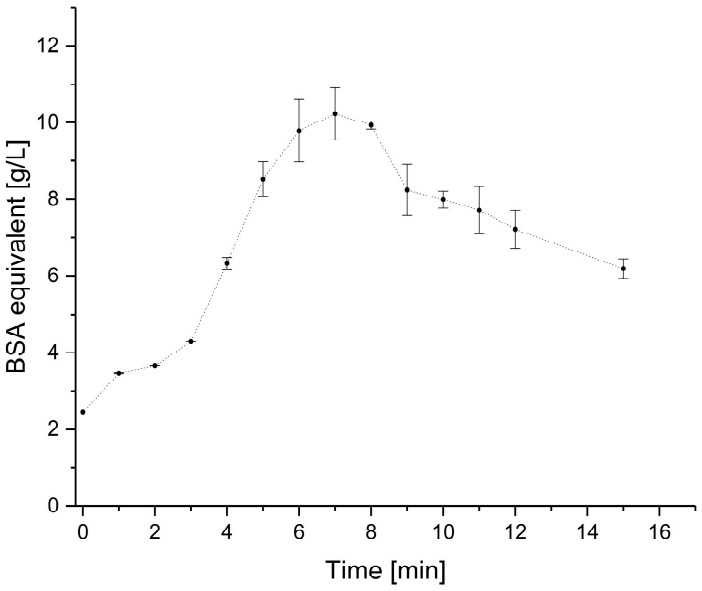


Figure 2.11: *Example of* Schizochytrium *sp. cell wall disruption by vortex bead milling a 40 g/L suspension using 1 mm diameter glass microbeads. Release of protein is represented as BSA equivalent as a function of the treatment time. Experiments were repeated in duplicate (n=2) and the standard deviation is represented as error bars.*

The release of protein reached a maximum between 5 min and 8 min and then declined (Figure 2.11). It is suspected that the complete disruption of *Schizochytrium* cells was achieved after an optimal time of approximately 8 min, afterwards the shear forces could cause a mechanical denaturation of the released protein. Under acidic conditions, the Coomassie dye used in the Bradford protein assay forms a strong, non-covalent complex with the protein carboxyl group by Van der Waals force and with the amino group through electrostatic interactions. It is suspected that due to the mechanical denaturation, the structure of the protein may change, which would explain the decline in BSA equivalent after 10 min represented in Figure 2.11. Cell counts were made on samples at 0 min and 15 min, indicating a cell disruption of 100%. As expected, the optimal conditions for cell wall lysis of *Chlorella* were also effective with *Schizochytrium*. The only significant difference was the requirement of 10 min vortexing with *Chlorella* (Figure 2.10) and only 8 min for *Schizochytrium*.

If the initial cell suspensions were stored at 4°C, the temperature in the test tubes after 15 min of vortexing was lower than 30°C. This suggest that with this method, thermosensitive compounds could be preserved.

Those experiments were carried out on microalgal suspensions not cultivated under elemental imbalance, therefore having a normal lipid composition ($< 30\%$). When the standard disruption procedure was carried out on microalgae with high lipid composition, the disruption of the cell walls was verified only by cell count and not by released soluble protein, since after centrifugation a layer of oil was formed impeding the correct measure of soluble proteins.

2.3.2 Solvent extraction of total lipids

The following solvent mixtures, were tested on the two microalgal strains aiming to investigate which solvent mixture was best for extraction of lipids. Solvent mixtures were chosen from various experiments described in the scientific literature [34] [92] [198] [174] [173] [199]. The expectation was that an efficient extraction method implies combining polar and non-polar solvents. The polar solvent acts on the cell membrane dissociating the protein-lipid complexes. Consequently lipids, become more available for the non-polar solvents which can also penetrate the cell membrane forming solvent-lipid complex [127].

- Chloroform methanol
- Dichloromethane - methanol
- Dichloromethane ethanol
- Hexane - isopropanol
- Ethyl acetate - methanol

2.3.2.1 *Schizochytrium* sp.

The analysis were started by extracting total lipid directly (without mechanical pre-treatment) from a suspension of *Schizochytrium*, cultivated in standard medium. Solvents listed above were tested three times to ensure reproducibility of the experiment. Results are represented as the percentage of total lipids as a proportion of of dry cell weight and they are shown in Figure 2.12.

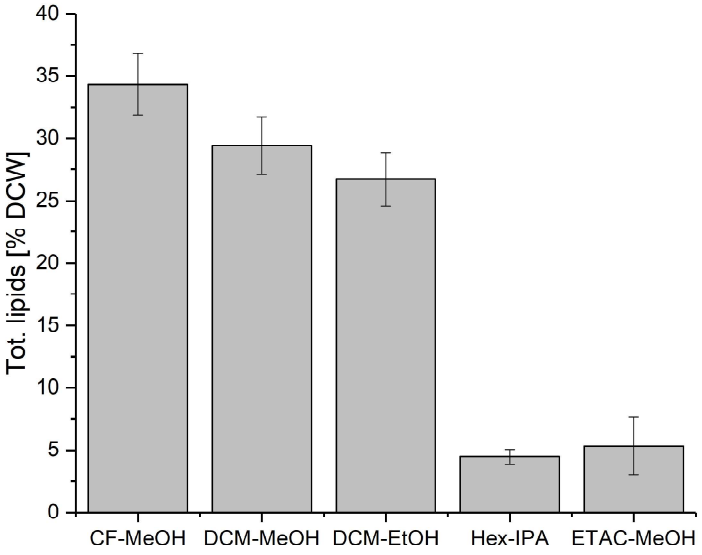


Figure 2.12: *Effect of different solvents mixtures on the total amount of lipids extracted from* Schizochytrium*, without mechanical pre-treatment. Experiments were repeated in triplicate (n=3) and the standard error is represented.*

Differences in the percentage of total lipids extracted from biomass shows that - as expected - lipid extraction seems to be dependent on the solvent or mixture used. After centrifugation only the organic phase, was taken to extract the lipids. Phase formation depends on the density of solvents, except Hex-IPA and ETAC-MetOH, the organic phase containing lipids corresponded to the lower layer, whereas the aqueous phase was the upper layer. Depending on the nature of the solvents, different amounts of lipid were extracted. Satisfying results were obtained with chloroform - methanol, total lipid composition of DCW was 34.32 % (SD= 2.455 %, n=3), followed by dichlormethane-methanol with 29.42% (SD= 2.318 %, n=3) and dichloromethane-ethanol 26.73 % (SD= 2.147%, n=3). Lower results were obtained from hexane-isopropanol and ethylene acetate-methanol with respectively 4.466 % (SD= 0.5988%, n=3) and 5.322 (SD= 2.309%, n=3).

2.3.2.2 *Chlorella protothecoides*

Solvent extraction experiments were repeated on suspensions of *Chlorella protothecoides* and the results are represented in Figure 2.13.

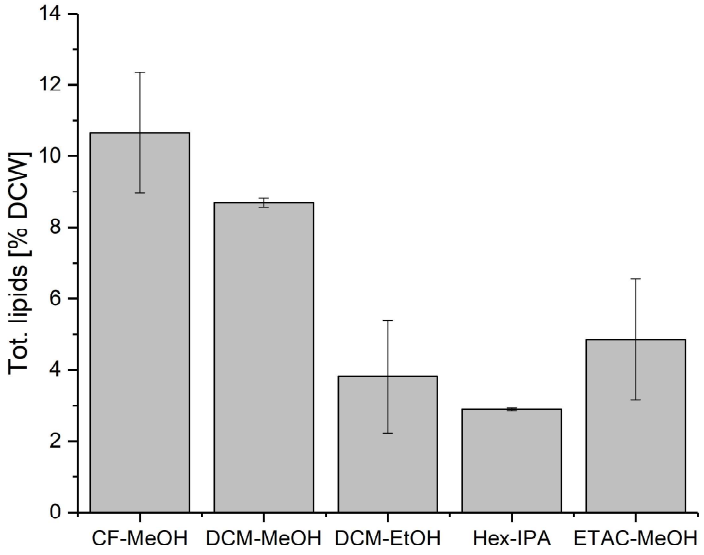


Figure 2.13: *Effect of different solvents mixture on the total amount of lipids extracted from* Chlorella*, without mechanical pre-treatment. Experiments were repeated in triplicate (n=3) and the standard error is represented.*

Figure 2.13 shows that chloroform-methanol and dichlormethane-methanol allowed to extract higher quantities of lipids, 10.65% (SD= 1.697 %, n=3) and 8.699 % (SD= 0.1287 %, n=3) respectively. Other solvents had a minor effect, showing a total lipid composition lower than 5% of the dry cell weight, therefore they were not considered for the continuation of the experiment.

2.3.2.3 Solvent extraction with and without disruption of the cell wall

Because of the rigid cell wall structure of some microalgal cells, it has been reported that without mechanical disruption, solvent extraction alone would have a low efficiency [201] [184]. Combining with mechanical cell disruption would facilitate lipid extraction by releasing lipids from the cellular matrix. However, the study of Ranjan et al., suggested that lipids diffuse across the cell wall when the biomass is suspended in a solvent with a high selectivity and solubility for lipids [175]. The aim of the experiment was to define whether the amount of lipid extracted by solvents, was improved by mechanical pre-treatment of the cell suspension (vortexing with microbeads). In Figure 2.14 is compared the total lipid extraction before and after mechanical pre-treatment of *Schizochytrium* sp. cell walls. The two previous best solvent

mixtures i.e chloroform-methanol and dichloromethane-methanol, were tested (Figure 2.12).

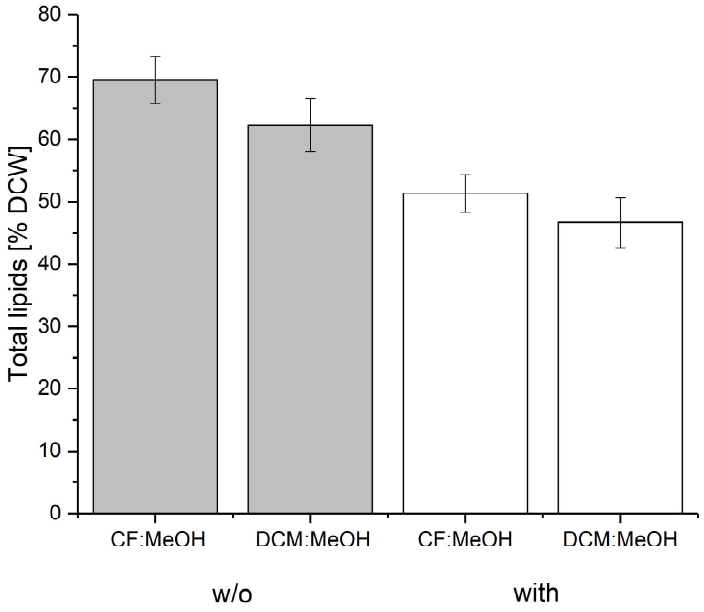


Figure 2.14: *Solvent extraction of total lipids from a* Schizochytrium *suspension with and without cell disruption pre-treatment. Two different solvent mixtures were used: chloroform-methanol and dichloromethane-methanol. The experiments were repeated five times (n=5) and the standard error was represented.*

Unexpectedly, mechanical disruption of *Schizochytrium* cell walls appeared to have a negative effect on total lipid extraction, when using dichloromethane-methanol, the total lipid was 46.62% (SD=4.008%, n=5) instead of 62.33% (SD= 4.269, n=5). When the solvent mixture used was composed of chloroform and methanol, the total lipid composition was 51.31 % (SD=3.008%, n=5) with cell walls disruption and 69.52 % (SD=3.747%, n=5) without (Figure 2.14). Since most lipids are stored in cells as triglycerides, it is possible that mechanical forces during the disruption process (shear forces) and/or intracellular lipases released into the medium, attacked the triglycerides freeing glycerol molecules in the aqueous phase during extraction [34] [202]. Previous experiments represented in Figure 2.11 showed that protein was also denatured. As a conclusion, mechanical disruption of *Schizochytrium* cell walls was not required with efficient lipid extraction achieved by solvent extraction alone.

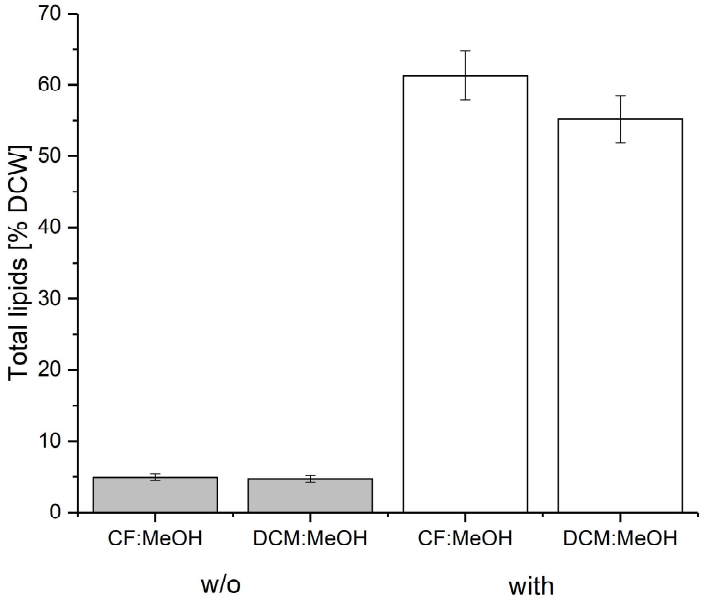


Figure 2.15: *Solvent extraction of total lipids from a* Chlorella *sp. suspension with and without cell disruption pre-treatment. Two different solvent mixtures were used: chloroform-methanol and dichloromethane-methanol. The experiments were repeated five times (n=5) and the standard error was represented.*

Figure 2.15 shows that the solvent mixture chloroform-methanol allowed the efficient recovery of lipids corresponding to 61.29 % (SD= 3.497 %, n=5) of dry cell weight and 55.18% (SD= 3.276 %, n=5) when using dichloromethane-methanol. Without mechanical pre-treatment, the amount was about 11-fold lower (Figure 2.15). It can be concluded that in order to efficiently extract intracellular lipids from *Chlorella*, it is necessary to first carry out mechanical lysis of the cells by vortexing with microbeads. It is important to point out that experiments carried out in Figure 2.12 and 2.13 were carried out on microalgae cultivated in a carbon-limited medium at the beginning of the project, when lipid accumulation was still not optimized. This explains the low composition in total lipids i.e $<$ 35% for *Schizochytrium* and $<$ 10% for *Chlorella*. Solvent extraction of total lipids represented in Figure 2.14 and 2.15 was carried out on microalgae cultivated in a medium limited in nitrogen, where the lipid accumulation was triggered.

As a conclusion for both studies, despite chloroform allowed a slightly higher extraction compared to dichloromethane (but less than 10% according to Figure 2.14 and 2.15), it was de-

cided to continue the experiment using dichlormethane. First of all, dichlormethane-methanol was evaporated much faster compared to the other solvents, due to its low boiling point of 39.6°C. This could be very helpful to avoid thermal degradation of sensitive fatty acids. Furthermore, dichloromethane is the recommended alternative to other chlorinated solvents (i.e. least worst). Solvent assessment is generally based on worker safety, process safety and environmental considerations [203]. Chloroform has acute potential health effects comprised mutagenic and carcinogenic effects, accidental spillage might cause considerable environmental damage. According to the principles of green chemistry, use of auxiliary substances should be made unnecessary wherever possible and innocuous when used [204]. Furthermore the faster evaporation is a considered as an advantage, when working with sensitive structures like long chain polyunsaturated fatty acids.

The study of Ranjan et al., hypothesized that lipids seems to diffuse across the cell wall when the biomass is suspended in a solvent with higher selectivity and solubility for lipids [175]. This seems to be the case for *Schizochytrium*, however cell walls of *Chlorella* seem to be more resistant, preventing the solvent to achieve the intracellular material. The cell wall was preventing efficient lipid extraction, thus, the cell wall need to be mechanically disrupted to liberate the lipids and allow them to come into contact with the solvent.

2.3.3 Enzymatic hydrolysis of *Chlorella protothecoides* cell wall

2.3.3.1 Single enzyme kinetics

The potential for enzymatic treatment of microalgal cell walls was explored as an alternative, sustainable and applicable at larger scale, method for lipid extraction. Enzyme studies were performed on *Chlorella protothecoides*, since this strain had a more robust cell wall, requiring mechanical pre-treatment prior to solvent extraction. The enzymes selected for the study - lysozyme, cellulase and sulfatase - were chosen based on literature reports [152] [58] [100] [179] [180] [181], whereas enzymes from *Talaromyces* sp. were produced in-house by Monaghan Biosciences, Ireland.

The product of the enzyme reaction was indirectly measured, based on the percentage of total lipids extracted from the microalgal suspension. Initially, enzymes were tested separately with the aim to define the single effects on the cell wall. A defined enzyme concentration was

added to a microalgal suspension and incubated under different conditions, according to Table 2.1 (Experiment A). Kinetic studies were carried out over 48 hours, samples were removed at intervals and total lipids were extracted. The results are expressed as extractable lipids, which is the ratio in percentage of the total lipid extracted after enzymatic hydrolysis on total lipid extracted from the positive control (Equation 2.5).

It was expected that the percentage of extractable lipids would increase over time until the reaction is complete (Figure 2.16).

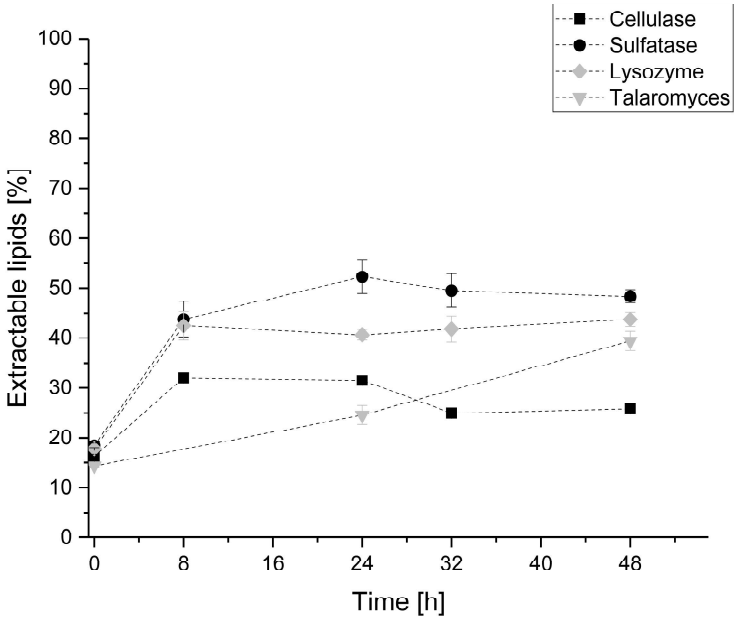


Figure 2.16: *Extractable lipids [%] as a function of time [h] for different enzymes i.e cellulase, sulfatase, lysozyme and Talaromyces enzymatic cocktail. Experiments were repeated 5 times (n=5) and standard deviation is represented as error bars.*

Figure 2.16 shows that among the enzymes tested, cellulase had the least effect, with a maximum of 32-35% of extractable lipids. On the other hand, sulfatase showed the greatest effect: maximum with 50-55% extractable lipids, followed by lysozyme with 42-47% and *Talaromyces* enzyme cocktail with 40%. With the exception of the *Talaromyces* enzyme cocktail, the greatest extraction was achieved after 8h incubation, with no further lipid release for a further 40 h. A negative control was carried out by incubating the algal suspension in Tris-HCl and acetate buffer without enzyme addition. The extractable lipids, after 48 hours, were constant at approximately 18%, showing that modifications in cell wall permeability were mainly due to enzyme hydrolysis (Results not shown).

2.3.3.2 Enzyme cocktails

The study of Gerken et al. reported that no single enzyme had a significant impact on the hydrolysis of algal cell walls of *Chlorella* sp. . Lysozyme appeared to expose inner cell wall substrates making them accessible to others enzymes [58]. Consequently, experiments were carried out by combining lysozyme with cellulase, sulfatase and *Talaromyces* enzyme. Details of the study are reported in Table 2.1 (Experiment B).

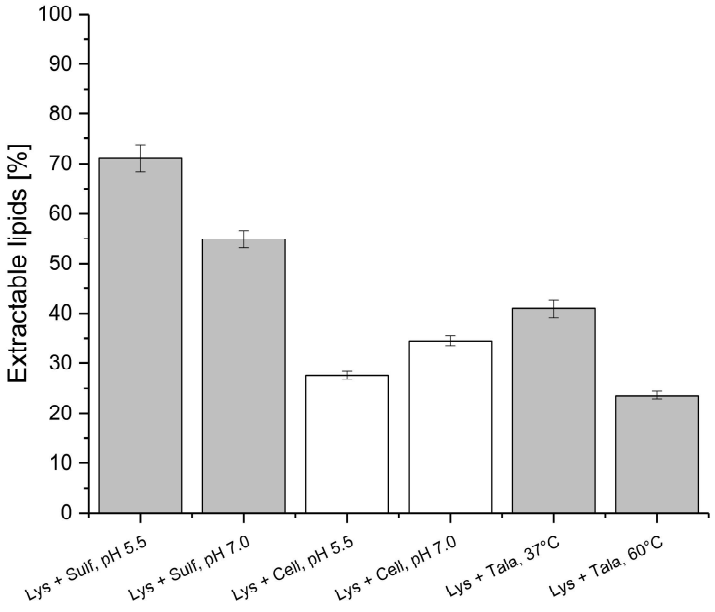


Figure 2.17: *Extractable lipids [%] obtained after an incubation of 24h under difference conditions and with different enzyme cocktails i.e Lysozyme + sulfatase, Lysozyme + Cellulase and Lysozyme + Talaromyces enzymes. Experiments were repeated 5 times (n=5) and propagation of standard deviation is represented as error bars.*

Figure 2.17 reports the results of enzyme hydrolysis and the effect on lipid extraction of a combination of lysozyme with other enzymes, after 24 hours of incubation. It can be seen that lysozyme combined with sulfatase showed a very promising effect. At pH 5.5, the cocktail enabled extraction of 71.12% (SD = 2.67%, n=5) of extractable lipids and only 54.86% (SD = 1.64%, n=5) at pH 7.0. A lower pH appears to have a better effect on the activity of sulfatase, which is reported to have a higher activity at pH 5, while lysozyme has a broader pH range. On the other hand, lysozyme combined with cellulase did not seem to have a comparable impact on the cell wall lysis. Experiments using lysozyme and cellulase were carried out at pH 5.5 and pH 7.0, showing 27.60% (SD = 0.77%, n=5) and 34.51% (SD = 0.94 %, n=5) of extractable lipids respectively. Surprisingly, when lysozyme was tested alone (Figure 2.16), the result was up

to 1.4-fold higher than the cocktail mixture with cellulase (Figure 2.17) i.e 42-47% instead of 27.60-34.51 %. The effect of cellulase may have been to change the molecular structure of the cell wall substrate of lysozyme, thereby reducing its effect. Extractable lipids obtained from a combination of *Talaromyces* enzymes and lysozyme were 23.64% (SD = 0.86 %, n=5) at 60°C and 40.94% (SD=1.78 %, n=5) at 37°C. As a consequence of the higher and faster extraction found with a combination of lysozyme and sulfatase at pH 5.5, 37°C, a kinetic study was carried out over a longer period of time - 48 hours - with two different enzyme concentrations i.e. 1-fold and 2-fold (Table 2.1, Experiment C, Chapter 3). Samples were taken regularly and total lipids extracted. The aim of the experiment was to describe the enzyme kinetics and define whether extractable lipids would increase further over time (Figure 2.18).

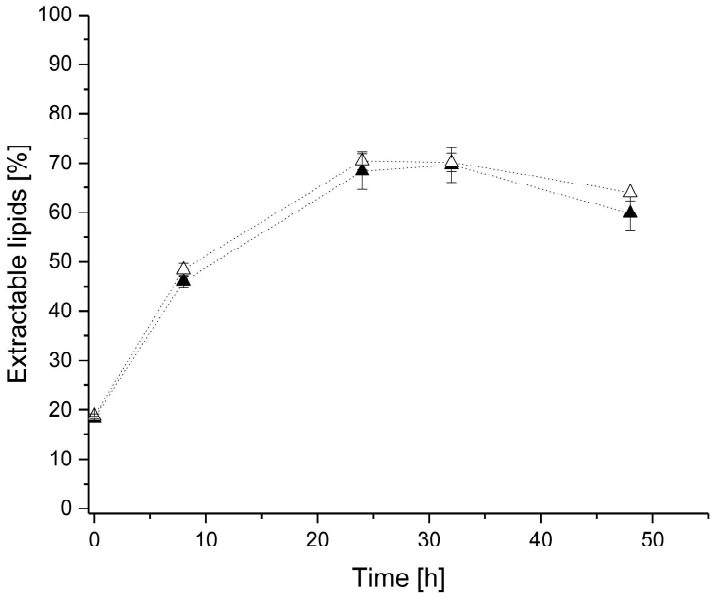
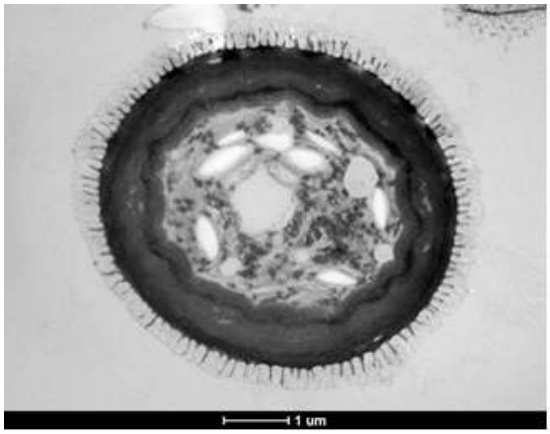


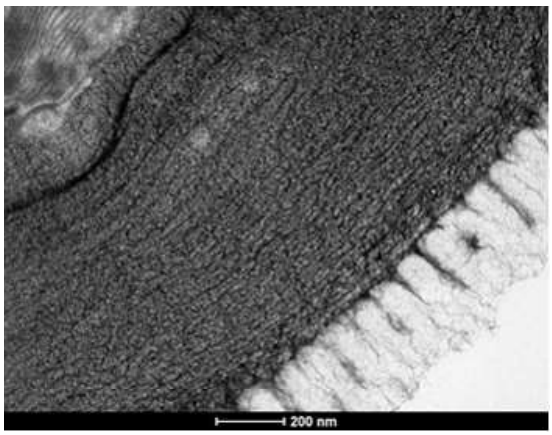
Figure 2.18: *Enzymatic treatment with a combination of lysozyme and sulfatase is represented as extractable lipids [%] as a function of time [h]. Two different concentrations were used (▲) 1-fold and (△) 2-fold (Table 2.1, Experiment C). This means that the concentrations of lysozyme and sulfatase in the 1-fold experiment were 4000 U/mL and 10 U/mL, respectively. In the 2-fold experiments were 8000 U/ml and 20 U/mL, respectively. Experiment were repeated 5 times (n=5) and propagation of standard deviation is represented as error bars.*

When the enzyme concentration was increased, by keeping substrate concentration constant, it was expected that a proportional increase in the reaction rate would be observed. Figure 2.18 shows that the concentration of enzyme did not have an effect on extractable lipids, for both concentrations, a maximum of 70% extractable lipids was reached after 24h. In terms of total lipid composition of the dry biomass, this corresponded to 47%, meaning an improvement of

7-fold compared to the negative control of 6.4 % (extracted with dichloromethane-methanol) shown in Figure 2.15. Cellulase treatment showed poor activity when applied to *Chlorella protothecoides*. This is in agreement with Gerken et al. who found that cellulase had a minimal effect on the permeability of *Chlorella vulgaris*, concluding that cellulose does not comprise a major component of cell walls [58]. The study of Takeda et al. demonstrated that most *Chlorella vulgaris* strains contain little or no glucose in the cell wall [159]. However, by contrast Fu et al. reported that cellulase - employed to hydrolyse the cell wall of *Chlorella* sp. - gave a very high hydrolysis yield [182]. Composition of cell walls is largely dependent on the conditions of cultivation method. Consequently the *Chlorella protothecoides* used for this study was cultivated under heterotrophic conditions and lead to a cell wall containing little cellulose. Particularly good results were obtained from sulfatase treatments, which could be explained by the presence of sulphated polysaccharides commonly found in algae [80]. Deniaud-Bouet et al. showed that fucans with a high content of sulphate substitutes are highly hygroscopic and it is thought that they contribute to regulate water potential at the outer cell membrane level [205]. The cell wall structure of *Chlorella protothecoides* is not completely defined, however it was claimed to be composed of a polysaccharidic matrix, containing in some cases fucans [58] [159] [205] [157] [158]. Sulfatases hydrolyse sulfate ester bonds, suggesting that sulfated polymers are integral to cell wall architecture in *Chlorella protothecoides*. It was hypothesized that sulfate groups are found in a key position which allow to act effectively on the cell wall. Figure 2.19 represents the intact cell wall of *Chlorella protothecoides* under transmission electron microscopy (TEM). It can be observed that the cell wall is surrounded by hair-like fibres, which according to Gerken et al. seem to be composed of hyaluron [58]. Lysozyme hydrolyses β-14 linkages between N-acetyl-muramic acid and N-acetyl-D-glucosamine residues. Therefore, it seems to remove the protective outer layer by efficiently removing the hair-like fibres. Consequently, more internal layers are exposed for further degradation by other enzymes that can reach new substrates present in more internal layers [152]. A combination of lysozyme and sulfatase resulted in the highest level of extractable lipids obtained in this study.



(a) Chlorella. HPF fixation. TEM medium power



(b) Chlorella. HPF fixation. TEM high power

Figure 2.19: *High-pressure freezing (HPF) fixation followed by Transmission Electron Microscopy (TEM) of Chlorella protothecoides. The pictures were provided by Monaghan Biosciences (Ireland).*

2.4 Conclusion

A standard analytical method to disrupt the cell wall of two different microalgal strains, *Chlorella protothecoides* and *Schizochytrium* sp. was developed and optimized by mix-vortexing a microalgal cell suspension with glass beads. As required, the method was applicable at lab-scale, it was relatively quick ($<$ 10 min), inexpensive, it was reproducible and more important, cell disruption was more than 99%. Results obtained from total lipid solvent extraction showed that the cell walls of *Schizochytrium* sp. were permeable and did not require mechanical disruption pre-treatment, whereas the mechanical disruption of cell walls of *Chlorella protothecoides* improved solvent extraction of 11-fold. *Chlorella protothecoides* cell walls were particularly resistant, therefore it was necessary to develop an alternative method which could be applic-

able at large scale that could weaken cell walls and improve disruption of *Chlorella*. Enzymatic hydrolysis of microalgal cell wall was explored, using different types and combinations of enzymes. and combinations. Pre-treatment with a combination of lysozyme-sulfatase, resulted in a total lipid extraction of 47% of the dry biomass. This corresponds to an improvement in total lipid extracted of 7-fold compared to untreated cells, which allowed a maximum total lipid extraction of only 6.4% of the dry biomass. More experiments are needed to optimize the enzymatic process and verify the application at larger scale; a drawback may be the cost of enzymes and the relatively long treatment time (8 hours). On the other hand, weakened cell walls could reduce solvent and energy requirements needed for lipid extraction by improving accessibility of the reagents. Not least, all of the methods presented in this research allowed to extract considerable amounts of total lipids efficiently directly from the wet and concentrated biomass. Wet extraction processes could improve larger scale lipid extraction, increasing the potential of microalgae bio-refineries.

Chapter 3

Characterization and optimization of a fermentation process for the production of high cell densities and lipids using heterotrophic cultivation of *Chlorella protothecoides*

3.1 Introduction

3.1.1 *Chlorella protothecoides*

High-cell-density cultivations are essential to increase the productivity of bio-products [188]. Therefore, the ultimate objective for any bioprocess is to achieve the highest product concentration in the shortest time [51]. The goal of this chapter is the development of an appropriate strategy to enhance *Chlorella protothecoides* biomass and target bio-product, in this case total lipids, by understanding the effect of different culture conditions, by altering the elemental composition of the cultivation medium.

The ”law of the minimum”, formulated by the chemist Justus von Liebig, states that only one nutrient limits the amount of biomass that can be produced in a biological system [206]. It is important to specify that the term nutrient-limited does not imply a nutrient depletion in the me-

dium, but refers to the nutrient that is depleted first (and whose depletion causes the cessation of growth). The growth-limiting substrate is often the carbon or nitrogen source, although in some cases it could be oxygen or other nutrients [85]. In fact, when a microorganism is grown under a substrate limitation, the initial substrate concentration is proportional to the biomass reached at the stationary phase. In this situation, the biomass yield on substrate is constant. After an optimal point, where the biomass is maximal, the initial substrate concentration does not give anymore a proportional increase in biomass, due to an accumulation of toxic products, substrate inhibition i.e osmotic stress, or exhaustion of another substrate [207].

The first step consisted in a strategy to achieve very high cell densities under carbon limitation. This study provides a description of the investigation of a standard cultivation medium and its optimization into a carbon limiting medium to promote cell proliferation. Parameters such as the biomass yield and the optimal concentration of glucose were determined during this study. Nitrogen deprivation conditions are frequently expressed in the literature as carbon-to-nitrogen mass ratio (C/N). This ratio becomes a critical factor in the development and optimization of the culture medium determining both the biomass and lipid accumulation [67]. The expectation is that with a low C/N ratio the medium will be carbon-limited, promoting cell proliferation and consequently the achievement of high cell densities whereas with a higher C/N ratio, under nitrogen starvation, lipids will be accumulated. Therefore, the influence of carbon-to-nitrogen ratio in the medium was examined and the determination of the threshold between carbon-limitation and nitrogen limitation was defined. Finally, a two-stage fed-batch cultivation process was developed, with the two stages characterized by the C/N mass ratio of the cultivation medium.

Chlorella protothecoides has been chosen as reference strain because of its successful large-scale cultivation history [58]. The comparison of the results, not only with the results obtained from the existing literature, but also with those obtained from a reference strain has a central importance in the characterization and optimization of a cultivation and production process. This project was developed in collaboration with an industrial partner Monaghan Bioscience (Ireland), which aim was to explore the potential of commercial exploitation of microalgae, involving the extraction of high value products such as lipids. *Chlorella* represented the ideal model to study and develop an industrial bioprocess based on the information collected and

Table 3.1: *Composition of the A5 Trace Solution*

Ingredient	Amount
H_3BO_3	2.86 g/L
$Na_2MoO_4\,2\,H_2O$	0.039 g/L
$ZnSO_4 \cdot 7\,H_2O$	0.222 g/L
$MnCl_2 \cdot 4\,H_2O$	1.81 g/L
$CuSO_4 \cdot 5\,H_2O$	0.074 g/L

selected from the existing literature. To summarize, the main aims of this chapter are as follow:

- Define a standard cultivation medium
- Study the effect of the C/N ratio on the growth
- Develop a carbon-limited medium to achieve high cell densities
- Define the highest cell density achievable in batch conditions
- Develop a two-stage fed-batch process

3.2 Material and methods

3.2.1 Materials and microalgal strain

All chemicals were purchased from either Sigma Aldrich (St. Louis, USA) or Thermo Fisher Scientific (Waltham, USA) if not stated otherwise. *Chlorella protothecoides* (ATCC 30411) was obtained from the ATCC (Manassas, USA) culture collection.

3.2.2 Standard medium preparation

The standard cultivation medium used here was previously described in the literature by Chen et al. [45]. The A5 Trace solution, which provides the micro-nutrients was firstly prepared dissolving the ingredients in DI water in the order explained in Table 3.1, to avoid precipitation. The A5 solution was added to the standard cultivation medium, the preparation of which is described in Table 3.2. A solution of 500 mg/L of Vitamin B1 was prepared and 200 μL were added into the medium. The pH of the medium was adjusted to 6.8 with 1M NaOH, followed by sterilisation through a 0.22 μm filter (Steritop, Millipore, Billerica, USA). The bottles used to store the media were previously autoclaved at 121°C for 20 min. The medium and the other solutions can be stored at 4°C for no longer than one month.

Table 3.2: *Composition of the standard cultivation medium for* Chlorella protothecoides

Ingredient	Amount
KH_2PO_4	0.7 g/L
K_2HPO_4	0.3 g/L
$MgSO_4 \cdot 7\,H_2O$	0.3 g/L
$CaCl_2$	25 mg/L
NaCl	25 mg/L
$FeSO_4 \cdot 7\,H_2O$	3 mg/L
Vitamin B1	0.01 mg/L
A5 Trace Solution	1 ml/L
Glucose	30 g/L
Yeast Extract	4 g/L

3.2.2.1 Effect of different nitrogen sources

Different sources of nitrogen such as yeast extract, glycine and urea were tested for the cultivation of *Chlorella protothecoides*. In Table 3.3 is represented the description of the preparation of the media for the experiments. In all of the experiments described, the same nitrogen concentration of 0.456 g/L was added. In part of the experiments, half of the nitrogen came from the yeast extract and the other half from the alternative nitrogen-source, glycine (N3) and urea (N5) whereas in some other experiments, YE was not added (N4, N6).

Table 3.3: *Description of the different experiments designed to define the optimum nitrogen source between yeast extract, glycine and urea.*

	N1	N2	N3	N4	N5	N6
Glucose [g/L]	10	10	10	10	10	10
YE [g/L]	4	0	2	0	2	0
N [g/L]	0.456		0.228		0.228	
Glycine [g/L]	-	-	1.22	2.44	-	-
N [g/L]			0.228	0.456		
Urea [g/L]	-	-	-	-	0.49	0.98
N [g/L]					0.228	0.456

3.2.2.2 Carbon to nitrogen ratio

The standard cultivation medium was prepared varying the ratio between the initial concentration of glucose (carbon) and yeast extract (nitrogen). The carbon to nitrogen (C/N) ratio is

calculated according to Equation 3.1, which represents a mass ratio, where 40% is the composition of carbon in glucose and 11.4% that of nitrogen in yeast extract. The percentage of nitrogen contained in yeast extract was calculated from the elemental composition of yeast which is $CH_{1.8}N_{0.2}O_{0.5}$ (24.6 g/mol) [85].

$$C/N = \frac{40\%\ Glucose\ [w/V]}{11.4\%\ Yeast\ Extract\ [w/V]} \tag{3.1}$$

3.2.2.3 Study of the carbon and nitrogen limitation

The threshold between carbon limitation and nitrogen limitation was determined by preparing the media for the cultivation of the two microalgal strains with different concentration of carbon and yeast extract. Their preparation is described in Table 3.4-= and Table .

Table 3.4: *Description of the preparation of a standard medium for the cultivation of Chlorella protothecoides with different C/N ratios. The yeast extract concentration was constant at 10 g/L and the glucose concentration was variable.*

C/N	1.75	5.25	10.53	11	13	15	17	19	21.05	31
Glucose [g/L]	5	15	30	31.35	37.05	42.75	48.45	57.15	60	90
YE [g/L]	10									

Table 3.5: *Description of the preparation of a standard medium for the cultivation of Chlorella protothecoides with different C/N ratios. The glucose concentration was constant at 20 g/L and the yeast extract concentration was variable.*

C/N	70	35	17.5	12	9
Glucose [g/L]	20				
YE [g/L]	1	2	4	6	8

3.2.3 Cultivation of *Chlorella protothecoides*

3.2.3.1 Cryopreservation

The optical density of a microalgal suspension in exponential phase was determined and depending on its concentration a sample was harvested and centrifuged at 478.8 x g for 10 min

(FL40R, Thermo Scientific, USA). The concentration was adjusted re-suspending the pellet in a certain amount of fresh medium in order to have a final OD_{540nm} of 10. In a 2 mL cryovial were added 1 mL of a solution 10% (v/v) methanol prepared in fresh media and 1 mL of the cell suspension and mixed well. The final OD_{540nm} should be 5 in 5% (v/v) methanol. As methanol is toxic for the cells, it was really important to work fast: no more than 10 min should pass before the freezing of the suspension. The cryovials were placed overnight into a cryogenic container (Mr. Frosty, Thermo Fisher Scientific, Waltham, USA) filled with isopropanol, where the rate of cooling is controlled at -1 °C/minute. Some cryovials (Master Cell Bank) were stored also in liquid nitrogen for backup. The method was adapted from the procedure developed by Monaghan Biosciences (Ireland).

3.2.3.2 Thawing

The cryovials were removed from the -80 °C freezer and placed in a water bath at 25°C (Salvis-Lab, Rotkreuz, Switzerland). When only a small pellet of ice remained, the solution (2 mL) was mixed with 5 mL of fresh medium and centrifuged at 478.8 x g for 10 min (FL40R, Thermo Scientific, Waltham, USA). This step is important to remove methanol, which can inhibit the cell growth. The supernatant was removed and the pellet was re-suspended in 50 mL of fresh culture medium in a 125 mL baffled flask. The initial OD_{540nm} in the flask should be approximately 0.2.

3.2.3.3 Baffled shake flasks preparation

The pre-inoculum cultivation and some other experiments were carried out in 500 mL baffled shake flasks with a working volume of 100 mL. The flasks were incubated in a shaking incubator (GFL, Germany) with orbital motion at 150 rpm.

3.2.3.4 Bioreactor preparation

Cultures were scaled-up to an autoclavable bench-top bioreactor (RALF Advanced, 2015, Bioengineering, Wald, Switzerland) adapted for microbial fermentations (5-L total volume); equipped with hollow baffles for temperature control, a Rushton agitator, a pH probe (Mettler Toledo, Columbus, USA), a temperature probe, and a dissolved oxygen probe (Mettler Toledo). The system is schematically represented in Figure 3.1. During the cultivation, the bioreactor was covered with a black plastic bag, to protected the culture against light and exclude photo-

trophic growth. pH was controlled with a conventional proportional-integral-derivative (PID) controller, which automatically added a flow of 1M NaOH or 0.5M H_2SO_4. A solution of 2% polypropylene glycol 2000 was used to control foam formation. Dissolved oxygen (DO) concentration was maintained $\geq$ 50% by increasing the air flow (from 0.75 vvm to 4 vvm) and the stirring rate (from 800 rpm to 1000 rpm). The temperature was kept constant at 25°C. The inoculum was made from a fresh pre-culture grown in a shake flask at exponential phase of growth. The initial working volume was 2,000mL and the initial cell concentration in the bioreactor had an optical density at 540nm of approximately 0.2.

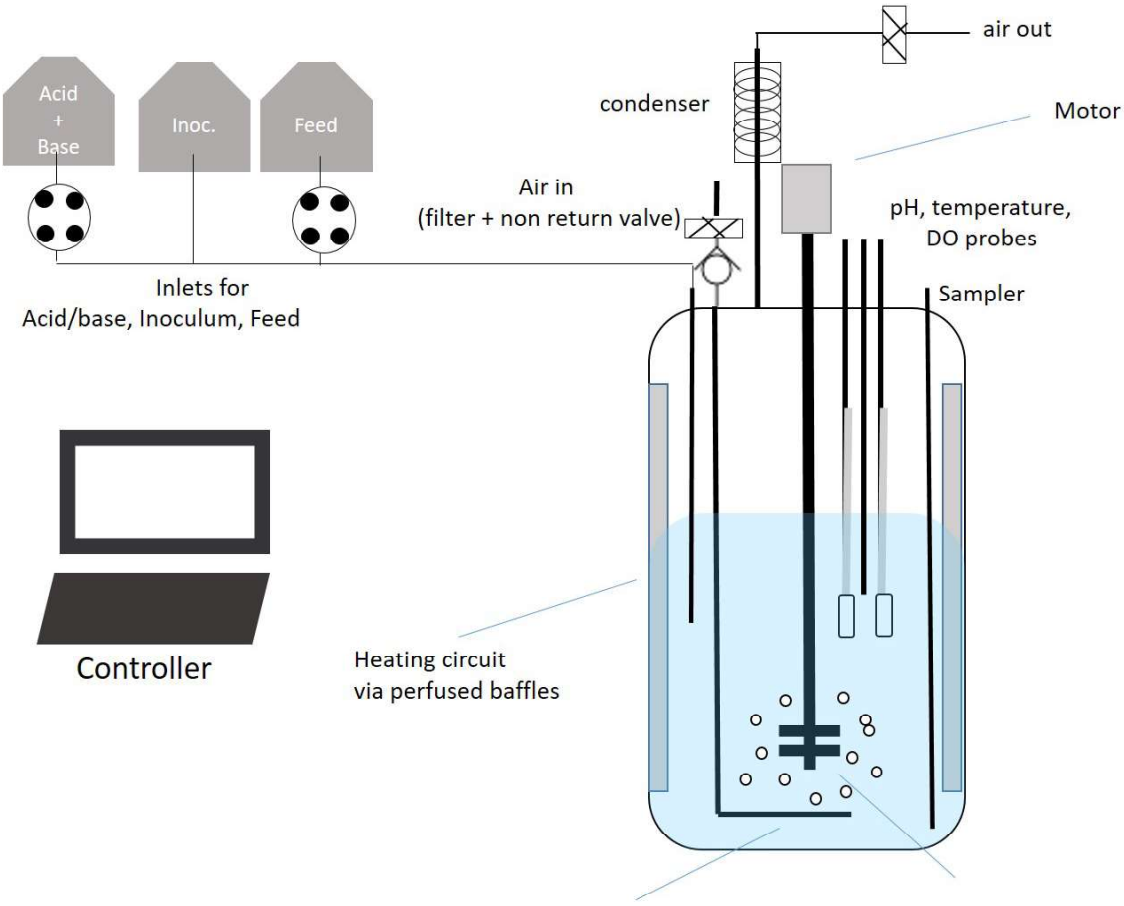


Figure 3.1: *Schematic representation of bench-top bioreactor RALF Advanced (Bioengineering, Switzerland). In the schema is represented: the air inlet, the ring sparger, the Rushton impeller, the air outlet provided with condenser, the inlets for the pH control with acid and base, the inlet for the inoculum, the probes for temperature, pH and dissolved oxygen (DO), the sampling system and the heating circuit via perfused baffles.*

3.2.3.5 Fed-batch

Microalgae showed a very high tolerance to glucose, thus a pulsed feed strategy was chosen instead of a complicated regulated system. This strategy consists of adding nutrients to the bioreactor based on the output signal of an indirect measurement of the substrate concentration,

in this case glucose. The feed was added manually as soon as the glucose was totally consumed. Over-feeding might lead to negative effects on cell growth, therefore the appropriate feeding strategy considered the glucose inhibitory level. The maximum concentration of glucose in the medium after feed addition was kept under 80 g/L for *Chlorella protothecoides*. The sterile feed was composed of 700 g/L glucose and 2-fold concentrations of other medium components excluding yeast extract (Section 3.2.2).

