THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Institut National Polytechnique de Toulouse (INP Toulouse)

Discipline ou spécialité :

Sciences des Agroressources

Présentée et soutenue par :

M. CARL SAFI

le mercredi 18 décembre 2013

Titre :

Microalgae biorefinery : proposition of a fractionation process

---

Ecole doctorale :

Sciences de la Matière (SM)

Unité de recherche :

Laboratoire de Chimie Agro-Industrielle (L.C.A.)

Directeur(s) de Thèse :

M. CARLOS VACA GARCIA

M. PIERRE YVES PONTALIER

Rapporteurs :

M. JAMES CLARK, UNIVERSITY OF YORK

M. YOON HO-SUNG, KYUNGPOOK NATIONAL UNIVERSITY

Membre(s) du jury :

M. CHRISTIAN CHERVIN, INP TOULOUSE, Président

M. AFIF ABDEL NOUR, KING ABDULAZIZ UNIVERSITY, Membre

M. CARLOS VACA GARCIA, INP TOULOUSE, Membre

M. MICHAEL KROEGER, DEUTSCHES BIOMASSEFORSCHUNGSZENTRUM, Membre

M. PIERRE YVES PONTALIER, INP TOULOUSE, Membre

Foreword

The last century witnessed an exponential increase of world population and an industrial revolution that consumed without limit the resources provided by our planet. Speculations expected that world population will exceed 9 billion by the end of this century, with an increase in life expectancy to 85 years per person. Moreover, due to the unlimited use of fossil fuel, estimations expected a complete depletion of these resources in 2050. This dangerous anthropogenic interference with the climate system resulted in unprecedented increase in global temperature, increase in oceans level, increase in natural disasters and other catastrophes. With this dynamic, our planet is incapable of holding this pressure anymore, and alternative measures are strongly required to prevent additional catastrophes in the future.

Biofuels are part of these measures. These plant-based liquid combustibles are readily obtained from starch or sugars (1^{st} generation bio-ethanol) or from vegetable oils (1^{st} generation bio-diesel). Due to their competition with food applications, intensive research has been done on 2^{nd} generation fuels (bio-ethanol and bio-diesel from cellulosic compounds). Nevertheless, many difficulties hindered their industrial development, which requires alternative renewable resources.

Microalgae are promising microorganisms that can take part of these measures, due to their important diversity and the numerous benefits they can provide; they can grow in autotrophy in fresh or marine water, with few geographical or weather limitations, they multiply rapidly and accumulate large amounts of organic components. For instance, *Chlorella vulgaris* can accumulate within two weeks twice as much oil as a soy plant can accumulate during three months. Moreover, non-arable lands can be used for industrial installations and deforestation can be avoided as well.

During the last two decades, interest in microalgal technology has been mainly focused on bio-energy purposes. But considering all the benefits this biomass can provide, it will be unfortunate to limit the outputs; especially that, so far, the production dedicated to bioenergy is uncompetitive in the market.

Nowadays scientists are convinced that the biorefinery concept (i.e., a sequence of unit operations to achieve the whole fractionation and/or transformation of biomass to produce multiple products) applied to microalgae would render this sector profitable. As it will be demonstrated later, a microalgae biorefinery could generate ten-fold more profit than the single use in biofuels.

For this study we will consider a model microalga: ***Chlorella vulgaris***. The challenge dwells in successfully separating each fraction with minimum cost (equipment and process) and minimum environmental footprint. The solution must be compatible with industrial realities. This challenge is not only ours but global for the microalgae community. Our work does not pretend to solve the whole problem but it will contribute to pursue this main goal.

* * *

This PhD thesis was done within the framework of the French project "**ALGORAFFINERIE**", financed by the French National Research Agency (ANR). It has been conducted at *Laboratoire de Chimie Agro-industrielle* (LCA) located in the Institut National Polytechnique Toulouse in France. It was conducted under the supervision of Professor Carlos Vaca-Garcia and Doctor Pierre-Yves Pontalier.