3.2.4 Cell growth analysis

3.2.4.1 Cell growth monitoring by optical density

The absorbance was measured with a spectrophotometer (Unispec2, LLG Labware, Meckenheim, Germany) at 540 nm, at this wavelength interference due to carotenoids or chlorophyll absorption are absent. The instrument was zeroed with a blank cuvette filled with DI water. If necessary the sample was diluted to be in the linear range and the measurement was repeated in duplicate. As a control, it is important to always check that the final absorbance is still between the linearity range and proportional to the first measurement. The cuvette must be dry, clean and without bubbles to avoid interference.

The higher is the cell number, the higher is the absorbance. However, in a concentrated suspension it is not possible to determine exactly the number of cells because it might happen that cells are overlapped. As a consequence, it is important to determine the linear range in which the cell number is directly proportional to the absorbance at a certain wavelength (Figure 3.2).

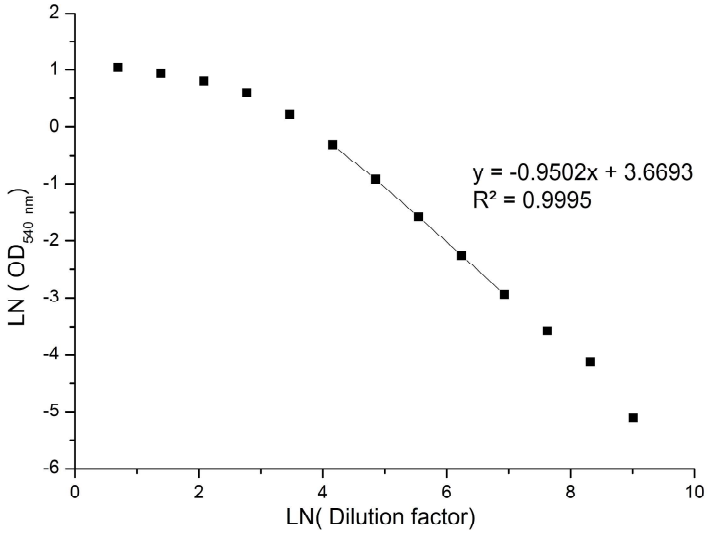


Figure 3.2: *Linear range of the measurement of Chlorella protothecoides by spectrophotometer with an optical density of 540 nm. The linear range is between an* OD_{540nm} *of 0.7 and 0.05.*

The linear range at an optical density of 540 nm determined for *Chlorella* was between 0.05 and 0.7 . However, a narrower range was defined for carrying out the experiments, 0.1 - 0.4. In this way the probability of a valid correlation is higher. Samples were diluted in deionized water to measure absorbance only between the linearity range and the results were calculated as follows:

$$OD_{540nm}\,[-] = dilution\,factor \cdot Absorbance \tag{3.2}$$

The spectrophotometrical measurements were repeated in duplicate (n = 2) to check the reproducibility of the results, the standard deviation error (SD) is reported in the experimental results or represented graphically as error bars.

3.2.4.2 Dry cell weight determination

To determine the DCW, 3mL of culture broth sample was transferred into a clean, dried, preweighed glass tube (W_0) and centrifuged (FL40R, Thermo Fisher Scientific) at 1,000 rpm for 10 min. The cell pellet was re-suspended in 10mL deionized water, centrifuged, and the supernatant discarded to remove medium residues from the biomass. Glass tubes were dried at

100°C for at least 24 h. The tubes were cooled in a dessicator and weighed (W_1). The measurements were repeated in duplicate (n = 2) to check the reproducibility of the results, the standard deviation error (SD) is reported in the experimental results or represented graphically as error bars. The dry cell weight calculation is described by Equation 4.1.
The dry cell weight calculation is the same as Equation 4.1.

$$DCW\,[g/L] = \frac{W_1 - W_0}{V_{sample}} \tag{3.3}$$

where:

DCW = dry cell weight of the biomass [g/L]

W_0 = weight of empty tube [g]

W_1 = weight of the tube with the biomass [g]

V_{sample} = volume of the sample ($3 \cdot 10^{-3}$ L)

3.2.4.3 Analysis of metabolites by high-performance liquid chromatography (HPLC)

The HPLC method was developed by the Laboratory of Integrated Bioprocessing (Dublin City University, Ireland) for metabolite detection in culture broths such as ethanol, glucose, phosphate, acetate, ammonium and lactate. The samples were prepared by filtering the culture broth through a 0.22 μm filter to remove cells and other insoluble particles. The HPLC (Dionex Ultimate 3000, Thermo Fisher, Waltham, USA) was coupled to a Refractive Index detector (1200 series, Agilent, Santa Clara, USA). An ion exchange column (Supelcogel C610H, Sigma Aldrich, St. Louis, USA) was used. The elution buffer was 0.027% (v/V) H_2SO_4 in ultra pure water with a flow rate of 0.5 ml/min (isocratic elution) at a temperature of 30°C and a maximum pressure of 68 bar. The run time of the method is 32 min. In Figure 3.3 and Figure 3.4 are reported the standard calibration curve of the metabolites analyzed. Each point of the calibration curve was measured in triplicate (n = 3) to check the reproducibility of the results, the standard deviation error (SD) is represented graphically as error bars.

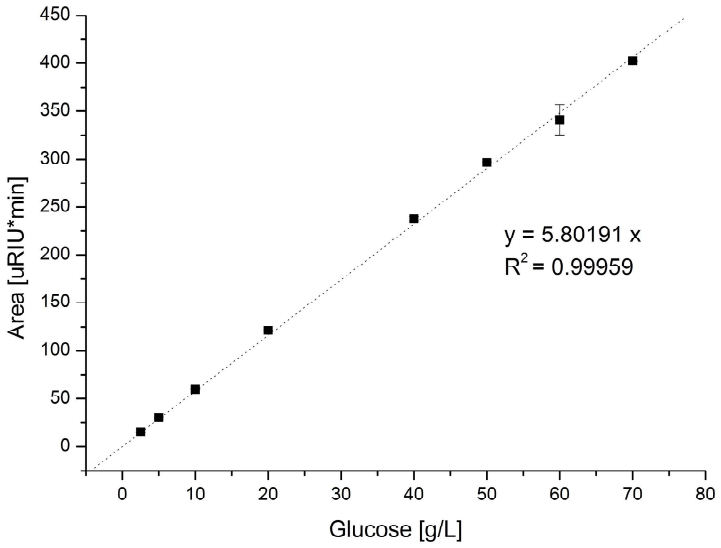


Figure 3.3: *Representation of the standard calibration curves of glucose analyzed by HPLC. The standards were prepared in the standard cultivation medium, each point was measured in triplicate (n=3) and the standard deviation error is represented as error bars.*

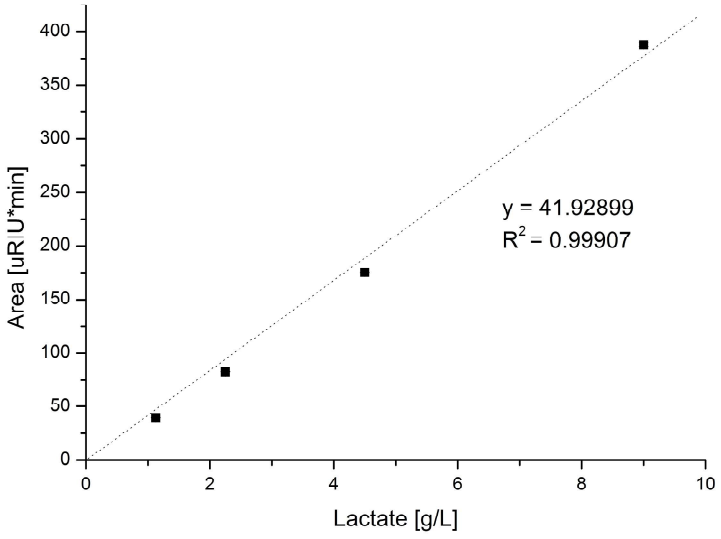


Figure 3.4: *Representation of the standard calibration curves of lactate analyzed by HPLC. The standards were prepared in the standard cultivation medium, each point was measured in triplicate (n=3) and the standard deviation error is represented as error bars.*

3.2.4.4 Analysis of glucose by Cedex Bio Analyzer

The glucose concentration during the fed-batch experiments was analyzed using a Cedex Bio Analyzer (Roche, Basel, Switzerland). The device is based on an enzymatic test using hexokinase, the rate of NADPH formation is measured UV-photometrically, which is directly proportional to the glucose concentration (Figure 3.5). Compared to membrane-based analyzers, the Cedex Bio Analyzers have significantly higher repeatability, accuracy and precision [208]. Calibrations were automatically carried out for all the new reagent sets and internal quality control tests were performed at regular intervals to check the integrity of the whole measuring system. For each test, up to three controls were defined. The results were automatically compared against predefined ranges or values and then interpreted accordingly. Because of the high data reliability, the analysis of the sample were not replicated (n=1).

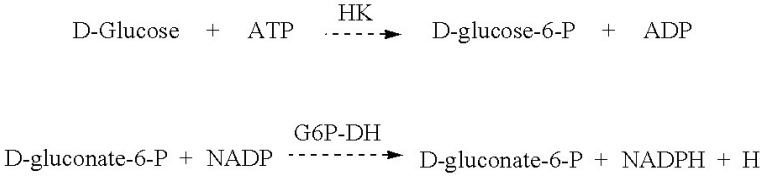


Figure 3.5: *Phosphorylation of glucose by ATP in presence of hexokinase (HK) to glucose-6-phosphate (G-6-P), which is oxidized by NADH in presence of glucose-6-phosphat dehydrogenase (G-6-PDH).*

3.2.4.5 Analysis of total lipids

Total lipids were extracted and measured according to the methods developed in Chapter 2.

3.2.5 Calculation of kinetic parameters and yields

The specific cell growth rate (μ), the biomass yield coefficient on substrate ($Y_{x/s}$), the productivity and the specific glucose consumption rate (q_s) were estimated from experimental data by Equation 3.4 to 3.7.

Specific growth rate

$$\mu = \frac{1}{x}\frac{dx}{dt} \qquad \mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \tag{3.4}$$

where:

μ = specific growth rate in h^{-1}

x_t = biomass at time t (based on OD_{540nm} values)

t = time in h

Biomass yield on substrate

$$Y_{x/s} = \frac{\Delta x}{\Delta s} \tag{3.5}$$

where:

$Y_{x/s}$ = biomass yield on substrate in gg^{-1}

x = biomass in g L^{-1}

s = substrate in g L^{-1}

Productivity

$$Productivity\,[g/L/day] = \frac{m_{product}}{V\,t} \tag{3.6}$$

where:

$m_{product}$ = product in g

V = total fermentation volume in L

t = time in day

Specific consumption

$$q_s = \frac{\mu_{max}}{Y_{x/s}} \tag{3.7}$$

where:

q_s = growth-linked specific substrate uptake rate in $gg^{-1}h^{-1}$

μ_{max} = maximal growth rate in h^{-1}

$Y_{x/s}$ = biomass yield on substrate in gg^{-1}

3.3 Results and discussion

3.3.1 Standard cultivation medium definition

A review of the existing literature has been made to define a suitable standard culture medium. In Annex 1 are summarized the different media compositions described in different scientific

studies aiming to cultivate *Chlorella* sp.. All media have similar compositions however, the one used by Chen and co-workers was selected because it is well described, it was developed specifically for *Chlorella*, particular components such as growth factors were not added, it used glucose as carbon source and there is only one source of nitrogen [45]. A cultivation medium prepared with few and relatively cheap components is ideal in prevision of a scale-up to industrial scale. Furthermore, this medium gave good results in terms of biomass and lipid productivity compared to other studies.

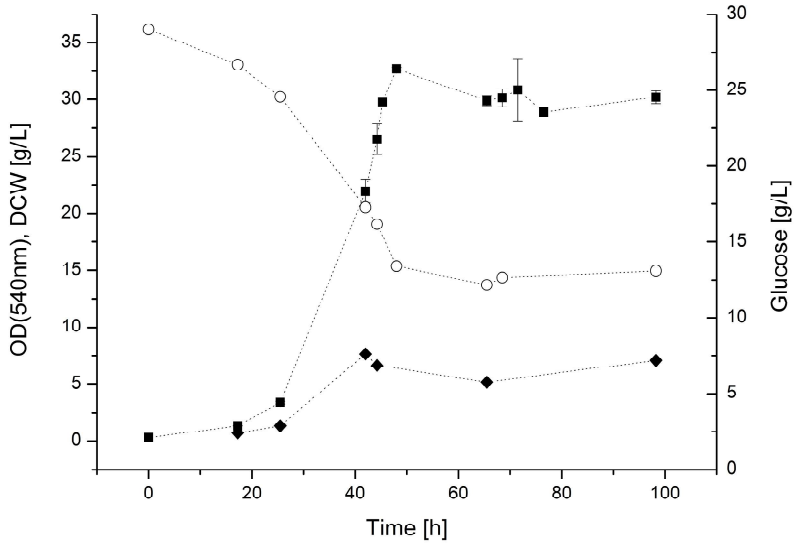


Figure 3.6: *Batch growth study of* Chlorella protothecoides *in standard medium. Results are represented as* ♦ *DCW in g/L,* ■ *optical density (OD_{540nm}) and* ○ *glucose concentration in g/L as a function of the fermentation time in h. The DCW and OD_{540nm} were measured in duplicate (n=2). The standard deviation is represented as error bars.*

In the study of Chen et al., the final DCW was 15.3 g/L [45]. Even though the same cultivation medium and conditions were applied, the result of the current work was 50% lower, 7 g/L (Figure 3.6). According to the published study, the total lipids constituted 50% of the biomass, therefore non-lipid biomass constituted the other half of the weight i.e. 7.5 g/L. It might be that the strain of *Chlorella* used in the experiment represented in Figure 3.6 did not accumulate lipids under these conditions. Furthermore, the stationary phase was reached after only 40-50 hours instead of 120 hours compared to the study of Chen. Results were not similar but it was shown that the standard medium was suitable for the cultivation of *Chlorella* and it will allow to carry out different experiments to set up the analytical methods and also to provide a good base to start the medium optimization. Another interesting result is that glucose was

not completely consumed at the beginning of the stationary phase, the concentration stabilized at 12 g/L, meaning that the limiting substrate was something other than carbon. Different experiments were planned to develop a medium under carbon limitation.

3.3.1.1 Effect of different nitrogen sources

An important aspect in heterotrophic algal cultivation is the selection of the right type and concentration of nitrogen in the culture medium. According to the publication of Xiong W. et al., organic sources of nitrogen i.e. yeast extract, glycine are better assimilated compared to inorganic sources i.e urea, potassium nitrate [62] because of the potential to enhance the growth and the formation of products. As stated in their study, yeast extract gave the best results in term of biomass accumulation. Yeast extract is a complex component that cannot be completely defined, especially in terms of nitrogen quantity. The composition may vary between the lots and between different suppliers. The ideal would be to have a reproducible, quantifiable and defined source of nitrogen. In this experiment, glycine and urea were tested as alternative nitrogen sources. In all of the experiments described in Table 3.3 the same nitrogen concentration of 0.456 g/L was added. In part of the experiments, half of the nitrogen came from the yeast extract and the other half from the alternative nitrogen-source, glycine (N3) and urea (N5) whereas in some other experiments, YE was not added (N4, N6). The expectation was to prove that the alternative nitrogen source could replace completely yeast extract. Theoretically, because of the same amount of nitrogen in every medium tested, it was expected to obtain the same results in term of cell density in all the media with the same nitrogen concentration despite the different sources.

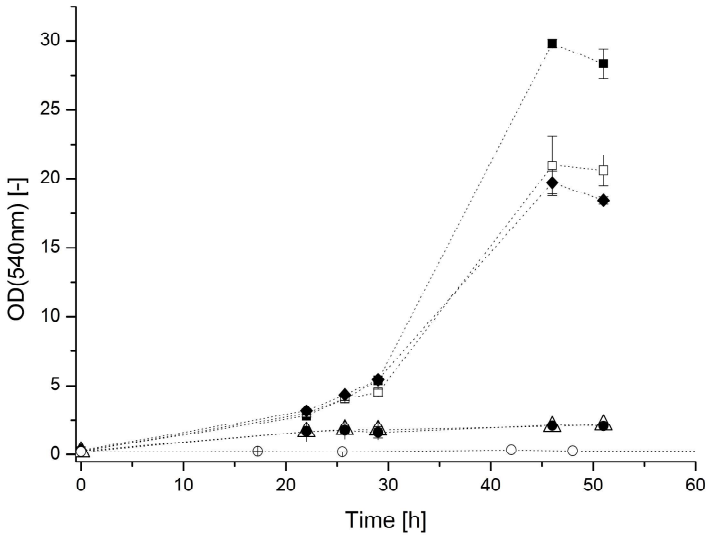


Figure 3.7: *Study of the influence of alternative nitrogen sources i.e glycine and urea, on* Chlorella *sp. cell growth. The cell growth is represented as the optical density at 540 nm as a function of the fermentation time. The composition of the medium is reported in Table 3.3 and the different experiments are indicated as the following:* ■ *N1,* ◯ *N2,* □ *N3,* ♦ *N4,* △ *N5,* ● *N6. The standard deviation of DCW (n=2) is represented as error bars.*

As reported in Figure 3.7, biomass obtained from the media prepared with yeast extract and glycine or urea (N3,N5) was lower than with nitrogen coming exclusively from YE (N1): maximum optical density was 20 instead of 30. Furthermore, when yeast extract was not added, cells did not grow (N2, N4, N6). The hypothesis was that this strain of *C. protothecoides* has not the necessary enzymes to metabolize the nitrogen present in the alternative sources. Lack of the enzyme urease can explain the inability of *Chlorella protothecoides* to metabolise urea [104]. It was also hypothesized that some essential nutrients such as vitamins or co-factors - essential for cell growth - were contained in yeast extract. Despite the complex composition, yeast extract was going to be used for all further experiments.

3.3.2 Study of the carbon-to-nitrogen (C/N) limitation

To define the threshold between carbon-limitation and nitrogen-limitation, different C/N ratios were tested and the effect on biomass yield studied. The medium preparation is described in Section 3.2.2 and only the amount of glucose and YE was modified as described in Table 3.4. In the first part of the work, the cultivation medium was prepared keeping the concentration of YE constant at 10 g/L and using increasing concentrations of glucose.

The threshold between carbon limitation and nitrogen-limitation was defined by cultivating cells under different C/N mass ratios and exploring the effect on biomass yield. It was expected that when the carbon substrate was limiting, the initial concentration would determine the final biomass achieved at stationary phase. The standard cultivation medium was modified by keeping the yeast extract concentration constant at 10 g/L and increasing the concentration of glucose (5; 15; 30; 31.35; 37.05; 42.75; 48.45; 57.15; 60; 90 g/L) (Table 3.4).

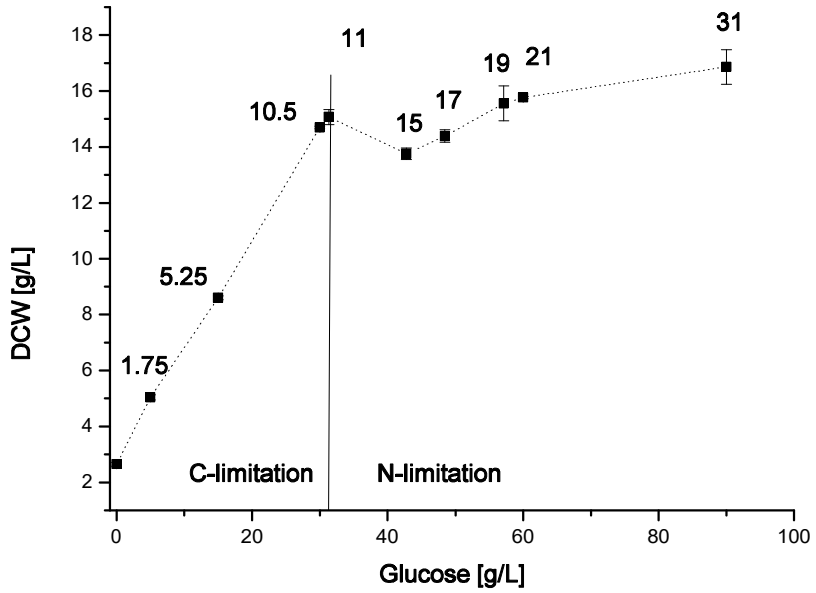


Figure 3.8: *Maximum biomass measured at the stationary growth phase as a function of the initial glucose concentration. Labels indicate the respective C/N ratio. The DCW corresponds to an average of two points (n = 2) measured during the stationary phase, the standard deviation is represented as error bars.*

Figure 3.8 represents the maximum biomass obtained by cultivating *Chlorella* in the growth medium as a function of different initial concentrations of glucose. Up to a C/N ratio of 11, the relation between the amount of DCW formed and the initial concentration of glucose was linear ($R^2 = 0.99$). Analysis confirmed that glucose was completely consumed at the beginning of the stationary phase and thus the culture was glucose limited (results not shown). C/N mass ratio of 11 corresponded to 31.35 g/L glucose and 10 g/L yeast extract, and glucose consumption with C/N ratios higher than 11 was always between 29 g/L and 31 g/L. Furthermore, with ratios higher than 11, DCW remains unchanged, reaching a plateau. The hypothesis is that this was due to the switch to nitrogen limitation. However, it may have been the case that the initial concentration of glucose (higher than 31 g/L) was having an inhibitory effect on growth.

Further experiments were needed to clarify the matter. Therefore, in the second part of the work, the culture experiments were repeated, keeping the glucose concentration constant at 20 g/L (Table 3.5) and varying the yeast extract (Figure 3.9).

Carbon forms about 50% of the yeast biomass. Therefore, for a better estimation of the C/N ratio and to define a carbon-limited medium, it was important to prove whether the microalgal strain was able to consume the carbon present in the YE. Figure 3.8 showed that without glucose the final DCW was only 2.7 g/L. The biomass yield on YE is very low indicating that *Chlorella* was not able to significantly uptake the carbon forming the yeast biomass. Furthermore, an HPLC analysis demonstrated that the YE extract was free of glucose (results not shown). In the continuation of the experiments the uptake of the carbon contained in the yeast extract would be considered as not significant.

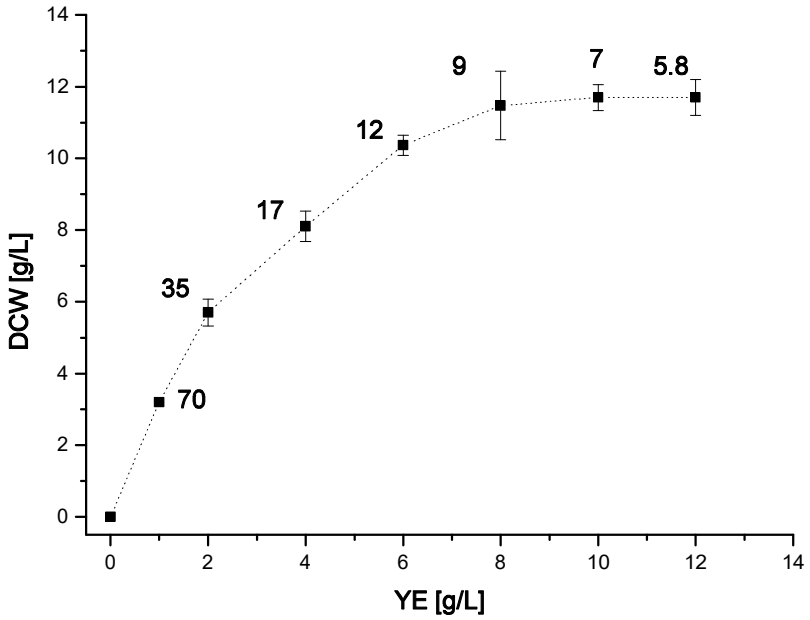


Figure 3.9: *Maximum biomass as a function of the initial glucose concentration. Labels indicate the respective C/N ratio. The DCW corresponds to an average of two points (n = 2) measured during the stationary phase, the standard deviation is represented as error bars.*

Results in Figure 3.9 show that, with a C/N ratio lower than 35, biomass increased proportionally with the amount of yeast extract in the medium. Subsequently, the curve increased slowly until forming a plateau at a C/N ratio between 9-11. In other words, with a lower concentration

of nitrogen (higher C/N ratio) the medium became limited in nitrogen and not in glucose. The biomass to yeast extract yield ($Y_{X/YE}$) is 3 g g^{-1} and corresponds to the slope of the linear part of Figure 3.9. The plateau was reached because of the new substrate limitation and not because of the inhibitory effect of glucose. The conclusion was that the threshold between carbon limitation and nitrogen limitation appears to be at some point between a C/N ratio of 9-11. It was difficult to set a precise limit value because of the slightly heterogeneous nitrogen concentration in yeast extract.

According to the literature, for the microalgal strain *Chlorella sorokiniana*, it was found that the switch from carbon to nitrogen limitation occurred at a C/N ratio of approximately 20. The cell lipid content was at a minimum below this value and increased at higher C/N values [209]. Singhasuwan and collaborators report that the biomass production of Chlorella sp. TISTR 8990 was maximized at a C/N ratio of 29, and with a ratio higher than 63 the accumulation of total fatty acids was enhanced. In order to obtain the target C/N ratio, they used glucose and potassium nitrate [87]. Cheng et al. showed that the maximum oil content was obtained when C/N was 19.8 [210]. It can be concluded that also in other studies, at high C/N ratios the cell lipid content increased. However, the specific ratio corresponding to the threshold between C-limitation and N-limitation is not comparable because of numerous factors, such as the microalgal strain, the composition of the medium and the source of nitrogen.

3.3.2.1 Relationship between maximal biomass and initial glucose concentration

With a carbon to nitrogen ratio lower than 12, it was shown that the medium was carbon limited. The next step was to confirm the proportional increase of the biomass as a function of the initial glucose concentration, which is typical characteristic of a carbon limitation. Experiments were carried out by preparing a series of media with a C/N ratio of 8.77 in shake flasks. The expectation was to obtain a linear relationship between the maximal biomass and the initial concentration of glucose.

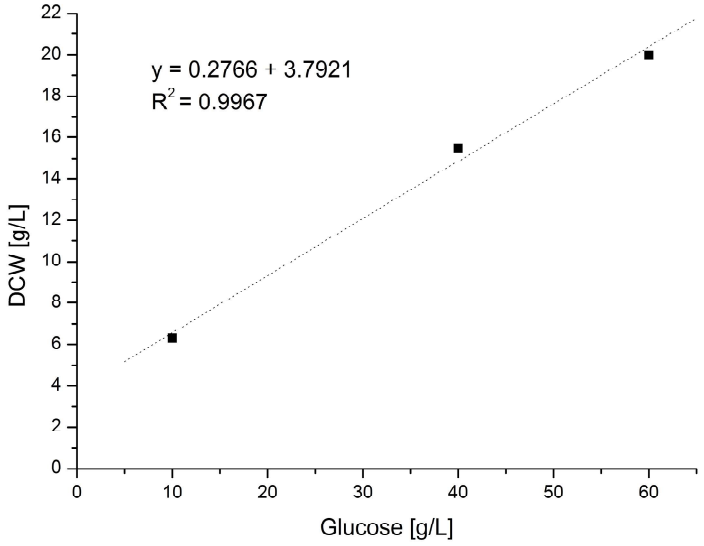


Figure 3.10: *Maximal dry cell weight (DCW) of* Chlorella protothecoides *achieved in shake flasks as a function of the initial concentration of glucose. The C/N ratio was constant at 8.77. The* DCW_{max} *corresponds to an average of three points (n=3) measured during stationary phase, the standard deviation is represented as error bars.*

In Figure 3.10, the final biomass concentration was proportional to the increase of glucose up to 60 g/L, as indicated by the correlation factors (R^2) very close to 1. Biomass yield on glucose ($Y_{X/S}$ was 0.276 g g^{-1}. Interestingly, in the experiment where 80 g/L of glucose and 32 g/L yeast extract were used (C/N 8.77), final biomass was lower than expected (22 g/L instead of ca. 25 g/L) and glucose was not totally consumed (Figure 3.11). A concentration of 1.48 g/L of lactate was detected in the samples taken during the stationary phase after 70 hours of fermentation. Physiological studies showed that the strain *Chlorella* has the enzymatic capacity to reduce pyruvate using NADH and produce lactate [211], however it was necessary to understand why this metabolite was produced.

3.3.2.2 Lactate and the effect of pH on microalgal growth

In shake flask culture, at the beginning of the stationary was observed a drop in pH to 4.5-5. It was hypothesized that lactate was responsible for the decrease. The aim of the experiment represented in Figure 3.12 was to investigate the effect of low pH on microalgal growth. The growth of *Chlorella* cultivated in the standard cultivation medium at pH 6.8 was compared to the growth in media prepared with lower pH values (4.0; 5.0; 6.0). Since the experiment was carried out in shake flasks, the pH was adjusted only after the preparation of the medium

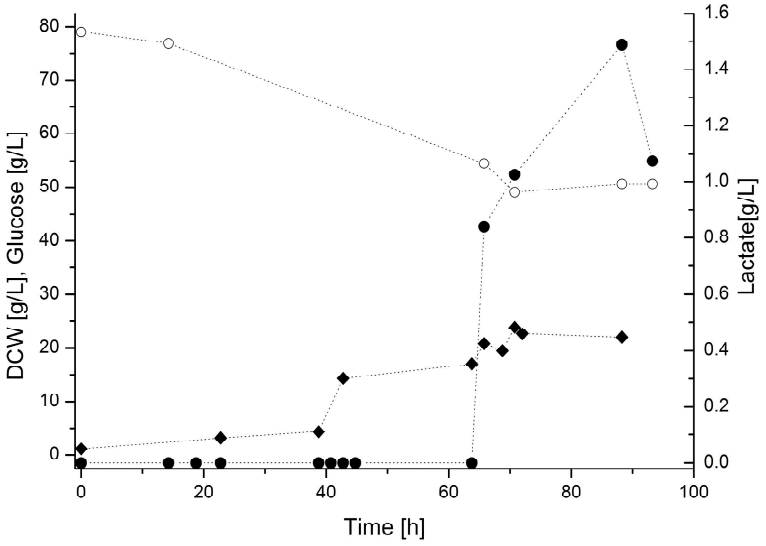


Figure 3.11: *Batch cultivation of* Chlorella protothecoides *in shake flask (C/N 8.77). Results are represented as* ♦ *DCW in g/L,* ◯ *glucose concentration in g/L and* • *lactate concentration in g/L as a function of the fermentation time in h. The DCW and* OD_{540nm} *were repeated in duplicate (n=2). The standard deviation is represented as error bars.*

and it was not controlled overtime in order to avoid contamination with the pH probe. The expectation was that the lower the level of pH, the lower the growth.

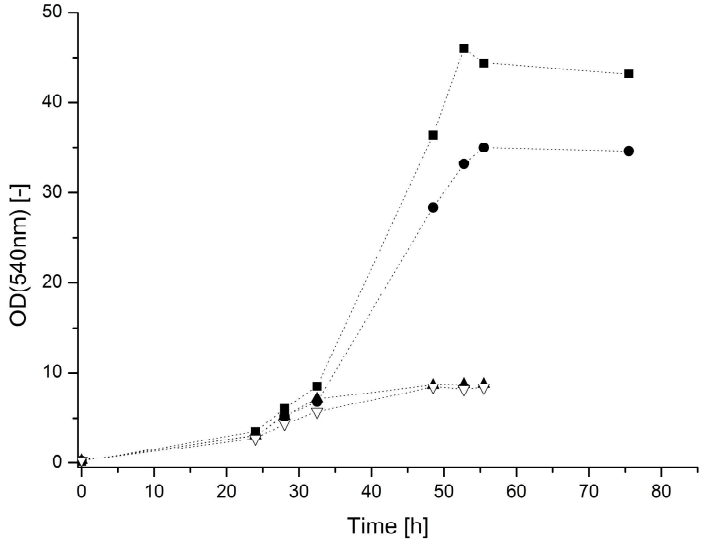


Figure 3.12: *Study of the influence of pH on the growth of* Chlorella protothecoides. *Results are represented as the optical density (*OD_{540nm}*) as a function of the fermentation time. Effect of pH value was studied at* △ *4,* ▲ *5,* • *6 and* ■ *6.8.*

As hypothesized, algal growth was higher when medium had a standard pH of 6.8. However, when cells were cultivated with a pH of 6, maximum optical density was 1.2-fold lower, i.e 35 instead of 43. A more drastic effect was obtained under very acidic pH of 4 and 5 where the level of growth was about 5-fold lower (Figure 3.12). Enzymes are extremely sensitive to pH, with a pH optimum, the activity is maximum. Lower - or higher - pH values affect the state of the amino acids altering the ionic bonds that determine the 3D shape of the protein. Shape of the active site, where the substrate binds, can be modified leading to an inactivation of the enzymes [212] [213]. The experiment proved that lactate, lowering the pH of the medium, had a negative impact on microalgal growth.

Mechanism of lactate production

Despite the proof that lactate had a negative impact on growth, it was still not clear why *Chlorella* accumulated it. The reasons could be:

1. When the amount of oxygen is limited, the electron transport chain availability is limited and pyruvate cannot be completely oxidized. Consequently the cells change the metabolism into another process called fermentation. After glycolysis, instead of entering in the citric acid cycle, the pyruvate undergoes a simple redox reaction, forming lactic acid with concomitant conversion of NADH to NAD^+. Increasing the oxygen transfer might be a solution to have a better glucose consumption and consequently obtain higher biomass yield [214].

2. The second reason could be metabolic overflow. This phenomenum happens when there is an excessive uptake of substrate resulting in the production of intermediate metabolites. Cells grown under conditions of substrate excess are often subject to substrate inhibition or product inhibition [215]. The overflow of the metabolism is well known for yeast, bacteria and mammalian cells and it typically occurs under glucose excess conditions with consequent excretions of by-products such as ethanol, acetate or lactate. The high rate of glycolysis reduces the respiration and induces fermentation because of catabolite repression on the the respiratory chain enzymes [216].

3.3.2.3 Study of the oxygen limitation

In order to investigate whether lactate was produced because of an oxygen limitation, a new experiment was carried out in a STR bioreactor. The conditions previously tested in shake flask (80 g/L glucose and 32 g/L YE, where 1.48 g/L of lactate was accumulated) were repeated. Oxygen is a major nutrient for the aerobic growth of microorganisms therefore, it is important to ensure an adequate delivery. Oxygen is poorly soluble in aqueous media and its solubility decreases with increasing temperature and salt concentration. Usually, with a concentration between 10-50 % the growth of microorganisms such as bacteria and yeast is limited. The dissolved oxygen (DO) concentration must be above a critical level otherwise growth can be limited. In bioreactors, the oxygen transfer is improved, compared to shake flasks, because of the mixing system and a better and controlled aeration through the sparger. The expectation was to achieve a higher level of biomass in order to respect the constant $Y_{X/S}$ ratio of 0.3 g g^{-1} obtained with lower glucose concentration (Figure 3.10) and also avoid lactate production. This will confirm whether cultures in bioreactors would be more indicated to achieve higher cells densities instead of continuing the experiment in shake flasks, where the oxygen transfer is limited and other parameters, such as the pH, cannot be controlled. The preparation of the bioreactor is described in Section 3.2.3.4.

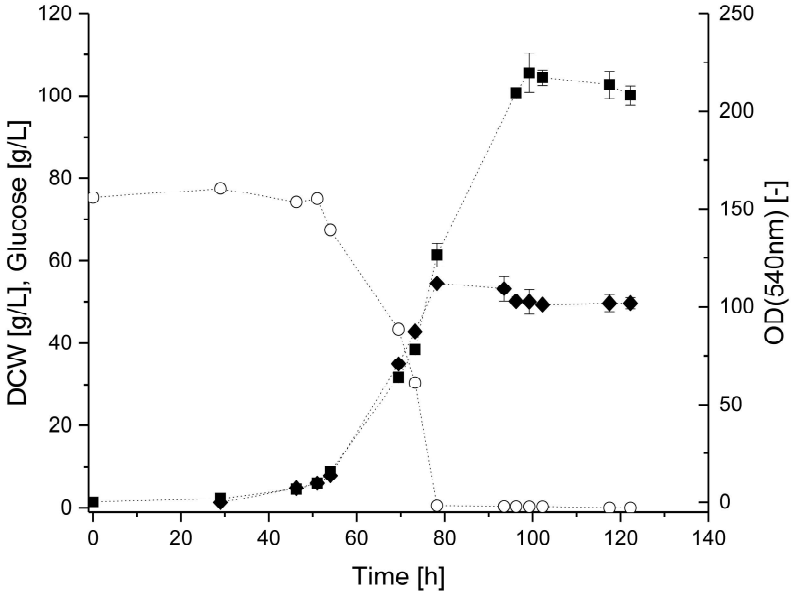


Figure 3.13: *Batch culture of* Chlorella protothecoides *in bioreactor. The medium had a C/N ratio of 8.77 and contained 80 g/L of glucose and 32 g/L of yeast extract. Results are represented as ♦ DCW in g/L, ■ optical density (OD_{540nm}) and ◯ glucose concentration in g/L as a function of the fermentation time in h. The DCW and OD_{540nm} were repeated in duplicate (n=2). The standard deviation is represented as error bars.*

Results shown in Figure 3.13 were interesting; as expected lactate was not produced during fermentation in the batch bioreactor (data not shown), confirming that in the shake flask there was an oxygen limitation rather than a metabolic overflow. Surprisingly, the biomass achieved a maximal concentration of 51.55 g/L (Figure 3.13) instead of about 25 g/L as expected (Figure 3.11). The same fermentation in bioreactors was repeated twice to confirm the results, good results in terms of reproducibility were obtained. The average of the final biomass obtained in the two batch cultivations was 51.24 g/L with a variation coefficient of 0.37%, in both lactate was not produced. The biomass yield on glucose ($Y_{X/S}$) was 0.64 g g^{-1} instead of 0.27 g g^{-1} obtained in shake flasks, corresponding to a 2.4-fold increase.

The biomass yield coefficient on glucose was close to the one defined in the literature by Singhasuwan et al., which was 0.62 g g^{-1} during exponential growth [87]. In the study of O'Grady et al., the biomass yield using glucose as substrate corresponded to 0.43 g g^{-1} but increased to 0.56 g g^{-1} using crude glycerol [217]. A typical value for *S. cerevisiae*, which is a microorganism extensively used to obtain very high cell density, is 0.49 g g^{-1} [218].
The biomass yield depends on stoichiometric limits determined by the amount of CO_2 lost and

the amount of energy required for biomass formation. The efficiency of mitochondrial energy generation, in the respiratory chain, has a strong effect on the cell yield. Various mechanisms may be involved, often affecting the maintenance energy requirement: nature of the carbon and nitrogen sources, pH, temperature, osmotic stress, heavy metals, oxygen and metabolites produced [219] [220]. Such explanations can justify the considerable increase in biomass yield on glucose from shake flasks to bioreactor.

3.3.3 Batch cultures in bioreactors

Experiments carried out in shake flasks (Figure 3.10) showed that with a carbon-to-nitrogen ratio lower than 12, the medium was carbon limited. The following experiment aimed to study the same proportionality between substrate and biomass under a carbon limited medium - but under controlled conditions in a batch bioreactor. The main objectives were (1) to prove that the biomass yield on glucose was constant at 0.6 g g^{-1} (Figure 3.13), and (2) to define the optimal concentration of initial glucose where the biomass is maximal by avoiding any inhibitory effect or metabolic overflow. The experiment was expected to demonstrate the highest biomass attainable in batch culture in a bioreactor.

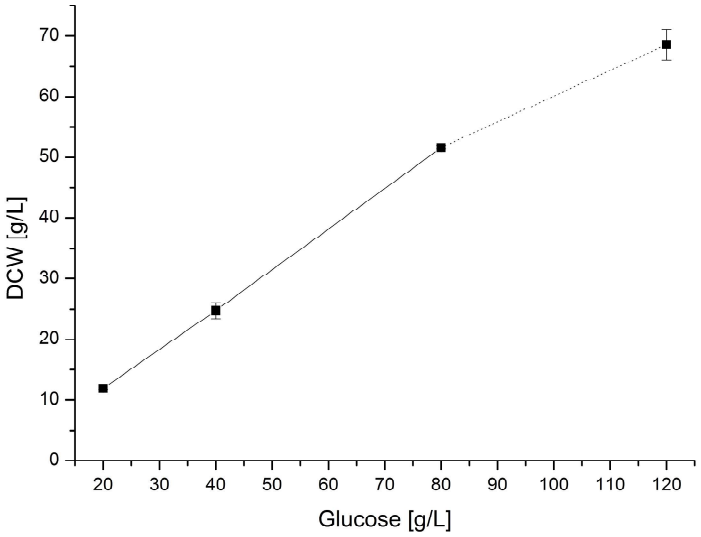


Figure 3.14: *Representation of the maximal dry cell weight (DCW) of* Chlorella protothecoides *achieved at stationary phase as a function of the initial concentration of glucose in the medium. The C/N ratio was constant at 8.77. The maximal DCW corresponds to an average of three points measured during the stationary phase, the deviation standard is represented as error bars.*

The slope of the linear part of the graph (R^2 = 0.999) represented in Figure 3.14 corresponds to the biomass yield on glucose ($Y_{X/S}$) with a value of 0.66 g g^{-1}. *Chlorella* was grown under glucose limitation (constant C/N 8.77), and the initial substrate concentration was completely consumed, resulting in a proportional increase of the biomass reached at the stationary phase. With 120 g/L of initial glucose, the yield decreased slightly, to 0.57 g g^{-1}, and during the fermentation, lactate was produced up to a concentration of 1.7 g/L (results not shown). These values suggested the beginning of a metabolic overflow, and it was suspected that this high concentration was beyond the optimal point. It could be seen in Figure 3.15 that the maximum specific growth rate occured at low glucose concentrations. When the initial concentration was 20 g/L, the maximum specific growth rate was 0.11 h^{-1}. With the increase of initial substrate concentration, the specific growth rate decreased. In fact, with higher concentrations of glucose, 167 g/L, cell growth rate was very low, 0.003 h^{-1}, which might be the result of an intense substrate inhibition. Between the specific growth rate and substrate concentration exists a hyperbolic relationship, described extensively by Monod [221]. The growth rate rises as a function of the initial substrate up to a maximal value (μ_{max}) however, beyond the maximal value, in case of substrate inhibition, the growth rate decreases [215] [222].

These results are in agreement with scientific literature; Xiong et al. defined that glucose concentration higher than 60 g/L had an inhibition on cell growth and, Doucha and Livansky claimed that *Chlorella* had good tolerance to glucose up to 80 g/L obtaining a cell yield on glucose of 0.55-0.69 g/g [169] [62].

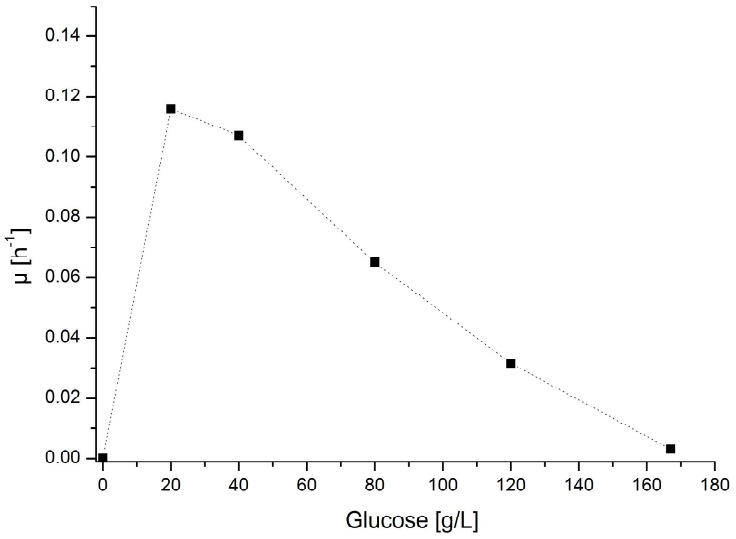


Figure 3.15: *Representation of the maximal growth rate of* Chlorella protothecoides *as a function of the initial concentration of glucose.*

The highest biomass concentration in batch culture was obtained growing cells in a medium where glucose was the limiting substrate (C/N 8.77); from 120 g/L of initial glucose, 69.83 g/L (SD= 3.535 10^{-3} g/L, n = 2) of DCW was produced.

Optimization of the biomass production is an important prerequisite to increase the productivity of lipids. According to the literature, higher cell densities, up to 117 g/L, were achieved with other *Chlorella* species, i.e. *Chlorella vulgaris*, by implementing fed-batch mode [186]. Most biotechnological production platforms of growth-associated products are based on fed-batch cultures because of advantages such as the achievement of high cell densities, the control over the production of byproducts, and improved productivity [223]. Fed-batch operation may be the solution to overcome the contradictory mechanism of cell proliferation and lipid accumulation by combining two different strategies. First, a high cell density may be achieved using a carbon-limited medium, and then, by changing the medium feed addition, lipid accumulation may be enhanced under nitrogen limitation.

3.3.4 Two-stage fed-batch cultivation

Heterotrophic bioreactor cultures of *C. protothecoides* were optimized to fed-batch operation with the aim of achieving high cell densities under a carbon-limitation medium and enhancing

the lipid accumulation under nitrogen limitation. The objective was to improve the overall lipid productivity. Microalgae showed a very high tolerance to glucose, thus a pulsed feed strategy was chosen instead of a complicated regulated system. This strategy consists of adding nutrients to the bioreactor based on the output signal of an indirect measurement of the substrate concentration, in this case glucose (Section 3.2.3.5). Over-feeding might lead to negative effects on cell growth, therefore the appropriate feeding strategy considered the glucose inhibitory level. The maximum concentration of glucose in the medium after feed addition was kept under 80 g/L (Figure 3.14).

A fed-batch fermentation with *C. protothecoides* was carried out with the cells initially cultivated in batch mode in medium with C/N ratio of 8.77 (Figure 3.16). When glucose was completely depleted, a feed composed of 700 g/L glucose and concentrated salts was added. A source of nitrogen was contained only in the initial cultivation medium and not in the feed, therefore it was expected that the continuous addition of glucose would increase the C/N ratio until complete nitrogen limitation was achieved which is expected to gradually enhance the lipid accumulation. Yeast extract concentration was 32 g/L, therefore approximately 100 g/L of biomass was expected ($Y_{X/YE}$ 3 g g^{-1}).

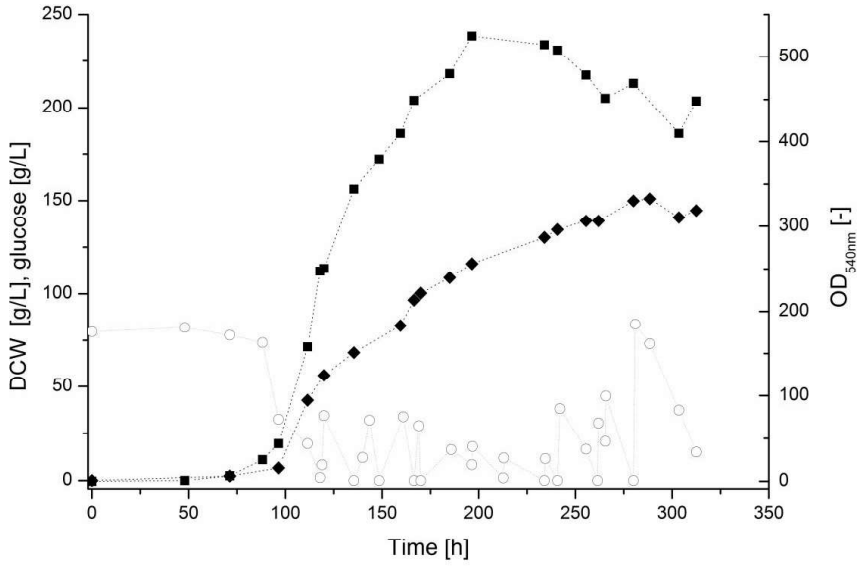


Figure 3.16: *Fed-batch cultivation of* Chlorella protothecoides. *Results are represented as ♦ DCW in g/L, ■ optical density (OD_{540nm}) and ◯ glucose concentration in g/L as a function of the fermentation time in h. The DCW and OD_{540nm} were repeated in duplicate (n=2). The standard deviation is represented as error bars.*

According to Figure 3.16, such a value corresponds to the point where the optical density measurements achieve a plateau, OD_{540nm} 500 [-]. After 170-190 h, the biomass was 100 g/L, which continued to increase up to about 150 g/L at the end of the culture. The hypothesis was that the difference of 50 g/L does not correspond to an increase in cell number but to an apparent lipid accumulation. The theoretical percentage of total lipid in the biomass corresponds to about 50% DCW. It was suspected that after 170-190 hours, cells stopped proliferating because of nitrogen limitation. Consequently, under such stressed conditions, lipids were accumulated. Three growth stages can be distinguished based on the growth rate, which was calculated from the DCW data: (1) batch phase 0.069 $h^{-}1$ (until 129 h); (2) fed-batch 0.01 $h^{-}1$ (between 130-190 h); (3) lipid accumulation 0.005 $h^{-}1$ (from 196-260 h). The growth rate during the hypothetical lipid accumulation phase was very low, corresponding to a cell doubling every 140 h. This indicates that in the last stage of cultivation, the biosynthesis of lipids was the major cellular activity, and cell division ceased. Total lipid percentage of biomass was determined only when cells were harvested (after 313 h), and corresponded to 55.18% (SD= 3.275%, n = 5), confirming that lipids were accumulated.

3.3.5 Improved Two-stage fed-batch cultivation

The aim of the next experiment was to achieve higher cell densities of *Chlorella protothecoides* by feeding more yeast extract (source of N) after the end of the batch phase. The expectation was that by adding more nitrogen, the carbon-limited phase would be prolonged, providing good conditions for cell proliferation and, consequently, higher cell densities. Based on the same suppositions applied for the previous culture, it was expected that by adding 15 g/L of yeast extract during the feed, 156 g/L of biomass would be produced.

Figure 3.17 shows that after 134 h, 168 g/L of biomass were achieved. At the same point, optical density measurements plateaued around OD_{540nm} 800 [-]. Hypothetically, cells entered the stationary phase due to the complete consumption of nitrogen. The difference in biomass yield between 134 h and 255 h of fermentation - 87 g/L - probably corresponds to total lipid accumulation, corresponding to approximately 52% of total lipids in the biomass.

Total lipid extraction was carried out at different intervals of the cultivation. The aim was to define the total lipid percentage of the biomass over time. Theoretically, cell growth and lipid accumulation can be divided into two stages based on the hypothetical C/N ratio. Under op-

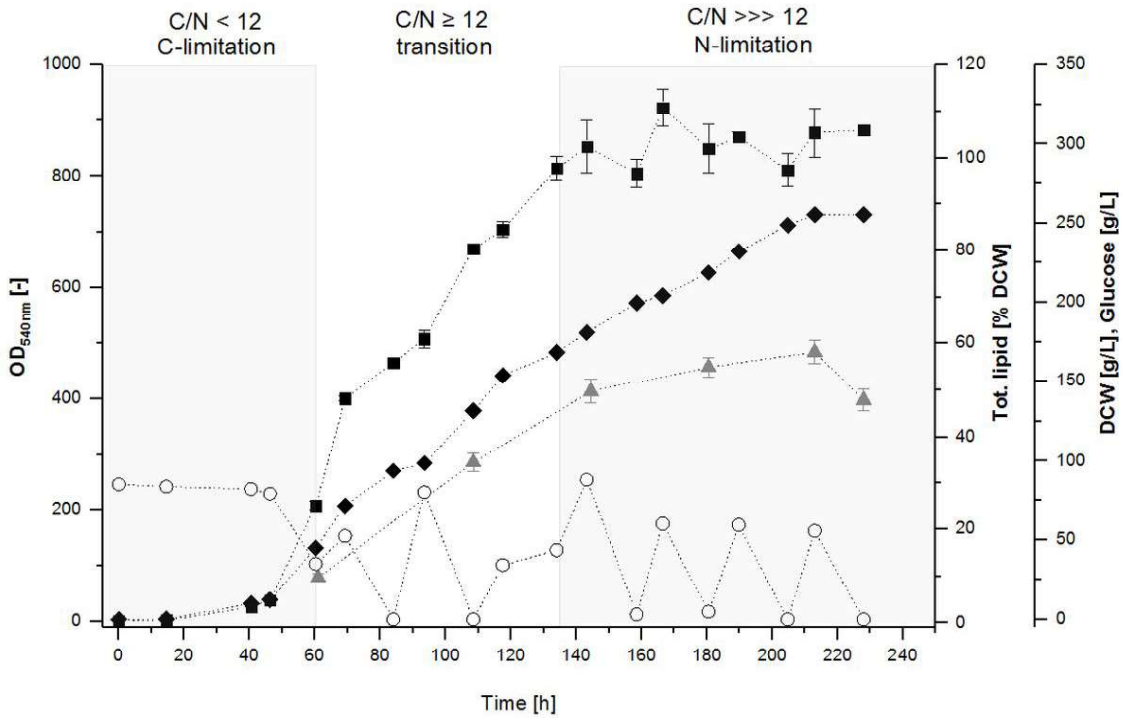


Figure 3.17: *Improved fed-batch cultivation of* Chlorella protothecoides. *Results are represented as ♦ DCW in g/L, ■ optical density (OD_{540nm}) and ◯ glucose concentration in g/L as a function of the fermentation time in h. Total lipid accumulation (▲) is represented as percentage of DCW. The DCW and OD_{540nm} were repeated in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6). The standard deviation is represented as error bars.*

timal growth conditions, algal cells have a relatively low lipid content, which constitutes about 5-20% of dry cell weight [192]. A similar result is expected when the C/N ratio is lower than 12, analogous to a carbon limitation. Cells will enter into a transition phase were nitrogen is present, but not in excess and continuously decrease. During the so-called transition phase, the C/N ratio is equal or slightly higher than 12. It is expected that under these conditions, cells accumulate lipids gradually, up to a point where nitrogen is not present anymore (C/N 12) and the maximum lipid level is attained.

Figure 3.17 shows that after about 60 h - at the end of the batch phase - biomass was composed of 9.982% (SD = 0.5046%,n = 5) of total lipids. This value was similar to that defined by the literature, under a carbon-limited medium [192] and it was assumed that even at the beginning of the culture the composition was similar. In fact, initial cultivation medium had a C/N of 8.77. Lipid percentage of biomass appeared to increase steadily until 140 h. When the lipid content

was equal to 33.75% (SD = 0.9332%, n = 5), the biomass corresponded to 108 g/L, meaning that hypothetically only 15 g/L of nitrogen were still available in the medium. Therefore, the theoretical C/N was about 20, a value situated in the transition phase. Only after 140 h, the percentage of total lipids in the biomass reached a maximum of 58.10% (SD = 2.487%, n = 5). This high percentage of lipids is strongly indicative that nitrogen was completely depleted. Analysis of results reported in Figures 3.16 - 3.17 revealed a linear relation between DCW values as a function of the OD_{540nm} measurements, until the beginning of the stationary phase (result not shown).

Under low C/N ratios, cell proliferation was enhanced and cell weight increased proportionally. After the achievement of stationary phase, where C/N ratio in the medium was higher, the DCW increased because of an accumulation of lipids and not because of an increase in cell number, therefore the correlation between optical density and DCW is no longer constant. The results of this experiment showed that under specific culture conditions- high O_2 mass transfer due to high agitation rate, leading to dissolved oxygen concentrations higher than 50%; specific C/N ratio in the medium; feeding more nitrogen after the batch phase - it was possible to achieve higher biomass, 255.3 g/L (SD = $2.828 \cdot 10^{-3}$ g/L, n = 2), and consequently accumulate more lipids. In term of lipid productivity, the maximum was 16.7 g/L/day. In a non-optimized fed-batch process, as the one represented in Figure 3.16, the total lipid productivity was 6 g/L/day, corresponding to a 2.8-fold improvement.

3.4 Conclusion

Color variations of *C. protothecoides* have been noticed in every fed-batch cultivations carried out. Initially, cells were bright green and in the course of fermentation color varied to yellow (Figure 3.18). Change occurred during the so-called C/N transition phase, where nitrogen was limiting. It was suspected that nitrogen limitation affected chlorophyll production, which is the pigment responsible of green color in microalgal cells. Despite chlorophyll being involved in photosynthesis process, there is evidence that chlorophyll is produced by *Chlorella* even under heterotrophic conditions [186].

There are different types of chlorophyll however, the basic structure of the molecule is a por-

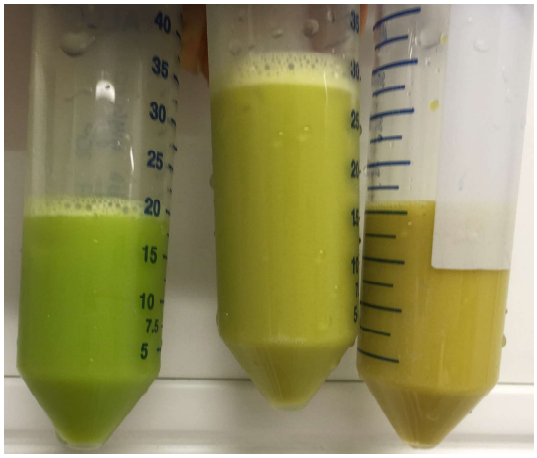


Figure 3.18: *Color variation in* Chlorella protothecoides *in a carbon-limited medium, in the transition phase and in a nitrogen-limited medium.*

phyrin ring, co-ordinated to a central atom of magnesium. It is a nitrogen-rich compound, containing four nitrogen atoms each molecule [137] (Figure 3.19).

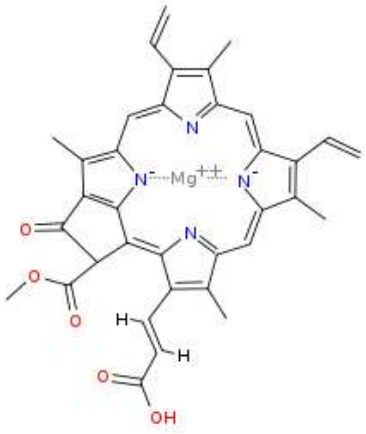


Figure 3.19: *Chemical structure of chlorophyll*

Fan et al, showed that in a normal cultivation medium under heterotrophic conditions, the chlorophyll content in *Chlorella* sp. is about 30mg/g DCW and decreased to less than 20 mg/g in nitrogen-limited medium [224]. Li et. al hypothesized that in *Neochloris oleoabundans*, when nitrogen is limiting growth, cells start to use the intracellular nitrogen pool to support the synthesis of cell material for further cell division. Chlorophyll seems to be one of the most accessible stocks of nitrogen. Their theory was supported by the observation of the sharp drop of chlorophyll cell content which coincided with the nitrate depletion in the medium. Chlorophyll content of microalage cultured in media with low nitrogen concentration was consenquently low [225]. According to Ling et al., the same seems to happen in *Chlorella*

sorokiniana: chlorophyll might act as a nitrogen pool which is consumed to support cell growth after nitrogen exhaustion [106]. Even under autotrophic conditions in photobioreactors, Fan et al. noticed that both nitrogen deficiency and phosphorus deficiency caused a sharp decline in chlorophyll [101]. In the research of Benavente-Valdes et al. is stated that nitrogen limitation in marine phytoplankton affects photosynthesis due to loss of chlorophyll and increases in non-photochemically active carotenoid pigments [103]. Nitrogen limitations have been reported to enhance the accumulation of secondary carotenoids such as lutein and beta-carotene in certain species of green algae, including *Chlorella* [226] [227] [126].

The present study has developed a fermentation strategy to obtain very high cell density cultivation of *Chlorella protothecoides*, 255.3 g/L, from an initial concentration of less than 2 g/L and with a high productivity of lipids (16.7 g/L/day). The specific growth rate of 0.078 h^{-1} was calculated under carbon-limited condition in the batch phase (low C/N ratio). Such results are considerably higher than reported in other studies for high cell density heterotrophic algal cultivation, Table 3.6 illustrates different examples. In all the presented experiments, starting cell densities were always lower than 10 g/L, making it possible to compare lipid productivities. Chen et al. reached 45.2 g/L of biomass after 197 h in fed-batch mode of culture, and the maximum lipid content was 54% of DCW. The basal medium was prepared with 30 g/L glycerol as carbon substrate and 4 g/L yeast extract, and the feed solution contained 150 g/L glycerol and 15 g/L yeast extract to maintain a certain C/N ratio [45]. Xiong et al. achieved cell density of 51.2 g/L in 167 h in a 5-L bioreactor by performing an improved fed-batch culture strategy. However, lipid content was 57.8% in batch culture and only 50.3% in fed-batch culture in a 5-L bioreactor [62]. Zheng et al. developed a two-stage fed-batch fermentation, resulting in 103.8 g/L biomass. In the first stage, the C/N ratio was 29, and during the second stage, only carbon was supplemented (similar to what done in the present study). The lipid content achieved a maximum of 38.7% after 228 h [228]. A major limitation of heterotrophic algal cultivation is the sustainability and cost of organic carbon source. Sugar cane juice hydrolysate (SCH) was used by Cheng et al. as an alternative carbon supply for lipid production. In fed-batch cultivation, Cheng et al. reported 48.52 g/L biomass with 54.7% of lipids after 170 h [210]. In another experiment, instead of enhancing the lipid accumulation by exposing cells to nutrient starved conditions, Palabhanvi et al. supplemented the medium with a lipid inducer [46]. According to this study, sodium acetate, being a simply structured molecule, seemed to be better

metabolized by cells into acetyl coA, the main metabolic precursor for lipid biosynthesis. As a result, 90.15 g/L biomass was achieved with a lipid productivity of 7.7 g/L/day. Interestingly, the biomass was composed of 70.2% w/w lipids [46] Acetate could be considered as an alternative substrate to glucose and for further optimisation of the lipid productivity. Other studies were focused mainly on producing a high yield of biomass and not lipids. Wu et al. obtained a cell density of 116.2 g/L, [229] whereas Doucha and Livansky achieved 117.18 g/L in only 40 hours [169]. Those results justify the more than doubling of biomass concentrations vs. previous users. In the study of Doucha and Livansky biomass was composed only of 9.7 % of lipids, if hypothetically cells would have been further cultured under N-limited conditions, biomass could have reached more than 200 g/L of biomass with a lipid composition of 50%. Focusing on achieving very high cell density before enhancing lipid accumulation is the key strategy to achieve high lipid productivity.

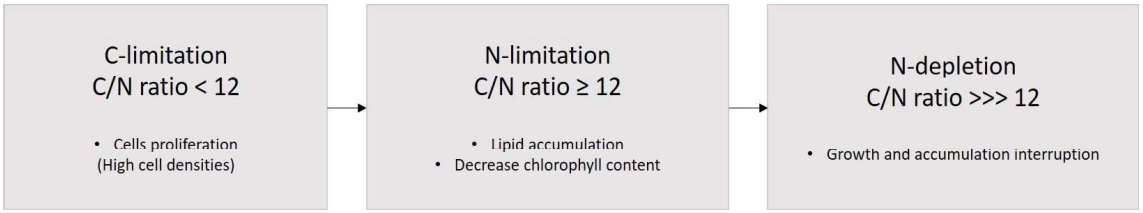


Figure 3.20: *Schematic representation of C/N evolution and effect on microalgal cells*

A carbon-limited medium created an efficient way to stimulate cell proliferation. At the end of this phase lipid accumulation was less than 10%. Nitrogen limitation resulted an efficient method to enhance lipid production. It was noticed that microalgae accumulated lipids during the so-called transition were nitrogen is present but not in excess and the C/N ratio is equal or higher than 12. When cells achieved the stationary phase, nitrogen in the medium was completely depleted and lipid accumulation achieved a maximum without increasing further. It is important to distinguish between N depletion and N limitation because the effect on cell metabolism is very different. If nitrogen supply is limited in proportion to other elements, growth seems continue but the intracellular compounds will include a smaller proportion of nitrogen-rich components such as chlorophyll and more energy-rich components such as lipids. Whereas under complete nitrogen depletion, microalgae cannot grow anymore and lipid accumulation is also interrupted [103]. Such a theory is schematically represented in Figure 3.20.

Table 3.6: *Overview of fed-batch strategies for heterotrophic high cell density culture of* Chlorella *sp.*

Strain	Substrate	DCW_{ini} [g/L]	Run Time [h]	DCW_{max} [g/L]	Total lipids [% DCW]	Lipid prod. [g/L/day]	Reference
C.protothecoides	Glc	<2	228	255.33	58	16.7	This study
C.protothecoides	Gro	<2[a]	197	45.2	54	2.99	[45]
C.protothecoides	Glc	5[a]	167	51.2	50.3	3.74	[62]
C.sorokiniana	Glc	<5[a]	228	103.8	38.7	4.2	[228]
C.protothecoides	SCH	<10[a]	170	48.52	45	3.28	[210]
Chlorella sp. FC6	Glc[b]	<5[a]	216	90.15	70.2	7.7	[46]
C.pyrenoidosa	Glc	<10[a]	120	116.2			[229]
C.vulgaris	Glc	6.25	32	117.18			[169]

[a]Data estimated from graph observation; [b]Medium was supplemented also with another C-source, sodium acetate

In the present study, the effect of C/N ratio on cell growth was explored and enabled the development of a two-stage, fed-batch fermentation process. The high levels of biomass obtained compared with previous studies (Table 3.6) was due to initially maintaining a low C/N, which provides very good conditions for cell proliferation, followed by a high C/N ratio to limit cell growth and promote lipid accumulation (58% w/w). As a result, a very high lipid productivity of 16.7 g/L/day was achieved. In this study of algal biomass (DCW), over 255 g/L was attained, which is 2-6 fold higher than previously reported. Furthermore, lipid productivity was 2-5 fold higher than previously published data. While the process allowed for only one run, it illustrates the potential for very high outputs. Next steps would be to confirm such improved yields in scale-up experiments. The process maybe further optimized by increasing the inoculum concentration, which will reduce fermentation time. This work showed also the importance of an organic nitrogen source, yeast extract. However, it would be beneficial to find cheaper organic sources for commercial development of the work. The higher biomass and increased lipid pro-

ductivity obtained in this work improve the potential of algal cultivation, which should find more applications with real-world benefits for uses as food ingredients, aquaculture and animal feed, bioenergy, and products for human health, nutrition and personal care.

Chapter 4

Characterization and optimization of a fermentation process for the production of high cell densities and lipids using heterotrophic cultivation of *Schizochytrium* sp.

4.1 Introduction

Schizochytrium, was chosen for this research because of the reported ability to accumulate lipids, particularly high concentrations of omega-3 fatty acids. *Schizochytrium* is a marine algae with different nutritional requirements to *Chlorella*, which is found in freshwater and land. *Schizochytrium* requires higher concentrations of Na^{+} ions to support its metabolic functions.

Nutrient limitation conditions can induce a significant increase in lipid content in many microalgal species [188]. There is evidence that the carbon-to-nitrogen ratio is a critical factor affecting the accumulation of lipids in *Schizochytrium* sp. [67] [66]. Jakobsen et al. noticed an accumulation of lipids in cells fed with an excess of glycerol, after the medium was depleted in nitrogen [75]. Likewise, results of Bowles et al. indicated that medium with a high C/N ratio stimulated lipid production [81]. Nutrient starvation conditions are particularly favorable

for lipid accumulation but not for cell proliferation. Two-stage fed-batch cultivations were proposed for the growth of *Chlorella protothecoides* in Chapter 3. In the first part, cells were grown under a C-limited medium, meaning that glucose was in excess and its depletion would have caused the cessation of growth. In a second stage, addition of carbon based feed, will gradually increase the C/N ratio - corresponding to a nitrogen limitation - until the complete depletion of nitrogen. It is expected that during the C/N transition phase, excess of carbon will be accumulated as storage lipids in the cells.

Experiments carried out with the reference strain *Chlorella protothecoides* were successful (Chapter 3). Very high cell densities with a good lipid productivity were achieved in heterotrophic two-stage fed batch cultivations. A similar process development will be applied to *Schizochytrium* in order to achieve the objectives listed below. The results and potential of both microalgal strains were compared and evaluated. The work involved:

- the definition of a standard cultivation medium
- study the effect of the C/N ratio on the growth
- define and verify the constant biomass yield on carbon
- define the highest cell density achievable under batch conditions
- develop a two-stage fed-batch process

The principal aim of this chapter was to achieve a good lipid productivity which involves, the achievement of high cell densities followed by a good lipid accumulation. It is expected that a high total lipid productivity involves also a high omega-3 fatty acids productivity. Chapter 6 focused on the specific production of omega-3 fatty acids.

4.2 Material and methods

4.2.1 Materials and microalgal strain

All chemicals were purchased from either Sigma Aldrich (St. Louis, USA) or Thermo Fisher Scientific (Waltham, USA) if not stated otherwise. *Schizochytrium* sp. (ATCC 20888) was obtained from the ATCC (Manassas, USA) culture collection.

Table 4.1: *Composition of the standard cultivation medium for Schizochytrium*

Ingredient	Amount
NaCl	25 g/L
$MgSO_4\,7\,H_2O$	7.5 g/L
KCl	0.5 g/L
$CaCl_2\,2\,H_2O$	0.5 g/L
$FeSO_4\,7\,H_2O$	80 mg/L
Glucose	40 g/L
Proteose peptone	10 g/L
Yeast Extract	5 g/L

4.2.2 Standard medium preparation

The ingredients of the cultivation medium were dissolved in DI water one after the other following the order showed in Table 4.1. The pH of the medium was adjusted to 7.2 with 1M NaOH followed by sterilisation through a 0.22 μm Steritop filter (Millipore, Billerica, USA). The bottles used to store the media were previously autoclaved at 121°C for 20 min. The medium and the other solutions can be stored in the fridge for no longer than one month.

4.2.2.1 Carbon to nitrogen ratio

The standard cultivation medium was prepared by varying the ratio between the initial concentration of glucose and yeast extract in order to modify the carbon to nitrogen ratio (C/N) as described in Section 3.2.2.2.

4.2.2.2 Study of the carbon and nitrogen limitation

The threshold between carbon limitation and nitrogen limitation was determined by preparing the media for the cultivation of the two microalgal strains with different concentration of carbon and yeast extract. Their preparation is described in Table 4.2.

Table 4.2: *Description of the preparation of a standard medium for the cultivation of Schizochytrium sp. with different C/N ratios. The yeast extract concentration was constant at 10 g/L and the glucose concentration was variable.*

C/N	0	1.75	5.3	10.5	17.5
Glucose [g/L]	0	5	15	30	50
YE [g/L]			10		

4.2.3 Cultivation of *Schizochytrium* sp.

The method developed to cultivate *Chlorella* in Section 3.2.3.5 were applied on *Schizochytrium*. In the fed-batch cultivation mode, the maximum concentration of glucose in the medium after feed addition was kept under 40 g/L for *Schizochytrium* sp.. The glucose concentration in the feed was 500 g/L, with 2-fold concentration of NaCl and MgSO4, and 4-fold the concentration of the other medium components - excluding yeast extract (Section 4.2.2).

4.2.4 Cell growth analysis

Methods to analyse *Schizochytrium* sp. cell growth are the same described for *Chlorella* sp. in Section . The only exception was the determination of the dry cell weight which is described below.

4.2.4.1 Dry cell weight - Filter method using microwave drying

By the means of forceps, a 0.22 μm filter was placed uncovered on a small Petri dish into the microwave. The microwave was run at the lowest intensity for 2 min. The Petri dish was transferred to a desiccator for about 10 min before being weighed on an analytical balance (W_0). The filter was placed in a vacuum filtration system, 3 ml of sample (V_{sample}) were filtered and washed three times with DI water. By the means of forceps, the filter was placed back into the Petri dish and dried in the microwave at lowest intensity for at least 2 minutes (until constant weight of the filter), dried in the desiccator and weighed on the analytical balance (W_1). The measurements were repeated in duplicate (n = 2) to check the reproducibility of the results, the standard deviation error (SD) is reported in the experimental results or represented graphically as error bars. The dry cell weight calculation is the same as Equation 4.1.

$$DCW\ [g/L] = \frac{W_1 - W_0}{V_{sample}} \tag{4.1}$$

where:

DCW = dry cell weight of the biomass [g/L]

W_0 = weight of empty filter [g]

W_1 = weight of the filter with the biomass [g]

V_{sample} = volume of the sample ($3 \cdot 10^{-3}$ L)

4.2.4.2 Analysis of total lipids

Total lipids were extracted and measured according to the methods developed in Chapter 2.

4.3 Results and discussion

4.3.1 Standard cultivation medium definition

A standard medium for the cultivation of *Schizochytrium*, rich in NaCl, was initially defined in-house by Monaghan Bioscience (Section 4.2.2). However, it contained two different sources of nitrogen i.e. proteose peptone and yeast extract. The aim of the first experiment, represented in Figure 4.1, was to study the effect of different nitrogen sources and to define the possibility to use only one of them.

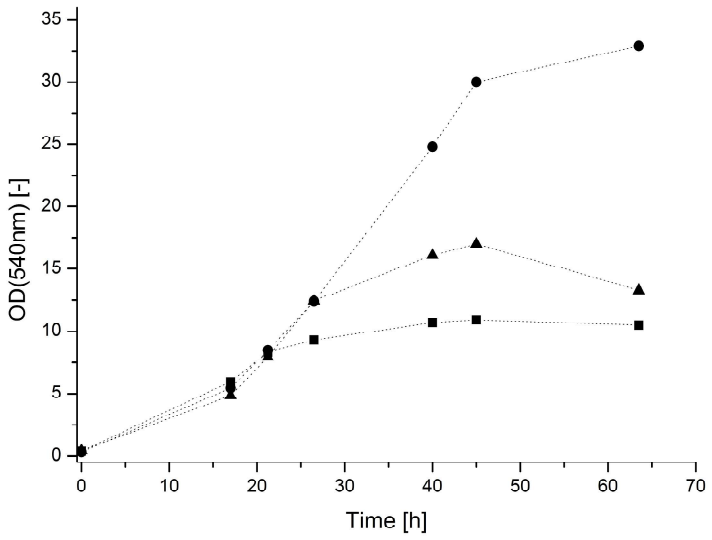


Figure 4.1: *Growth study of* Schizochytrium *sp. in normal standard medium prepared by* ▲ *Monaghan Bioscience, in the medium* ● *without proteose-peptone and in the one* ■ *without yeast extract.*

Cultivation in a medium without proteose peptone achieved higher cell densities (2-fold improvement) compared to the medium where it was present. The hypothesis was that something in the composition of proteose peptone or the high concentration of amino acids had an inhibitory effect. Therefore, further experiments were carried out preparing the standard cultivation medium without the addition of proteose peptone. Yeast extract was considered an ideal nitrogen source for this work: it has been used previously with *Chlorella* and it will provide a

good comparison for the two microalgae cultivations. Furthermore, when cells were cultivated without yeast extract, the optical density was approximately 30% lower than experiments without it, i.e 10.05 instead of 32 (Figure 4.1). A new standard medium formulation was similar to those reported elsewhere. Yokochi et al. used a medium containing 3% glucose, 1% yeast extract and artificial sea water [230]. Ren et al. prepared a medium adding 40 g/L glucose, 0.4 g/L yeast extract, 2 mL/L of a trace element solution and 2 ml/L of a vitamin solution to an artificial sea water solution [188]. Artificial seawater is a mixture of dissolved mineral salts - mainly NaCl ca. 25 g/L - and sometimes vitamins that simulates seawater. A high salt concentration is essential for the marine protist *Schizochytrium* [69] [43].

4.3.2 Study of carbon-to-nitrogen limitation

The aim of the following series of experiments was to define a carbon-limited medium for the high cell density cultivation of *Schizochytrium*. The development was based on the variation of the carbon to nitrogen ratio to define the threshold between carbon-limitation and nitrogen-limitation. The experiments were carried out in shake-flasks by varying the concentrations of glucose and using a constant concentration of 10 g/L of yeast extract. Preparation of the medium and respective C/N ratios are represented in Table 4.2. Under substrate limitation, the initial substrate concentration is proportional to the biomass reached at stationary phase. Increase in biomass is no longer proportional when for example, an exhaustion of another nutrient occurs. In this case, it is assumed that the carbon limitation range would be similar to *Chlorella*, meaning with a C/N ratio lower than 9-11; with higher ratios under nitrogen limitation expected.

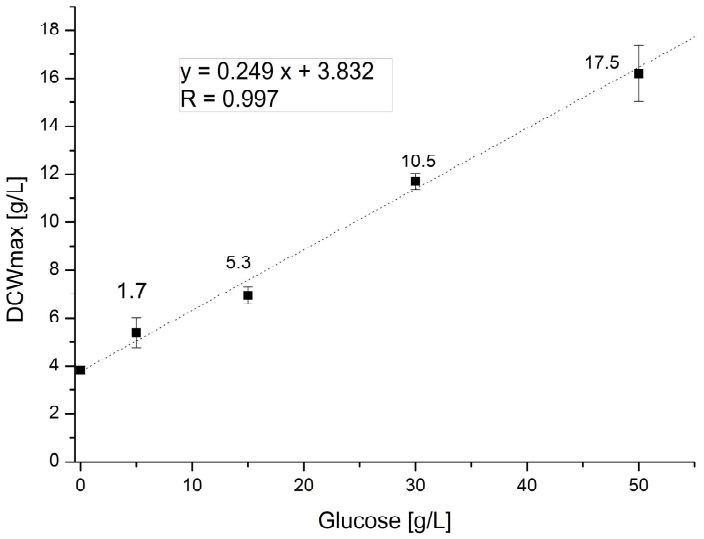


Figure 4.2: *Maximal dry cell weight (DCW) achieved in shake flasks as a function of the initial concentration of glucose. The concentration of yeast extract was constant at 10 g/L and the C/N ratios are indicated with the labels. The DCW max corresponds to an average of three points (n=3) measured during stationary phase, the standard deviation is represented as error bars.*

Results displayed in Figure 4.2 showed a linear correlation (R^2 = 0.997) between the initial concentration of glucose and the dry cell weight achieved during the stationary phase, confirming a carbon limitation up to a C/N ratio of 17.5. HPLC results demonstrate that glucose was completely consumed at the beginning of the stationary phase (results not shown). Biomass yield on glucose was 0.249 gg^{-1}, compared to a yield of 0.27 gg^{-1} for *Chlorella* in shake flasks (Fig 3.10, Chapter 3). As expected, *Schizochytrium* and *Chlorella* showed a carbon limitation within the same C/N ratio range. Results at a C/N ratio of 17.5 for *Schizochytrium* sp., indicated that the medium was still carbon limited, therefore it was suspected that the nitrogen-limitation range will start at a higher C/N ratio, compared to *Chlorella*. Similar results were obtained in the study of Chatdumrong and al. which showed that the C/N ratio at 20 was suitable for *S. limacinum* BR212 to improve the cell concentration to 38.0 g/l [231]. The publication of Kimura demonstrated that Thraustochytrids species appear to have a C/N ratio of about 10.4 for biomass accumulation, whereas oil accumulation requires higher C/N ratio [232]. Moreover, according to Ganuza and collaborators, the biomass of *Schizochytrium* G13/2S increased with an elevation of glutamic acid monosodium (N-source), whereas the fatty acids accumulation was highest at the lowest glutamate concentrations [233].

In previous experiments, the cultivation of *Chlorella* was scaled-up to bioreactors due to the limited aeration in the shake-flasks, which caused an accumulation of lactate in the medium. Even though lactate was not accumulated in experiments carried out in Figure 4.2, it was decided to scale-up the cultivation of *Schizochytrium*. This choice was motivated by different reasons: better control of the conditions, in particular of the pH of the culture, expectations to improve the $Y_{X/S}$ as happened with *Chlorella*, better oxygenation and also higher total amount of biomass to employ for other tests (i.e disruption methods).

4.3.3 Batch cultures in bioreactors

The aim of the next experiment was to define the highest biomass achievable in a controlled batch process in bioreactor with *Schizochytrium*. It was decided to carry out the batch cultivation using a medium with constant C/N ratio of 8.77, which was the same adopted for *Chlorella*. Previous tests demonstrated a carbon limitation also with *Schizochytrium* under this condition (Table 4.2). Different cultivations were carried out in batch bioreactors by varying the initial concentration of glucose and yeast extract but keeping the C/N ratio constant. When a microorganism is grown under substrate limitation, the initial substrate concentration is proportional to the biomass obtained at stationary phase. It is expected that the biomass yield on substrate is constant up to a certain optimum point, where the substrate concentration is maximum without having an inhibitory effect on cells.

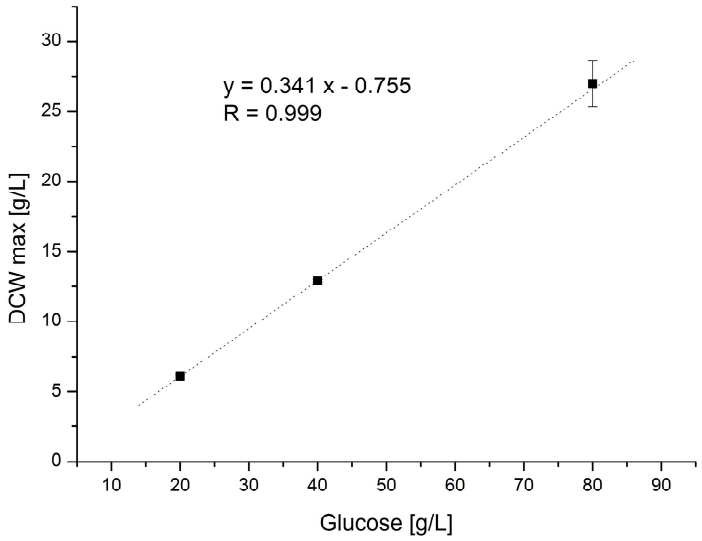


Figure 4.3: *Maximal dry cell weight (DCW) achieved in batch bioreactors as a function of the initial concentration of glucose. The C/N mass ratio was constant at 8.77. The DCW max corresponds to an average of three points (n=3) measured during stationary phase, the standard deviation is represented as error bars.*

Data provided by the study represented in Figure 4.3 showed that in bioreactors, as expected, biomass yield on glucose improved 1.4-fold, from 0.24 g g^{-1} to 0.34 g g^{-1}. The same experiment performed with *Chlorella* showed a $Y_{X/S}$ of 0.66 g g^{-1}, with an improvement of 2.4-fold compared with shake flasks experiments (Figure 3.13, Chapter 3). It was likely that the oxygen demand of microorganism could not be met during the process in shake flasks, reducing the biomass yield on glucose. In batch bioreactors, maximal biomass at stationary phase was 27 g/L, obtained with 80 g/L of glucose and 32 g/L of yeast extract. With the same medium composition i.e 80 g/L glucose - 32 g/L YE, *Chlorella* achieved a maximum dry biomass of 50 g/L. This shows that the two strains cannot convert substrate at the same efficiency to form biomass. Such a difference is represented by the $Y_{X/S}$ with the one calculated for *Chlorella* is being approximately twice the one of *Schizochytrium*. This reason is probably related to different metabolic pathways.

Cultures with higher concentrations of glucose were not tested since the fermentation in medium prepared with 80 g/L of glucose was difficult to control, because of high foam formation, despite anti-foam addition. It was hypothesized that this was caused by the reaction of high salt concentration with proteins present in yeast extract or some metabolites produced by

Schizochytrium. De Swaaf et al. observed during the cultivation of another Thraustochytrids, *Crypthecodinium cohnii*, production of a viscous extracellular polysaccharides [234]. Consequently, higher concentrations of glucose were not tested. The maximum growth rate corresponded to 0.17 h^{-1} when glucose concentration was 20-40 g/L and decreased to 0.13 h^{-1} when 80 g/L was used. It wa sobserved, that when using the same amount of glucose i.e 20 g/L, *Schizochytrium* growth is 1.5 times faster than *Chlorella*, which in terms of doubling time is 4 hours and 6 hours respectively. When this study is compared to the literature is found that Ren et al. analyzed *Schizochytrium* sp. specific growth rate as a function of the fermentation time: maximum was between 0.125 h^{-1} and 0.15 h^{-1}, with a doubling time of 5.5-4.6 hours [188]. Ganuza et al. determined a maximum specific growth rate of *Schizochytrium* sp. on glucose of 0.071 $^{-1}$ whereas Ethier et al. found a maximum specific growth rate of 0.029 h^{-1} when *Schizochytrium limacinum* was cultivated in glycerol [235] [51] [236].

Figure 4.4 shows an example of a batch cultivation of *Schizochytrium* in a medium with 40 g/L of glucose and 16 g/L of yeast extract (C/N 8.77). Stationary phase occurred after about 30 hours of fermentation with a maximal cell density of 12 g/L. Glucose was completely consumed, indicating a C-limitation. During the cultivation, the cells tended to aggregate, making it difficult to analyze spectrophotometrically the samples. Despite this problem, the standard deviation represented in the graph is relatively low.

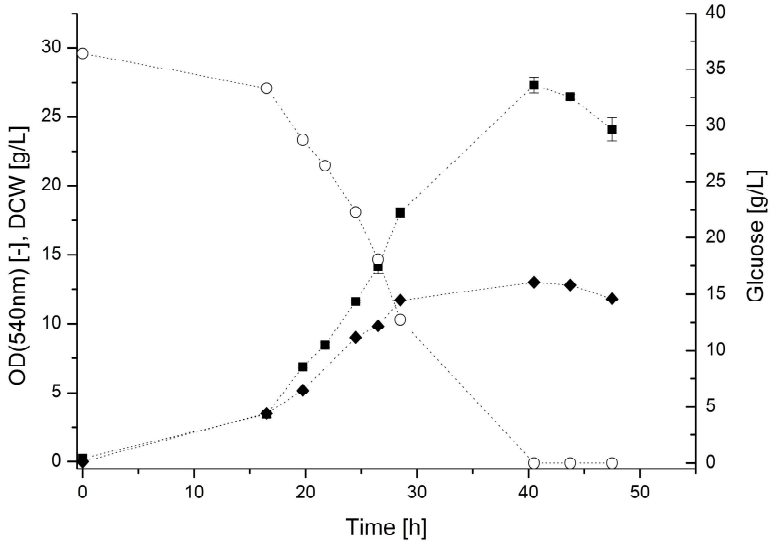


Figure 4.4: *Growth study of* Schizochytrium *sp. in batch bioreactor. The medium had a C/N ratio of 8.77 and contained 40 g/L of glucose and 16 g/L of yeast extract. The ♦ dry cell weight in g/L, the ◯ glucose concentration in g/L and the ■ optical density measured at 540 nm are represented as a function of the fermentation time. The measurements of the DCW and OD(540nm) were repeated in duplicate (n=2) and the standard deviation is represented as error bars.*

In the initial part of work, cultivation of *Schizochytrium* sp. was optimized, by defining an optimal standard cultivation medium, with only one source of nitrogen and by determining the effect of C/N ratio on cell growth. Experiments showed that a carbon limitation appeared up to a ratio of 17.5 and with higher C/N ratios, a nitrogen limitation was hypothesized. In bioreactor, the biomass obtained at stationary phase as a function of the initial glucose concentration was studied, showing a proportional yield ($Y_{X/S}$) of 0.341 gg^{-1}. The maximum dry cell weight in batch bioreactor, 27 g/L, was obtained from an initial concentration of 80 g/L of glucose, under a C/N ratio of 8.77 and after less than 48 hours.

Results are promising and the same approach adopted in the study of *Chlorella* appears to work also for such strain. Therefore, process may be further optimized, from batch to fed-batch. This is justified not only by the need to achieve high cell densities but also by the necessity of developing a two-stage process, where the second part of the bioprocess aims to accumulate lipid under a nitrogen limited medium. Fed-batch is a good solution to (1) achieve higher cell densities, (2) change the composition of the medium and (3) increase the overall lipid productivity.

Scientific studies of microalgae cultivations support the view that fed-batch operation can be an ideal solution. In the study of Huang et al., *Schizochytrium* sp. was first grown in shake flask batch cultures using glycerol as carbon source. When the initial glycerol concentration was 50 g/L, biomass was maximal at 10.85 g/L and a growth rate of 0.03 $^{-1}$. Experiments were continued in fed-batch mode with the intention of studying the effect of different C/N ratios and the influence of oxygen supply. After 180 hours, algal biomass reached 61.76 g/L by feeding with 100 g/L glycerol, 40 g/L YE and 40 g/L peptone.
Despite the inconvenience of long culture periods and being relatively more complicated, it was proven that fed-batch operation allows a two-stage mode of culture which stimulates first the cell growth and second the lipid accumulation [67]. Ganuza et al. pointed out that fed-batch cultures are indeed preferred for industrial production, production of lipids and secondary metabolites in general are bi-phasic processes [233].

4.3.4 Fed-batch cultivations

Two-stage fed-batch cultivation processes were firstly developed on the reference strain *Chlorella protothecoides* in Chapter 3, showing very good results in term of biomass and lipid productivity. Therefore, same approach is going to be applied to *Schizochytrium* sp.