Part of this work was done in close collaboration with Université Blaise Pascal (Polytech) - Clermont Ferrand, partner of the ANR project. In addition, a successful collaboration was created with the Biomolecular and Bioengineering department of The University of Melbourne (Australia) with Doctor Gregory Martin who hosted me during four months in his laboratory.

The manuscript is dressed with four chapters accessorised with seven publications (published, accepted or submitted by the time of writing) that reflect the fruit of the results obtained:

Chapter one presents an overview on *Chlorella vulgaris* under the form of a review paper followed by a discussion of the algorefinery challenges and concludes on the adopted strategy of our work.

Chapter two is composed of one publication related to the extraction of lipids from *Chlorella vulgaris* investigating whether it is necessary to conduct a cell disruption before applying a supercritical carbon dioxide extraction.

Chapter three compiles four publications that deal with the characterization and extraction of *Chlorella vulgaris* proteins (other microalgae were also analysed for comparison).

Chapter four includes one publication, and it is concentrated on fractionating the aqueous phase by ultrafiltration after breaking its cell wall. The study was conducted on *Tetraselmis suecica* for the reasons that will be described in the introduction of this chapter.

* * *

Albert Einstein quoted: **"*The grand aim of all science is to cover the greatest number of empirical facts by logical deduction from the smallest number of hypothesis or axioms*"**. This quote mirrors my scientific convictions and had inspired the spirit of the following research.

Chapter 1: State of the art on microalgae: scopes and challenges

1.1 Introduction

Considering all microalgal species in the bibliography is almost an impossible task, due to their wide diversity that exceeds one million species. Indeed, microalgae and their technological advancements are still in their infancy, but so far 40.000 have been already isolated and analysed. Therefore, we cannot afford to talk into details about all these species. Consequently, we voluntarily limited the global bibliography in the introduction of the submitted review that gives a general history on microalgae and then detailed bibliography related to all the aspects of *Chlorella vulgaris*. This species has been long exploited and is one of the most grown and consumed microalga in the world. It appears in almost all the publications exposed in this manuscript.

1.2 Morphology, Composition, Production, Processing and Applications of *Chlorella vulgaris*: A review

Carl Safi [1,2], Bachar Zebib [1,2], Othmane Merah[1,2], Pierre-Yves Pontalier [1,2], Carlos Vaca-Garcia [1,2,3]

[1] Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), F-31030 Toulouse, France.
[2] INRA, UMR 1010 CAI, F-31030 Toulouse, France.
[3] King Abdulaziz University, Jeddah, Saudi Arabia.

E-mail : carl.safi@ensiacet.fr

This review has been submitted to ***Renewable & Sustainable Energy Reviews***

Abstract

Economic and technical problems related to the reduction of petroleum resources require the valorisation of renewable raw material. Recently, microalgae emerged as promising alternative feedstock that represents an enormous biodiversity with multiple benefits exceeding the potential of conventional agricultural feedstock. Thus, this comprehensive review article spots the light on one of the most interesting microalga *Chlorella vulgaris*. It assembles the history and a thorough description of its ultrastructure and composition according to growth conditions. The harvesting techniques are presented in relation to the novel algorefinery concept, with their technological advancements and present and potential applications in the market.

Keywords *Chlorella vulgaris*, Algorefinery, Growth conditions, Morphology, Primary composition, Production

1.3 Introduction

Microalgae have an ancient history that left a footprint 3.4 billion years ago, when the oldest known microalga, belonging to the group of cyanobacteria, fossilised in rocks of Western Australia. Studies confirmed that until our days their structure remains unchanged and, no matter how primitive they are, they still represent rather complicated and expertly organised forms of life [1]. Nevertheless, other reports estimated that the actual time of evolution of cyanobacteria is thought to be closer to 2.7 billion years ago [2, 3]. Hence, evolutionary biologists estimate that algae could be the ancestors of plants. Thus, through time algae gave rise to other marine plants and moved to the land during the Palaeozoic Age 450 millions years ago just like the scenario of animals moving from water onto the land. However, evolutionists need to overcome multiple obstacles (danger of drying, feed, reproduction, protection from oxygen) to definitely confirm this scenario complemented with more scientific evidence.