The strategy of two-stage fed-batch cultivation is to maximize biomass accumulation under a carbon limitation and consequently enhance lipid accumulation under a nitrogen limitation. It is important to understand that carbon-limitation medium implies that in the first part of process, glucose is going to be added in excess and its depletion has as consequence the cessation of cell growth (C/N ratio $<$17.5). In the second stage, it is expected that nitrogen will be depleted progressively, favouring lipid accumulation. As shown in Figure 4.3 with a C/N ratio of 8.77, substrate concentration was proportional to biomass with an $Y_{X/S}$ of 0.34 gg^{-1}. By adding only glucose with the feed, the C/N is going to increase until a complete nitrogen starvation. Feed is going to be added manually as soon as the glucose will be totally consumed i.e stepwise/pulsed feed (Section 4.2.3). Maximal glucose concentration in the medium was kept under 40 g/L to avoid undesired inhibitory effects on cell growth.

4.3.4.1 Two-stage fed-batch # 1 - No addition of yeast extract during the feed

The first fed-batch experiment was conducted by initially cultivating cells in a carbon-limited medium composed of 40 g/L of glucose and 16 g/L of yeast extract - C/N ratio of 8.77. When glucose was completely depleted, feed composed of 500 g/L glucose and concentrated salts was added. Yeast extract was not fed, thus the only source of nitrogen was present in the initial medium. The expectation was that by adding only glucose cells will consume completely nitrogen entering gradually in a N-limitation phase under which lipid are expected to be accumulated.

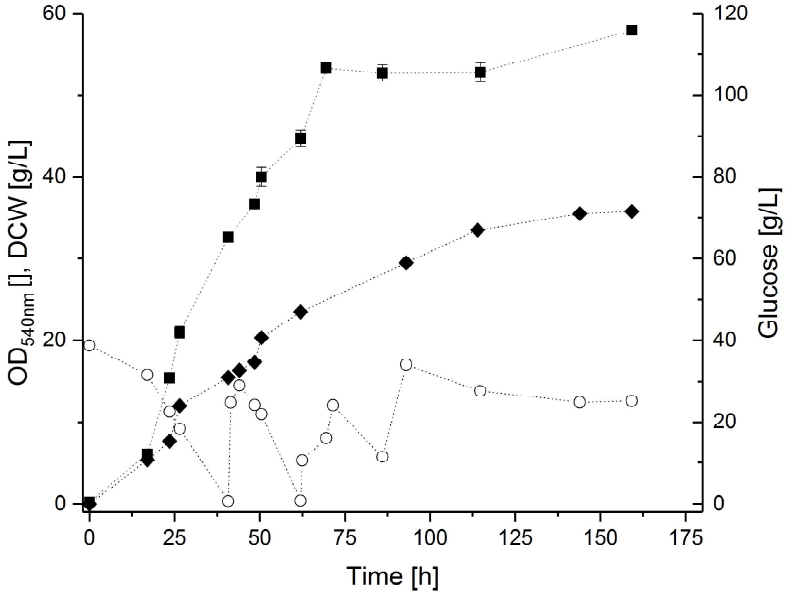


Figure 4.5: *Fed-batch cultivation of* Schizochytrium *sp. with 0 g/L YE feed. Results are represented as ♦ dry cell weight (DCW) in g/L, ■ optical density (OD_{540nm}) and ◯ glucose in g/L as a function of the cultivation time in h. The DCW and OD_{540nm} were measured in duplicate (n=2), the standard deviation is represented as error bars.*

Figure 4.5 shows that the carbon source present in the initial medium, i.e 40 g/L glucose, was completely depleted after 41 hours, hence feed composed of 500 g/L glucose and concentrated salts was added to the medium. At the end of the batch phase, dry biomass corresponded to 15.5 g/L. Cells continued to grow exponentially up to an optical density at 540 nm of 53, after 69.5 hours of fermentation. After this point, OD values did not further increase, remaining stable until the end of the culture. Despite the apparent stationary phase, dry cell weight continued to increase from 23.5 g/L (69.5 hours) to a maximum of 35.8 g/L after 160 hours. After 45 hours, it was noticed that biomass was difficult to separate during centrifugation to prepare dry cell weight. After 70 hours of cultivation, instead of a precipitate, a stable emulsion was

formed making it difficult to wash biomass to prepare dry cell weight, therefore it was decided to measure dry biomass by filtration, as explained in Section 4.2.4.1.

Dry cell weight was observed to increase despite a constant optical density indicating a stationary phase. The hypothesis was that after about 70 hours, the medium was nitrogen limited, enhancing the intracellular lipid accumulation in microalgal cells. Emulsion formation could be interpreted as a signal of the effect of the variation of C/N ratio on cells. Lipid accumulation affects the chemico-physical properties of the cells by modifying the density. In simple terms: oil forms an upper layer i.e. emulsion, when mixed with water due to its lower density; the same seems to occur with microalgal cells full of lipids. Because of the addition of feed rich in salts, the density of the medium will be slightly higher than water, increasing the density difference between the two phases. Figure 4.6 shows a microalgal suspension after centrifugation, an emulsion was clearly visible.

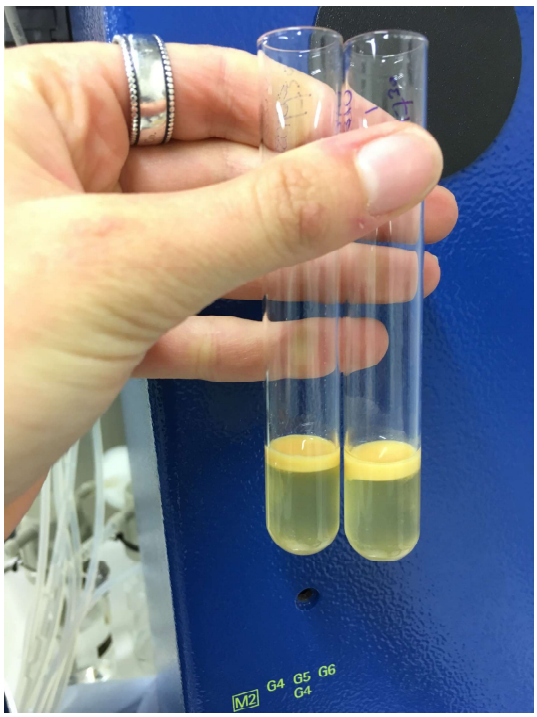


Figure 4.6: Schizochytrium *sp. emulsion after centrifugation*

Based on optical density data, three different growth rates are distinguished over the process: (1) during the batch phase, from 0 h to 41 hours of 0.172 h^{-1}; (2) during the beginning of fed-batch from 41-69.5 hours of 0.016 h^{-1} and (3) close to zero during the stationary phase. The growth rate indicated that after 69.5 hours no cell proliferation occurs. The hypothesis is

that the main metabolic activity is lipid accumulation, which is reflected by the dry cell weight measurements. The difference in biomass from the beginning of the apparent stationary phase and the end of the process is 12.3 g/L, which corresponds to a percentage of lipid accumulation in DCW of 34.35%. Experimental results, subsequent to total lipid extraction from harvested biomass, showed that dry biomass was composed of 46.99 % (SD= 3.9286 %, n=5) total lipids. As seen with *Chlorella protothecoides*, lipid were probably accumulated also during the transition phase as a function of the increase of C/N ratio and not only when nitrogen was completely depleted.

Ren et al. also noticed three different stages when cultivating *Schizochytrium* sp. HX-308. They defined the first stage (0-16 h) as the early lipid accumulation stage, where cells were growing slower in the new cultivation environment with little lipid accumulated i.e $<$10 %. The next stage (16-40 h) was defined as a rapid lipid accumulation stage, cell growth was fast achieving the maximum biomass of 58.5 g/L after 40 hours and lipid accumulation almost reached the maximum. In this stage, depletion of nitrogen was hypothesized. In the last stage (40-50 h), cells entered the stationary phase, biomass was constant with little lipid accumulation [68].

4.3.4.2 Two-stage fed-batch # 2 - Addition of 10 g/L of yeast extract in the feed

In the previous experiment (Fed-batch # 1), yeast extract i.e. source of nitrogen, was added only in the initial medium (16 g/L). The second experiment aimed to achieve even higher cell densities of *Schizochytrium* sp. by feeding yeast extract towards the late exponential phase of batch growth. The expectation was that by adding more nitrogen, the carbon-limited phase would be prolonged allowing to achieve a higher cell density. During this extended feed phase, lipid accumulation should be enhanced with consequent increase of lipid productivity. A concentration of 10 g/L of yeast extract was added after the batch phase.

As can be seen in Figure 4.7, after 70 hours, optical density values stabilized around 150 corresponding to a dry biomass of 62.5 g/L. In a previous experiment (Figure 4.5) it was clear that dry cell weight continued to increase despite an apparent stationary phase represented by OD. The dry cell weight increased only up to 69.5 g/l after 77.5 hours and remained at an average of 65 g/L until the end of the culture. In terms of growth rate, the results are similar: (1) During the batch phase, from 0 h to 31.5 hours, the growth rate was 0.18 h^{-1}; (2) during the beginning

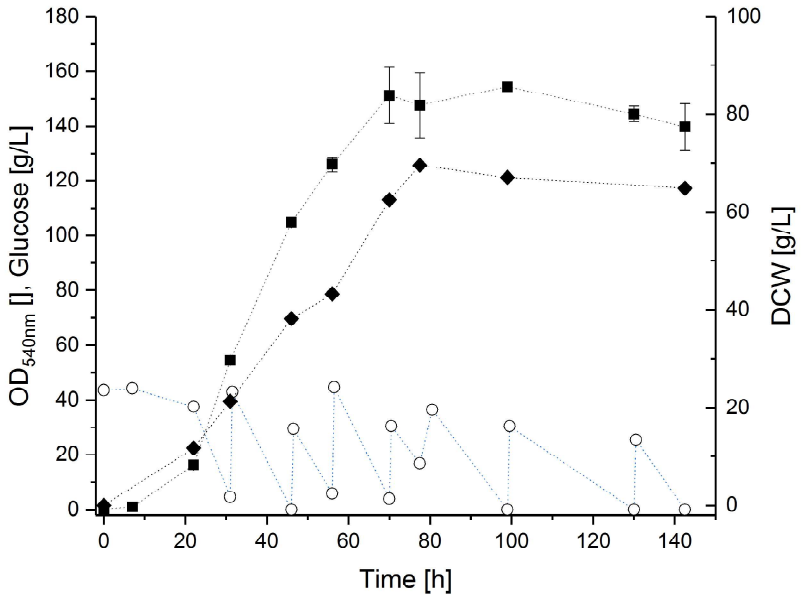


Figure 4.7: *Fed-batch cultivation of* Schizochytrium *sp. with a total of 10 g/L YE fed. Results are represented as* ♦ *dry cell weight (DCW) in g/L,* ■ *optical density (OD_{540nm}) and* ◯ *glucose in g/L as a function of the cultivation time in h. The DCW and OD_{540nm} were measured in duplicate (n=2), the standard deviation is represented as error bars.*

of fed-batch from 46-70 hours this decreased to 0.015 h^{-1} and (3) close to zero during the stationary phase. Lipid analysis showed that dry harvested biomass was composed of 62.33 % (SD=4.268 %, n=5).

It is important to remember that *Schizochytrium* has a tendency to form aggregates which might lead to errors when measured spectrophotometrically. Optical density measurements are very useful to monitor cell growth because of the rapidity and easiness of the method, however it seems that the method is not sufficiently accurate to obtain information about the growth and/or accumulation stages of the cells, especially at very high cell densities.

Overall results are positive, high DCW where achieved, 69.5 g/L instead of 35.8 g/L obtained in experiment # 1, which corresponds to an improvement of 1.9-fold. Lipid accumulation also increased by 1.3-fold.

4.3.4.3 Two-stage fed-batch # 3 - Addition of 15 g/L of yeast extract in the feed

In the third experiment, an even higher concentration of yeast extract was added during the feed. The aim was to support the hypothesis that by adding more nitrogen it is possible to prolong the carbon-limitation phase and achieve higher cell densities. Therefore, after the batch phase, 15 g/L of yeast extract were additionally fed. Samples of the culture were taken over time and lipids were extracted in order to define the evolution of total lipids. Another goal was to describe the lipid accumulation and define the optimal moment to harvest cells and extract lipids.

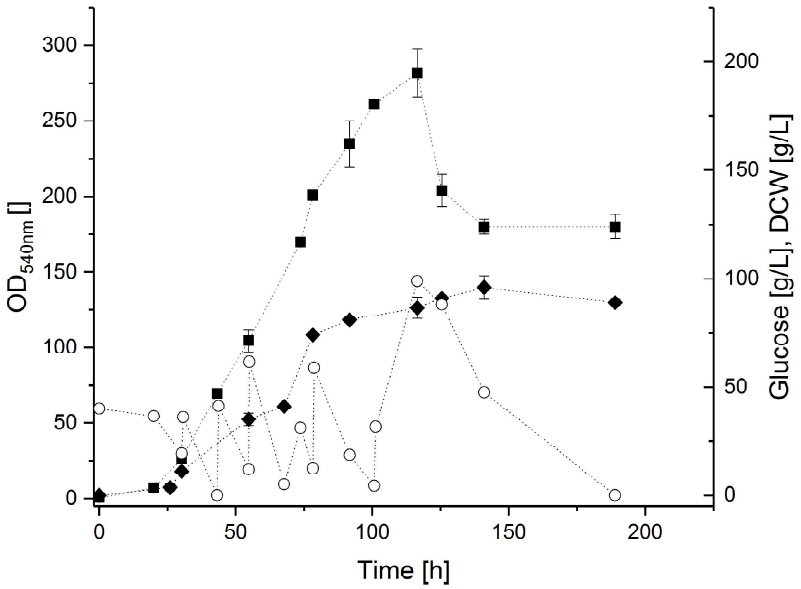


Figure 4.8: *Fed-batch cultivation of* Schizochytrium *sp. with a total of 15 g/L YE feeded. Results are represented as* ♦ *dry cell weight (DCW) in g/L,* ■ *optical density (OD_{540nm}) and* ◯ *glucose in g/L as a function of the cultivation time in h. The DCW and OD_{540nm} were measured in duplicate (n=2), the standard deviation is represented as error bars.*

Dry cell weight achieved an average of about 90 g/L after 120 hours of fermentation. Optical density values achieved a maximum of 281.6 after 116.5 hours and dropped at 180 one day later. Optical density measurements between 75 and 125 hours appear to be overestimated and do not show changes in biomass. The hypothesis is that, as noticed in experiment # 2, at high cell densities, spectrophotometric analysis is inaccurate. Overall results showed that the third experiment was successful: lipid extraction indicated that at the end of fermentation, cells were composed of 68.90 % (SD=3.948 %, n=5) total lipids. Dry cell weight improved of 2.5-fold

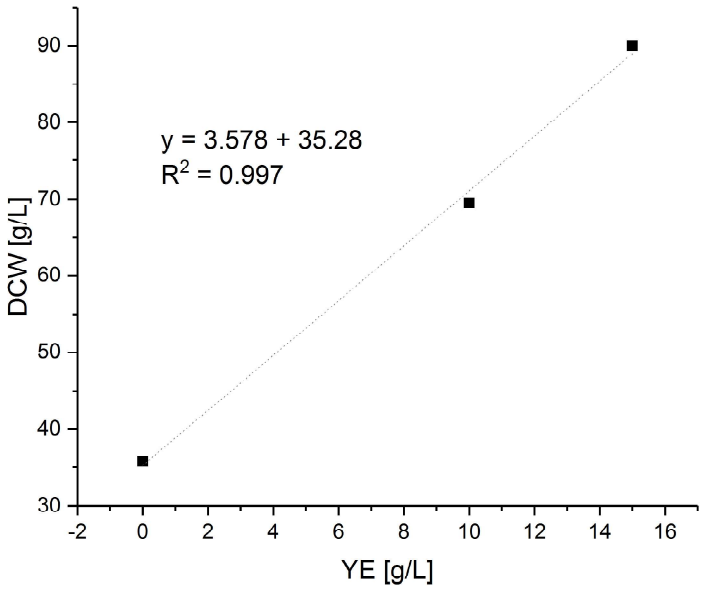


Figure 4.9: *Representation of the relation between yeast extract added during the feed and final dry cell weight in g/L.*

from the first fed-batch experiment.

Figure 4.9 shows the maximum DCW as a function of the yeast extract fed to the cultivation medium. Increased concentrations of nitrogen added during the feed were proportional to the maximum biomass achieved. As expected, results indicate that the carbon-limited phase was prolonged i.e more carbon needed to be consumed before the increase of C/N ratio, allowing the achievement of higher cell densities.

Analysis of Figures 4.5, 4.7, 4.8 showed a linear relation between DCW values as a function of the optical density up to a certain point of the growth. It was hypothesized that since cells started to accumulate lipids - under nitrogen limitation - the correlation between DCW and OD was no longer constant. As hypothesized already in Chapter 3 for *Chlorella protothecoides*, DCW increased because of an accumulation of lipids and not because of an increase in cell number. The correlation was constant up to 49 hours for fed-batch # 1, 56 hours for # 2 and 80 hours for # 3. In the case of the last two experiments, it was noticed that optical density values were not representative of cell growth after that point. Values representing the DCW/OD correlation for all fed-batch cultivations were reproducible (results not shown). Spectrophotometric assay for microalgal cell number quantification has the advantage of being

fast and easy of use. However, as specified in Section 3.3.5, this method is not reliable when cells accumulate lipids, it offers a good way to estimate the beginning of metabolic changes in algae, due to nitrogen limitation effects.

4.3.5 Total lipid analysis

Total lipids were measured gravimetrically after solvent extraction. In the first two fed batch experiments (Figures 4.5 and 4.7), total lipids were extracted only from the harvested biomass, whereas for the third fed batch experiment (Figure 4.8), lipids were analysed as a function of fermentation time. Defining the percentage of total lipid accumulated by cells as a function of cultivation time will (1) define the maximal lipid accumulation achievable by *Schizochytrium* during a two stage fed-batch fermentation, (2) sustain the hypothesis that nitrogen limitation enhanced lipid accumulation, (3) define when the best moment to harvest cells.
Figure 4.10 shows the third fed-batch experiment, where lipids were extracted throughout the fermentation.

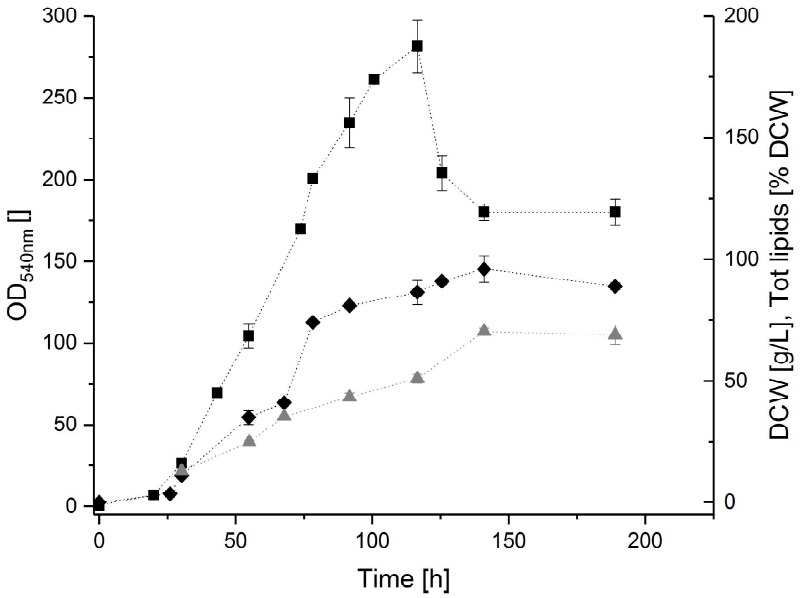


Figure 4.10: *Improved fed-batch cultivation of* Schizochytrium *sp.. Results are represented as ♦ dry cell weight (DCW) in g/L and ■ optical density (OD540 nm) as a function of the cultivation time in h. Total lipids accumulation (▲) is represented as percentage of DCW. The DCW and the OD540nm were measured in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6), the standard deviation is represented as error bars.*

Before the batch phase, total lipids constitute only 12.98% (SD=0.06703, n=5) of the dry biomass, which was in accordance with other studies carried out in the project. In fact, under

normal growth conditions, algal biomass is mainly produced, with relatively low lipid contents i.e about 5-20% of their dry cell weight [192] [188]. After 140 hours the percentage of total lipids achieved a maximum of 70.35% (SD=1.2802, n=5) of the biomass. Dry cell weight continued to increase slightly as a function of the increase of lipids, until about 90 g/L (Figure 4.10). Maximum lipid composition of dry biomass, obtained in the three different experiments is represented in Figure 4.11.

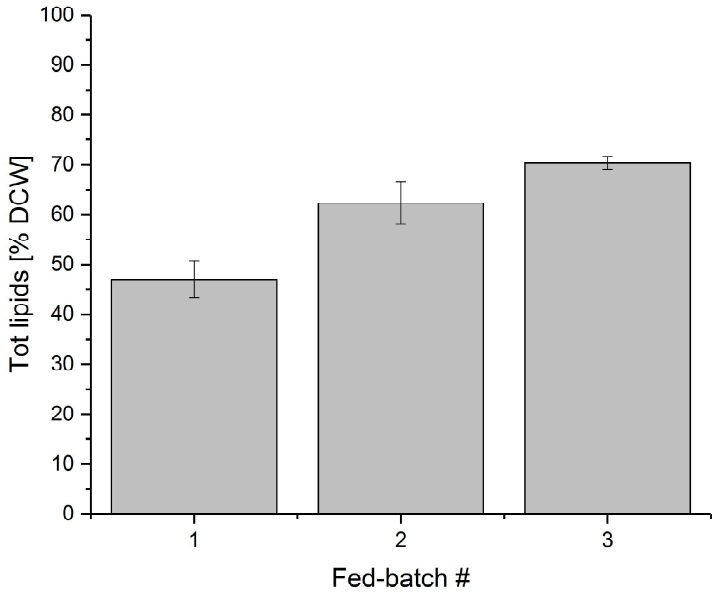


Figure 4.11: *Representation of total lipids as percentage of the dry cell weight, produced in three different two-stage fed-batch experiments. Total lipid percentage of the DCW was measured in sextuplicate (n=6), the standard deviation is represented as error bars.*

Lipid accumulation as percentage of dry weight was 46.99 % for fed-batch #1, 62.33 % for fed-batch #2 and 70.35% for fed-batch #3. The main difference between the three fed-batch cultivations was the amount of yeast extract i.e source of nitrogen, added in the medium during the feed. Results indicated not only an increase of dry biomass proportional to the amount of nitrogen added (Figure 4.9) but also seemed to have an effect on lipid accumulation. The hypothesis is that a longer transition phase, from nitrogen limited medium (C/N ratio $\leq$ 17.5) to complete nitrogen depletion, has an impact on the maximum total lipid composition of biomass.

The productivity of total lipids corresponded to 2.53 g/L/day for fed-batch #1 and 6.80 g/L/day for fed-batch #2. Calculations were made based only on lipids extracted at the moment of

harvesting, therefore it could be that productivity of lipid was at its maximum before. On the other hand, productivity was calculated at different times for fed-batch #3 (Figure 4.12). After 91.75 hours total lipid productivity achieved 9.17 g/L/day. The tendency was to reach a plateau of 11.5 g/L/day. The harvest of microalgae appeared to be optimal after 91.75 hours. For comparison, the maximum lipid productivity of *Chlorella* was 16.7 g/L/day and the maximum lipid content was 58.10 % (SD=2.487 %, n=5) of the dry cell weight(Chapter 3).

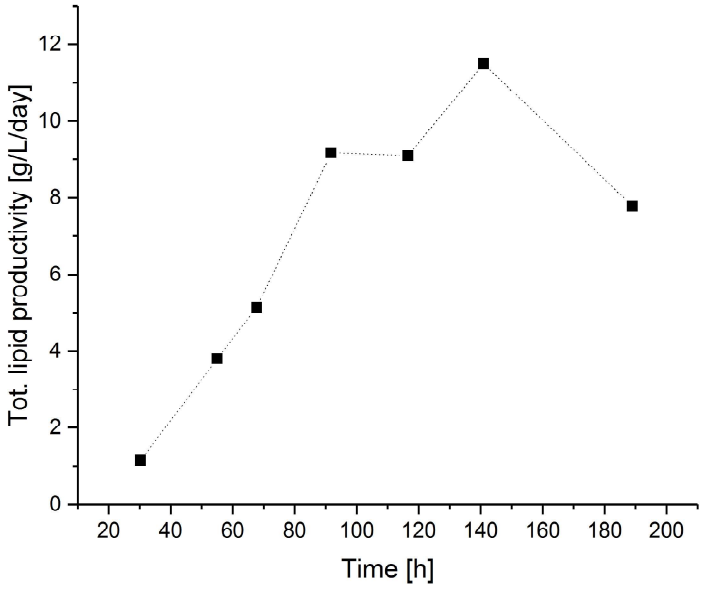


Figure 4.12: *Productivity of lipids as a function of the cultivation time in h.*

Results showed that *Schizochytrium* starts to accumulate storage lipids when cells are in a medium with an excess of carbon but are limited in nitrogen. When nitrogen is completely depleted algal growth is interrupted and lipid are not further accumulated. Also Bajpai et al. did not detect any major lipid accumulation in the post-exponential growth phase of *Thraustochytrium aureum* ATCC 34304; it was stated that accumulation of lipids seems to happen during the growth period where only carbon source is fed, thereby increasing C/N ratio. Several studies have shown that a high C/N ratio is advantageous for high- level lipid accumulation in Thraustochytrids grown in complex media containing yeast extract and the present study is in agreement [75] [81] [70] [65]. Specific reasons of lipid accumulation are not totally clear. However, it is suspected that when there is insufficient nitrogen for protein synthesis, required by algal growth, excess carbon is channeled into storage molecules such as triacylglycerols.

When cell division is not possible, fatty acids provide a useful mechanism to convert harvested energy [41]. Furthermore, Stephenson et al. claimed that the most effective strategy to achieve high lipid content in *C. vulgaris* was to allow cells to naturally deplete the nitrogen source in the medium, rather than transferring them into one devoid of that element [237]. Very positive results show that a two-step fed-batch fermentation is the ideal solution for lipid production.

4.3.5.1 Summary

A two-stage fed-batch cultivation was successfully applied to the microalgal strain *Schizochytrium* sp.. Optimization of the process was successful: in third experiment, after 120 hours of cultivation, 90 g/L of dry biomass containing 70.35% of total lipids were produced. Maximum lipid productivity was between 9-10 g/L/day (Figure 4.8). Results of the present study are compared with scientific literature in Table 4.3.

Table 4.3: *Overview of fed-batch strategies for heterotrophic high cell density culture of* Schizochytrium *sp.. Parameters marked with ∗ were calculated from information founded in the respective publications.*

Strain	Substr.	DCW_{ini} [g/L]	Run time [h]	DCW_{max} [g/L]	Tot. lipids [% DCW]	Lipid prod. [g/L/day]	Ref.
Schizochytrium sp.	Glc	<3	189	90	70.35	9.17 (after 92 h)	This study
Aurantiochytrium limacinum SR21	Gro	<2 *	190 *	61.76	65.2	7.92 *	[67]
Schizochytrium sp. LU310	Glc	10 *	200 *	88.6	55-60*	9.8 * (after 120 h)	[66]
Schizochytrium sp.	Glc	<10 *	160	92.72	50.26	6.99*	[238]
Aurantiochytrium sp. T66	Gro	<5 *	180-190	90-100	54-58	6.48-7.32*	[75]
Schizochytrium sp. M209059	Glc	<10 *	132	71	49.5	6.39	[188]

The study of Huang et al., 61.76 g/L of *Aurantiochytrium limacinum* biomass with a total lipid content of 65.2 % were obtained by feeding 100 g/L glycerol, 40 g/L yeast extract and 40 g/L peptone (C/N ratio of 1.25) [67].

Schizochytrium sp. LU310 was cultivated in shake-flask by Ling et al., the highest DCW of 88.6 g/L was achieved with intermittent glucose feeding and by adding 0.4 g/L of nitrogen after 72 hours. After 120 hours, accumulation of lipids was maximal at about 55 % of the

dry weight. Results showed that strategy of carbon and nitrogen addition can maintain cells at nutrient-replete growth and promote the lipid accumulation [66]. Qu et al. developed a two-stage oxygen supply control strategy achieving a total lipid content in the cell dry weight of 50.26 % and a maximum cell dry weight of 92.72 g/L after 160 hours of cultivation. The source of nitrogen, glutamate, was consumed after less then 40 hours, when the lipid content was about 10%. In their study, the volumetric oxygen mass transfer coefficient (KLa) was controlled and its effect on microalgae was studied. Apparently, a value of 88.5 h^{-1} favourised lipid accumulation rather than 150 h^{-1} [238].

Comparable results, to the present study, were obtained from the experiments of Jackobsen et al. carried out on the Thraustochytrids microalgal strain *Aurantiochytrium* sp. T66. The source of nitrogen i.e monosodium glutamate was only supplied at the beginning, whereas glycerol was continuously fed (batch addition). When glutamate was completely depleted, total lipid content corresponded to 13%, at the end of fermentation it increased to 58% of cell dry weight after 190 hours of fermentation. A maximum of 90-100 g/L of biomass were achieved. When nitrogen limitation was combined with oxygen limitation, maximum dry biomass attained 88-90 g/L and lipid content improved to 63% after 190 hours [75]. Studying the effect of aeration on *Schizochytrium* sp., Ren et al. achieved a high cell density (71 g/L) with high lipid content (35.75 g/L) in a 1,500-L fed-batch bioreactor using a stepwise aeration control strategy [188].

4.4 Conclusion

One of the aims of this Chapter was to compare and evaluate the potential and the application of *Chlorella protothecoides* and *Schizochytrium* sp. in the production of high cell densities as a prerequisite for high productivity of lipids.

The first consideration was that steps carried out in the development and optimization of *Chlorella* were possible to apply to *Schizochytrium*. Results of the main objectives and of the optimised two-stage fed-batch cultivations are represented in Table 4.4.

Table 4.4: *Comparison results obtained with microalgal strains* Chlorella protothecoides *and* Schizochytrium *sp.*

	Schizochytrium sp.	*Chlorella protothecoides*
C/N ratio C-limited	<17.5	<12
$Y_{X/S}$ [g g^{-1}] (C/N 8.77)	0.34	0.66
Max. growth rate [h^{-1}]	0.17	0.11
Max. DCW [g/L] (batch bioreactor)	27	50
Max DCW [g/L] (fed-batch bioreactor)	90	255
Max. total lipid (w/w) [% DCW]	70	58
Lipid productivity [g/L/day]	9-10	16.7

Both strains could consume glucose and therefore grow heterotrophically to high cell density, in the absence of a light source. Such a characteristic is not associated to numerous microalgal strains and this particularity was an essential pre-requiste in the advancement of this project. The definition of the threshold between carbon limitation and nitrogen limitation was a key-step in the development of microalgal cultivation. Experiments were carried out by testing the effect of different proportions of glucose, the carbon source and of yeast extract, the nitrogen

source. Media prepared with a C/N ratio between 0-12, glucose was the limiting substrate. In the case of *Schizochytrium* this range was broader and N-limitation is suspected with a C/N ratio slightly higher than 17.5. Biomass yield on glucose, under a C/N ratio of 8.77, was $Y_{X/S}$ 0.66 g g $^{-1}$ for *Chlorella* and 2-fold lower for *Schizochytrium*, 0.34 g g $^{-1}$. This is further reflected in the maximum biomass achieved in bioreactors: in batch cultivation this was 50 g/L for *Chlorella* and 27 g/L for *Schizochytrium*. On the other hand, *Schizochytrium* has a faster growth rate: 0.17 h^{-1} instead of a maximum of 0.11 h^{-1} for *Chlorella*. Development and application of two-stage fed batch cultivation was very successful for both microalgae. *Chlorella* achieved a very high dry biomass of 255.3 g/L composed of 58% of total lipids, whereas *Schizochytrium* dry biomass achieved 90 g/L with 70 % total lipids. The principal aims were successfully achieved: maximum total lipid productivity was 16.7 g/L and 9-10 g/L respectively, values which are considerably higher than previously reported studies.

The present work proposed a promising technique for the cultivation of *Schizochytrium* sp.. High productivity of biomass and total lipids can be harnessed for various profitable and ecological industrial purposes.

Chapter 5

Alternative carbon sources for the sustainable cultivation of *Chlorella protothecoides*

5.1 Introduction

Chapter 3 explored the potential of *Chlorella protothecoides* for high lipid productivity under heterotrophic conditions. Very high cell densities were obtained by cultivating microalgae under a carbon limited medium; transition towards complete nitrogen starvation enhanced the mechanism of lipid accumulation. Results appeared to be very promising, such high production of lipid-rich algal biomass could have many industrial applications.

The main bottleneck of the microalgal industry is found in downstream processing, which has a significant impact on the production costs of microalgal oil. However, the high cost of the fermentation substrate also causes restrictions in production [210]. It was estimated from Li et al. that the cost of organic carbon (e.g. glucose), nutrients such as nitrogen (e.g. yeast extract) and phosphorous and enough water for cell growth accounted for around 50-80% of the total medium costs [106]. Optimizing the medium has a critical importance not only in terms of lipid productivity - by balancing the C/N ratio - as shown in Chapter 3, but also to lower the cost in view of an industrial scale-up. A conceivable approach is to use alternative carbon sources instead of pure glucose. The cost of raw materials is very high when pure glucose is used as main carbon source. The goal is to optimise product productivity while reducing the total investment in nutrients.

On of the aim of this Chapter was to grow *Chlorella protothecoides* with different carbon sources with the purpose to define the effect on cell growth and lipid accumulation. The growth rate, biomass and lipid yields, and the cellular overall lipid profile were examined and compared to those of *Chlorella protothecoides* grown on pure glucose. The influence of the substrate on the total lipid profile is going to be characterized, to define whether lipid production and composition was dependent on the substrate. According to the literature presented below, some strains of *Chlorella protothecoides* can grow on alternatives substrates such as glycerol, acetate and molasses. Another objective of this study is to verify the capacity of the particular strain of *Chlorella protothecoides* used in this project to metabolise such substrates and to evaluate their application in the two-stage fermentation process developed in Chapter 3.

5.1.1 Glycerol

A major by-product of biodiesel production from microalgae is crude glycerol (Figure 5.1). In general, for 4.5 kg of biodiesel produced, 0.45 kg of crude glycerol is produced as co-product [239] [240].

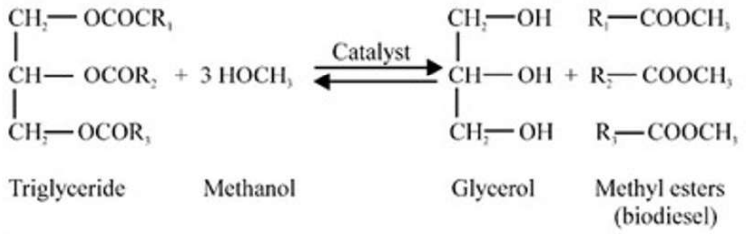


Figure 5.1: *Methylation of triglycerides for the production of biodiesel. Glycerol is a side product of the reaction.*

Recycling waste glycerol for the cultivation of microalgae presents interesting opportunities to convert it into higher-valuable products [217]. The cost is very competitive compared to glucose and it supports the philosophy of bio-refinery: all microalgal products are valorised and re-used to maximize the profitability of the biomass [131]. This capacity allows for an increased flexibility in methods of algal production, it is more environmentally friendly and economically advantageous for large-scale biomass production. Care must be taken when using waste glycerol for the production of products intended for human/animal consumption,

toxicological analysis would be required to guarantee the safety of the final product.

Several studies examined the possibility to use glycerol as an alternative carbon source for heterotrophic algal growth, the outcome suggests that there is potential for such a cultivation strategy for commercial applications. Multiple strains of algae, including *Chlorella protothecoides*, are able to uptake and grow rapidly on glycerol. Feng et al., studied the growth of *Chlorella protothecoides* on a mixed waste substrate from brewer fermentations, rich in nitrogen and crude glycerol. Biomass achieved a maximum of 14.07 g/L showing that the microalgal strain can utilize effectively the carbon, nitrogen and other trace elements from biodiesel crude glycerol and brewer fermentation waste [165]. O'Grady et al. showed that in a medium containing a mixture of glycerol and glucose, *Chlorella protothecoides* was able to simultaneously uptake the two substrates. According to their results, lipid production was 0.077 g lipid l^{-1} h^{-1} during growth on glycerol and 0.098 g lipid l^{-1} h^{-1} on a mixture of both glucose and glycerol [217]. Using crude glycerol as C-source, in fed-batch mode, Chen et al. obtained 45.2 g/L of *Chlorella protothecoides* biomass and 24.6 g/L of total lipid after 8.2 days of cultivation. Pure glycerol and crude glycerol from biodiesel production (62% purity) were compared showing that the latter could be used directly; results obtained were similar to those with pure glycerol [45].

Metabolism of glycerol has not have been extensively studied in microalgal systems. Research on bacterial and higher plant systems suggests that glycerol enters the cell by simple diffusion [241]. In the cytoplasm, glycerol is oxidized to glyceraldehyde triphosphate (GA3P), which is an intermediate in the glycolytic pathway. It can be converted to pyruvate and the to acetyl-CoA, which can enter the Krebs cycle to produce energy or it can enter a different metabolic pathway e.g. fatty acid synthesis [241] [35].

5.1.2 Acetate

Acetate is a readily available and inexpensive substrate derived from many industrial applications [35]. Overflow metabolism is well known for yeast, bacteria and mammalian cells and it typically occurs under glucose excess conditions and is characterized by excretion of by-products such as acetate, ethanol or lactate. Uptake of carboxylic acids, such as acetic, citric, fumaric, glycolic, lactic, malic, pyruvic, and succinic, is well known since decades in micro-

bial cultivations [242]. Acetate is one of the most common carbon sources for many microbial species, including most of the microalgal species capable of heterotrophic growth [51].
However, acetate does not always promote growth. It can be toxic for many microorganisms at high concentrations, despite its common use for buffering high pH levels in bioreactors, with the exception of *Chlamydomonas mundana*, which grew rapidly using acetate [35]. Kumar et al. added sodium acetate to *Chlorella sorokiniana* FC6 IITG after the stationary phase of cell growth was reached to enhance lipid production under mixotrophic conditions; maximum lipid productivity was 0.550 g/L/day [46]. Huang et al. stated that *Chlorella sorokiniana* GXNN01 cultivated under mixotrophic conditions favored acetate under high light but glucose under low light [243].
Alternative fermentation strategies were studied for the cultivation of heterotrophic marine alga *Crypthecodinium cohnii*. Glucose and acetic acid were compared as carbon sources. After 400 hours of an optimized fed-batch - controlled with feed consisting of pure acetic acid - resulted in a concentrations of dry biomass and lipids of 109 g/L and 61 g/L (56% of DCW) respectively. Use of glucose, showed lower biomass and lipid (26 g/L, 15% total lipids of DCW) [64]. In the case of *C. cohnii* fatty acid synthetase was shown to be cytosolic, therefore lipid synthesis in this alga occurs in the cytosol. During growth on glucose, the export of acetyl-CoA from the mitochondrial matrix to the cytosol is required to make it available for lipid synthesis. However, it seems that acetic acid can directly be converted into acetyl-CoA in the cytosol by acetyl-coenzyme A synthetase. The hypothesis is that the under this metabolic pathway, lipid production is generally favourised [35] [64].

5.1.3 Molasses

Sugar from sugar beet (*Beta vulgaris)* is mainly produced in Europe, Russia, Turkey and in some states of the US; because of the more temperate zone. In contrast, sugar cane grows exclusively in tropical zones, major producers being Brazil, India, China, Thailand and Mexico. Molasses is the by-product of refining sugarcane or sugar beet into sugar. This syrup comes from the final crystallization stage where further extraction of sugar is not possible with traditional processes.

Molasses from sugar beet contains 40-50% of sucrose by dry weight and low amounts of raffinose, glucose and fructose. The non-sugar content includes relatively high concentrations of

minerals, such as calcium, phosphorus, potassium and sodium. Accumulation of minerals is a result of sucrose concentration from the original plant material, chemicals are added during the process making sugar beet molasses unpalatable to humans unlike from sugar cane molasses which is used for sweetening and flavoring foods. Beet molasses is marketed as animal feed or used as fermentation feedstock, mainly for yeast. It is the most economical source of carbohydrate for ethanol and citric acid fermentations [244]. The main disadvantage is the varying chemical composition of molasses, which could affect the reproducibility of the cultivation. Ideally it would be required to evaluate every new lot and consider pre-treatments to standardize the substrate concentration [245].

Liu et al. [246] investigated the influence of carbon sources on lipid production in *Chlorella zofingiensis*, culturing the cells in the dark with different carbon sources. Glucose gave the highest growth rate (0.03 h^{-1}), cell biomass (10.1 g/L), lipid content (0.52 g/L) and lipid yield (5.27 g/L); results obtained with sucrose where slightly lower. Sucrose being the main sugar present in molasses, such results show the potential of *Chlorella* sp. to consume sucrose obtaining similar results to glucose [246]. Alternatively, different studies showed the possibility to convert sucrose present in molasses to glucose by chemical or enzymatic techniques. Cheng et al. [210] studied the feasibility of using sugar cane juice hydrolysate (SCH) as an effective carbon source for algal fermentation and oil production. Sugar cane molasses were were enzymatically hydrolysed to from SCH. Results showed that algal cells grew faster and provided a higher yield of biomass and oil with SCH than that with glucose. In terms of biomass 48.52 g/L and 43.12 g/L respectively was obtained and in terms of oil yield was 21.88 g/L and 20.72 g/L [210]. Sugar cane molasses was pre-treated in the study of Liu et al., using a cation exchange resin in order to remove metal ions. Higher biomass, lipid and astaxanthin productivities (11.3 g/L, 0.71 g L^{-1} day^{-1} and 1.7 mg L^{-1} day^{-1}, respectively) were obtained using treated molasses, compared to glucose (9.9 g/L, 0.66 g L^{-1} day^{-1} and 1.6 mg L^{-1} day^{-1}). Untreated molasses gave a much lower cell dry weight, specific growth rate and sugar utilization than that based on glucose or treated molasses. This suggests that metal ion removal provides better growth conditions [246]. Yan et al. [247] reduced the cost of oil production by 50% using molasses instead of glucose. Under optimized conditions, the maximum algal cell density, oil content, and oil yield were respectively 70.9 g/L, 57.6%, and 40.8 g/L. Sugar cane molasses was previously hydrolyzed by invertases [247]. Alternatively, acidic treatment

of molasses can be applied to hydrolyse sucrose into glucose and fructose, prior to fermentation [248] [249].

Most of the studies using molasses were carried out in Asia, where sugar is produced from sugar cane and not from beet, as a result little or no research has been reported on the growth of *Chlorella protothecoides* on molasses from sugar beet. In this work, molasses was going to be evaluated. Cell growth and lipid profiles were studied and compared. The requirement for a pre-treatment was evaluated. According to the literature, some strains of *Chlorella* are able to consume fructose and sucrose with similar results to glucose. This experiment determined whether *Chlorella protothecoides* can metabolize sucrose and if it is possible to achieve yields comparable to pure glucose.

5.2 Material and methods

All the methods describing the cultivation of *Chlorella* and the analysis of the cell wall are described in Section 3.2 in Chapter 3.

5.2.1 Material

All chemicals were purchased from either Sigma Aldrich (St. Louis, USA) or Thermo Fisher Scientific (Waltham, USA) if not stated otherwise. *Chlorella protothecoides* (ATCC 30411) was obtained from the ATCC (Manassas, USA) culture collection. Sugar beet molasses was obtained from Schweizer Zucker AG (Aarberg, Switzerland). The composition is found in Annexe 4.

5.2.2 Analysis of metabolites by high-performance liquid chromatography (HPLC)

The HPLC method was described in Section 3.2.4.3 of Chapter 3 and in Figure 5.2 and Figure 5.3 are reported the standard calibration curve of acetate and glycerol, respectively. Each point of the calibration curve was measured in triplicate (n = 3) to check the reproducibility of the results, the standard deviation error (SD) is represented graphically as error bars.

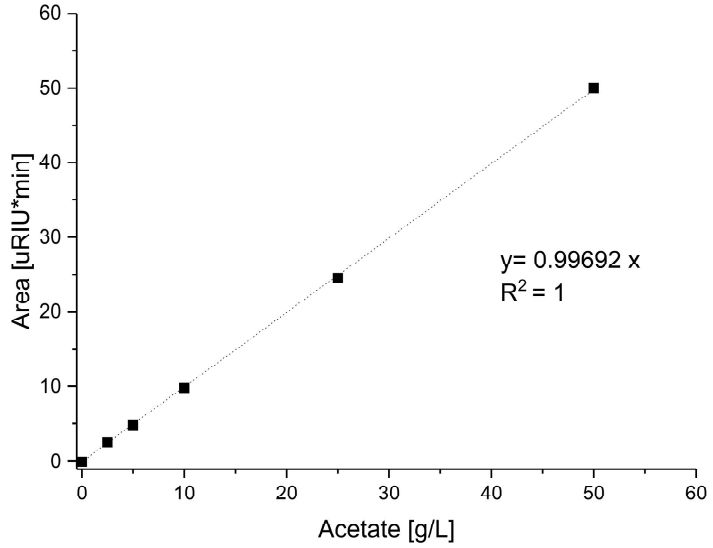


Figure 5.2: *Representation of the standard calibration curves of acetate analyzed by HPLC. The standards were prepared in the standard cultivation medium, each point was measured in triplicate (n=3) and the standard deviation error is represented as error bars.*

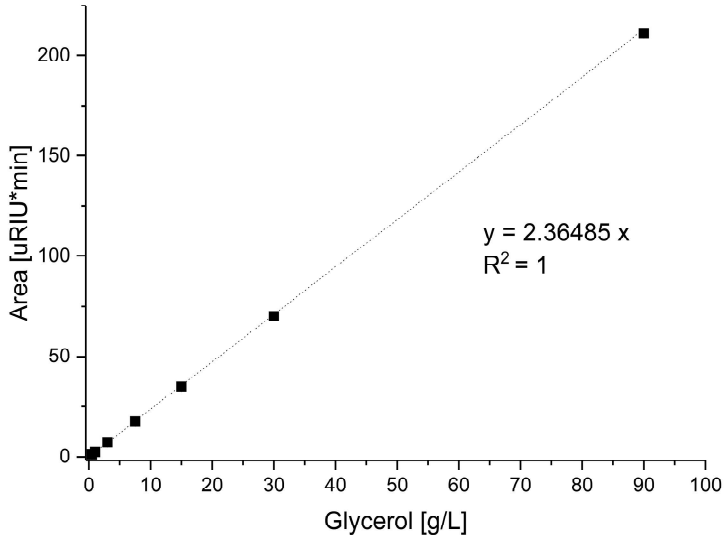


Figure 5.3: *Representation of the standard calibration curves of glycerol analyzed by HPLC. The standards were prepared in the standard cultivation medium, each point was measured in triplicate (n=3) and the standard deviation error is represented as error bars.*

5.2.3 Acid hydrolysis of molasses

Pre-treatment of molasses aimed to hydrolyse sucrose into glucose and fructose under low pH and high temperature, as represented in Figure 5.4.

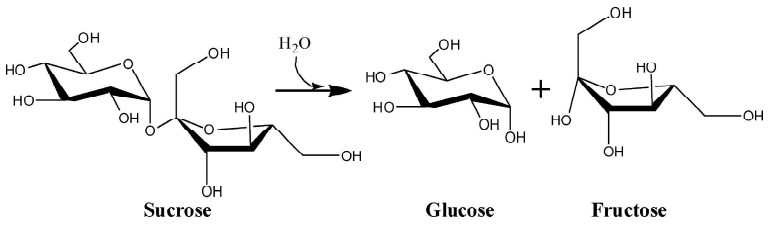


Figure 5.4: *Hydrolysis of sucrose into glucose and fructose [4]*

A 40% (m/M) molasses solution was adjusted to pH 3.5 with H_2SO_4 and autoclaved at 121°C for 20 min. Under sterile conditions it was neutralized to pH 7 with NaOH. Samples before and after the treatment were taken and analysed by HPLC (Section 3.2.4.3).

Annexe 4 shows that the sucrose content of molasses was 48.1 % [w/w], therefore according to Equation 5.1 it was expected to obtain about 111 g/L of glucose.

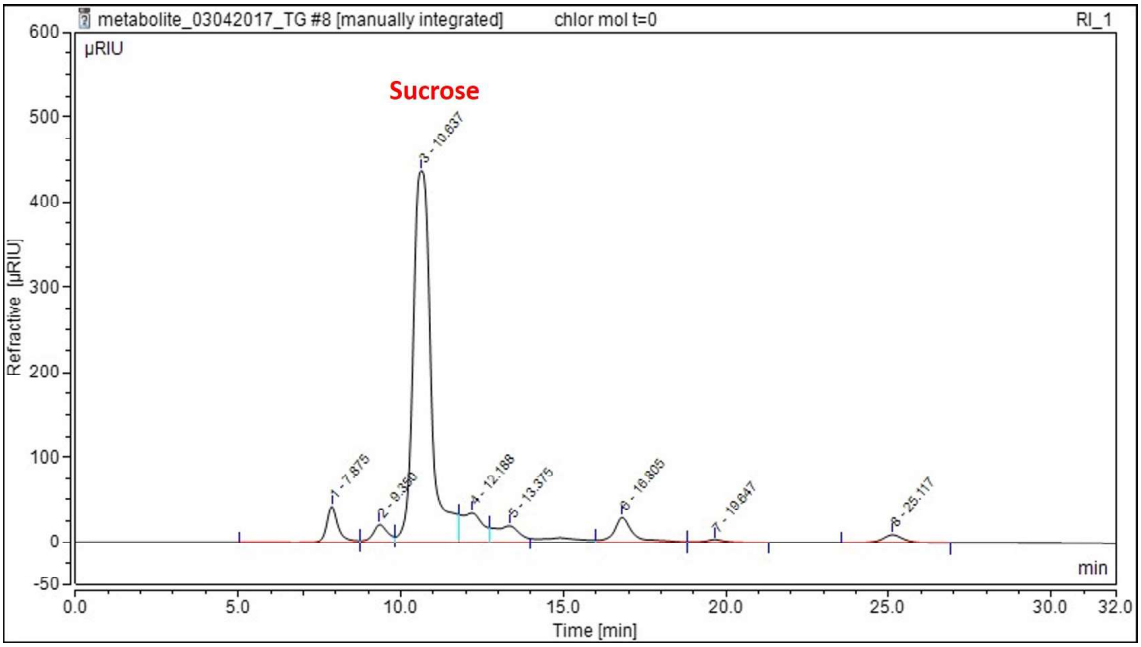
$$Glucose[g] = \frac{Conc_{molasses} \cdot Sucrose}{2} \tag{5.1}$$

where:

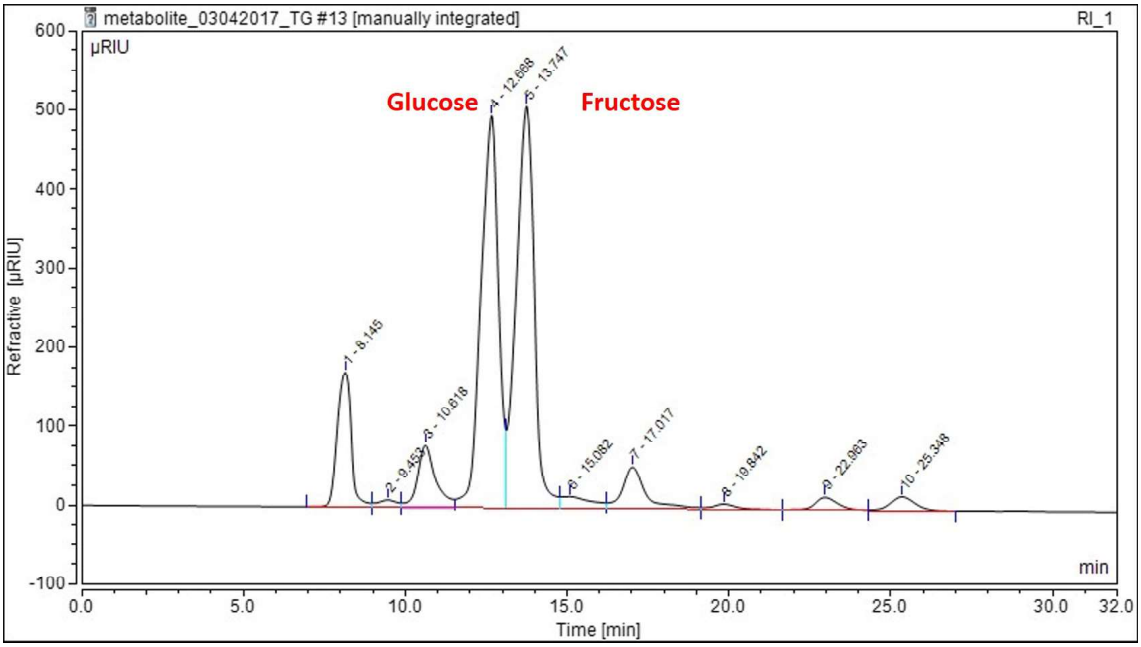
$\text{Conc}_{molasses}$ = Concentration of molasses (w/W) in DI water

Sucrose = Percentage of sucrose in molasses [%]

HPLC chromatograms of molasses before and after acidic hydrolysis are shown in Figure 5.5. Peak at 10.6 min corresponds to sucrose, the main sugar present in molasses (Figure 5.5(a)). After acidic treatment, the sucrose peak area was reduced by more than 80% and two new peaks appeared at 12.5 min (glucose) and 13.7 min (fructose) (Figure 5.5(b)). The glucose concentration in the pre-treated solution corresponded to 108 g/L. Such methods were repeated every time fresh pre-treated molasses was required for new experiments, reproducible results in terms of glucose concentration were obtained.



(a) Before treatment



(b) After treatment

Figure 5.5: *HPLC chromatograms of a molasses solution before and after acid hydrolysis.*

5.2.4 Preliminary study

Chlorella protothecoides was grown in 500 mL shake flasks with 100 mL of fresh medium. Standard medium was prepared as indicated in Section 3.2.2, glucose was replaced by various carbon sources. The modifications are described in Table 5.1. Flasks were incubated in a shak-

ing incubator (GFL, Burgwedel, Germany) with orbital motion of 120 rpm at 25°C. Growth was monitored by measuring dry cell weight (DCW).

Table 5.1: *Description of media composition of preliminary study*

Carbon source	Concentration C-source	Yeast extract [g/L]
Glycerol	20 g/L	8
Sodium acetate	20 g/L	8
Molasses	4 % (w/W) (20 g/L sucrose)	-
Molasses + YE	4 % (w/W) (20 g/L sucrose)	8
Pre-treated molasses	20 g/L glucose	8

5.2.5 Two stage fed-batch cultivation

Fed-batch cultures were carried out as described in Section 3.2.3.5. Modifications to the standard medium are represented in Table 5.2, as well as the percentage carbon of the substrate and the composition of the feed.

Table 5.2: *Description of media composition of two-stage fed-batch cultivations*

C-source	C [%]	C-source [g/L]	YE [g/L]	Feed [g/L]
Glycerol	39.1	40.92	16	350
Sodium acetate	29.3	20.00	8	125
Pre-treated molasses (Glucose)	40.0	10	4	100

5.3 Results and discussion

5.3.1 Preliminary batch study

The first step of this part of project aimed to define whether *Chlorella protothecoides* was able to grow on different sources of carbon such as glycerol, acetate (from sodium acetate) and molasses. Studies were carried out in 500 mL shake flasks containing 100 ml of fresh medium. Cell growth was monitored, growth rate and yields were to be compared.

According to the supplier, molasses contains raw proteins (135 g/kg), sources of nitrate (1.4

g/kg), nitrite (0.2 g/L) and various minerals.(Annexe 4) It was suspected that microalgae could metabolise nitrogen present in the various molecules, avoiding the need for supplementary yeast extract. Several studies were carried out: (A) *Chlorella* was cultivated in a 4% (w/w) molasses solution in DI water in which the sucrose concentration was about 20 g/L. In another flask, (B) a final concentration of 4% (w/w) molasses was prepared in a standard medium containing 8 g/L of yeast extract. In a third flask, (C) the effect of pre-treated molasses by acidic hydrolysis was studied. The glucose concentration - derived from acidic hydrolysis - was diluted to 20 g/L (Table 5.1). One of the objectives was to observe if cells grew better on sucrose or on glucose as main C-source. In all the studies, the concentration of substrates was kept at 20 g/L, use of low concentrations aimed to avoid undesirable inhibitory effects.

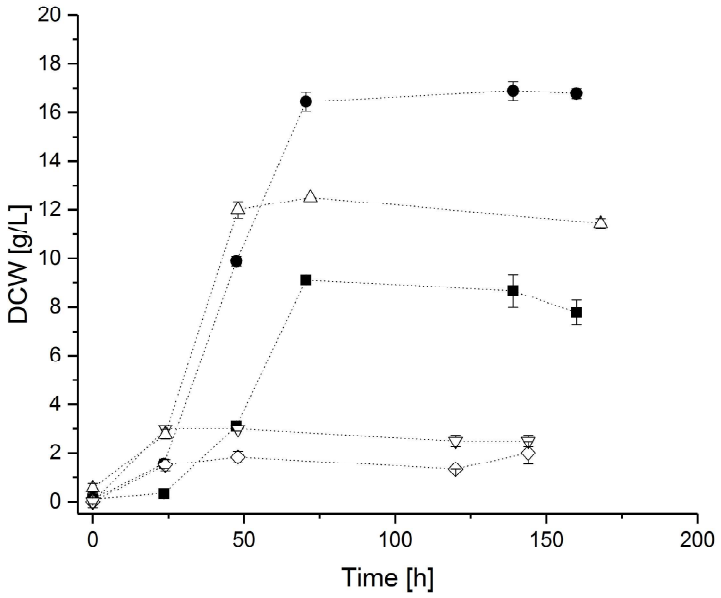


Figure 5.6: *Cultivation of* Chlorella protothecoides *under different carbon-sources:* • *glycerol* ■ *acetate,* △ *pre-treated molasses,* ∇ *molasses + YE,* ◇ *untreated molasses.*

At first glance, in Figure 5.6, microalgal cells grew well in glycerol (16.5 g/L), followed by pre-treated molasses (12.5 g/L) and acetate (9 g/L). The growth rate on glycerol and acetate was 0.092 h^{-1} and 0.070 h^{-1} respectively. Final biomass was < 4 g/L when cells were grown on untreated molasses, suggesting that sucrose might not be the ideal substrate to grow *Chlorella protothecoides*.

Biomass yield is the ratio of the amount of biomass produced to the amount of substrate consumed (g biomass/g substrate). It is has a considerable relevance to the economic evaluation of the biotechnological processes, as it depends strongly on the microorganism and on the growth substrates. In this study, biomass yields on carbon ($Y_{X/C}$) were evaluated and compared. Yield based on carbon is particularly important, considering that one of the main parameters for microalgae cultivation is the mass ratio between carbon and nitrogen (C/N ratio). Furthermore, it allows a better comparison of the different substrates. Glucose results were obtained from previous experiments represented in Chapter 3.

Table 5.3: *Comparison of biomass yields on carbon.*

	Glycerol	**Acetate**	**Glucose**
	$C_3H_8O_3$	$C_2H_3NaO_2$	$C_6H_{12}O_6$
Carbon [%]	39.125	29.282	40.001
$Y_{X/C}$ [gg^{-1}]	2.1	1.4	1.5

Table 5.3 shows that the addition of glycerol achieved the highest yield of 2.1 gg^{-1}. Glucose and acetate resulted in a lower yield of 1.4 gg^{-1} and 1.5 gg^{-1} respectively. Results of such a preliminary study were positive, showing that *Chlorella protothecoides* was able to metabolise glycerol and acetate while molasses needed to be pre-treated by acid-hydrolysis to hydrolyse sucrose into glucose prior to utilization. These alternative substrates were then used to develop two-stage fed-batch fermentations. The aim was to evaluate and compare results in term of biomass yield, lipid productivity and lipid profile.

5.3.2 Two-stage fed-batch cultivations

Optimization of a two-stage fed-batch cultivation of *Chlorella protothecoides* was extensively described in Chapter 3. Although glucose was the carbon source used, it is expected that the same mechanism could be repeated with alternative substrates. In the first phase, cell proliferation will occur under carbon limitation and under N-depleted conditions lipids are accumulated. In fact, with carbon substrate pulsed-feed, a high C/N ratio is formed in the culture medium, due the consumption of nitrogen and the new addition of substrate, resulting in a favorable condition for lipid accumulation within cells [250]. Differences among the substrates were expected in term of biomass yield, as seen preliminary studies reported in Table 5.3. The

description of the experiments is reported in Table 5.2.

5.3.2.1 Glycerol

Chlorella protothecoides was grown in an initial medium composed of 40.92 g/L of glycerol and 16 g/L of yeast extract (C/N 8.77). Feed was composed of 350 g/L of glycerol and concentrated salts. Yeast extract i.e source of nitrogen, was added only at start and not with the feed. Figure 5.7 represents the results of the culture.

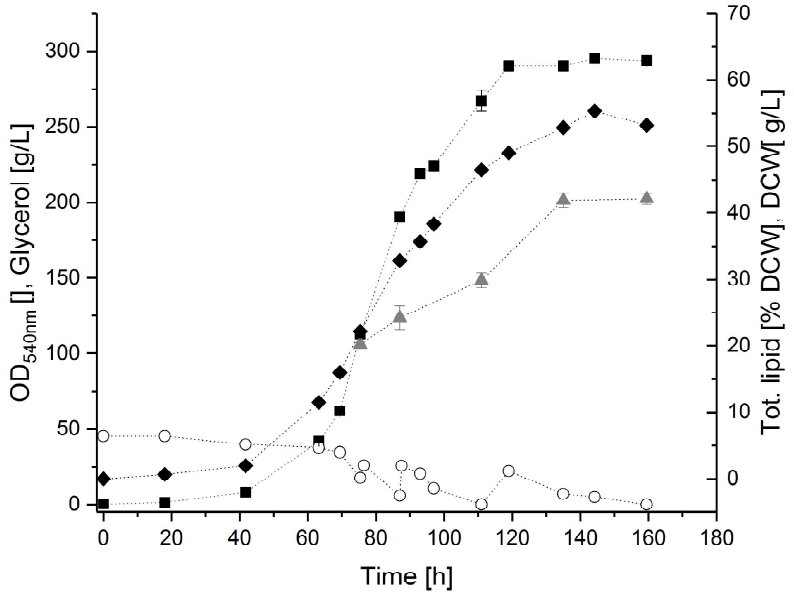


Figure 5.7: *Fed-batch cultivation of* Chlorella protothecoides *with glycerol as C-source. Results are represented as* ♦ *dry cell weight (DCW) in g/L,* ■ *optical density (OD_{540nm}) and* ◯ *glucose in g/L as a function of the cultivation time in h. Total lipids accumulation (▲) is represented as percentage of DCW. The DCW and the OD540nm were measured in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6), the standard deviation is represented as error bars.*

Fed batch started after 76 hours, when glycerol concentration was 17.5 g/L. During the batch phase, $Y_{X/C}$ was 2.04 gg^{-1}, such results are comparable to those obtained during the study in shake flasks of 2.10 gg^{-1}, demonstrating reproducibility of the experiments (Table 5.3). The maximum growth rate during the carbon limited phase was 0.076 h^{-1} and the maximal OD_{540nm} of 292 was achieved after 120 hours of fermentation, remaining constant until the end of the fermentation. Cell dry weight was 48 g/L after 110 hours and increased slightly up to 55.33 g/L after 145 hours.

Before the beginning of the feed, lipids were 20% of the dry cell weight and after the feed was started, it was suspected that C/N ratio increased gradually allowing to enhance a lipid accumulation up to a maximum of 42% after 135hours. Highest lipid productivity was 3.9 g/L/day after 135 hours. Optical density and dry cell weight show a linear correlation only up to 120 hours of cultivation, it is suspected that the further increase in DCW is related to an intracellular-lipid accumulation and not because of an increase in cell number. During the transition phase, where nitrogen is limiting but not completely depleted, the percentage of lipids in the biomass doubled between the beginning of the feed and 135 hours. Since the biomass yield on yeast extract ($Y_{X/YE}$) is 3 gg^{-1} (Figure 3.9, Chapter 3), from 16 g/L of yeast extract about 48 g/L of DCW are expected. Such values correspond to when the beginning of nitrogen limitation is suspected (i.e 70 hours). Furthermore, during the so-called transition phase, the color of cells changed from bright green to yellow, suggesting that nitrogen deficiency caused a sharp decline in chlorophyll content. Similar effect were observed previously in Chapter 3, when cells were cultivated on glucose (from 90 hours), with a similar mechanism of lipid accumulation appears to be repeated with glycerol [101]. These results suggest that that lipids are mainly accumulated during the transition phase between 75 hours and 135 hours.

5.3.2.2 Acetate

Preliminary studies showed the ability of *Chlorella protothecoides* to grow on acetate, therefore experiment were further optimized by carrying out a two-stage fed-batch cultivation. *Chlorella protothecoides* was initially cultivated in a medium composed of 20 g/L of acetate and 8 g/L yeast extract (C/N 6.42). Higher concentrations of acetate could not be prepared as an inhibitory effect on growth was observed (results not shown). Feed composed of 125 g/L acetate and concentrated salts was started after 56 hours and during the fermentation it was kept below 20 g/L for the same reason. Figure 5.8 represents the two-stage fed-batch cultivation of *Chlorella* on acetate.

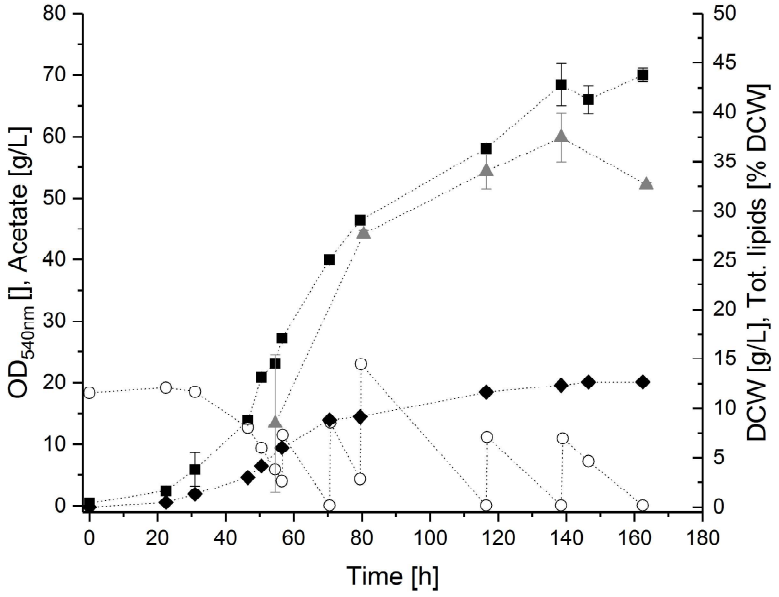


Figure 5.8: *Fed-batch cultivation of* Chlorella protothecoides *with acetate as C-source. Results are represented as* ♦ *dry cell weight (DCW) in g/L,* ■ *optical density (OD_{540nm}) and* ◯ *acetate in g/L as a function of the cultivation time in h. Total lipids accumulation (▲) is represented as percentage of DCW. The DCW and the OD540nm were measured in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6), the standard deviation is represented as error bars.*

Feed was started after 57 hours of fermentation when acetate was at 4 g/L. During the batch phase, $Y_{X/C}$ was 1.2 gg^{-1}, such a result is comparable to that obtained in batch cultures, as was the maximum growth rate of 0.074 h^{-1} (Table 5.3). Figure 5.8 shows that after 140 hours the OD_{540nm} achieved a maximum of 65-70 whereas DCW was at 8 g/L after 57 hours and increased slightly to a maximum of 12.5 g/L after 140 hours.

According to biomass yield on yeast extract ($Y_{X/YE}$ 3 gg^{-1}), estimated in Chapter 3, from 8 g/L of YE about 24 g/L of biomass were expected however, the result obtained is about 2-fold lower. Other factors might have restrained cell growth, accumulation of Na^{+} contained in sodium acetate might have had a negative effect on growth, since *Chlorella* is a fresh water microalgal strains. This theory is also sustained by the fact that relatively low concentration of sodium acetate ($\geq$ 20 g/L) are toxic for cells.

Despite the lower final DCW, dry biomass increased from 8.46 % total lipids (before the beginning of the feed) to a maximum total lipid composition of 37.45 % after 140 hours. It was

suspected that C/N mass ratio increased due to the acetate feed, allowing to accumulate lipids under a nitrogen-limitation phase. Other typical signs of N-limitation where identified in the change of microalgal color from green to yellow after about 100 hours and in the correlation between OD and DCW, which was non-linear after 116 hours (Figure 5.8) The highest lipid productivity was 0.82 g/L/day calculated after 116 hours.

5.3.2.3 Molasses

Preliminary studies developed in shake flasks, showed that sucrose was not consumed by *Chlorella protothecoides*. Acid treatment of molasses was necessary to hydrolyse sucrose into fructose and glucose. The aim of this experiment was to perform a fed-batch cultivation using as substrate, glucose derived from hydrolized sucrose contained in molasses. Initial medium was prepared with a pre-treated solution of molasses diluted in standard medium to a final concentration of 10 g/L of glucose; yeast extract concentration was 4 g/L yeast extract (C/N 8.77). Feed was composed of 50% (w/w) pre-treated molasses, in which the glucose concentration was 100 g/l glucose. In the initial medium and in the feed, no further salts were added, due to an already high concentration in minerals of molasses. Figure 5.9 represents the two-stage fed-batch cultivation of *Chlorella* on glucose derived from molasses.

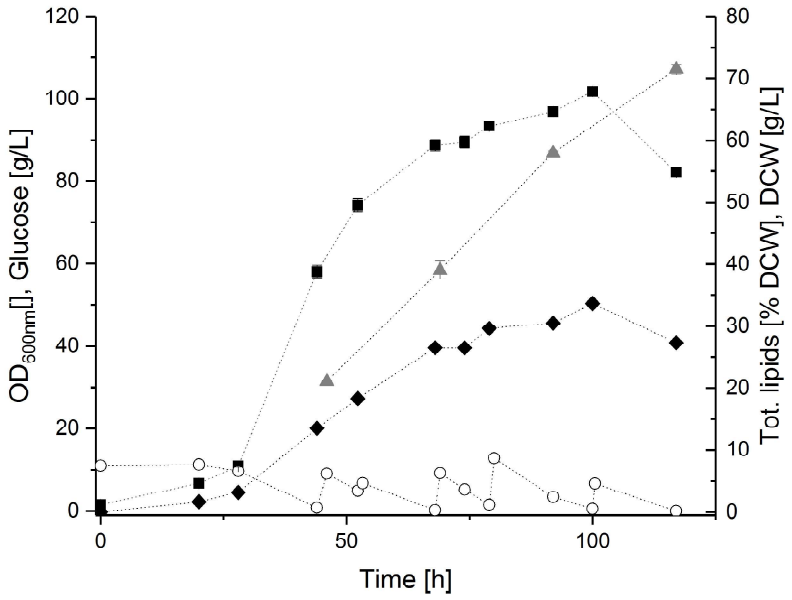


Figure 5.9: *Fed-batch cultivation of* Chlorella protothecoides *with glucose from molasses as C-source. Results are represented as ♦ dry cell weight (DCW) in g/L, ■ optical density (OD_{540nm}) and ◯ glucose in g/L as a function of the cultivation time in h. Total lipids accumulation (▲) is represented as percentage of DCW. The DCW and the OD540nm were measured in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6), the standard deviation is represented as error bars.*

The feed was started after 46 hours, when the glucose concentration was close to 0 g/L. The maximum growth rate was 0.08 h^{-1} whereas growth rate of *Chlorella* grown on an equal mass of pure glucose (10 g/L) was close to 0.12 h^{-1} (Chapter 3). This difference may be due to the richer composition of molasses.

The OD_{540nm} achieved a value of 80 after 68 hours, increasing slightly to a maximum of 101 after 100 hours, whereas DCW achieved a maximum of 30-34 g/L after 100 hours. Very promising results were obtained in terms of lipid production: before the beginning of the feed, cell dry weight was composed of 21% total lipids, which increased up to 71% at the end of the cultivation. A critical evaluation results in the conclusion that the maximum lipid percentage of biomass is between 58% and 71%. Such result is very high, in Chapter 3 maximum lipid composition was 55.18 % of biomass. Highest lipid productivity was 4.6 g/L/day after 92 hours. The expected level of biomass from 4 g/L of yeast extract (12 g/L DCW), changes in microalgae color, relation between OD_{540nm} and DCW indicated that the transition phase, favorable to lipid accumulation, seems to begin after about 52 hours. HPLC analysis of the

cultivation medium, showed that since sucrose was not consumed it was accumulated in the medium however, in addition to glucose, fructose appeared to be consumed during growth.

This study examined the use of glycerol, acetate and pre-treated molasses as an alternative carbon source for *Chlorella protothecoides* growth. For a better comparison, in all fed-batch batch experiments, yeast extract was supplemented only at the start and was not added with the feed. The main results are reported in Table 5.4.

Table 5.4: *Comparison of* Chlorella protothecoides *grown on different C-sources.* $Y_{X/C}$ *was calculated during the batch part i.e before the addition of the feed, while* $Y_{lipid/S}$ *was calculated from the total amount of substrate used for the experiment; n.d not determined.*

	Glycerol	**Acetate**	**Pre-treated molasses**
Growth rate [h^{-1}]	0.076	0.074	0.080
Tot. lipids [% DCW]	42	37.5	> 58
$Y_{X/C}$ [gg^{-1}]	2.04	1.43	n.d
$Y_{lipid/S}$ [gg^{-1}]	0.13	0.025	0.19

Overall results were positive; *Chlorella protothecoides* was able to take-up and grow rapidly on the different carbon sources. The doubling time was 9.12 hours on glycerol, which is only 1.4-fold lower compared to glucose on an equal amount of initial substrate (μ 0.11 h^{-1}). Cells grown on 10 g/L of glucose derived from molasses showed a similar growth rate (0.080 h^{-1}) however, such results are lower when compared to the same amount of "pure" glucose, probably because of the rich and complex composition of molasses.

Among the various carbon sources tested, pre-treated molasses resulted in the highest total lipid accumulation in biomass (58%), followed by glycerol with 42%. Lipid productivity was maximum at 4.6 g/L/day for pre-treated molasses and 3.9 g/L/day for glycerol. Results were considerably lower when acetate was used as substrate, DCW was composed of 37.45% of total lipids with a maximum productivity of only 0.82 g/L/day. The cause of the low productivity is related to the low biomass achieved, 12.6 g/L. Biomass on acetate yield is lower compared to other substrates.

Biomass yield on carbon was calculated for every cultivation in the batch phase. This value allows to compare the uptake of carbon for every substrate used. The best yield was obtained using glycerol 2.04 gg^{-1}, which is 1.2-fold higher then with glucose as substrate ($Y_{X/C}$ 1.65 gg^{-1} glucose). On the other hand, acetate was not as good, showing a yield of 1.43 gg^{-1}. In experiments where pre-treated molasses was used, the yield on carbon was not estimated, more experiment are needed to define the repartitioning of the two carbon sources, fructose and glucose.

In general, all cells need energy for anabolism i.e. synthesis of complex molecules, as well as for cell maintenance. In the case of lipid accumulation, product formation is directly coupled to energy metabolism. Lipids are defined as growth-linked products, being dependent on biomass itself [251]. The accumulation of lipids from carbon substrates occur as a mean of creating an energy deposit that can be readily utilized in response to a more favorable environment allowing for rapid growth [252] [253]. Part of the substrate is used by the cell to carry out other metabolic activities and complete conversion of carbon into lipids is unachievable. Studies showed the Krebs cycle, part of cellular respiration and fatty acid (FA) synthesis share the same molecules of acetyl-CoA. As a consequence, lipid yield on substrate will always be lower than 100%. Table 5.4 shows that approximately 13% of glycerol and 19% of glucose from molasses were converted to lipids whereas only 2.5 % of acetate was transformed into the bio-product. An optimized two-stage fed-batch culture on glucose resulted in a conversion of 12% (Chapter 3). Chen et al. studied lipid accumulation under heterotrophic conditions of *Chlorella zofingiensis*. The lipid yield on glucose was maximum at 0.18 gg^{-1} after 4 days, when cells were composed of 40% lipid and biomass was close to 8 g/L. After 6 days of culture lipid yield decreased constantly to a constant value of 0.13 gg^{-1} until the end of the culture. Such results are in the same range of this study [254]. When growing *Chlorella protothecoides* on glycerol, O'Grady et al. obtained a specific growth rate and a lipid yield of 0.1 h^{-1} and 0.31 g lipid/g substrate. Conditions were more favorable to those observed in growth on glucose alone (0.096/h and 0.24 g lipid/g substrate) [217]. The hypothesis is that in the first part of the culture (C-limited medium), substrate is consumed to achieve high cell densities. The percentage of lipid in the biomass is usually lower than 20%. In the second stage of the process - during the nitrogen-limitation phase - most of the carbon source is converted to lipids, $Y_{lipid/S}$ is higher in that part.

An improved two-stage fed-batch fermentation, characterized and developed in Chapter 3 (Section 3.3.5), resulted in 255.3 g/L (SD=2.828 10^{-3} g/L, n=2) composed for a maximum of 58.10% (SD = 0.9332%, n=6), which resulted in a maximum lipid productivity of 16.6 g/L/day. Such high results were obtained by feeding more nitrogen in the medium after the batch phase. In this way, the carbon limited phase and the transition phase were expected to be prolonged allowing the achievement of higher cell densities and higher lipid accumulation. Indeed, in the two-stage fed-batch where no additional nitrogen was added, biomass was 1.7-fold lower. It is expected that cultures carried out with alternative substrate will improve by adding further nitrogen.

5.3.3 Total lipid profile comparison

Different metabolic pathways in the uptake of alternative substrates are expected to show differences in lipid production. Indeed, the aim of the experiment was to define whether the lipid profile is algae-specific or it can be influenced by the substrate change. Furthermore, the variation of lipid profile was defined over the course of fermentation. Total lipids were extracted at different times during the course of fermentation, then derivatized into fatty acid methyl esters (FAME) and analysed by GC-FID. (Section 6.2.2 and 6.2.3)

In Figures 5.10 to 5.13 is represented the relative composition of the main fatty acids present in *Chlorella protothecoides* cultivated with different substrates: glucose, glycerol, acetate and molasses and at different times of the cultivation. The first bar in each graph represents values of the last sample taken before the beginning of the feed, meaning before the beginning of the nitrogen limitation. Generally it is possible to notice that the lipid profile does not appear to change over the course of the fermentation. However, it appears that the quantity of lipids increased over time, as well as the ratio between the lipids produced.

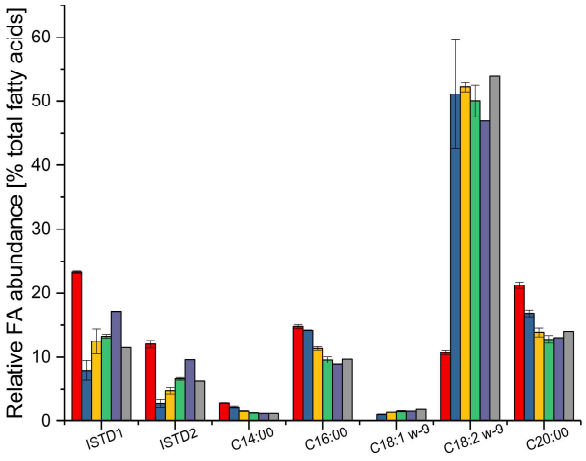


Figure 5.10: *Relative fatty acid (FA) composition of* Chlorella protothecoides *cultivated in glucose. Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars. The fatty acids reported are the following: myristic acid (C14:00), palmitic acid (C16:00), oleic acid (C18:1 ω-9), linoleic acid (C18:2 ω-9) and arachidic acid (C20:00).*

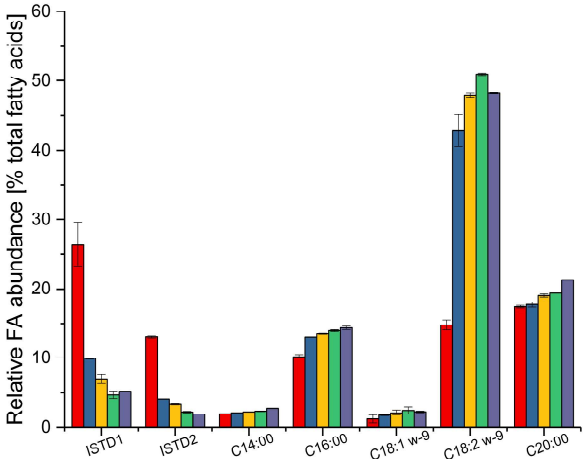


Figure 5.11: *Relative fatty acid (FA) composition of* Chlorella protothecoides *cultivated in glycerol. Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars. The fatty acids reported are the following: myristic acid (C14:00), palmitic acid (C16:00), oleic acid (C18:1 ω-9), linoleic acid (C18:2 ω-9) and arachidic acid (C20:00).*

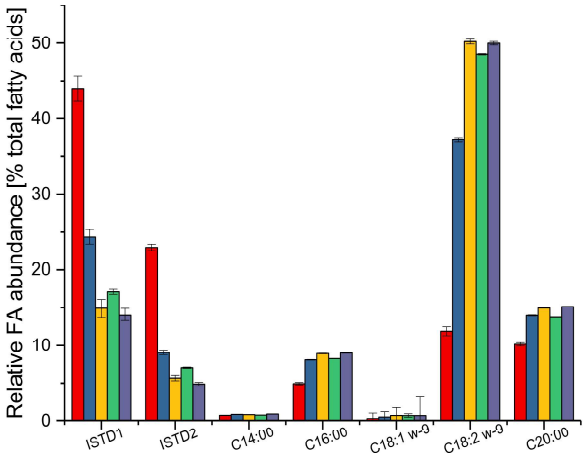


Figure 5.12: *Relative fatty acid (FA) composition of* Chlorella protothecoides *cultivated in acetate. Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars. The fatty acids reported are the following: myristic acid (C14:00), palmitic acid (C16:00), oleic acid (C18:1 ω-9), linoleic acid (C18:2 ω-9) and arachidic acid (C20:00).*

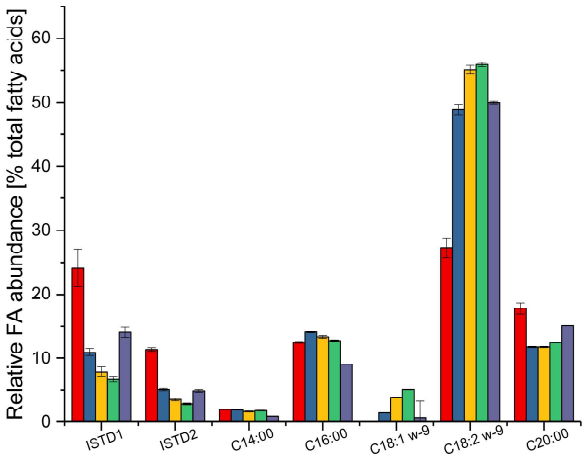


Figure 5.13: *Relative fatty acid (FA) composition of* Chlorella protothecoides *cultivated in pre-treated molasses. Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars. The fatty acids reported are the following: myristic acid (C14:00), palmitic acid (C16:00), oleic acid (C18:1 ω-9), linoleic acid (C18:2 ω-9) and arachidic acid (C20:00).*

The amount of the two different internal standards, pentadecane ($C_{15}H_{32}$, ISTD1) and methyl tridecanoate (C13:0 methy esther, ISTD2) decreased as more other fatty acids accumulated.

It was found that palmitic acid (C16:0), oleic acid (C18:1 ω-9) and linoleic acid (C18:2 ω-6) were the major fatty acids, which corresponds to what reported in the literature [255] [61] [250]. Oleic acid was predominantly produced, increasing up to more than 50% under every cultivation conditions. Palmitic acid and linoleic acid were also accumulated to high levels, except when glucose (comprised molasses) was used as carbon source; in this case it appeared to decrease after nitrogen limitation began.

As an example, in Table 5.5 reports the composition of a sample of *Chlorella protothecoides* cultivated in glucose. The methods is described in Section 6.2.4. The 52.7% of the total lipid composition may corresponds to other lipid compounds such as carotenoids, glycolipids, etc. Oleic acid was the main fatty acid (30.6%) followed by linoleic acid (9.2%) and palmitic acid (5.7%).

Table 5.5: *Lipid composition of* Chlorella protothecoides *cultivated in glucose. The fatty acids reported are the following: myristic acid (C14:00), pentadecanoic acid (C15:00), palmitic acid (C16:00), palmitoleic acid (C16:1 ω-7), heptadecanoic acid (C17:00), stearic acid (C18:00), oleic acid (C18:1 ω-9), linoleic acid (C18:2 ω-9) and arachidic acid (C20:00).*

FAME	**Quantity of FAME** [μg]	**Composition** [%]
C14:00	77.8 $\pm$ 0.7	0.6
C15:00	18.9 $\pm$ 1.1	0.1
C16:00	792.6 $\pm$ 30.2	5.7
C16:1	60.4 $\pm$ 2.9	0.4
C17:00	19.8 $\pm$ 2.8	0.1
C18:00	39.3 $\pm$ 11.8	0.3
C18:1 ω-9	4282.4 $\pm$ 285.9	30.6
C18:2 ω-6	1292.5 $\pm$ 83.0	9.2
C20:00	35.3 $\pm$ 3.2	0.3
Others	-	52.7

5.4 Conclusion

The use of glycerol, acetate and molasses as alternative carbon sources, showed potential for the heterotrophic cultivation of *Chlorella protothecoides*. Glycerol was shown to be a very promising substrate with the biomass on carbon yield of 2.04 gg^{-1}, being almost 24% higher than that obtained with glucose i.e $Y_{X/C}$ 1.65 gg^{-1}. Final biomass attained was 55.33 g/L after

145 hours and was composed of 42% total lipids. Lipid productivity was maximum after 135 hours with a value of 3.9 g/L/day. Since glycerol is a low-value by-product from biodiesel production, it could provide a suitable, inexpensive substrate for the heterotrophic growth of microalgae.

Molasses, a by-product of the sugar production, is rich in sucrose. An acidic hydrolysis was necessary to hydrolyse it into glucose and fructose. *Chlorella* was able to grow principally on glucose resulting in a final biomass of 34 g/L after 100 hours, this biomass was composed of more than 58% lipids. Lipid productivity was maximum at 4.6 g/L/day after 92 hours. The rich composition of molasses in terms of minerals was suspected to inhibit growth at higher concentrations, as a results the concentration of glucose was maintained below 10 g/L.

Acetate was shown to be less effective compared to other substrates tested. After 140 hours, only 12.5 g/L of biomass were produced with a total lipid composition of 37.45%. Consequently lipid productivity was very low, with a value of 0.82 g/L/day.

In general, nitrogen limitation resulted in an increased lipid accumulation despite the use of different carbon sources. It is expected that even better results could be achieved with these substrates by optimizing the fed-batch process, as carried out previously with glucose in Chapter 3. It was shown that by adding more nitrogen after the batch cultivation phase, the transition phase, in which cells continue to proliferate and at the same time accumulate higher amounts of intracellular lipids, could be prolonged.

Analysis of fatty acids composition indicates that the lipid profile appears to be algae specific, with certain fatty acids produced and others consumed, depending on the carbon-substrate. The main fatty acids produced were oleic acid, linoleic acid and palmitic acid.

The capacity of growing on different carbon sources allows for an increased flexibility in the methods of production of *Chlorella protothecoides*. Alternative carbon sources in heterotrophic cultivation of microalgae reduce the cost of substrate, which is one of the most significant costs, demonstrating a partial solution to the difficulties currently facing the microalgae production.

Chapter 6

Analysis and optimization of production of omega-3 fatty acids in *Schizochytrium* sp.

6.1 Introduction

In the last few years, there has been an increased interest in using microalgal lipids in various industries, in particular in food products, nutraceuticals and animal feeds [143]. Microalgal oil is particularly rich in long chain polyunsaturated fatty acids (lcPUFAs), which are important constituents of human diets [256].

Among the PUFAs are found omega-3 fatty acids, which include alpha-linolenic acid (ALA, 18:3 n-3) and the longer chain PUFAs eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid DHA (22:6 n-3). ALA is typically found in plants such as walnuts, flaxseed oil, soybean, and canola oil; since the human body cannot synthesize it, it is considered as an essential fatty acids [1] [96]. ALA can be converted by the body into EPA and DHA, but the amounts are not significant [143] [96] [144]. DHA is the most abundant component of structural lipids in the brain, it has important functions in the retina and its consumption has been attributed to anti-inflammatory responses. It is also involved in the prevention of cardiovascular and neurological diseases [77] [230]. EPA has been shown to be beneficial in coronary heart disease, high blood pressure, anticoagulation and in some inflammatory disorders such as rheumatoid arthritis [144].

The main sources of omega-3 LC-PUFA are cold-water fish and their oils [257]. As for humans, fish have a limited capacity to synthesize LC-PUFAs, therefore they have to acquired them through the consumption of zoo-plankton and microalgae. Moreover, the quality of the fish oil is variable, it can contain environmental pollutants such as heavy metals, and the typical fish smell and unpleasant taste are not suitable for many industrial preparations [258]. The requirements for alternative and more environmental friendly sources of LC-PUFAs is clear [257] [141]. Marine Thraustochytrid and in particular *Schizochytrium* strains are reported to contain a higher proportion of DHA in total fatty acids [259] [67].

Chapter 4 focused on the optimization of the production of high cell densities of *Schizochytrium* sp. as a pre-requisite for high lipid productivity. The results were very successful showing high biomass production (90 g/L) with a composition of total lipids up to 70%. The total lipid productivity was 9-10 g/L/day. Such high results provide an opportunity to investigate the production potential of valuable bio-products such as DHA and EPA.

The present chapter is focused on answering the following questions:

- Which other conditions stimulate total lipid accumulation?
- What influences the production of Omega-3 fatty acids, in particular DHA and EPA?
- Can the lipid profile be influenced or is it algae-specific?

Two-stage fed-batch cultivations of *Schizochytrium* were extensively described in Chapter 4 and it was found that nitrogen limitation could trigger microalgal lipid accumulation. Sun et al. reported that nitrogen limited conditions could also help to improve the DHA content of lipids [195]. Ren et al. described that the DHA percentage of total fatty acids fluctuated with the glucose addition, indicating that glucose feeding would influence for DHA production [188].