Like any other phytoplankton, microalgae have a nutritional value. The first to consume the blue green microalga were the Aztecs and other Mesoamericans, who used this biomass as an important food source [4]. Nowadays, these microscopic organisms are still consumed as food supplement such as *Chlorella vulgaris* and *Spirulina platensis* [5] and their products are also used for different purposes like dyes, pharmaceuticals, animal feed, aquaculture and cosmetic. For the last two decades, microalgae started to take a new course with enlarging applications motivated by the depletion of fossil fuel reserves, the consequent increase in oil prices and the global warming concern. These dramatic thresholds are forcing the world to find global strategies for carbon dioxide mitigation by proposing alternative renewable feedstocks and intensifying researches on third-generation biofuels. In this context, microalgae are regarded nowadays as a promising sustainable energy resource due to their capacity to accumulate large quantities of lipids suitable for biodiesel production that performs much like petroleum fuel [6, 7]. They also proved to be a source of products such as proteins, carbohydrates, pigments, vitamins and minerals [8]. In addition, microalgae capture sunlight and perform photosynthesis by producing approximately half of atmospheric oxygen on earth and absorbing massive amounts of carbon dioxide as a major feed. Therefore, growing them next to combustion power plants is of major importance due to

their remarkable capacity to absorb carbon dioxide that they convert into potential biofuel, food, feed and highly added value components [9-14].

Microalgae can grow in both fresh and marine water as well as in almost every environmental condition on earth from frozen lands of Scandinavia to hot desert soil of the Sahara [15]. If production plants were installed in an intelligent way, microalgae would not compete with agricultural lands, no conflict with food production [16] and especially would not cause deforestation.

Microalgae represent an enormous biodiversity from which about 40.000 are already described or analysed [17]. One of the most remarkable is the green eukaryotic microalga *Chlorella vulgaris*, which belongs to the following scientific classification: Domain: Eukaryota, Kingdon: Protista, Divison: Chlorophyta, Class: Trebouxiophyceae, Order: Chlorellales, Family: Chlorellaceae, Genus: Chlorella, Species: *Chlorella vulgaris*. Hence, Martin Willem Beijerinck, a Dutch researcher, first discovered it in 1890 as the first microalga with a well-defined nucleus [18]. The name *Chlorella* comes from the Greek word *chloros* (Χλωρός), which means green, and the latin suffix *ella* referring to its microscopic size. It is a unicellular microalga that grows in fresh water and has been present on earth since the pre-Cambrian period 2.5 billion years ago and since then its genetic integrity has remained constant [1]. By the early 1900s, *Chlorella* protein content (>55% dry weight) attracted the attention of German scientists as an unconventional food source. In the 1950s, the Carnegie Institution of Washington [19] took over the study and managed to grow this microalga on a large scale for CO_2 abatement. Nowadays, Japan is the world leader in consuming *Chlorella* and uses it for medical treatment [20, 21] because it showed to have immune-modulating and anti-cancer properties [22-26]. After feeding it to rats, mice and rabbits in the form of powder, it showed protection properties against hematopoiesis [27] age-related diseases like cardiovascular, hypertension, and cataract; it lowers the risk of atherosclerosis and stimulates collagen synthesis for skin [28, 29]. Furthermore, *C. vulgaris* is also capable of accumulating important amounts of lipids especially after nitrogen starvation with a fatty acid profile suitable for biodiesel production [30, 31].

The available reviews have focused so far on evaluating microalgae as an important source of lipids for biofuel production [32, 33] and also explained in details the different production processes and harvesting techniques. The following review covers larger

information about *C. vulgaris*, including not only production and harvesting techniques already conducted on this microalga, but also detailed information about its ultrastructure and chemical composition accompanied by cell wall breaking techniques and extraction processes. The last section focuses on the multiple applications and potential interests of this microalga in different areas and not only on the production of fatty compounds.

1.4 Morphology

Chlorella vulgaris is a spherical microscopic cell with 2-10 mm diameter [33-35] and has many structural elements similar to plants (Fig 1).

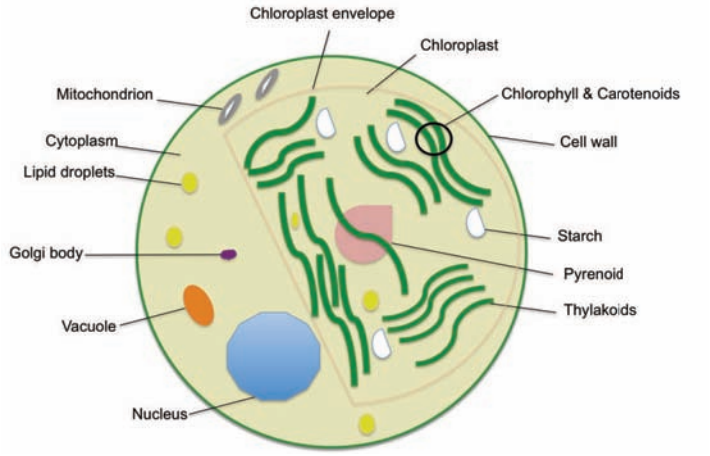

Figure 1: Schematic ultrastructure of *C. vulgaris* representing different organelles

1.4.1 Cell wall

The rigidity preserves the integrity of the cell and is basically a protection against invaders and harsh environment. It varies according to each growth phase. During its early formation in its autosporangia, the newly formed cell wall remains fragile forming a 2 nm thin electron-dense unilaminar layer [33, 36]. The cell wall of the daughter cell gradually increases in thickness until it reaches 17-21 nm after maturation [33, 35], where a microfibrillar layer is formed representing a chitosan-like layer composed of glucosamine [36, 37], which accounts for its rigidity. In the mature stage, cell wall thickness and composition are not constant because they can change according to different growth and environmental conditions. Furthermore, some reports [38, 39]explained the rigidity of the cell wall by focusing on the presence of a sporopollenin layer, even though it is generally

accepted that *C. vulgaris* has a unilaminar cell wall that lacks sporopollenin,which is an extremely resistant polymerised carotenoid found on the cell wall of *Haematococcus pluvialis* [40] and *Chlorella fusca* [41]. However, a contradictory study conducted on *C. vulgaris* by Martinez et al. [42] reported the presence of sporopollenin by observing an outer trilaminar layer and by detecting resistant residues after being submitted to acetolysis.

1.4.2 Cytoplasm

It is the gel-like substance residing within the cell membrane and composed of water, soluble proteins and minerals. It hosts the internal organelles of *C. vulgaris* such as mitochondria, a small nucleus, vacuoles [43], a single chloroplast and the Golgi body [44].

1.4.3 Mitochondrion

Every mitochondrion contains some genetic materials, the respiratory apparatus and has a double-layer membrane; the outer membrane surrounds the whole organelle and is composed of an equal ratio of proteins and phospholipids. Nevertheless, the inner membrane is composed of thrice more proteins than phospholipids; it surrounds the internal space called the matrix, which contains the majority of mitochondrial proteins [44].

1.4.4 Chloroplast

C. vulgaris has a single chloroplast with a double enveloping membrane composed of phospholipids; the outer membrane is permeable to metabolites and ions, but the inner membrane has a more specific function on proteins transport. Starch granules, composed of amylose and amylopectin, can be formed inside the chloroplast, especially during unfavourable growth conditions. The pyrenoid contains high levels of Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) and is the centre of carbon dioxide fixation. The chloroplast also stores a cluster of fused thylakoids where the dominant pigment chlorophyll is synthesised masking the color of other pigments such as lutein. During nitrogen stress, lipid globules mainly accumulate in the cytoplasm and the chloroplast [15, 45].