In this study, the strategy was to maintain the procedure of the developed two-stage fed-batch process and combine it with other conditions that were anticipated to enhance PUFAs accumulation, in particular of omega-3 fatty acids, DHA and EPA. Two-stage fed-batch cultivation developed in Chapter 4 was shown to be very efficient for total lipid productivity therefore it was also expected to achieve a high DHA productivity. The different conditions tested with the

aim of enhancing specific omega-3 fatty acid production are outlined below.

For better comparison, all of the fed-batch experiments are going to be prepared with the same initial medium composition (40 g/L glucose and 16 g/L yeast extract). The nitrogen source was added only at start and it was not fed during the cultivations. Results reported in this Chapter were compared to the experiments carried out in Section 4.3.4.1 (Chapter 4). The additional conditions are going to be applied after the end of the batch phase, when the medium was suspected to enter the so-called transition phase. Here, the nitrogen was limiting but not totally depleted (C/N $>$ 12) and total lipid accumulation was enhanced.

6.1.0.1 Effect of Oxygen Radicals

Reactive oxygen species (ROS) are chemical compounds that are normally formed as by-products of aerobic cellular metabolism. They include free radicals, such as superoxide anion, hydroxyl radical and non-radical molecules like hydrogen peroxide, singlet oxygen, etc (Figure 6.1). They are mainly formed during the electron transport of oxygen in mitochondria, chloroplasts or plasma membranes. Under normal conditions they are involved in cell signaling, playing an important role in the immune system and other physiological processes. However, when cells are subjected to environmental stress such as UV, heat exposure, salinity, heavy chemicals, the ROS concentration can increase becoming extremely harmful for cells. This state is defined as "oxidative stress" and the ROS can cause peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to death of the cell [260] [261] [262].

Studies showed that in diatoms and green algae, nitrogen depletion resulted in increased lipid accumulation, but also of ROS species [263] [264]. The hypothesis is that there is a connection between the increase of ROS and lipid accumulation in algae species. Yilancioglu et al. showed that in the microalgal strain *Dunaliella salina*, nitrogen limitation increased cellular lipid content up to 35% of dry cell weight and they observed an increased oxidative stress by measuring important markers such as malondialdehyde, catalase, ascorbate peroxidase and superoxide dismutase antioxidant enzymes. In these experiments, when oxidative stress was directly induced by the addition of H_2O_2, cellular lipid content increased up to 44% [263]. Exogenous application of hydrogen peroxide (H_2O_2) was also studied by Burch et al., showing

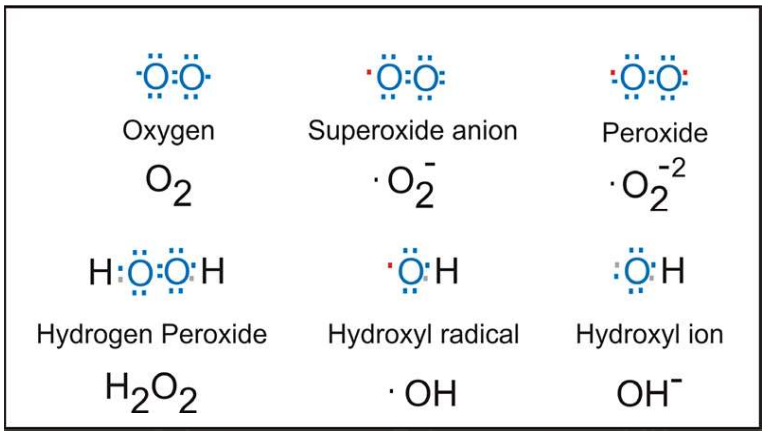


Figure 6.1: *Examples of Reactive Oxygen Species (ROS)*

an increase in neutral lipid production in *Phaeodactylum tricornutum*. Treatment with H_2O_2 increased the total levels of neutral lipids harvested, from 64 mg/L to 96 mg/L [264].

The effect of H_2O_2 is going to be tested not only to increase lipid production but because it is suspected that this could favourise the specific accumulation of lcPUFAs. In fact, PUFAs are usually used by the cells to repair membrane damage and, since they contain many double bonds, they also act as antioxidants by scavenging free radicals [114].

6.1.0.2 Effect of low temperature

Every microorganism has specific growth temperature requirements, which are largely determined by those of its enzymes. Temperature is considered to be a major factor for cell growth and PUFAs synthesis [256]. It has been shown that a shift to lower temperature varied the composition of the cell membrane [96]. The hypothesis was that this occurred as a response to adjust the membrane fluidity of photosynthetic membranes, in fact PUFAs are important for adjusting membrane fluidity in cell membranes [96]. In general, cells can alter the composition of their membranes to maintain fluidity across a range of temperatures. The tails of phospholipids are composed of fatty acids, which have different levels of mobility in the membrane. As a response to low temperatures, the composition of fatty acids changes with an increased amount of unsaturated fatty acids. The double bonds of unsaturated fatty acids have kinked hydrocarbon tails which are more difficult to pack together [214], as represented in Figure 6.2 [265].

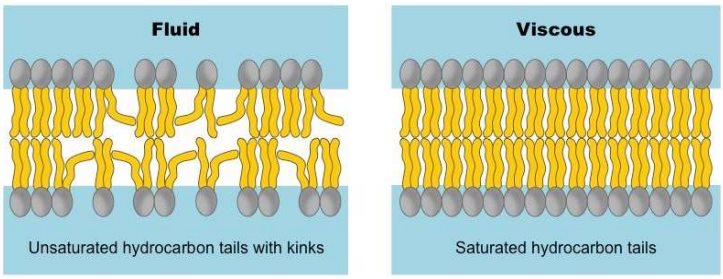


Figure 6.2: *Representation of the fluidity of a phospholipid bilayer of a cell membrane, depending on the length and relative saturation of the fatty acid tails.*

Studies reported that lowering the cultivation temperature can be applied to trigger PUFA production in microalage [114]. Jiang et al. showed that a temperature-shift from 25°C to 10°C enhanced the proportion of PUFAs, especially EPA in *P. tricornutum* [266]. *Pavlova lutheri* increased the content of EPA by 50% when the culture temperature was reduced to 15°C [114]. Temperatures between 25°C and 30°C are generally indicated for optimal growth in *Schizochytrium* species [71] [70] however, it is reported that low temperatures stimulate PUFA production and in particular DHA [114] [260]. The strains of *Schizochytrium* isolated from cold water, contained the largest amounts of PUFA, this characteristic has been noted for other organisms from low-temperature environments [81].

6.1.0.3 Effect of salt concentration

The osmotic pressure plays significant roles in cell growth and the cell osmotic adjustment is one of the most important biological processes, especially for marine microorganisms [81] [267] [268]. According to Fan et al., Thraustochytrids are found in a wide variety of marine environments with different total salt concentrations. Some strains isolated from mangrove environments grew better in low salt concentrations of 5-8 g/L whereas for other strains, it was found that the optimum salt concentrations was between 15-30 g/L [268]. Despite, the wide tolerance of salinity of *Schizochytrium* strains [230], in the study of Hong et al. it was reported that the concentration of NaCl in the medium appears to affect the DHA yield. The concentration of NaCl was optimum at 1.50% and with higher concentrations (3.00%), the DHA yield decreased from 1.23 g/L to 0.5 g/L [256]. In the study of Chen et al., *Schizochytrium* sp. S056 highest biomass and DHA yield (34.76 and 6.61 g/L, respectively) were obtained when sea salt concentration was 2% [269]. Lowering salt concentrations to the minimum required may be beneficial to maximize biomass and the biosynthesis of DHA [270] [267] [70]

[256]. High composition of PUFAs in the cell membrane is important for adjusting membrane fluidity not only with low temperatures but also during shifts in salinity (Figure 6.2).

6.2 Material and methods

All the methods describing the cultivation of *Schizochytrium* and the analysis of the cell wall are described in Section 3.2 in Chapter 3 or in Section 4.2 of Chapter 4.

6.2.1 Materials and microalgal strain

All chemicals were purchased from either Sigma Aldrich (St. Louis, USA) or Thermo Fisher Scientific (Waltham, USA) if not stated otherwise. *Schizochytrium* sp. (ATCC 20888) was obtained from the ATCC (Manassas, USA) culture collection.

6.2.2 Preparation of fatty acid methyl esters

A commercial solution of HCl 37% was diluted with 41.5 ml of methanol to a total volume of 50 mL in a graduated flask. Extracted microalgal oil was dissolved in 200 μL of toluene (recommended final concentration of 0.010 g/ 200 μL). To the lipid solution, 1.5 mL of methanol, 0.30 mL of 8% HCL and 50 μL of 10 g/L methyl tridecanoate (C13:0 methyl ester, Matreya, USA) were added in the order described. The tube containing the mixture was covered with silver foil and parafilm and vortexed. The tube was placed in a water bath at 45°C and incubated overnight for 14 hours. The tube was then cooled at room temperature and 1 mL of hexane and 1 mL of DI water were added, mixed well and centrifuged at 1000 rpm for 5 min (FL40R, Thermo Fisher Scientifi, Waltham, USA). The upper layer (850 μL) composed of hexane containing the fatty acid methyl esters (FAMEs) - were extracted and mixed with 50 μL of 10 g/L pentadecane (Thermo Scientific, Waltham, USA) in a GC vial and analysed by GC-FID (Section 6.2.3). A reagent blank, along with the samples, was prepared with the internal standards. The method was described in the publication of Ichichara et al. [271].

6.2.3 Gas chromatography with Flame Ionization Detector analysis

The method was adapted from the one developed by the U.S. National Renewable Laboratory [272].The FAMEs were separated by gas chromatography (Thermo Trace 1300, Thermo Scientific, Waltham, USA) and analyzed by flame ionization detector (FID) (Thermo Scientific,

Waltham, USA). The column ZB-Wax (Phenomenex, Torrance, USA) of length 30 m x 0.25 mm and 0.25 μm film thickness was used with the following temperature program: 100°C for 1 min, 25°C /min up to 200°C, hold 1 min, 5°C/min up to 250°C, hold 7 min (Estimated time per sample, 23 min). The split ratio was 10:1 at 10 ml/min, the injection volume was 1 μl with an inlet temperature of 250°C. The carrier gas was helium at a constant flow of 1 mL min. The detector gases were: 350 ml/min air, 40 ml/min H_2 and 35 ml/min hydrogen and the temperature detector was set to 280°C. The measurements were repeated in triplicate (n = 3) to check the reproducibility of the results, the standard deviation error (SD) is reported in the experimental results or represented graphically as error bars.

6.2.4 Determination of total fatty acids

The analysis of total fatty acids was carried by the HEIA-FR (Haute ecole d'ingenierie et d'architecture de Fribourg, Switzerland). The total fatty acids were analyzed using a standard mix of 19 FAME (500 μL, CRM47801, Sigma Aldrich, USA). The comparisons of the lipid samples co-injected with the FAME mix are found in Annexe 7.2 and 7.3. The FAME mix did not contain DHA and EPA fatty acids.

6.2.5 Determination of DHA and EPA

Methyl tridecanoate (Matreya, State College, USA) was used as internal standard. The calibration standards of DHA and EPA fatty acids were in a methylated form purchased also from Matreya (Matreya, State College, USA). The standard calibration curves of DHA methyl ester and EPA methy ester are represented in mol/L in Figure 6.3 and Figure 6.4.

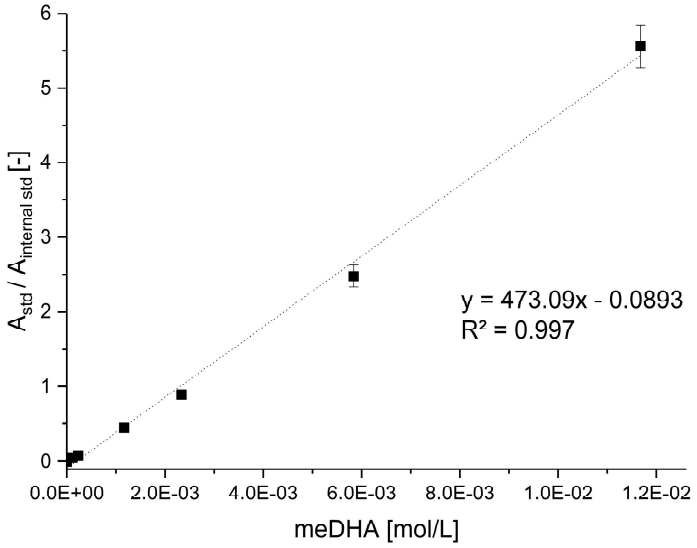


Figure 6.3: *Representation of the standard calibration curve of meDHA by GC. The samples were prepared in hexane and the experiments were repeated three times (n=3) and the standard deviation error is represented.*

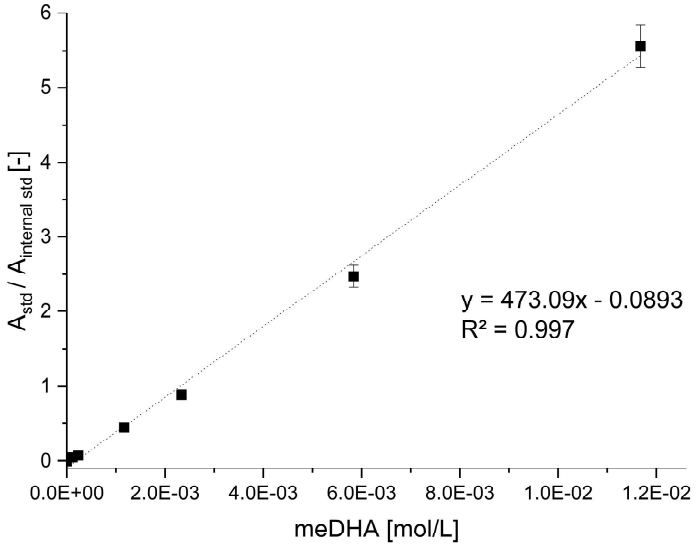


Figure 6.4: *Representation of the standard calibration curve of meEPA by GC. The samples were prepared in hexane and the experiments were repeated three times (n=3) and the standard deviation error is represented.*

Equations 6.1 to 6.3 describe the steps necessary to calculate the percentage of DHA or EPA in the lipids.

$$Area_{FAME,normalized} = \frac{A_{FAME}}{Area_{int.std}} \tag{6.1}$$

where:

$\text{Area}_{FAME,normalized}$ = Corrected peak area

A_{FAME} = Area of analyte from the sample chromatogram

$\text{Area}_{int.std}$ = Area of internal standard (methyl tridecanoate) from the sample chromatogram

$$Conc._{FAME,sample} = (A_{FAME,normalized} - b) \cdot a \tag{6.2}$$

where:

$\text{Conc.}_{FAME,sample}$ = concentration of methylated fatty acid in the GC sample [mol/L]

a = slope of the methylated fatty acid calibration curve

b = y-intercept of the methylated fatty acid calibration curve

$$\%FA = 100 \cdot (Conc._{FAME,sample} \cdot Dilution \cdot MM_{FA} \cdot V_{toluene})/Tot.lipids \tag{6.3}$$

where:

FA = Percentage of fatty acid in total lipids [%]

Dilution = $\text{V}_{hexane}/\text{V}_{toluene}$ = 6.25 [-]

$\text{MM}_{FattyAcid}$ = Molar mass of fatty acid [g/mol]

$\text{V}_{toluene}$ = $2 \cdot 10^{-4}$ [L]

Tot. lipids = Amount of derivatized total lipids [g]

As an example, in Figure 6.5 is reported a GC-FID chromatrogram of a derivatized commercial sample of microalgal oil of *Schizochytrium* sp. (Omega-3, Actilife, Migros, Zuerich, CH).

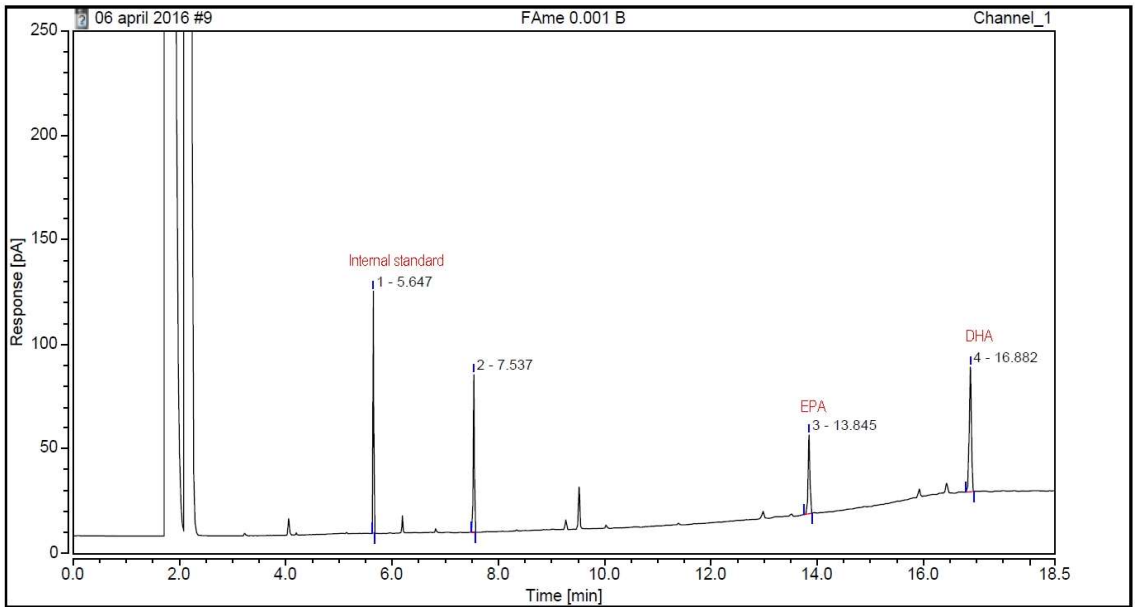


Figure 6.5: *Representative GC-FID chromatogram of a commercial solution of microalgal oil from* Schizochytrium *sp. (Omega-3, Actilife, Migros, Zuerich, CH) The peaks corresponding to EPA and DHA are represented.*

6.2.6 Proton nuclear magnetic resonance (H-NMR)

Samples of derivatized microalgal fatty acids were analyzed by nuclear magnetic resonance (H-NMR) to estimate the yield of the trans-methylation reaction of fatty acids. It is important to verify whether the derivatization reaction is complete, since the aim is to quantify DHA and EPA by gas chromatography. NMR spectroscopy is an analytic technique that allows to determine the content and purity of a sample, as well as its molecular structure. Information about the nucleus chemical environment can be derived from its resonant frequency, which is represented by the peaks of the chromatograms. The analysis was performed by the analytical service of the HEIA-FR (Haute ecole d'ingenierie et d'architecture de Fribourg, Switzerland) and the chromatograms of two derivatized samples of *Schizochytrium* oil are represented in Figures 6.6 and 6.7.

Figures 6.6 and 6.7 show a full-range H-NMR spectra of derivatized microalgal lipids samples. For the first sample, 0.008 g of *Schizochytrium* total lipids were derivatized and extracted in 1 mL of hexane, whereas for the second, the initial concentration of lipids was 0.024 g. The derivatization method is described in Section 6.2.2.

Fatty acids are found in the cells in the form of triglycerides (TAGs), therefore the derivatization procedure involves first the hydrolysis of triglycerides into glycerol and fatty acids and then the methylation of the free fatty acids (Figure 6.8). When the derivatization of triglycerides is

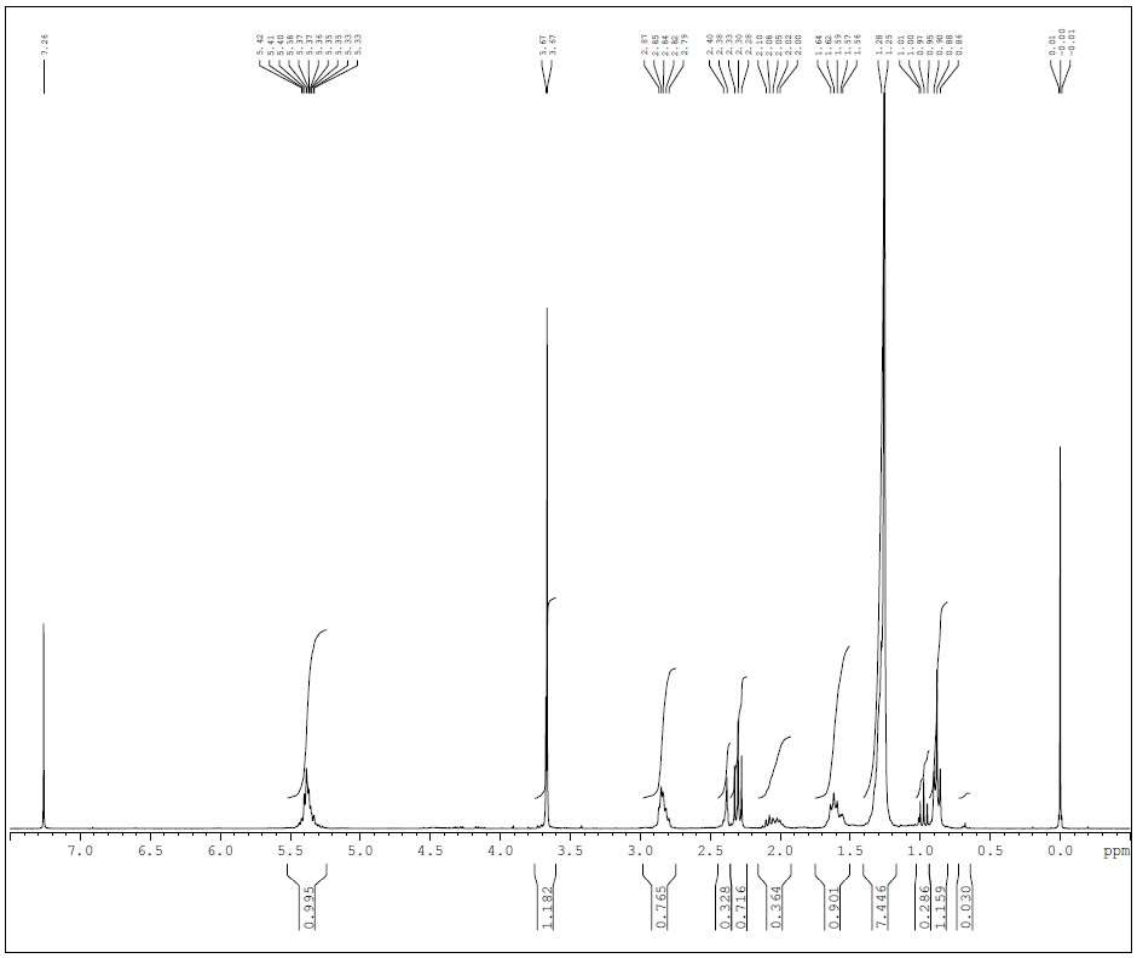


Figure 6.6: *H-NMR spectra of 0.008 g/mL derivatized microalgal lipids from* Schizochytrium *sp. . The analysis was performed by the analytical service of the HEIA-Fr, Switzerland. Peaks characteristic of glycerol residues (4.1 ppm, 4.3 ppm and 5.26 ppm) are not present. At 3.7 ppm it can be seen the peak corresponding to the chemical shift of the ester methoxy group which indicates the presence of methyl fatty acids.*

complete, characteristic peaks corresponding to molecular structures found in glycerol or in triglycerides should not be present. It is important to mention that free glycerol is soluble in water and it should not be contained in the hexane layer after extraction (Section 6.2.2). The samples were washed twice with hexane prior to analysis in order to remove glycerol and other impurities. Results represented in Figure 6.7 shows that peaks appear at 4.1 ppm and 4.3 ppm. This is where H-1 and H-3 protons of glycerol residues (glyceridic protons) have a shift, with the corresponding CH at 5.26 ppm (Figure 6.8). However, peaks at 4.1 ppm and 4.3 ppm are not found in the sample represented in Figure 6.6. In both samples is found the peak corresponding to the chemical shift of the ester methoxy group, situated approximately at 3.7 ppm. This molecular group indicates the presence of methyl fatty acids. The other not mentioned signals represent the shift of various molecular groups found in the derivatized sample. As an

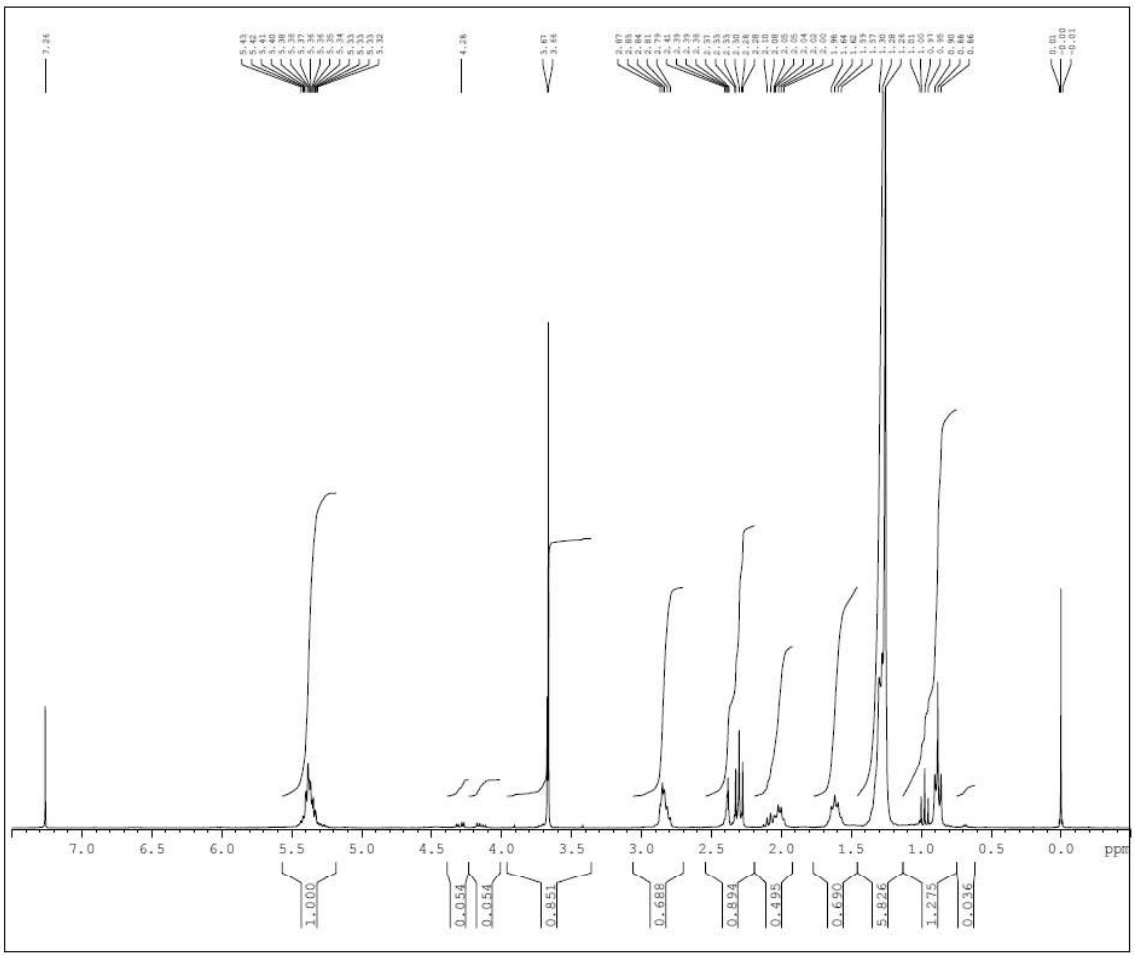


Figure 6.7: *H-NMR spectra of 0.024 g/mL derivatized microalgal lipids from* Schizochytrium *sp. . The analysis was performed by the analytical service of the HEIA-Fr, Switzerland. Very small peaks characteristic of glycerol residues appear at 4.1 ppm, 4.3 ppm and 5.26 ppm. At 3.7 ppm it can be seen the peak corresponding to the chemical shift of the ester methoxy group which indicates the presence of methyl fatty acids.*

example at 5.3 ppm the chemical shift of olefinic carbons, indicating the significant presence of polyunsaturated fatty acids [273] [274] [275] [276].

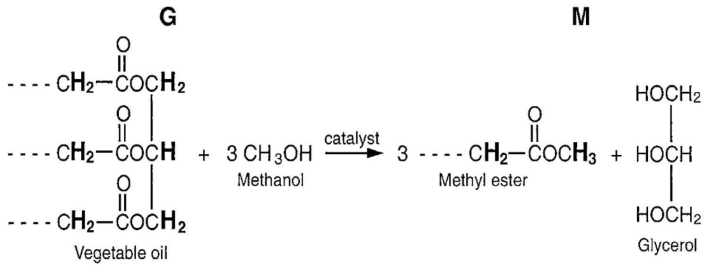


Figure 6.8: *Schematic representation of a trans-methylation reaction. The letters G and M denote the glyceridic and methyl ester protons, respectively.*

The glyceryl moiety of a triglyceride has five protons and the three methyl ester moieties resulting from one triglyceride molecule have nine protons, these factors need to be considered when calculating the conversion yield [275] [276]. The conversion (in %) of microalgal triglycerides into their methylated form can be calculated from the integration values of the glyceridic and methyl ester protons by the following equation :

$$Conversion\,[\%] = 100 - (\frac{5I_ME}{5I_ME + 9I_TAG}100) \tag{6.4}$$

where:

I_{ME} = Integration value of the methyl ester peak at 3.7 ppm

I_{TAG} = Sum of integration values of the glyceridic peaks at 4.1 ppm and 4.3 ppm

Based on the integral values reported in Figures 6.6 and 6.7, the conversion yield was 100% and 87%, respectively. (Equation 6.4) In order to have a complete conversion of fatty acids contained in the triglycerides, it is necessary to dilute the microgal lipid sample in toluene to a final concentration of 0.010 g/200 μL.

6.3 Results and discussion

6.3.1 Oxidative stress induction

The aim of the experiment was to investigate the effect of hydrogen peroxide on the total lipids accumulation in *Schizochytrium* sp. The first part of the experiment was carried out in 500 mL shake flasks. Different concentrations of H_2O_2 (0 mM, 0.1 mM, 0.2 mM, 0.6 mM, 1 mM, 2 mM and 50 mM) were added to a *Schizochytrium* cell suspension in exponential growth phase. The negative control was carried out by adding a concentration of 50 mM H_2O_2: such a high concentration was expected to inhibit cell growth [263]. The results of the study were analyzed by determining the maximum dry cell weight, the total lipid accumulation and the consumption of glucose as a function of the hydrogen peroxide concentration. It was expected to define an optimum concentration, which causes enough cellular stress to increase intracellular lipid accumulation without having an inhibitory effect on cell proliferation. In other words, the hypothesis was that an over-diluted concentration will not show any effect on cells and a too

concentrated solution i.e 50mM H_2O_2 would be cytotoxic.

Figure 6.9 represents the results of the three parameters analyzed during the experiment: the percentage of total lipids extracted, the maximum dry cell weight and the glucose consumption as a function of the concentration of H_2O_2 in the medium.

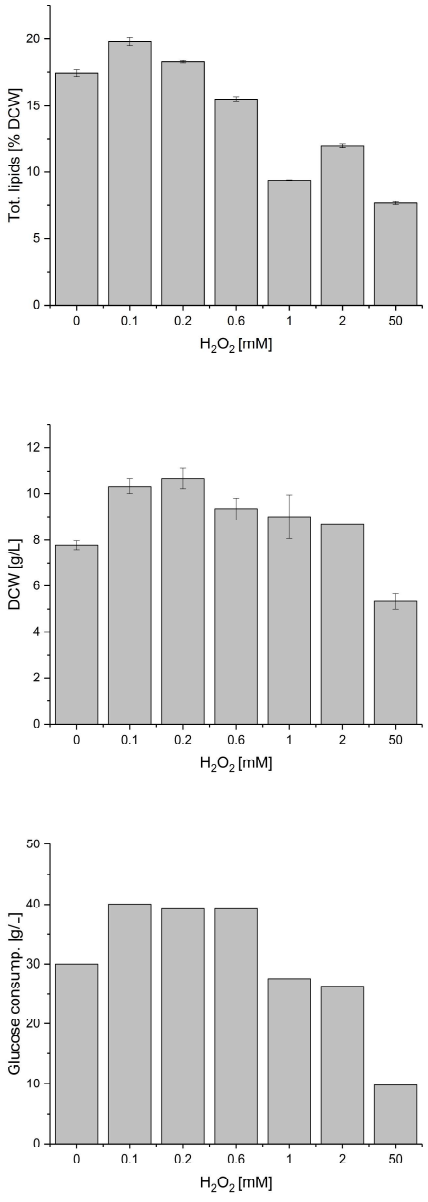


Figure 6.9: *Effect of different concentrations of H_2O_2 (0.1 mM, 0.2 mM, 0.6 mM, 1 mM, 2 mM and 50 mM) on a suspension of* Schizochytrium *sp. in exponential growth phase. The average of the results are represented as the total lipid accumulation (n=6), the final dry cell weight (n=3) and the glucose consumption (n=1). The standard deviation is represented as error bars.*

Figure 6.9 shows that the dry biomass of cells supplemented with 0.1 mM and 0.2 mM of H_2O_2

was composed of 19 and 18 % total lipids, respectively. These values were slightly higher than those obtained in the positive control (0 mM H_2O_2), where the DCW was composed of 16% of total lipids. It was observed that the lipid accumulation decreased when H_2O_2 concentration was higher than 0.2 mM. In the negative control (50 mM H_2O_2), the dry biomass was composed of 7.7 % lipids. Similar results were obtained when the maximum dry cell weight was represented as a function of the concentration of H_2O_2. The highest amount of biomass was obtained with H_2O_2 concentrations of 0.1 mM, 0.2 mM and 0.6 mM: 10.33 g/L, 10.67 g/L and 9.33 g/L respectively. The cells in the positive control flask achieved a maximum of 7.78 g/L and the values decreased to 5.33 g/L in the negative control experiment. The initial cultivation medium was composed of 40 g/L of glucose, 16 g/L of yeast extract and other salts. A complete glucose consumption occurred when cells were cultivated with 0.1 mM, 0.2 mM and 0.6 mM of H_2O_2, whereas in the positive control, 30 g/L glucose were consumed. In the negative control flask, as expected, only 9.76 g/L were consumed meaning that after the addition of 50 mM H_2O_2 the cell growth was inhibited (Figure 6.9).

These experiments allowed to analyse the effect of an oxidative stress inducer on different parameters: total lipid accumulation, maximum dry cell weight and glucose consumption. It was observed that concentrations between 0.1 mM and 0.2 mM H_2O_2 have an effect on lipid accumulation (Figure 6.9). The results obtained in the positive control were lower whereas high H_2O_2 used in negative control seemed to inhibit cell growth. The response of the control experiments were in agreement with initial expectations and they support the hypothesis that a concentration of H_2O_2 between 0.1 and 0.2 mM may have a positive effect on lipid accumulation.

6.3.2 Fed-batch cultivation with addition of H_2O_2

A two-stage fed-batch cultivation of *Schizochytrium* sp. was carried out by initially cultivating microalgal cells in a medium composed of 40 g/L glucose and 16 g/L yeast extract. Along with the glucose feed, a solution of 1M H_2O_2 was supplied to a final concentration of 0.1 mM in the bioreactor. Hydrogen peroxide is unstable, decomposing readily to oxygen and water, therefore it was added at several intervals in the culture medium. Nitrogen was added only to the initial medium, therefore a nitrogen limitation leading to enhanced lipid accumulation was expected. The hypothesis was that a combination of the effects of nitrogen limitation combined

with the additional oxidative stress inducer, would increase accumulation of lipids.

Figure 6.10 represented the results of the two-stage fed-batch cultivation of *Schizochytrium* sp. with addition of H_2O_2.

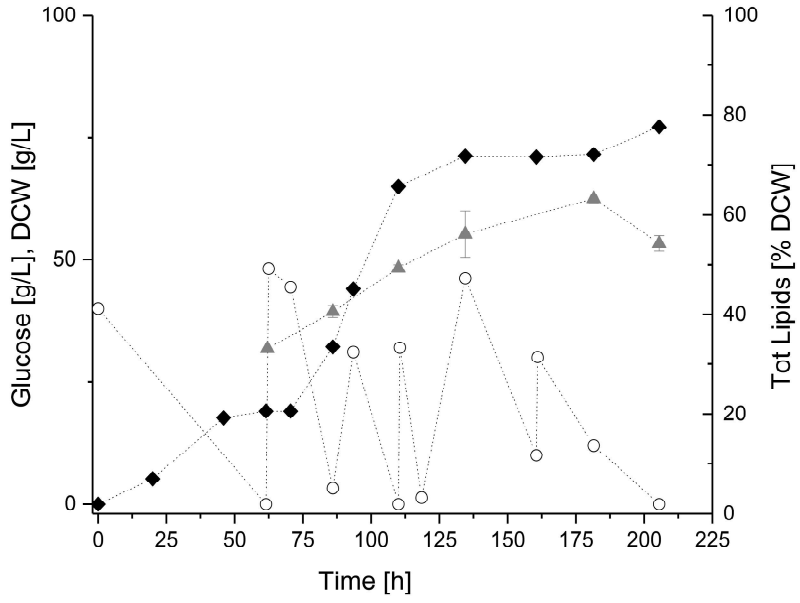


Figure 6.10: *Two-stage fed-batch cultivation of* Schizochytrium *sp.. Hydrogen peroxide was added at different intervals (i.e 65 h, 93.50 h, 110.5 h, 135 h, 161h). Results are represented as ♦ dry cell weight (DCW) in g/L and ◯ glucose concentration in g/L as a function of the cultivation time in h. The total lipids accumulation (▲) is represented as percentage of DCW. DCW was measured in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6), the standard deviation is represented as error bars.*

Hydrogen peroxide was added at different intervals (i.e 65 h, 93.50 h, 110.5 h, 135 h, 161h). Dry cell weight increased steadily until achieving 71 g/L after 135 hours then values remained constant. Lipid accumulation before peroxide addition was 33.14 % (SD=15.83 $\cdot$ 10^{-3} %, n=6) of dry cell weight, maximum of 63.27 % (SD=85.38 $\cdot$ 10^{-3}, n=6) was attained after 181.5 hour.

6.3.3 Fed-batch cultivation with temperature shift

A two-stage fed-batch cultivation of Schizochytrium sp. was carried out - as in the previous experiment - by initially cultivating microalgal cells in a medium composed of 40 g/L glucose and 16 g/L yeast extract, no further source of nitrogen was fed to the medium. After the batch cultivation phase, the temperature was lowered from 25°C to 18°C. The aim was to study the

effect of nitrogen deprivation combined with a temperature shift, which according to literature may increase lipids and omega-3 fatty acid production [114].

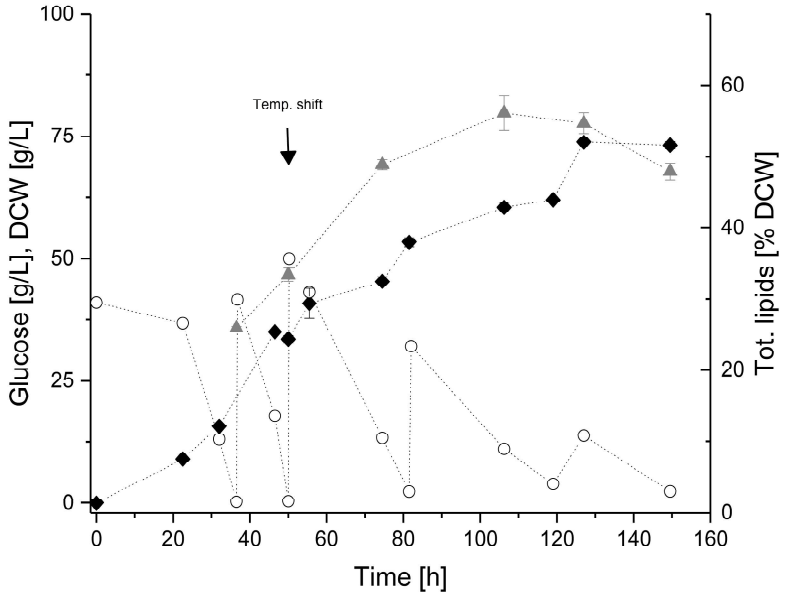


Figure 6.11: *Two-stage fed-batch of* Schizochytrium. *The temperature of the process was changed from 25°C to 18°C after 52 h. Results are represented as ♦ dry cell weight (DCW) in g/L and ◯ glucose in g/L as a function of the cultivation time in h. The total lipids accumulation (▲) is represented as percentage of DCW. The DCW was measured in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6), the standard deviation is represented as error bars.*

In the previous experiment, reported in Section 4.3.4.1 of Chapter 4, similar conditions as this study were used and it was suspected that the transition phase, where lipids are accumulated, was between 40 and 70 hours, therefore the temperature shift was applied after 52 hours. The dry cell weight (33.5 g/L) was composed of 33.40 % (SD= 8.782 10^{-1} %, n=6) total lipids. After 127 hours, cells achieved a maximum dry biomass of 73.8 g/L with a lipid content of 47.87% (SD = 1.166%, n=6). Cells had a maximum lipid content of 56.11% (SD= 2.430%, n=6) after 150 hours.

6.3.4 Fed-batch cultivation with lower salt concentration

Some of the studies reported in the literature suggest that high concentrations of salt, and in particular of sodium, may not be optimal for the production of DHA. In previous experiments, the feed was composed of glucose (500 g/L) and a high concentration of various salts: 50 g/L

NaCl, 15 g/L $MgSO_4$ and 2 g/L of KCl, 2 g/L $CaCl_2$ and 3.2 g/L $FeSO_4$. These concentrations were increased proportionally based on the composition of the standard cultivation medium (Section 4.2.2). In order to avoid an excessive accumulation of salts, the concentration of salts in the feed was maintained as in the standard medium: 25 g/L NaCL, 7.5 g/L $MgSO_4$ and 0.5 g/L of KCl, 0.5 g/L $CaCl_2$ and 80 mg/L $FeSO_4$. Figure 6.12 represents the results of the fed-batch cultivation of *Schizochytrium*.

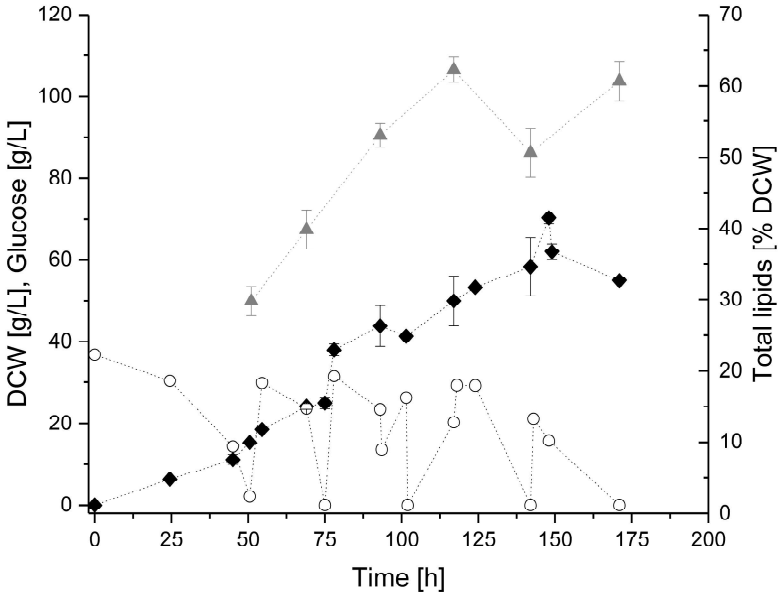


Figure 6.12: *Two-stage fed-batch cultivation of* Schizochytrium *sp. with low salts concentration feed. Results are represented as ♦ DCW in g/L and ○ glucose concentration in g/L as a function of the fermentation time in h. The total lipid accumulation (▲) is represented as percentage of DCW. The DCW measurements were repeated in duplicate (n=2) and the total lipid percentage of the DCW in sextuplicate (n=6). The standard deviation is represented as error bars.*

Feed, containing a lower concentration of salts, began after 51 hours, when the dry cell weight was 15 g/L and with a total lipid composition of 29.78 % (SD=2.064, n=6). The stationary growth phase was achieved after 140 hours with a maximum dry cell weight of approximately 60 g/L. Total lipid composition was maximum after 117 hours with a value of 62.26 (SD=1.769%, n=6).

6.3.5 Comparison

The results of the dry biomass accumulation obtained from the "normal" two-stage fed-batch cultivation of *Schizochytrium* described in Section 4.3.4.1 (Chapter 4), where compared to

the modified fed-batch experiments reported in this Chapter. The effect of the addition of an oxidative inducer addition, the effect of the temperature shift to 18°C and the effect of decreasing the salt concentration in the feed were compared. In all the experiments, the initial medium composition was the same i.e 40 g/L glucose, 16 g/L yeast extract and no further nitrogen source was fed in the medium after the batch phase. In Figure 6.13, the evolution of dry cell weight as a function of time is compared .

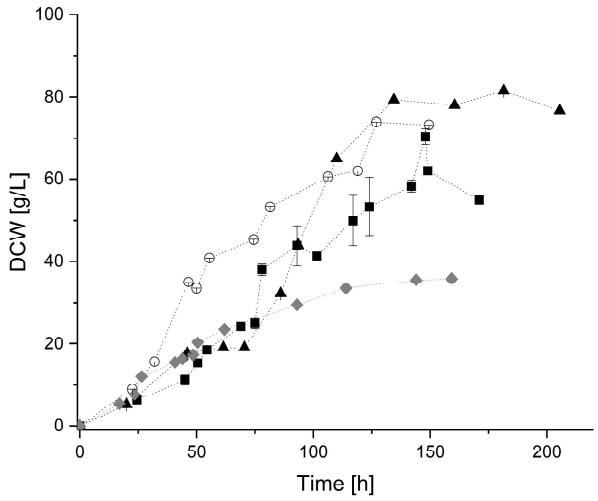


Figure 6.13: *Comparison of a ♦ normal two-stage fed-batch to three modified fed-batch experiments. The effect of the addition of H_2O_2 (▲), the effect of the temperature shift (◯) and the effect of lowering the salt concentration in the feed (■) were studied. The results are represented as the amount of dry cell weight as a function of the cultivation time. The measurements were repeated in duplicate (n=2) and the standard deviation is represented as error bars.*

At a first glance, in Figure 6.13, it is observed that in all the three modified fed-batches, dry cell weight achieved higher values (between 1.5-fold to 2.2-fold) compared to the normal fed batch.

In the normal fed-batch cultivation, maximum dry cell weight obtained after 160 hours was 35.8 g/L, with a total lipid composition of 47% whereas with the addition of hydrogen peroxide, the final biomass was doubled (71 g/L) with a maximum of 63.27 % total lipids. When the concentration of salts in the feed was kept lower, the maximum biomass was approximately 60 g/L with a maximum total lipid composition of 60% while when the temperature shift was applied the maximum was at 73 g/L with 55% total lipids (Figure 6.13 and 6.14). In each cultivation, the first extraction of total lipids was made just before the beginning of the feed and the lipid composition of dry biomass was approximately 30%. The maximum accumula-

tion is situated between 55% and 65%. The results of normal two-stage fed-batch could not be represented because the extraction of lipids was made only at the end of the cultivation. The final lipid composition was 47% of the dry cell weight, which is slightly lower than the results of this study. In Section 4.3.4.3 (Chapter 4) are reported the results of the optimized two-stage fed-batch, the only difference being that yeast extract was also added after the batch phase allowing to have a longer phase where lipid was accumulated. Total lipid accumulation was monitored over time, initially the content was low, between 5-20 % and the it increased to a maximum of 70.35%. A summary of the results is represented in Table 6.1.

Table 6.1: *The results obtained from five different two-stage fed-batch cultivations were compared in term of maximal biomass in g/L, total lipid accumulation as percentage of the dry cell weight and total lipid productivity in g/L/day.*

	Normal Two-stage fed-batch	Optimized Two-stage fed-batch	H_2O_2 addition	Salts conc.	Temperature
Biomass [g/L]	35.8	90.0	71.0	60.0	73.8
Tot. Lipid [% DCW]	47.0	70.3	63.3	62.3	56.1
Lipid productivity [g/L/day]	2.5	9.2	7.9	6.4	7.7

In terms of total lipid productivity in the optimized two-stage fed-batch (Section 4.3.4.3, Chapter 4), the maximum was 9.17 g/L/day whereas with the effect of H_2O_2 it was 7.94 g/L/day after 134.5 hours, when temperature was lowered to 18°C it was 7.7 g/L/day after 106h and when the concentration of salt in the feed was reduced it was 6.38 g/L/day after 117 hours. In the "normal" two-stage fed batch (Section 4.3.4.1, Chapter 4), at the end of the cultivation productivity was 2.53 g/L/day. The combination of nitrogen limitation with other particular conditions, tested in this Chapter, showed that biomass accumulation and lipid accumulation can be increased allowing to achieve higher total lipid productivity. Such productivities are comparable to that obtained in an optimized two-stage fed-batch where yeast extract was fed during the cultivation. Therefore, it can be concluded that adding H_2O_2, reducing salt concentration in the medium or lowering the temperature have similar effects to "optimizing" the fed-batch with increased addition of nitrogen source.

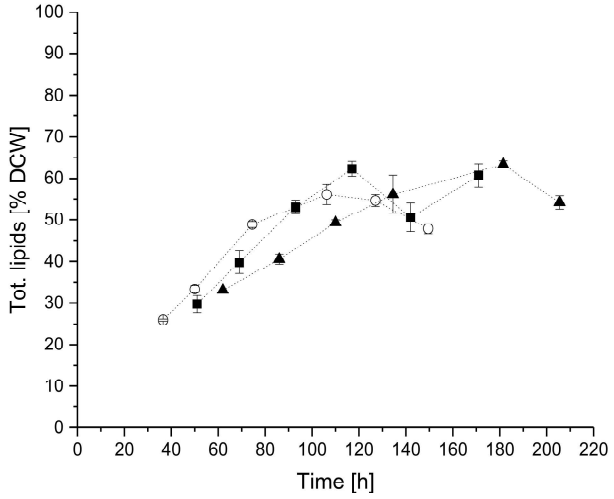


Figure 6.14: *Comparison of three modified fed-batch experiments, in which the effect of the addition of H_2O_2 (▲), the effect of the temperature shift (◯) and the effect of lowering the salt concentration in the feed (■) were studied. Results are represented as the amount of total lipids contained in the dry cell weight as a function of the cultivation time. The measurements were repeated in sextuplicate (n=6) and the standard deviation is represented as error bars.*

6.3.6 Analysis of DHA and EPA

It was anticipated that the different cultivation conditions applied in this Chapter may have enhanced not only the total lipid production, as seen in the first part of the study, but in particular the percentage of EPA and DHA in proportion to other fatty acids. Total lipids were extracted from algal biomass at different stages of the cultivation and derivatized into fatty acid methyl esters (FAME) to make them volatile for separation and analysis using GC-FID.

In Figure 6.15 the percentage of DHA in the total lipid composition is presented. The optimized two-stage fed-batch carried out in Section 4.3.4.3 (Chapter 4) is compared to the three modified fed-batch cultivations, where the DHA percentage was expected to increase.

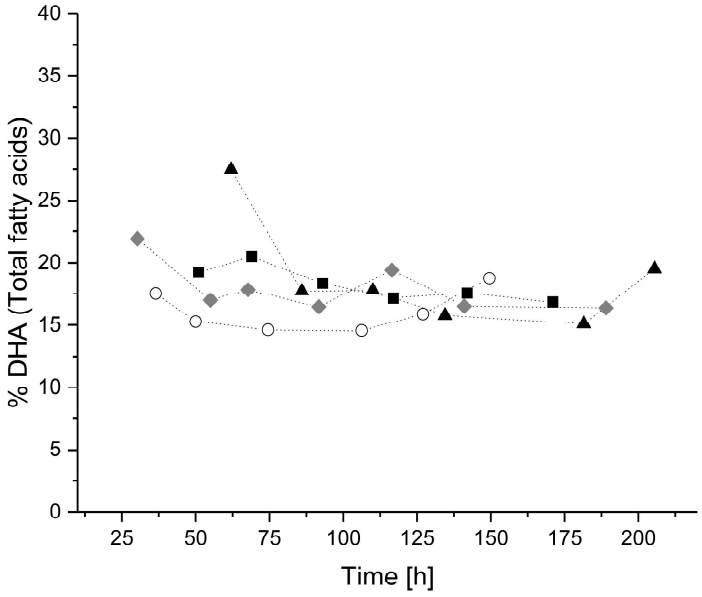


Figure 6.15: *Comparison of the percentage of DHA in total lipids for different experiments: ♦ optimized two-stage fed-batch, effect of the addition of oxidative inducer (H_2O_2) ▲, the effect of the temperature shift ◯ and the effect of lowering the salt concentration in the feed ■ were studied. In order to compare the values with the literature, the percentage of the internal standards was removed from the total. The experiment was repeated in triplicate (n=3).*

The analysis of microalgal oil extracted from *Schizochytrium* showed that EPA was less than 1% of the total fatty acids in each experiment (data not shown), therefore in Figure 6.15 are reported only the results of the relative content of DHA in the total fatty acids. Such results are in agreement with those reported in the literature, where it is described that, depending on the specifity of the enzyme complex PUFA synthases, which carry out *de novo* synthesis of specific long-chain PUFAs, the main product can be a single PUFA or a mix of PUFAs [73] [96]. In general, PUFA sythases characterized in marine bacteria appear to produce predominantly a single product: either DHA or EPA. This may explain why the concentration of EPA is absent or very low. Thraustochytrids appears to naturally produce palmitic acid, docosapentaenoic acid and DHA as major fatty acids, while accumulating EPA to very low levels [73]. Several studies have reported similar characteristics, in general microalgal oil produced from *Schizochytrium* contains lower levels of EPA [259]. Shirasaka et al. showed that in *Schizochytrium* the addition of ρ-toluic acid increased the EPA content in total fatty acids, from 4.99% to 6.51%. The percentage of DHA decreased slightly from 51.6% to 50.3% [77]. Ganuza et al. in batch cultivation of *Schizochytrium* G13/2S with glucose (40g/L) and glutamate (2g/L) produced a maximum of 15 g/L dry cell weight composed of 35% total fatty acids. At stationary phase, the

percentage of DHA in total fatty acid was between 43% and 47% while EPA was not detected.

In each experiment represented in Figure 6.15, the first lipid extraction was carried out on the microalgal suspensions sampled before the beginning of the feed. During the batch phase of the process, the medium was carbon-limited whereas with the beginning of the glucose feed the medium was nitrogen depleted. In Figure 6.15, it is observed that the initial concentration of DHA in total lipids was slightly higher compared to the ulterior results. As an example, in the case of the optimized two-stage fed-batch (Section 4.3.4.3, Chapter 4), after 30.25h the percentage of DHA was 20% and decreased after 24 hours to about 16%, which remained constant until the end of the fermentation (Figure 6.15). Such results, in terms of DHA composition of total fatty acids, were lower than some reported studies. For instance, *Schizochytrium* sp. T18 is used by Martek to produce microalgal oil with a mixture of triglycerides containing mostly polyunsaturared fatty acids (PUFA). The predominant fatty acid is DHA, with a minimum amount of 35% DHA in the total fatty acids whereas EPA had a very low concentration ($<$ 2% of total fatty acids).

In order to increase the concentration of DHA in the oil, several experiments were carried out by combining the developed two-stage fed-batch process to specific conditions, which were expected to enhance the production of PUFAs and in particular of DHA. In Figure 6.15 are reported the results of DHA analysis in *Schizochytrium* produced under the effect of lower temperature, of the addition of an oxidative inducer and of the reduction of salt in the feed. All the results are very similar, following the same tendency to decrease the percentage of DHA after the addition of the feed, to a value between 15% and 20 % of the total fatty acids. Despite that the two-stage fed-batch process was combined with other specific conditions to enhance the production of DHA, the results did not seem to improve further. The main fatty acids composing the microalgal oil were identified and the results are reported in Figures 6.16 - 6.19.

It can be observed that a common fatty acid profile emerged between the different modified fed-batch cultivations (Figures 6.16 - 6.19). Palmitic acid (C16:00) appeard to be the main fatty acid ($>$ 20-25%) followed by myristic acid (C14:00) with more than 20%. Both are saturated fatty acids and their concentration seemed to increase throughout the cultivation. Pentadecanoic acid (C15:00) was present in a lower proportion which decreases to approxim-

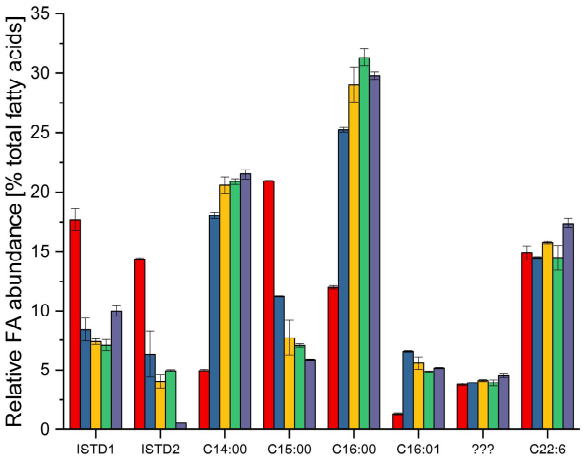


Figure 6.16: *Relative fatty acid (FA) composition of* Schizochytrium *cultivated in the optimized two-stage fed-batch cultivation (Chapter 4). Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The fatty acids reported are the following: myristic acid (C14:00), pentadecanoic acid (C15:00), palmitic acid (C16:00), palmitoleic acid (C16:1 ω-7) and docosahexaenoic acid i.e DHA (C22:6 ω-3).The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars.*

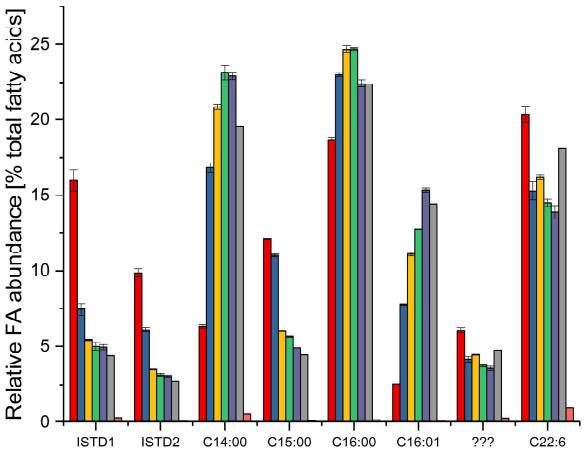


Figure 6.17: *Relative fatty acid (FA) composition of* Schizochytrium *cultivated in a two-stage fed-batch cultivation with the addition of oxidative inducer (H_2O_2). Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The fatty acids reported are the following: myristic acid (C14:00), pentadecanoic acid (C15:00), palmitic acid (C16:00), palmitoleic acid (C16:1 ω-7) and docosahexaenoic acid i.e DHA (C22:6 ω-3).The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars.*

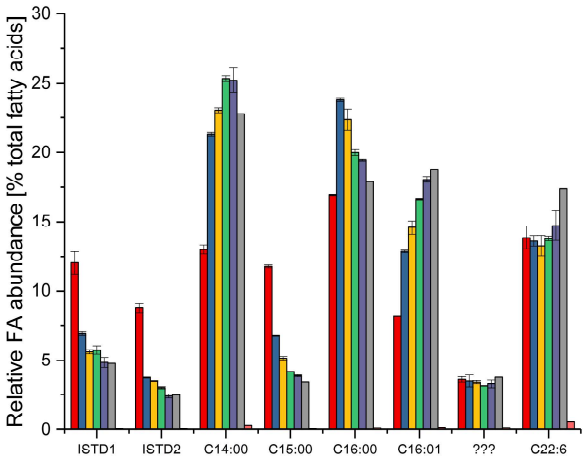


Figure 6.18: *Relative fatty acid (FA) composition of* Schizochytrium *cultivated in a two-stage fed-batch cultivation with the effect of a temperature shift. Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The fatty acids reported are the following: myristic acid (C14:00), pentadecanoic acid (C15:00), palmitic acid (C16:00), palmitoleic acid (C16:1 ω-7) and docosahexaenoic acid i.e DHA (C22:6 ω-3).The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars.*

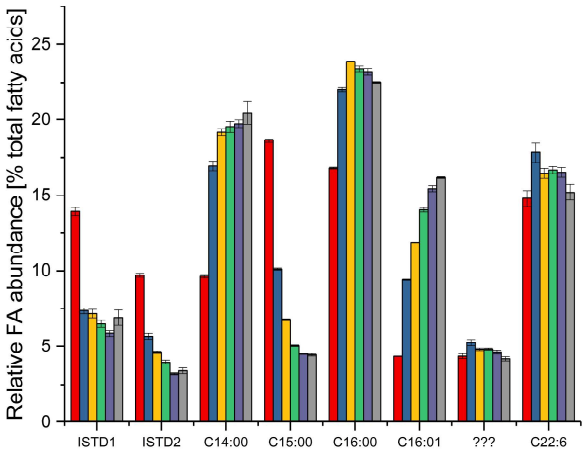


Figure 6.19: *Relative fatty acid (FA) composition of* Schizochytrium *cultivated in a two-stage fed-batch cultivation with reduction of salt concentration. Each bar corresponds to the cultivation time when total lipids were extracted from the algal sample. The internal standards, methyl tridecanoate (ISTD1) and pentadecane (ISTD2) are represented. The fatty acids reported are the following: myristic acid (C14:00), pentadecanoic acid (C15:00), palmitic acid (C16:00), palmitoleic acid (C16:1 ω-7) and docosahexaenoic acid i.e DHA (C22:6 ω-3).The same oil sample was analyzed in triplicate (n=3) and the standard deviation is represented as error bars.*

ately 5% at the end of the cultivation. In the sample is also present a mono-unsaturated fatty acids, the omega-7 palmitoleic acid (16:1), with a final concentration of approximately 15%. The only fatty acid not identified, which has a retention time of approximately 14 min, is suspected to correspond to docosapentaenoic acid (DPA, C22:5), which was reported in several publications analyzing *Schizochytrium* sp. extracted oil [258] [235]. In general, these results are similar to lipid profiles obtained by others studies for this species [105] [277] [73].

Triacylglyceride accumulation mostly occurs under sub-optimal environmental conditions and they are stored in lipid bodies in the cytoplasm [96]. Most algae accumulate saturated and monounsaturated FAs in the TAGs under certain stress conditions such as nitrogen limitation, as shown in Chapter 3 with *Chlorella protothecoides* and in Chapter 4 with *Schizochytrium* sp. However, it appears that omega-3 LC-PUFA are not accumulated appreciably in TAGs [96] but instead are mainly found in the cell membrane, where they are involved in the regulation of membrane fluidity [278]. As mentioned previously, the effect of nitrogen limitation on DHA accumulation resulted in contradictory conclusions between the different studies reported in the scientific literature [81] [230] [233] [195] [188]. For example, in *N. oceanica* under stressful conditions, the content of the major LC-PUFA, eicosapentaenoic acid (EPA), was significantly reduced while TAG reached 25% of the biomass [278]. Contrary to this, an increase in TAGs and incorporation of EPA in TAGs was observed in the diatom *T. pseudomonas* in the stationary growth phase [96].

In this study, it is observed that the proportion of DHA in total fatty acids is higher before the beginning of the feed and therefore before the beginning of the transition phase where nitrogen is limiting the growth (high C/N ratio) and it allows very high lipid accumulation. At the end of the fermentation the main fatty acids in *Schizochytrium* were saturated fatty acids such as palmitic acid, myristic acid, pentadecanoic acid) and a monounsaturated fatty acid, palmitoleic acid (Figures 6.16 - 6.19).

Although the two-stage fed-batch cultivation process developed in Chapter 4 is very effective for increasing total lipid productivity, nitrogen limitation does not appear to be favorable for DHA and EPA production. This mechanism might have stimulated the accumulation of saturated and mono-unsaturated fatty acids in TAGs but not the omega-3 PUFAs. Since PUFAs are

contained in the cell membrane, different methods (oxidative inducer, temperature and salinity) were combined with the developed fed-batch and resulted in increased biomass and accumulation of total lipids but not of DHA. It may be that the composition of the initial cultivation medium was not favorable since it contained 25 g/L of NaCl. In the study of Song et al. it was reported that the main fatty acids in cells were palmitic acid and docosahexaenoic acid which were 34.30% and 44.54% of the total fatty acids, respectively [279]. In the research of Kang et al., it was concluded that the reduction in NaCl concentration reduced the formation of palmitic acid (PA, C16:0) but enhanced the production of PUFAs in *T. aureum*. It appeared that low salt concentration stimulates a desaturation of fatty acids, and an increase in the cellular quantities of PUFAs, in particular DHA [270]. Many other different reasons could explain the difference in the results, from the particular type of microalgal strain to the source of nitrogen, etc.

Despite the lower results, Figure 6.20 shows the accumulation of DHA in g/L in the culture. The maximum production of DHA (4.6 g/l) was obtained in the optimized two-stage fed-batch (Section 4.3.4.3, Chapter 4), meaning that the objective of producing DHA in *Schizochytrium* was attained but more effort is needed to improve the production.

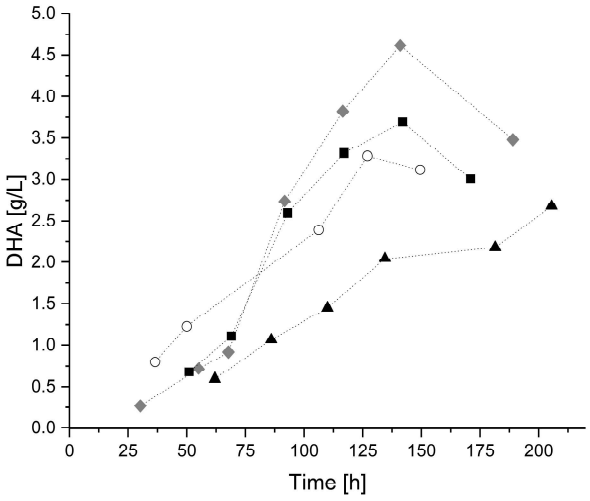


Figure 6.20: *Concentration of DHA in g/L different experiments: ♦ optimized two-stage fed-batch, effect of the addition of oxidative inducer (H_2O_2) ▲, the effect of the temperature shift ◯ and the effect of lowering the salt concentration in the feed ■ were studied.*

6.4 Conclusion

Lipids need to be first derivatized into another compound to enable chromatographic separation and detectability. The derivatization reaction makes the analyte sufficiently volatile, masking the polar group, to be eluted at high temperature without decomposing it. The derivatization is also needed to distinguish between the very slight differences exhibited by unsaturated fatty acids. In this study, an efficient method to derivatize samples of microalgal oil was developed, using a methanolic HCl solution. The derivatization yield was verified by H-NMR and when initial diluted concentrations of lipids were used (0.10 g in 200 μL toluene) the conversion yield was 100%. Gas chromatography analysis coupled with FID detector allowed to identify the composition of the main fatty acids in the sample by using a standard mix of 19 FAMEs and the standards of DHA and EPA.

Only very small amounts of eicosapentaenoic acid (EPA) were produced by *Schizochytrium* used in the study. However, such results are in agreement with the reported literature and it could be in some cases beneficial since EPA is considered as contra-indicatory in breast milk substitutes. In fact, DHA is the main omega-3 needed during early development and only a low quantity of EPA is necessary [81].

Production of DHA in *Schizochytrium* was possible, achieving a maximum of 4.6 g/L in the optimized two-stage fed-batch cultivation developed in Section 4.3.4.3 of Chapter 4. The aim of producing DHA from *Schizochytrium* was achieved, however more effort is needed to improve the specific accumulation of DHA in the total fatty acids. In order to regulate DHA production, it is important to understand the mechanisms that determine in what lipid fraction DHA is present [96]. It was suspected that the best method to accumulate PUFA is the remodeling of phospholipid fatty acid tails, since PUFAs are involved in the mechanism of membrane fluidity. The developed two-stage fed-batch cultivation was combined with some particular conditions suspected to enhance PUFA accumulation. The expectation was to combine high lipid productivity to high DHA accumulation. However, saturated and mono-unsaturated fatty acids were mainly produced.

The optimized two-stage fed-batch cultivation allowed to combine two contradictory mechan-

ism: a high cell density production to a high lipid accumulation, resulting in a very high lipid productivity. However, it appears that other process factor need to be considered to enable increased DHA productivity.

Chapter 7

Conclusion and perspectives

This thesis focused on the production of lipids from microalgae, which are a promising and sustainable sources for replacing the traditional feedstocks, used for food, agricultural and petrochemical industries. The main findings will be summarized and compared to the initially stated objectives. There are other relevant studies that in the future will deserve research, an outlook of these perspectives will be described.

7.1 Conclusion

- **Increase of lipid productivity**

  So far, low yield of microalgal lipids has been a critical obstacle preventing competitive commercial production in commodities markets. The major finding of this research was the successful optimization of the lipid productivity in *Schizochytrium* and particularly in *Chlorella protothecoides*. A heterotrophic fermentation strategy was developed by combining two contradictory mechanisms in the same process: enhanced cell proliferation and increased intra-cellular lipid accumulation. Efforts have been made in developing first a carbon limited medium (low C/N ratio) that stimulated the cell proliferation allowing to produce very high cell densities. Second, a nitrogen limited medium (higher C/N ratio) was achieved by feeding in the medium only carbon sources. During the "transition-phase", where nitrogen was present but not in excess, lipid were mostly accumulated.

  The optimized two-stage fed-batch cultivation process resulted in 255 g/L dry cell weight

of *Chlorella protothecoides*, composed by 58% (w/w) of lipids, which is 2 to 6 times higher than previously reported studies. Indeed, the lipid productivity was also very high, 16.7 g/L/day.

The two-stage fed-batch fermentation process was applied successfully to *Schizochytrium* sp. achieving 90 g/L of dry biomass composed of 70% of total lipids, the maximum lipid productivity was 9-10 g/L/day. The analysis of total lipids by gas chromatography, showed that the omega-3 fatty acid EPA was not produced by *Schizochytrium* whereas DHA composed approximately 20% of the total fatty acids, resulting in a maximum accumulation of 4.7 g/L. Despite the fed-batch process was combined to particular conditions suspected to enhance DHA production, its accumulation was lower than those reported in the literature. Nitrogen limitation may not be the best method to accumulate PUFAs and more effort is needed to find an optimal balance between biomass, lipid and DHA productivity.

- **Development in downstream processing**

  A number of technical challenges remain in the downstream processing part of microalgal process, impeding the widespread commercial implementation. [137] All of the methods developed in this research were directly applied to wet concentrated biomass, allowing to extract considerable amounts of total lipids. More than 99% of the cell wall of *Chlorella* was broken by vortex-mixing a cell suspension with glass beads. As required, the method was applicable at lab-scale, it was relatively quick ($<$ 10 min) and inexpensive. The pre-treamtment of *Chlorella* cell walls allowed to improve the lipid extraction with solvents of 10-fold whereas with *Schizochytrium* the mechanical pretreatment was not necessary. Such a result shows that microalgal cell walls are not comparable between strains and that it is necessary to develop methods that meet individual requirements. Since *Chlorella protothecoides* cell walls were particularly resistant, an alternative method applicable at large scale was developed, aiming to weaken the cell walls improving the disruption of *Chlorella*. The best results were obtained from a combination of lysozyme-sulfatase and resulted in an improvement in total lipid extracted of 7-fold compared to intact cells. Indeed, lysozyme seems to expose more internal layers of the cell wall, which allows for further degradation by other enzymes that can reach

new substrates present in these internal layers.

- **Alternative substrates for heterotrophic cultivation**

  Microalgae cultivated heterotrophically have generally higher oil yield compared to phototrophic growth however, obtaining lipids from expensive carbon sources is not ideal. Besides the economic cost, glucose is obtained from starch produced from plants grown under phototrophic conditions, which could create an inherent conflict with food supply and land use, generating a threat to food security. [42] The potential of alternative carbon source such as glycerol, molasses and acetate was tested on*Chlorella protothecoides* and their effect on cell growth and lipid composition was studied. It concluded that glycerol and molasses could be a very promising alternative, showing comparable growth rates (0.076 h^{-1} and 0.080 h^{-1}, respectively) to glucose (0.11 h^{-1}) and a relatively good productivity (3.9 g/L/day and 4.6 g/L/day, respectively). It is important to consider that the process requires optimisation, as in the case of glucose in Chapter 3. Now that the potential of these sources is confirmed, more effort is needed to optimize the productivity.

In Figure 7.1 is schematized the process flow and the major findings of the project.

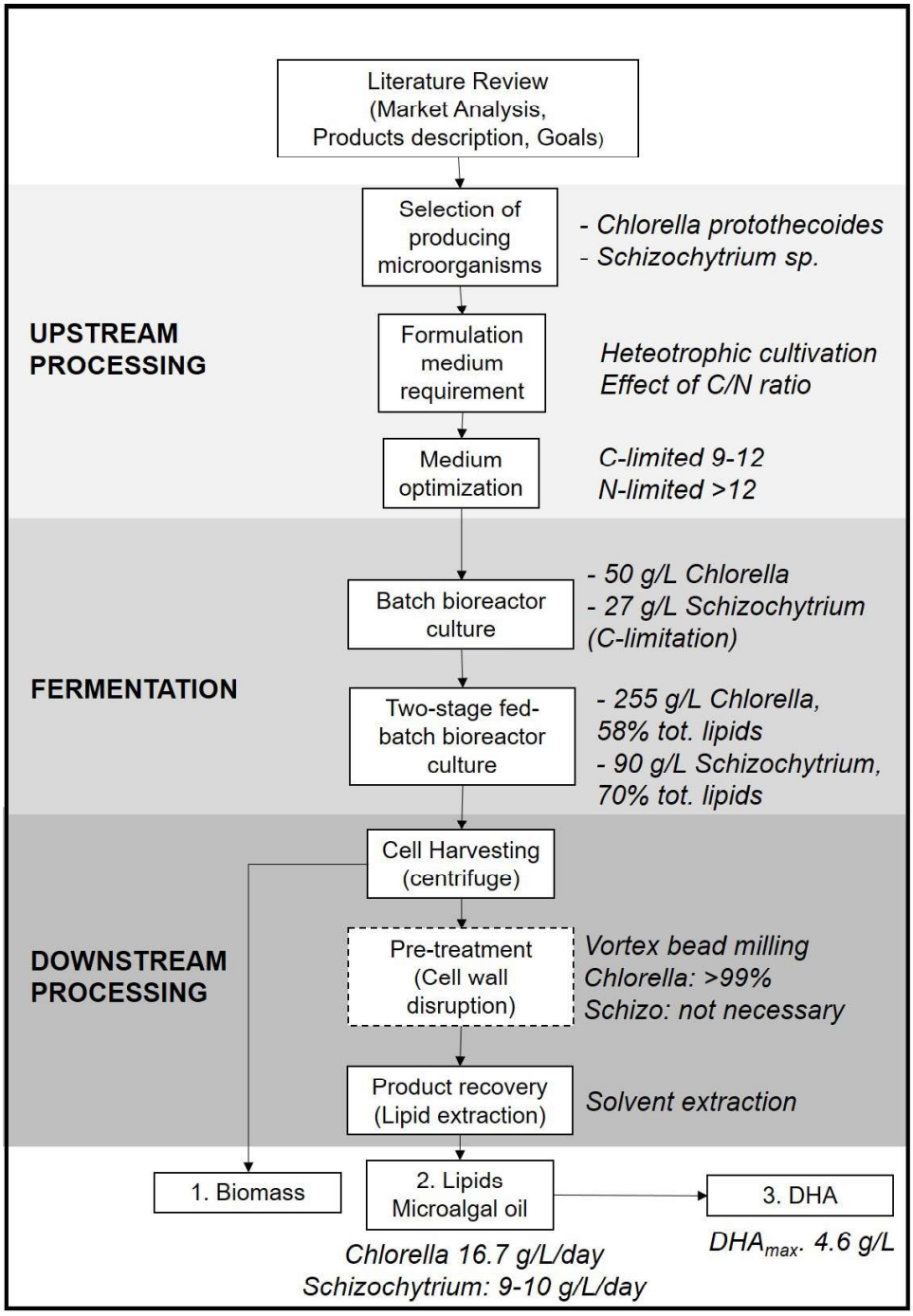


Figure 7.1: *Flowchart process plan reporting the main results*

7.2 Perspectives

A small-scale industrial bioprocess was developed based on information collected and adapted from several scientific studies described by the literature. The results presented in this thesis led to increased insights into the process parameters that determine microalgal productivity and provided a solid basis for assessing the commercial potential of microalgae. It is expected to contribute to both basic science and towards the commercial utilization of microalgae and it is believed that this is on the right track to achieve economically feasible microalgal lipid production. However, more research needs to be carried out - mostly in view of an industrial scale-up - and the following lists some directions to expand the present work further:

- Lipid productivity could be further optimized by improving the seed-train development for inoculation. In this study, the initial concentration of cells was relatively low, less than 1 g/L. However, the production bioreactor could be inoculated with about 10% volume of an actively growing microalgae suspension, a common practice carried out in industrial biotechnology. [251]

- Yeast extract provided the optimum nitrogen source however, it is relatively expensive for the process. Ammonia and glycine were unsuccessfully tested for the growth of *Chlorella*, consequently more effort is needed to find cheaper alternatives or eventually to define a valid combination of nitrogen sources .

- The two-stage fed-batch process needs to be up-scaled toward industrial process, as well as the downstream processing part. When scaling-up a fermentation process to large scale, it is important that the physical and chemical conditions in the bioreactor are maintained similar. The processes up-scaled to large scale bioreactors face often problems with mixing and mass transfer. [280] Therefore, it is expected that oxygen limitation may be a critical parameter, especially due to the viscosity of the highly concentrated microalgal suspension. It is important to preserve a similar geometrical configuration i.e impellers to achieve a good mixing. In the DSP part, more experiments are needed to optimize the enzymatic process and to verify the application at larger scale; a drawback may be the cost of enzymes and the relatively long treatment time (8 hours).

- Since *Schizochytrium* was chosen as strain to produce high added value molecules such

as DHA, and not as feedstock for commodity market as *Chlorella*, alternative carbon sources were not tested. However this could be interesting to define the effect on DHA production. In general, more studies are needed to optimize the DHA production. Eventually, other high valuable bio-products such as carotenoids could be characterized and studied.

- Finally, a cost analysis of the microalgal lipid obtained from the heterotrophic cultivation of microalgae, ideally with alternative substrates, should be compared to the results of a phototrophic cultivation aiming to define price attractiveness.
- Further work is required to determine whether other microalgae may be cultivated heterotrophically under similar conditions to produce unsaturated fatty acids.

Bibliography

1. Lee J. M, Lee H., Kang S. B., et al. Fatty Acid Desaturases, Polyunsaturated Fatty Acid Regulation, and Biotechnological Advances, *Nutrients*, 8(1):23, 2016

2. Andrews B. A., Asenjo J. A. Enzymatic lysis and disruption of microbial cells, *Trends in Biotechnology*, 5(10): 273-277, 1987

3. Jungbauer A. Continuous downstream processing of biopharmaceuticals, *Trends in Biotechnology*, 31(8): 479-492, 2013

4. He Y., Hoff T. C., Emdadi L., et al. Catalytic consequences of micropore topology, mesoporosity, and acidity on the hydrolysis of sucrose over zeolite catalysts, *Catalysis Science & Technology*, 4(9):3064, 2014

5. Living Planet Report - Biodiversity, biocapacity and better choices, www.footprintnetwork.org (May 2016)

6. United Nations News Service Section, http://www.un.org/en/globalissues/food/ (May 2016)

7. Demirbas A., Demirbas M. F. Algae Energy - Algae as a New Source of Biodiesel, Springer-Verlag, London (2010)

8. Chisti Y. Biodiesel from microalgae.*Biotechnol. Adv.*, 25(3):294-306, 2007

9. United Nation, Global Issues - Food, www.un.org

10. Jeong S., Kang D., Lim M., et al. Risk assessment of growth hormones and antimicrobial residues in meat, *Toxicol. Res.*, 26(4):301-13, 2010

11. Worldwatch Report 2009, Livestock and Climate Change - www.worldwatch.org/files/pdf/Livestock and Climate Change.pdf (May 2016)

12. FAO, Livestock and the environment - www.fao.org/livestock-environment/en/ (May 2016)

13. Hoekstra A. Y. The hidden water resource use behind meat and dairy. *Anim. Front.*, 2(2):3-8, 2012

14. McCauley D. J., Pinsky M. L., Palumbi S. R., et al. Marine defaunation: Animal loss in the global ocean, *Science*, 347(6219):1255641-1255641, 2015

15. FAO, General situation of world fish stocks, www.fao.org (May 2016)

16. FAO, The State of World Fisheries and Aquaculture, 2014 - www.fao.org/3/a-i3720e.pdf (May 2016)

17. Brown L. R. Full Planet, Empty Plates : the new geopolitics of food scarcity, W.W. Norton & Company, New York, (2012)

18. Forum for the future, The Protein Challenge 2040, www.forumforthefuture.org (June 2016)

19. Solazyme, Sustainability principles and commitments, Technical report, 2015

20. Safi C., Zebib B., Merah O., et al. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review., *Renew. Sustain. Energy Rev.*, 35:265-278, 2014

21. Kim S. Handbook of Marine Microalgae: Biotechnology Advances, Academic Press, 2015

22. Brennan L., Owende P. Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products, *Renew. Sustain. Energy Rev.*, 14(2):557-577, 2010

23. Sheehan J., Dunahay T., Benemann J., et al. Look back at the U.S. Department of Energy aquatic species program: Biodiesel from algae; Close-out report. Technicalreport, National Renewable Energy Laboratory (NREL), Golden, CO, 1998

24. Hannon M., Gimpel J., Tran M., et al., Biofuels from algae: challenges and potential, *Biofuels*, 1(5):763-784, 2010

25. Hamilton G. Kingdoms of Life - Fungi (eBook), Lorenz Educational Press, 2006

26. Heimann K., Huerlimann R. Microalgal classification: major classes and genera of commercial microalgal species, Handbook of Marine Microalgae, Academic Press (2015) 25-41

27. Garay L. A., Boundy-Mills K. L., German J. B. Accumulation of high-value lipids in single-cell microorganisms: A mechanistic approach and future perspectives, *Journal of Agricultural and Food Chemistry*, 62(13):2709-2727, 2014

28. Gupta A., Barrow C. J., Puri M. Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils, *Biotechnology Advances*, 30(6):1733-1745, 2012

29. Yokoyama R., Honda D. Taxonomic rearrangement of the genus *Schizochytrium* sensu lato based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes), *Mycoscience*,48(4):199-211, 2007

30. Kazama F., The zoospore of *Schizochytrium aggregatum*. *Canadian Journal of Botany*, 58(23):2434-2446, 1980

31. Rosenberg J. N., Kobayashi N., Barnes A., et al. Comparative analyses of three *Chlorella* species in response to light and sugar reveal distinctive lipid accumulation patterns in the microalga *C. sorokiniana*, *PLoS One*, 9(4):92460, 2014

32. Baudelet P. H., Ricochon G., Linder M., et al. A new insight into cell walls of Chlorophyta, *Algal Research*, 35:333-371, 2017

33. Doran P., Bioprocess Engineering Principles, Academic Press, London (1995)

34. Ryckebosch E., Muylaert K., Foubert I. Optimization of an analytical procedure for extraction of lipids from microalgae, *Journal of the American Oil Chemists Society*, 89(2):189-198, 2011

35. Perez-Garcia O., Escalante F., De-Bashan L., et al. Heterotrophic cultures of microalgae: metabolism and potential products, *Water Research*, 45(1):11-36, 2011

36. Lee Y. K. Microalgal mass culture systems and methods: Their limitation and potential, *Journal of Applied Phycology*, 13(4):307-315, 2001

37. Wasanasathian A., Peng C. A., Algal photobioreactor for production of lutein and zeaxanthin in bioprocess, Bioprocessing for Value-Added Products from Renewable Resources, Elsevier, Amsterdam (2007) 491-505

38. Ruiz J., Olivieri G., de Vree J., et al. Towards industrial products from microalgae, *Energy & Environmental Science*, 9(10):3036-3043, 2016

39. Das D. Algal Biorefinery: An Integrated Approach, Springer International Publishing, 2015

40. Del Rio E., Armendariz A., Garcia-Gomez E., et al. Continuous culture methodology for the screening of microalgae for oil, *Journal of Biotechnology*, 195:103-107, 2015

41. Amaro H. M., Guedes A. C., Malcata F. X. Advances and perspectives in using microalgae to produce biodiesel, *Applied Energy* 88(10):3402-3410, 2011

42. Morales-Sanchez D., Martinez-Rodriguez O. A., Martinez A. Heterotrophic cultivation of microalgae: production of metabolites of commercial interest, *Journal of Chemical Technology & Biotechnology*, 92(5):925-936, 2017

43. Patil K. P., Gogate P. R. Improved synthesis of docosahexaenoic acid (DHA) using *Schizochytrium limacinum* SR21 and sustainable media. *Journal of Chemical & Engineering Data*, 268:187-196, 2015

44. Harrison S., Griffiths M. J., Richardson C., et al. Advantages and challenges of microalgae as a source of oil for biodiesel, INTECH Open Access Publisher, 2011

45. Chen Y. H., Walker T. H. Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol, *Biotechnology Letters*, 33(10):1973-1983, 2011

46. Kumar V., Muthuraj M., Palabhanvi B., et al. High cell density lipid rich cultivation of a novel microalgal isolate *Chlorella sorokiniana* FC6 IITG in a single-stage fed-batch mode under mixotrophic condition, *Bioresource Technology*, 170:115-124, 2014

47. Liang I., Sarkany N., Cui Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions, *Biotechnology Letters*, 31(7):1043-1049, 2009

48. Heredia-Arroyo T, Wei W., Hu B. Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*, *Biotechnology and Applied Biochemistry*, 162(7):1978-1995, 2010

49. Hammed A. M., Prajapati S. K., Simsek S., et al. Growth regime and environmental remediation of microalgae, *Algae*, 31(3):189-204, 2016

50. Zheng Y., Chi Z., Lucker B., et al. Two-stage heterotrophic and phototrophic culture strategy for algal biomass and lipid production, *Bioresource Technology*, 103(1):484-488, 2012

51. Bumbak F., Cook S., Zachleder V., et al. Best practices in heterotrophic high-cell-density microalgal processes: Achievements, potential and possible limitations, *Applied Microbiology and Biotechnology*, 91(1):31-46, 2011

52. Olsvik E., Kristiansen B. Rheology of filamentous fermentations, *Biotechnology Advances*, 12(1):1-39, 1994

53. Yamamoto M., Fujishita M., Hirata A. Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris*, *Journal of Plant Research*, 117(4):257-264, 2004

54. Bewicke D., Potter B. *Chlorella*: The emerald food, Ronin Publishing (1984)

55. Trentacoste E. M., Martinez A. M., Zenk T. The place of algae in agriculture: policies for algal biomass production, *Photosynthesis research*, 123(3):305-315, 2015

56. Guccione A., Biondi N., Sampietro G., et al. *Chlorella* for protein and biofuels: From strain selection to outdoor cultivation in a Green Wall Panel photobioreactor, *Biotechnology for Biofuels*, 7(1):84, 2014

57. Manuel M. E. The cultivation of *Chlorella* sp., *Plant physiology*, 19(2):359-369, 1944

58. Gerken H. G., Donohoe B., Knoshaug E. P. Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production, *Planta*, 237(1):239-253, 2013

59. Spolaore P., Joannis-Cassan J., Duran E., et al. Commercial applications of microalgae, *Journal of bioscience and bioengineering*, 101(2):87-96, 2006

60. Day A. G., Brinkmann D., Franklin S., et al. Safety evaluation of a high-lipid algal biomass from *Chlorella protothecoides*, *Regulatory toxicology and pharmacology*, 55(2):166-180, 2009

61. Xu H., Miao X., and Qingyu Wu. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters, *Journal of biotechnology*, 126(4):499-507, 2006

62. Xiong W., Li X., Xiang J., et al. High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production, *Applied Microbiology and Biotechnology*, 78(1):29-36, 2008

63. Jiang Y, Chen F. Effects of medium glucose concentration and pH on docosahexaenoic acid content of heterotrophic, *Cell*, 35:1205-1209, 2000

64. De Swaaf M. E., Sijtsma L., Pronk J. T. High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*, *Biotechnology and Bioengineering*, 81:666-672, 2003

65. Yaguchi T., Tanaka S., Yokochi T., et al. Production of high yields of docosahexaenoic acid by *Schizochytrium* sp. strain SR21., *Journal of the American Oil Chemists Society*, 74:1431-1434, 1997

66. Ling X., Guo J., Liu X., et al. Impact of carbon and nitrogen feeding strategy on high production of biomass and docosahexaenoic acid (DHA) by Schizochytrium sp. LU310, *Bioresource Technology*, 184:139-147, 2014

67. Huang T. Y., Lu W. C., Chu I. M. A fermentation strategy for producing docosahexaenoic acid in *Aurantiochytrium limacinum* SR21 and increasing C22:6 proportions in total fatty acid, *Bioresource Technology*, 123:8-14, 2012.