1.5 Reproduction

Chlorella vulgaris is a non-motile reproductive cell (autospore) that reproduces asexually and rapidly. Thus, within 24 h, one cell of *C. vulgaris* grown in optimal conditions multiplies by autosporulation, which is the most common asexual reproduction in algae. In this manner, four daughter cells having their own cell wall are formed inside the cell wall of the mother cell (Fig 2,3) [33, 35]. After maturation of these newly formed cells, the mother cell wall ruptures allowing the liberation of the daughter ones and the remaining debris of the mother cell will be consumed as feed by the newly formed daughter cells.

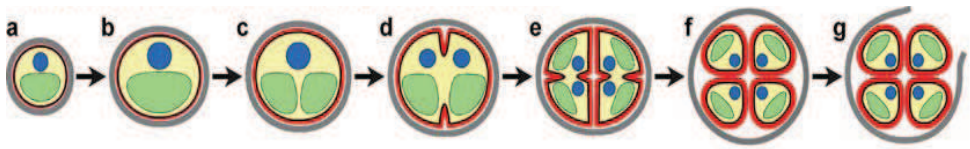

Figure 2: Drawings showing the different phases of daughter cell-wall formation in Chlorella *vulgaris*. a Early cell-growth phase. b late cell-growth phase. c Chloroplast dividing phase. d Early protoplast dividing phase. e Late protoplast dividing phase. f Daughter cells maturation phase. g Hatching phase [35].

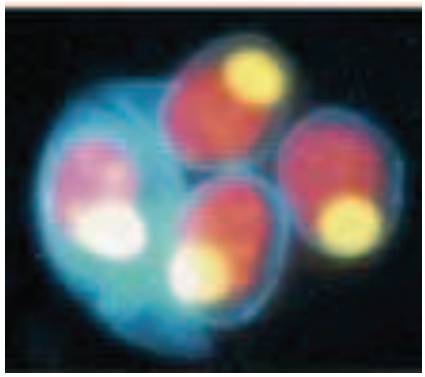

Figure 3: Newly formed cells emerging outside the cell wall of the mother cell after hatching [33].

1.6 Production

Annual production of *Chlorella* reached 2000 tonnes (dry weight) in 2009, and the main producers are Japan, Germany and Taiwan [46]. This microalga has a rapid growth rate and responds to each set of growth condition by modifying the yield of a specific component. *C. vulgaris* is ideal for production because it is remarkably resistant against harsh conditions and invaders. On the one hand, lipid and starch content increases and biomass productivity ceases or decreases [47] during unfavourable growth conditions such as nitrogen and

phosphorus limitation, high CO_2 concentration, excessive exposure to light [30, 48-50], excess of iron in the medium [51], or increase in temperature [52]. On the other hand, protein content increases during normal and managed growth conditions (nitrogen supplementation). Therefore, many growth techniques have been tested in order to voluntarily target biomass productivity, lipid, proteins, carbohydrates and pigments content.

1.6.1 Autotrophic growth

1.6.1.1 Open pond systems

Open ponds are the most common way of production and are the cheapest method for large-scale biomass production. These systems are categorised into natural waters (lakes, lagoons and ponds) or wastewater or artificial ponds or containers. They are usually built next to power plants or heavy industry with massive carbon dioxide discharge where the biomass absorbs nitrogen from the atmosphere in the form of NO_x. The optimal pond depth is 15 to 50 cm [46, 52] in order to allow easy exposure of all the cells to sunlight, especially at the end of the exponential growth phase. On the other hand, open pond systems have some limitations because they require a strict environmental control to avoid the risk of pollution, water evaporation, contaminants, invading bacteria and the risk of growth of other algae species. In addition, temperature differences due to seasonal change cannot be controlled and CO_2 concentration and excess exposure to sunlight are difficult to manage. Moreover, near the end of the exponential growth phase, some cells are not sufficiently exposed to sunlight because other cells floating near the surface cover them, leading to lower mass yields. Therefore, stirring of the medium is preferable and currently practiced.