68. Ren L. J., Huang H., Xiao A., et al. Enhanced docosahexaenoic acid production by reinforcing acetyl-CoA and NADPH supply in *Schizochytrium* sp. HX-308, *Bioprocess and biosystems engineering*, 32(6):837-43, 2009

69. Unagul P., Assantachai C., Phadungruengluij S., et al. Biomass and docosahexaenoic acid formation by *Schizochytrium mangrovei* Sk-02 at low salt concentrations, *Botanica Marina*, 49(2):182-190, 2006

70. Burja A. M., Radianingtyas H., Windust A., et al. Isolation and characterization of polyunsaturated fatty acid producing Thraustochytrium species: screening of strains and optimization of omega-3 production, *Applied microbiology and biotechnology*, 72(6):1161-9, 2006

71. Perveen Z., Ando H., Ueno A., et al. Isolation and characterization of a novel thraustochytrid-like microorganism that efficiently produces docosahexaenoic acid, *Biotechnology Letters*, 28(3):197-202, 2006

72. Sharma R., Singh G. P., Sharma V. K. Comparison of different media formulations on growth, morphology and chlorophyll content of green alga, *Chlorella vulgaris*, *International Journal of Pharma and Bio Sciences*, 2(2):509-516, 2011

73. Truksa M., Wu G., Vrinten P., et al. Metabolic engineering of plants to produce very long-chain polyunsaturated fatty acids, *Transgenic Research*, 15(2):131-137, 2006

74. Barclay W. R., Food product containing *Thraustochytrium* and/or *Schizochytrium* microflora and an additional agricultural based ingredient, Patent US5908622, 1999

75. Jakobsen A. N., Aasen I., Josefsen K. D., et al. Accumulation of docosahexaenoic acid-rich lipid in thraustochytrid *Aurantiochytrium* sp. strain T66: effects of N and P starvation and O_2 limitation, *Applied Microbiology and Biotechnology*, 80(2):297-306, 2008

76. Lippmeier J., Fatty acid metabolism of marine microalgae, PhD Thesis, University of Hull - UK, 2007

77. Shirasaka N., Hirai Y., Nakabayashi H., et al. Effect of cyanocobalamin and p-toluic acid on the fatty acid composition of *Schizochytrium limacinum* (Thraustochytriaceae, Labyrinthulomycota), *Mycoscience*, 46(6):358-363, 2005

78. Hauvermale A., Kuner J., Rosenzweig B., et al. Fatty acid production in *Schizochytrium* sp.: Involvement of a polyunsaturated fatty acid synthase and a type I fatty acid synthase, *Lipids*, 41(8):739-747, 2006

79. Mara, Renewables and Corporation, GRAS Determination of DHA Algal oil for use in infant formula, Technical report, 2016

80. Domozych D. S., Ciancia M., Fangel J. U., Dalgaard Mikkelsen M., et al. The cell walls of green algae: A journey through evolution and diversity, *Frontiers in plant science*, 3:82, 2012

81. Bowles R., Hunt E., Bremer G. B., et al., Long-chain n-3 polyunsaturated fatty acid production by members of the marine protistan group the thraustochytrids: screening of isolates and optimization of docosahexaenoic acid production, *Journal of Biotechnology*, 70:193-202, 1999

82. Siegenthaler P. A., Belsky M. M., Goldstein S. Phosphate uptake in an obligately marine fungus: a specific requirement for sodium, *Science*, 155(3758):93-4, 1967

83. Hiremath S., Mathad P. Impact of salinity on the physiological and biochemical traits of *Chlorella vulgaris*, *Journal of Algal Biomass*, 1(2):51-59, 2010.

84. Moheimani B., McHenry M., de Boer K. Biomass and Biofuels from Microalgae: Advances in Engineering and Biology, Springer, 2015.

85. Doran P. Bioprocess Engineering Principles, Academic Press, 1995.

86. Croft M. T., Warren M. J., Smith A. G., Algae need their vitamins, *Eukaryotic Cell*, 5(8):1175-1183, 2006

87. Singhasuwan S., Choorit W., Sirisansaneeyakul S., et al. Carbon-to-nitrogen ratio affects the biomass composition and the fatty acid profile of heterotrophically grown *Chlorella* sp. TISTR 8990 for biodiesel production, *Journal of Biotechnoly*, 216:169-177, 2015

88. Finkle B. J., Appleman D. The effect of magnesium concentration on growth of *Chlorella*, *Plant physiology*, 28(4):664-673, 1953

89. Liu Z., Wang G., Zhou B. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*, *Bioresource Technology*, 99(11):4717-22, 2008

90. Becker E. W., Microalgae: Biotechnology and Microbiology, Cambridge University Press, 1994

91. Prabandono K., Amin S., Biofuel Production from Microalgae, Handbook of marine microalgae : biotechnology and advances, Academic Press, (2015) 145-158

92. Li Y., Naghdi F. G., Garg S., et al. A comparative study: the impact of different lipid extraction methods on current microalgal lipid research, *Microbial cell factories*, 13:14, 2014

93. Tonon T., Harvey D., Larson T. R., et al. Long chain polyunsaturated fatty acid production and partitioning to triacylglycerols in four microalgae, *Phytochemistry*, 61(1):15-24, 2002

94. Martins D., Custodio L., Barreira L., et al. Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae, *Marine Drugs*, 11(7):2259-2281, 2013

95. Blatti J., Michaud J., Burkart M. D. Engineering fatty acid biosynthesis in microalgae for sustainable biodiesel, *Current Opinion in Chemical Biology*, 17(3):496-505, 2013

96. Muhlroth A., Li K., Rokke G., et al. Pathways of lipid metabolism in marine algae, co-expression network, bottlenecks and candidate genes for enhanced production of EPA and DHA in species of Chromista, *Marine Drugs*, 11(11):4662-97, 2013

97. Voet D., Voet J. Biochemistry, John Wiley & Sons, 2nd edition, 1995

98. Kong F., Torres I., Warakanont J., et al. Lipid catabolism in microalgae, *New Phytologist*, 218(4):1340-1348, 2018

99. Li Y., Xu H., Han F., Regulation of lipid metabolism in the green microalga Chlorella protothecoides by heterotrophy-photoinduction cultivation regime, *Bioresource Technology*, 192:781-791, 2015

100. Taher H., Al-Zuhair S., Al-Marzouqi A., et al. Effective extraction of microalgae lipids from wet biomass for biodiesel production, *Biomass and Bioenergy*, 66:159-167,2014

101. Fan J., Cui Y., Wan M., et al. Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors, *Biotechnology for Biofuels*, 7(1):17, 2014

102. Griffiths M. J., van Hille R., Harrison S. The effect of nitrogen limitation on lipid productivity and cell composition in *Chlorella vulgaris*, *Applied Microbiology and Biotechnology*, 98(5):2345-2356, 2014

103. Benavente-Valdes J., Aguilar C., Contreras-Esquivel J., et al. Strategies to enhance the production of phoosynthetic pigments and lipids in Chlorophycae species, *Biotechnology Reports*, 10:117-125, 2016

104. Hodson R. C., Thompsn J. F., Metabolism of urea by *Chlorella vulgaris*, *Plant Physiology*, 44(5):691-6, 1969

105. Calvey C., Su Y., Willis L. B., et al. Nitrogen limitation, oxygen limitation, and lipid accumulation in *Lipomyces starkeyi*, *Bioresource Technology*, 200:780-788, 2016

106. Li Y., Zhao F., Yu D., et al. Effect of nitrogen limitation on cell growth, lipid accumulation and gene expression in *Chlorella sorokiniana*, *Brazilian Archives of Biology and Technology*, 58(3):462-467, 2015

107. Goncalves E. C., Koh J., Zhu N., et al. Nitrogen starvation-induced accumulation of triacylglycerol in the green algae: Evidence for a role for ROC40, a transcription factor involved in circadian rhythm, *Plant Journal*, 85(6):743-757, 2016

108. Tan K., Lee Y. The dilemma for lipid productivity in green microalgae: Importance of substrate provision in improving oil yield without sacrificing growth, *Biotechnology for Biofuels*, 9(1):255, 2016

109. Morales-Sanchez D., Kim Y., Terng E. A multidomain enzyme, with glycerol-3-phosphate dehydrogenase and phosphatase activities, is involved in a chloroplastic pathway for glycerol synthesis in *Chlamydomonas reinhardtii*, *Plant Journal*, 90(6):1079-1092, 2017

110. Beopoulos A., Cescut J., Haddouche R. *Yarrowia lipolytica* as a model for bio-oil production, *Progress in Lipid Research*, 48(6):375-387, 2009

111. Cohen Z., Ratledge C. Single cell oils-microbial and algal oils. AOCS Press, 2010.

112. Verlinden R., Hill D., Kenward M, et al. Bacterial synthesis of biodegradable polyhydroxyalkanoates, *Journal of Applied Microbiology*, 102(6):1437-1449, 2007

113. Vadivelan G., Venkateswaran G. Production and enhancement of Omega-3 fatty acid from *Mortierella alpina* CFR-GV15: Its food and therapeutic application, *BioMed Research International*, 2014:657414, 2014

114. Adarme-Vega C., Lim D., Timmins M., et al. Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production, *Microbial Cell Factories*, 11:96, 2012

115. Pereira S. L., Leonard A. E., Huang Y, et al. Identification of two novel microalgal enzymes involved in the conversion of the omega3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid, *The Biochemical journal*, 384(2):357-66, 2004

116. Tocher D. R., Agaba M., Hastings N., et al. Biochemical and molecular studies of the polyunsaturated fatty acid desaturation pathway in fish, The Big Fish Bang, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, 2003

117. Metz J. G., Kuner J., Rosenzweig B., et al. Biochemical characterization of polyunsaturated fatty acid synthesis in *Schizochytrium*: Release of the products as free fatty acids, *Plant Physiology and Biochemistry*, 47(6):472-478, 2009

118. PUFA Synthases, Zirkle R., www.lipidlibrary.aocs.org/Biochemistry (Novemeber 2016)

119. Son I., Tan S., Kao P., et al. Recent developments on genetic engineering of microalgae for biofuels and bio-based chemicals, *Biotechnology Journal*, 12(10):1600:1644, 2017

120. Pulz O., Gross W. Valuable products from biotechnology of microalgae, *Applied microbiology and biotechnology*, 65(6):635-648, 2004

121. Remmers I., Wijffels R., Barbosa M., et al. Can we approach theoretical lipid yields in microalgae?, *Trends in Biotechnology*, 36(3):265-276, 2018

122. Zaslavskaia L., Lippmeier J., Shih C., et al. Trophic conversion of an obligate photoautotrophic organism through metabolic engineering, *Science*, 292(5524):2073-2075, 2001

123. Flynn K., Mitra A., Greenwell H., et al. Monster potential meets potential monster: pros and cons of deploying genetically modified microalgae for biofuels production, *Interface Focus*, 3(1):20120037-20120037, 2012

124. Matos J., Cardoso C., Bandarra N. M., et al. Microalgae as healthy ingredients for functional food: a review, *Food & Function*, 8(8):2672-2685, 2017

125. Alsenani F., Ahmed F., Schenk P. Nutraceuticals from microalgae, Nutraceuticals and Functional Foods in Human Health and Disease Prevention, CRC Editors, 1st edition, (2015) 673-684

126. Borowitzka M. High-value products from microalgae - their development and commercialisation, *Journal of Applied Phycology*, 25(3):743-756, 2013

127. Bahadar A., Khan M. B. Progress in energy from microalgae: A review, *Renewable and Sustainable Energy Reviews*, 27:128-148, 2013

128. Hill J., Nelson E., Tilman D., et al. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels, *Proceedings of the National Academy of Sciences of the United States of America*, 103(30):11206-11210, 2006

129. Sheehan J., Dunahay T., Benemann J., et al., Look back at the U. S. Department of Energys Aquatic Species Program: Biodiesel from Algae - Close-Out Report, Technical report, NREL, 1998

130. Park J., Park M., Lee Y., et al. Advances in direct transesterification of algal oils from wet biomass, *Bioresource Technology*, 184:267-275, 2015

131. Gonzalez-Delgado A., Kafarov V., El-Halwagi M. Development of a topology of microalgae-based biorefinery: process synthesis and optimization using a combined forward-backward screening and superstructure approach, *Clean Technologies and Environmental Policy*, 17(8):2213-2228, 2015

132. Vanthoor-Koopmans M., Wijffels R., Barbosa M. J., et al. Biorefinery of microalgae for food and fuel, *Bioresource Technology*, 135:142-149, 2013

133. Wijffels R., Barbosa M. J., Eppink M. Microalgae for the production of bulk chemicals and biofuels, *Biofuels, Bioproducts & Biorefininf*, 4:287-295, 2010

134. Belay A., Ota Y., Miyakawa K., et al. Current knowledge on potential health benefits of *Spirulina*., *Journal of Applied Phycology*, 5(2):235-241, 1993

135. Cell-wall disruption and lipid/astaxanthin extraction from microalgae: *Chlorella* and *Haematococcus*, *Bioresource Technology*, 199:300-310 2015.

136. Bernstein P., Li B., Vachali P., et al. Lutein, zeaxanthin, and meso-zeaxanthin: The basic and clinical science underlying carotenoid-based nutritional interventions against ocular disease, *Progress in retinal and eye research*, 50:34-66, 2016

137. Halim R., Hosikian A., Lim S., et al., Chlorophyll extraction from microalgae: A review on the process engineering aspects, *International Journal of Chemical Engineering*, 2010

138. Nunes M., The Science of Sustenance: Silicon Valley wants to start a food revolution, Solazyme Report 2014 (June 2016)

139. Milne G. Animal Diseases and their Threat to Food and Food Security, Carlton University, 2018

140. Tuttle B. Bird Flu Outbreak Brings on Higher Egg Prices, Shortages at Supermarket, Money, 2015.

141. Lane K., Derbyshire E., Li W., et al. Bioavailability and potential uses of vegetarian sources of omega-3 fatty acids: a review of the literature, *Critical reviews in food science and nutrition*, 54(5):572-9, 2014

142. Gerster H. Can adults adequately convert alpha-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)?, *International journal for vitamin and nutrition research*, 68(3):159-73, 1998

143. Simopoulos A. P. The importance of the ratio of omega-6 / omega-3 essential fatty acids, *Biomedicine and Pharmacotherapy*, 56:365-379, 2002.

144. Swanson D., Block R., Mousa S. Omega-3 fatty acids EPA and DHA: health benefits throughout life, *Advances in nutrition*, 3(1):1-7, 2012

145. Suphioglu C., De Mel D., Kumar L., et al. The omega-3 fatty acid, DHA, decreases neuronal cell death in association with altered zinc transport, *FEBS Letters*, 584(3):612-8, 2010

146. Arab-Tehrany E., Jacquot M., Gaiani C., et al. Beneficial effects and oxidative stability of omega-3 long chain polyunsaturated fatty acids, *Trends in Food Science & Technology*, 25(1):24-33, 2012

147. Mendes A., Reis A., Vasconcelos R., et al. *Crypthecodinium cohnii* with emphasis on DHA production: A review, *Journal of Applied Phycology*, 21(2):199-214, 2009

148. Hashimoto M., Hossain S., Yamasaki H., et al. Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells, *Lipids*, 34(12):1297-304, 1999

149. Cordain L., Eaton S., Sebastian A., et al. Origins and evolution of the Western diet: health implications for the 21st century. *The American Journal of Clinical Nutrition*, 81(2):341-354, 2005

150. Ryckebosch E., Bruneel C., Muylaert K., et al. Microalgae as an alternative source of omega-3 long chain polyunsaturated fatty acids, *Lipid Technology*, 24(6):128-130, 2012

151. Samarasinghe N., Fernando S., Lacey R., Algal cell rupture using high pressure homogenization as a prelude to oil extraction, *Renewable Energy*, 48:300-308, 2012

152. Guenerken E., DHondt E., Eppink E., et al. Cell disruption for microalgae biorefineries, *Biotechnology Advances*, 33(2):243-260, 2015

153. Halim R., Harun R., Danquah M., et al. Microalgal cell disruption for biofuel development, *Applied Energy*, 91(1):116-121, 2012

154. Byreddy A., Gupta A., Barrow C., et al. Comparison of cell disruption methods for improving lipid extraction from Thraustochytrid strains, *Marine Drugs*, 13(8):5111-5127, 2015

155. Voigt J., Woestemeyer J., Frank R. The chaotrope-soluble glycoprotein GP2 is a precursor of the insoluble glycoprotein framework of the *Chlamydomonas* cell wall, *The Journal of biological chemistry*, 282(42):30381-92, 2007

156. Harun R., Danquah M. K. Enzymatic hydrolysis of microalgal biomass for bioethanol production, *Chemical Engineering Journal*, 168(3):1079-1084, 2011

157. Loos E., Meindl D. Composition of the cell wall of *Chlorella fusca*, *Planta*, 156(3):270-273, 1982

158. Loos E., Meindl D. Cell wall-lytic activity in *Chlorella fusca*, *Planta*, 160(4):357-362, 1984

159. Takeda H., Sugar composition of the cell wall and the taxonomy of *Chlorella* (Chlorophyceae), J. Phycol., 27(2):224-232, 1991

160. Zepp R., Sonntag C. The role of non-living organic matter in the earths carbon cycle. John Wiley & Sons, 1995.

161. Borowitzka M. A., Beardall J., Raven J. A., The Physiology of Microalgae. Springer International Publishing, 2016

162. Darley W., Porter D., Fuller M. S. Cell wall composition and synthesis via Golgi-directed scale formation in the marine eukaryote, *Schizochytrium aggregatum*, with a note on Thraustochytrium sp.,*Archiv fuer Mikrobiologie*, 90(2):89-106, 1973.

163. Margulis L., Chapman M. J., Kingdom Protoctista. In Kingdoms and Domains, Elsevier, (2009) 117-230

164. Fan K., Chen F. Production of high-value products by marine microalgae Thraustochytrids, Production of High-Value Products by Marine Microalgae Thraustochytrids, Elsevier, (2007) 293-323

165. Feng X., Walker T., Bridges W., et al. Biomass and lipid production of *Chlorella protothecoides* under heterotrophic cultivation on a mixed waste substrate of brewer fermentation and crude glycerol, *Bioresource Technology*, 166:17-23, 2014

166. Gerde J. A., Montalbo-Lomboy M., Yao L., et al. Evaluation of microalgae cell disruption by ultrasonic treatment, *Bioresource Technology*, 125:175-81, 2012

167. Rakesh S., Dhar D., Prasanna R., et al. Cell disruption methods for improving lipid extraction efficiency in unicellular microalgae, *Engineering in Life Sciences*, 15(4):443-447, 2015

168. Stang M., Schuchmann H., Schubert H. Emulsification in High-Pressure Homogenizers, *Engineering in Life Sciences*, 1(4):151, 2001

169. Doucha J., Livansky K. Influence of processing parameters on disintegration of *Chlorella* cells in various types of homogenizers, *Applied Microbiology and Biotechnology*, 81(3):431-440, 2008

170. DYNO-Mill, www.glenmills.com (June 2016)

171. Postma P. R., Miron T.L., Olivieri G., et al. Mild disintegration of the green microalgae *Chlorella vulgaris* using bead milling, Bioresource Technology, 184:297-304, 2015

172. McMillan J. R., Watson I. A., Ali M., et al. Evaluation and comparison of algal cell disruption methods: Microwave, waterbath, blender, ultrasonic and laser treatment, *Applied Energy*, 103:128-134, 2013

173. Kumar R. R., Rao P. H., Arumugam M. Lipid extraction methods from microalgae: a comprehensive review, *Frontiers in energy research*, 2:1-9, 2015

174. Lee J., Yoo C., Jun S., et al. Comparison of several methods for effective lipid extraction from microalgae, *Bioresource technology*, 101:75-77, 2010

175. Ranjan A., Patil C., Moholkar V. Mechanistic assessment of microalgal lipid extraction, *Industrial & Engineering Chemistry Research*, 49(6):2979-2985, 2010

176. Yao L., Gerde J. A., Lee S. L., et al. Microalgae lipid characterization, *Journal of Agricultural and Food Chemistry*, 63(6):1773-1787, 2015

177. Asenjo J. A., Separation Processes in Biotechnology, CRC Press, 1990.

178. Al-Zuhair S., Ashraf S., Hisaindee S., et al. Enzymatic pre-treatment of microalgae cells for enhanced extraction of proteins, *Engineering in Life Sciences*, 17(2):1-11, 2016

179. Liang K., Zhang Q., Cong W, Enzyme-assisted aqueous extraction of lipid from microalgae, *Journal of Agricultural and Food Chemistry*, 60(47):11771-11776, 2012

180. Demuez M., Mahdy A., Tomas-Pejo E., et al. Enzymatic cell disruption of microalgae biomass in biorefinery processes, *Biotechnology and Bioengineering*, 112(10):1955-1966, 2015

181. Salazar O., Asenjo J., Enzymatic lysis of microbial cells, *Biotechnol Letters*, 29(7):985-994, 2007

182. Fu C., Hung T., Chen J., et al, Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction, *Bioresource Technology*, 101(22):8750-8754, 2010

183. Choi S. P., Nguyen M. T., Sim S. J. Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production, Bioresource Technology, 101(14):5330-5336, 2010

184. Zheng H., Yin J., Gao Z., et al. Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: A Comparison of grinding, ultrasonication, bead Milling, enzymatic lysis, and microwaves, *Applied Biochemistry and Biotechnology*, 164(7):1215-1224, 2011

185. Benvenuti G., Ruiz J., Lamers P., et al. Towards microalgal triglycerides in the commodity markets, *Applied Biochemistry and Biotechnology*, 10(1):188, 2017

186. Doucha J. and Livansky K. Production of high-density *Chlorella* culture grown in fermenters, *Journal of Applied Phycology*, 24(1):35-43, 2012

187. Zhang T. Y., Hu H. Y., Wu Y., et al. Promising solutions to solve the bottlenecks in the large-scale cultivation of microalgae for biomass/bioenergy production, *Renewable and Sustainable Energy Reviews*, 60:1602-1614, 2016

188. Ren L., Ji X., Huang H., et al. Development of a stepwise aeration control strategy for efficient docosahexaenoic acid production by *Schizochytrium* sp., *Applied microbiology and biotechnology*, 87(5):1649-56, 2010

189. Greenwell H., Laurens L., Shields R. Placing microalgae on the biofuels priority list: a review of the technological challenges, *Journal of the Royal Society*, 7(46):703-726, 2010

190. Lam G., Vermue M., Eppink M. H., et al. Multi-product microalgae biorefineries: from concept towards reality, *Trends in Biotechnology*, 2017

191. Murthy G. Enzymatic degradation of microalgal cell walls, *Planta*, 237(1):239-53, 2009

192. Sharma K., Schuhmann H., Schenk P. M. High lipid induction in microalgae for biodiesel production, *Energies*, 5(12):1532-1553, 2012

193. Goldberg E. Handbook of downstream processing, Springer Netherlands, 1997

194. Gimenez A., Gonzalez M. J., Medina A., et al. Downstream processing and purification of eicosapentaenoic (20:5n-3) and arachidonic acids from microalga *Porphyridium cruentum*, *Bioseparation*, 7(2):89-99, 1998

195. Sun L., Ren L., Zhuang X., et al. Differential effects of nutrient limitations on biochemical constituents and docosahexaenoic acid production of *Schizochytrium* sp., *Bioresource Technology*, 159:199-206, 2014

196. Cho H. S., Oh Y., Park S. C., et al. Effects of enzymatic hydrolysis on lipid extraction from *Chlorella vulgaris*, *Renewable Energy*, 54:156-160, 2013

197. Sigma-Aldrich, Bradford Reagent (B6916), Technical Bulletin (2016)

198. Rezende Dos Santos R., Moreira D., Kunigami C., et al. Comparison between several methods of total lipid extraction from *Chlorella vulgaris* biomass, *Ultrasonics sonochemistry*, 22:95-99, 2014

199. Bligh E. G., Dyer W. J. A rapid method of total lipid extraction and purification, *Canadian Journal of Biochemistry and Physiology*, 37(8), 1959

200. Umetrics, User Guide to Modde 9, (2009)

201. Aguirre A. M., Bassi A. Investigation of high pressure steaming (HPS) as a thermal treatment for lipid extraction from *Chlorella vulgaris*, *Bioresource Technology*, 164:136-142, 2014

202. Breuer G., Evers W., de Vree J., et al. Analysis of fatty acid content and composition in microalgae, *Journal of Visualized Experiments*, 80, 2013

203. Alfonsi K., Colberg J., Dunn P. J., et al. Green chemistry tools to influence a medicinal chemistry and research chemistry based organisation, *Green Chemistry*, 10(1):31-36, 2008

204. Warner P., Green Chemistry: Theory and Practice, Oxford University Press, (1998) 135

205. Deniaud-Bouet E., Kervarec N., Michel G., et al. Chemical and enzymatic fractionation of cell walls from Fucales: Insights into the structure of the extracellular matrix of brown algae. *Annals of Botany*, 114(6):1203-1216, 2014

206. Brock W., Von Liebig J., The chemical gatekeeper, Cambridge University Press, 2002

207. Stanbury P., Whitaker P., Hall S., Principles of Fermentation Technology, Elsevier, 2013

208. Roche, Cedex Bio and Cedex Bio HT Analyzers, Advance the potential of your cell culture (2018)

209. Chen F., Johns M. R. Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic *Chlorella sorokiniana*, *Journal of Applied Phycology*, 3(3):203-209, 1991

210. Cheng Y., Lu Y., Gao C., et al. Alga-based biodiesel production and optimization using sugar sane as the feedstock, *Energy& Fuels*, 23(8):4166-4173, 2009

211. Gruber P. J., Frederick S. E., Tolbert N. E. Enzymes related to lactate metabolism in green algae and lower land plants, *Plant Physiology*, 53(2):167-170, 1974

212. Wong C., Whitesides G. M. Enzymes in synthetic organic chemistry, Academic Press, 1994

213. Panesar P., Marwaha S., Chopra H., et al. Enzymes in Food Processing: Fundamentals and Potential Applications, I. K. International Pvt Ltd, 2010

214. Alberts B., Johnson A., Lewis J., et al. Molecular Biology of the Cell, 4th edition, Gardland Science, New York (2002)

215. Xiu Z., Zeng A., Deckwer W. Multiplicity and stability analysis of microorganisms in continuous culture: growth inhibition, *Biotechnology and bioengineering*, 57:251-261, 1998

216. Paczia N., Nilgen A., Lehmann T., Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms, *Microbial cell factories*, 11(1):122, 2012

217. O Grady J., Morgan J. Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol, *Bioprocess and biosystems engineering*, 34(1):121-5, 2011

218. Van Hoek P., Van Dijken J., Pronk J. T. Effect of specific growth rate on fermentative capacity of bakers yeast, *Applied Environmental Microbiolology*, 64(11):4226-4233, 1998

219. Verduyn C., Physiology of yeasts in relation to biomass yields, *Antonie Van Leeuwenhoek*,60(3-4):325-353, 1991

220. Cohen Z. Chemicals from Microalgae, CRC Press, 1999

221. El-Mansi E., Fermentation Microbiology and Biotechnology, 3rd edition, CRC Press, (2011)

222. Yao H., Ren Y., Wei C, et al. Biodegradation characterisation and kinetics of m-cresol by *Lysinibacillus cresolivorans*, *Water SA*, 37(1):15-20, 2011

223. Agrawal P., Koshy G., Ramseier M. An algorithm for operating a fed-batch fermentor at optimum specific-growth rate, *Biotechnology and bioengineering*, 33(1):115-25, 1989

224. Fan J., Cui Y., Wan M. Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors, *Biotechnology for biofuels*, 7(1):17, 2014

225. Li Y., Horsman M., Wang B., et al. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*, *Applied Microbiology and Biotechnology*, 81(4):629-636, 2008

226. Shi X., Jiang Y., Chen F. High-yield production of lutein by the green microalga *Chlorella protothecoides* in heterotrophic fed-batch culture, *Biotechnology progress*, 18(4):723-7, 2002

227. Sansawa H., Endo H. Production of intracellular phytochemicals in *Chlorella* under heterotrophic conditions. *Journal of bioscience and bioengineering*, 98(6):437-44, 2004

228. Zheng Y., Li T., Yu X., et al. High-density fed-batch culture of a thermotolerant microalga *Chlorella sorokiniana* for biofuel production, *Applied Energy*, 108:281-287, 2013

229. Wu Z., Shi X. Optimization for high-density cultivation of heterotrophic *Chlorella* based on a hybrid neural network model, *Letters in Applied Microbiology*, 44:1-18, 2007

230. Yokochi T., Honda D., Higashihara T., et al. Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21, *Applied Microbiology and Biotechnology*, 49:72-76, 1998

231. Chatdumrong W., Yongmanitchai W., Limtong S., Optimization of docosahexaenoic Acid (DHA)production and improvement of astaxanthin content in a mutant *Schizochytrium limacinum* isolated from mangrove forest in Thailand, *Kasetsart Journal - Natural Science*, 41(2), 2007

232. Kimura H., Fukuba T., Naganuma T. Biomass of *Thraustochytrid protoctists* in coastal water. *Marine Ecology Progress Series*, 189:27-33, 1999

233. Ganuza E., Izquierdo M. S., Lipid accumulation in *Schizochytrium* G13/2S produced in continuous culture. *Applied Microbiology and Biotechnology*, 76(5):985-990, 2007

234. Swaaf M., Grobben G., Eggink G., et al. Characterisation of extracellular polysaccharides produced by *Crypthecodinium cohnii*, *Applied Microbiology and Biotechnology*, 57(3):395-400, 2001

235. Ganuza E., Benitez-Santana T., Atalah E., et al. *Crypthecodinium cohnii* and *Schizochytrium* sp. as potential substitutes to fisheries-derived oils from seabream (*Sparus aurata*) microdiets, *Aquaculture*, 277(1-2):109-116, 2008

236. Ethier S., Woisard K., Vaughan D. Continuous culture of the microalgae *Schizochytrium limacinum* on biodiesel-derived crude glycerol for producing docosahexaenoic acid, *Bioresource Technology*, 102(1):88-93, 2011

237. Stephenson A., Dennis J., Howe C., Influence of nitrogen-limitation regime on the production by *Chlorella vulgaris* of lipids for biodiesel feedstocks, *Biofuels*, 1(1):47-58, 2010

238. Qu L., Ji X., Ren L., et al. Enhancement of docosahexaenoic acid production by *Schizochytrium* sp. using a two-stage oxygen supply control strategy based on oxygen transfer coefficient, *Letters in applied microbiology*, 52(1):22-7, 2011

239. Xiu S., Shahbazi A., Wallace C. W., et al. Enhanced bio-oil production from swine manure co-liquefaction with crude glycerol, *Energy Convers. Manag.*, 52(2):1004-1009, 2011

240. Thompson J. C., He B. Characterization of crude glycerol from biodiesel production from multiple feedstocks, *Applied Engineering in Agriculture*, 22(2):261-265, 2006

241. Neilson A. H., Lewin R. A. The uptake and utilization of organic carbon by algae: an essay in comparative biochemistry, *Phycologia*, 13(3):227-264, 1974

242. Bollman R. C., Robinson G. The kinetics of organic acid uptake by three Chlorophyta in axenic culture, *Journal of Phycology*, 13(1):1-5,1977

243. Huang A., Sun L., Wu S., et al. Utilization of glucose and acetate by Chlorella and the effect of multiple factors on cell composition, *Journal of Applied Phycology*, 29(1):23-33, 2017

244. Adasi M. Beet-sugar handbook, John Wiley & Sons, 2006

245. Kristiansen B., Linden J., Mattey M. Citric acid biotechnology, CRC Press, 1999

246. Liu J., Huang J., Jiang Y., et al. Molasses-based growth and production of oil and astaxanthin by *Chlorella zofingiensis*, *Bioresource Technology*, 107:393-398, 2012

247. Yan D., Lu Y., Chen Y., et al. Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. 2011.

248. Veana F., Martinez-Hernandez J., Aguilar C.N., et al. Utilization of molasses and sugar cane bagasse for production of fungal invertase in solid state fermentation using *Aspergillus niger* GH1, *Brazilian Journal of Microbiology*, 45(2):373-377, 2014

249. Morsy F., Hydrogen production from acid hydrolyzed molasses by the hydrogen overproducing *Escherichia coli* strain HD701 and subsequent use of the waste bacterial biomass for biosorption of Cd(II) and Zn(II), *International Journal of Hydrogen Energy*, 36(22):14381-14390, 2011

250. Liu J., Huang J., Fan K., Production potential of *Chlorella zofingienesis* as a feedstock for biodiesel, *Bioresource Technology*, 101:8658-8663, 2010

251. Kuck H., Frankenberg-Dinkel N. Biotechnology, Walter de Gruyter, 2015

252. Tan K. W., Lee Y. K. The dilemma for lipid productivity in green microalgae: importance of substrate provision in improving oil yield without sacrificing growth, *Biotechnology for biofuels*, 9(1):255, 2016

253. Breuer G., Lamers P., Janssen M., et al. Opportunities to improve the areal oil productivity of microalgae, *Bioresource Technology*, 186:294-302, 2015

254. Chen T., Liu J., Guo B., Light attenuates lipid accumulation while enhancing cell proliferation and starch synthesis in the glucose-fed oleaginous microalga *Chlorella zofingiensis*, *Scientific Reports*, 5:14936, 2015

255. OGrady J., Morgan J. Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol, *Bioprocess and biosystems engineering*, 34(1):121-5, 2011

256. Hong D., Anh H., Thu N. Study on biological characteristics of heterotrophic marine microalga *Schizochytrium mangrovei* pq6 isolated from Phu Quoc Island, Kien Giang Province, Vietnam, *Journal of Phycology*, 47(4):944-954, 2011

257. Shene C., Leyton A., Rubilar M., Production of lipids and docosahexasaenoic acid (DHA) by a native *Thraustochytrium* strain, *European Journal of Lipid Science and Technology*, 115(8):890-900, 2013

258. Sijtsma L., De Swaaf M. E., Biotechnological production and applications of the omega-3 polyunsaturated fatty acid docosahexaenoic acid, *Applied Microbiology and Biotechnology*, 64:146- 153, 2004

259. Winwood R. J. Algal oils: Properties and processing for use in foods and supplements, Specialty Oils and Fats in Food and Nutrition, Woodhead publishing, (2015) 160-172

260. Raghukumar S. Thraustochytrid marine protists: Production of PUFAs and other emerging technologies, *Marine Biotechnology* 10(6):631-40, 2008

261. Hancock J. T., Desikan R., Neill S. J. Role of reactive oxygen species in cell signalling pathways, *Biochemical Society Transactions*, 29(2):345, 2001

262. Mallick N., Mohn F. Reactive oxygen species: Response of algal cells, *Journal of Plant Physiology*, 157(2):183-193, 2000

263. Yilancioglu K., Cokol M., Pastirmaci I., et al. Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain, *PLoS One*, 9(3):e91957, 2014

264. Burch A. R., Franz A. Combined nitrogen limitation and hydrogen peroxide treatment enhances neutral lipid accumulation in the marine diatom *Phaeodactylum tricornutum*, *Bioresour. Technol.*, 219:559-565, 2016

265. Membrane fluidity - Phospholipid structure, www.bioninja.com.au (December 2017)

266. Jiang H., Gao K. Effects of lowering temperature during culture on the production of

polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum* (Bacillariophyceae), *Journal of Phycology*, 40(4):651-654, 2004

267. Hu X., Ren L., Chen S., et al. The roles of different salts and a novel osmotic pressure control strategy for improvement of DHA production by *Schizochytrium* sp., *Bioprocess and biosystems engineering*, 38(11):2129-2136, 2015

268. Fan K., Vrijmoed L., Jones E. Physiological studies of subtropical mangrove *Thraustochytrids*, *Botanica Marina*, 45(1):50-57, 2002

269. Chen W., Zhou P., Zhu Y., et al. Improvement in the docosahexaenoic acid production of *Schizochytrium* sp. S056 by replacement of sea salt, *Bioprocess and Biosystems Engineering*, 39(2):315-321, 2016

270. Kang D., Jeh E. J., Seo J., et al. Effect of salt concentration on production of polyunsaturated fatty acids in *Thraustochyhium aureum* ATCC 34304, *Korean Journal of Chemical Engineering*, 24(4):651-654, 2007

271. Ichihara K., Fukubayashi Y. Preparation of fatty acid methyl esters for gas-liquid chromatography, *Journal of Lipid Research*, 51(3):635-640, 2010

272. Van Wychen S., Laurens L. Determination of total lipids as Fatty Acid Methyl Esters (FAME) by in situ transesterification, Laboratory Analytical Procedure (LAP), Technical Report December, National Renewable Energy Laboratory, 2013

273. Mello V., Oliveira F., Fraga W., et al. Determination of the content of fatty acid methyl esters (FAME) in biodiesel samples obtained by esterification using 1H-NMR spectroscopy. *Magnetic Resonance in Chemistry*, 46(11):1051-1054, 2008

274. Nieva-Echevarria B., Goicoechea E., Manzanos M., et al. A method based on 1H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures, *Food Research International*, 66:379-387, 2014

275. Anderson L., Franz A. Real-time monitoring of transesterification by 1H NMR spectroscopy: Catalyst comparison and improved calculation for biodiesel conversion, *Energy and Fuels*, 26(10):6404-6410, 2012

276. Knothe G. Monitoring a progressing transesterification reaction by fiber-optic near infrared spectroscopy with correlation to 1H nuclear magnetic resonance spectroscopy, *Journal of the American Oil Chemists' Society*, 77(5):489-493, 2000

277. Hadley K. B., Bauer J., Milgram N. The oil-rich alga *Schizochytrium* sp. as a dietary source of docosahexaenoic acid improves shape discrimination learning associated with visual processing in a canine model of senescence,*Prostaglandins Leukotrienes and Essential Fatty Acids*, 118:10-18, 2017

278. Boelen P., van Dijk R., Damste J., et al. On the potential application of polar and temperate marine microalgae for EPA and DHA production, *AMB Express*, 3(1):1-9, 2013

279. Song X., Zang X., et al. Production of high docosahexaenoic acid by *Schizochytrium* sp. using low-cost raw materials from food industry, *Journal of oleo-science*, 64(2):197-204, 2015

280. Ye Q., Bao J., Jian-Jiang Z. Cell Factories, Bioreactor Engineering Research and Industrial Applications I, Springer-Verlag Berlin, 2016.

281. Ho J. The heterotrophic culture of *Chlorella protothecoides* for the production of lipids, PhD thesis, Swinburne University of Technology, 2012

282. Gao C., Zhai Y., Ding Y., et al. Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*, *Applied Energy*, 87(3):756-761, 2010

Appendix

1 Media composition for the culture of *Chlorella protothecoides*

	C.protothecoides UTEX 250 [281]	*C.protothecoides UTEX 249 [48]*	*C.protothecoides UTEX 256 [45]*	*C.protothecoides UTEX 255 [217]*	*C.protothecoides [62]*	*C.protothecoides [282]*
Composition medium						
KH_2PO_4	0.70	0.70	0.70	0.70	0.70	0.70
K_2HPO_4	0.30	0.30	0.30	0.30	0.30	0.30
$MgSO_4\,7H_2O$	0.30	0.30	0.30	0.30	0.30	0.30
$CaCl_2$				0.025		
NaCl				0.025		
$FeSO_4\,7H_2O$	3	0.003	0.003	0.003	0.003	0.003
Glycine	0.10			0.10		
Vitamin B1	0.01E-3		0.01E-3	0.01E-3	0.01E-3	0.01E-3
Glucose	10	30	30	30	15	10
Yeast extract		4	4	4	4	
Chlorella GF					1	
A5 Trace Mineral Sol.	1 mL/L	1 ml/L	1 ml/L	1 ml/L	1 ml/L	-
H_3BO_3	2.86	2.86	2.86	2.86	2.86	
$Na_2MoO_4\,2H_2O$	0.039	0.039	0.039	0.039	0.039	
$ZnSO_4\,7H_2O$	0.222	-	0.222	0.222	0.222	
$MnCl_2\,4H_2O$	1.81	1.81	1.81	1.81	1.81	
$CuSO_4\,5H_2O$	0.074	0.074	0.074	0.074	0.074	
$ZnCl_2$		0.105				
$CoCl_2$		0.030				
Conditions	25°C	26°C, pH 6.5	28°C, pH 6.8	28 °C	28°C	28°C
DCW [g/L]	2.33	10	15	ca. 5	16.35	1.59
Lipid [%]	13	22	50		46	53
Lipid prod [g/L/day]	0.6		1.5	2.3	1.3	0.4

2 Worksheet of the experimental design and results

Exp. Name	*Conc. suspension* [g/L]	*Diameter Microbead* [mm]	*Microbeads volume fraction* [%]	*DCW/BSA equivalent* [-]	*Disruption* [%]
N1	40	0.10	50	0.034	44.1
N2	120	0.10	50	0.004	28.2
N3	40	1	50	0.091	99.1
N4	120	1	50	0.071	97.2
N5	40	0.10	80	0.020	57.1
N6	120	0.10	80	0.002	0
N7	40	1	80	0.071	99.6
N8	120	1	80	0.070	95.7
N9	80	0.5	65	0.036	85.1
N10	80	0.5	65	0.036	88.6
N11	80	0.5	65	0.035	90.3

3 Analysis of total fatty acids

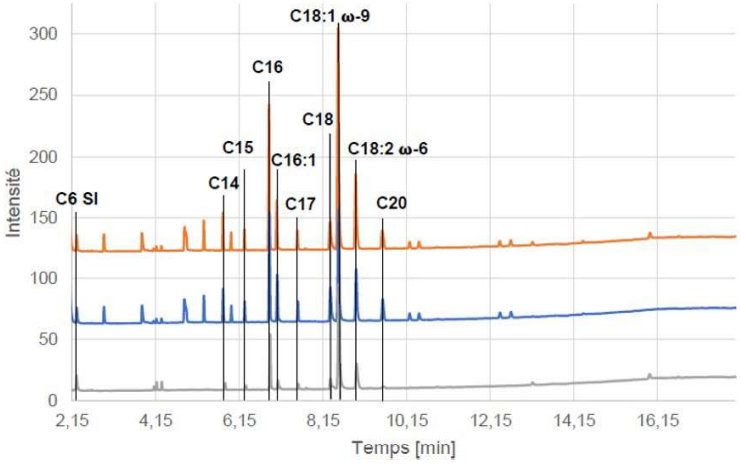


Figure 7.2: *GC-FID analysis of Chlorella fatty acids. (A) Lipid sample + Standard mix 19 FAME + Internal standard; (B) Standard mix 19 FAME; (C) Lipid sample + internal standard. The different FAME were identified and represented on the graphic.*

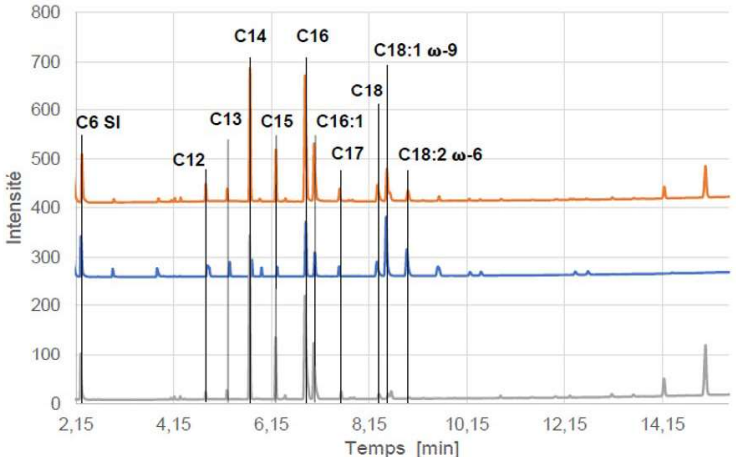


Figure 7.3: *GC-FID analysis of Schizochytrium fatty acids. (A) Lipid sample + Standard mix 19 FAME + Internal standard; (B) Standard mix 19 FAME; (C) Lipid sample + internal standard. The different FAME were identified and represented on the graphic.*

4 Composition of molasses

eurofins

Eurofins Scientific AG
Parkstrasse 10
Postfach
CH–5012 Schönenwerd

Telefon +41 (0)62 858 71 00
Fax +41 (0)62 858 71 09
www.eurofins.ch
info@eurofins.ch

Untersuchungsbericht Nr
AR-17-TT-001174-01

Schweizer Zucker AG
Herr Philipp Steiner
Radelfingerstrasse
Postfach

3270 Aarberg

Email: p.steiner@zucker.ch
Fax: 0041 32 391 62 70

Bestellung EB-78087

Eurofins Probe Nr. **107-2016-50075430**
Probenbezeichnung **Melasse**
Kampagne 2016
20.12.2016

Probeneingang 06.01.2017 Eingangstemperatur 22°C ± 2°C

Test	Ergebnis	Methode
Rohprotein	135 g/kg TS	SOP GCh 02
Rohfaser	<0.1 g/kg TS	SOP MC 219
Rohfett	<0.1 g/kg TS	SOP MC 148
Rohasche	141 g/kg TS	SOP MC 177
Rohwasser	21.3 g/100 g	SOP MC 177
Trockensubstanz (TS)	78.7 g/100 g	Interne Methode
Organische Substanz (OS)	859 g/kg TS	Interne Methode
NEL	7.4 MJ/kg TS	Interne Methode
NEV	8.2 MJ/kg TS	Interne Methode
APDE	73 g/kg TS	Interne Methode
APDN	78 g/kg TS	Interne Methode
NfE	724 g/kg TS	Interne Methode
VES	13.6 g/kg TS	Interne Methode
Glucose	<0.5 g/100 g	Internal method
Fructose	4.1 g/100 g	Internal method
Saccharose	48.1 g/100 g	Internal method
Lactose	<0.5 g/100 g	Internal method
Maltose	<0.5 g/100 g	Internal method
Gesamtzucker (ber. als Summe)	52.2 g/100 g	calc.
*Calcium (Ca)	14 mg/kg	DIN EN ISO 11885, mod.
*Phosphor (P)	190 mg/kg	DIN EN ISO 11885, mod.
*Magnesium (Mg)	21 mg/kg	DIN EN ISO 11885, mod.
*Kalium [K]	40 000 mg/kg	DIN EN ISO 11885, mod.
*Natrium (Na)	5 700 mg/kg	DIN EN ISO 11885, mod.
*Fluorid (F)	<10 mg/kg	PV 01369
*°Nitrat (NO3)	1 441.2 mg/kg	Internal Method based on EN 12014-7
*°Nitrit (NO2)	247.1 mg/kg	Internal Method based on EN 12014-7