1.6.1.2 Closed photo-bioreactor

This technology was implemented mainly to overcome some limiting factors in the open pond systems, thus, growing the biomass in a managed environment (pH, light intensity, temperature, carbon dioxide concentration) to obtain higher cell concentration as well as products that are more suitable for the production of pure pharmaceuticals, nutraceuticals and cosmetics. In addition, these systems are more appropriate for sensitive strains that cannot compete and grow in harsh environment. Feeding the biomass with CO_2 comes by bubbling the tubes. Fluorescent lights are used in case the tubes are not or not sufficiently exposed to sunlight. The tubes, are generally 20 cm or less in diameter [32] and

the thickness of their transparent walls is few millimetres allowing appropriate light absorption. Hence, multiple designs have been used and tested: flat-plate photo-bioreactor [53, 54], tubular photo-bioreactor [55] and column photo-bioreactor [56]. Degen et al. [57] achieved 0.11 $g.L^{-1}.h^{-1}$ dry biomass productivity after growing the cells of *C. vulgaris* in a flat panel airlift photobioreactor under continuous illumination (980 mE $m^{-2}.s^{-1}$). Nonetheless, the main disadvantages of closed system are the cost of the sophisticated construction, small illumination area and sterilizing costs [58].

1.6.2 Heterotrophic growth

This technique does not require light and the biomass is fed with organic carbon source. Thus, microalgae are grown in a stirred tank bioreactor or fermenter where higher degree of growth are expected as well as low harvesting cost due to the higher dry biomass productivity achieved (up to 0.25 $g.L^{-1}.d^{-1}$) and high accumulation of different components such as lipids 22-54 $mg.L^{-1}.d^{-1}$ [42, 59, 60]. The carbon sources used for *C. vulgaris* are glucose, acetate, glycerol and glutamate with maximum specific growth rate obtained with glucose. Nevertheless, the major disadvantage of this system is the price and availability of sugars, which compete with feedstocks for other uses such as food and biofuel productions.

1.6.3 Mixotrophic growth

C. vulgaris is capable of combining both autotrophic and heterotrophic techniques by performing photosynthesis as well as ingesting organic materials such as glucose, which is the most appropriate for *C. vulgari*s [59-63]. Hence, the cells are not strictly dependent on light or organic substrate to grow. This technique competes favourably with autotrophic systems and according to Yeh and Chang [63] mixotrophic conditions showed high dry biomass productivity (2-5 $g.L^{-1}.d^{-1}$) and lipids productivity (67–144 $mg.L^{-1}.d^{-1}$). The main advantages of mixotrophic metabolism are limiting the impact of biomass loss during dark respiration and reducing the amount of organic substrates used for growing the biomass.

1.6.4 Other growth techniques

Growth of *C. vulgaris* can take an additional growth dimension by co-immobilizing it with plant growing bacterium *Azospirillum brasilense* in alginate beads [64, 65]. This

technique has been extrapolated to *C. vulgaris* and other microalgae from the hypothesis that *A. brasilense* promotes terrestrial plant growth performance by interfering with the host plant hormonal metabolism and provides O_2 for the bacteria to biodegrade pollutants and then the microalga consumes CO_2 released form bacterial respiration [66]. Consequently, depending on the strain of *C. vulgaris* [67] this technique has an impact on prolonging its life span, enhancing biomass production, cell size (62% larger), pigments and lipids accumulation. Simultaneously, uptake of zinc, cadmium, phosphorus, nitrogen and other heavy metals from wastewater increases. On the other hand, growing *C. vulgaris* with its associative bacterium *Phyllobacterium myrsinacearum* also has a different impact by ceasing its growth or cell death [68]. Furthermore, mixing and shear stress has an effect on increasing the photosynthetic activity and growth of *C. vulgaris*. Thus, optimal conditions (tip speed of 126 $cm.s^{-1}$ and friction velocity 2.06 $cm.s^{-1}$) increased the photosynthetic activity by 4-5% with 48-71% stronger growth compared to null tip speed or friction velocity. Nevertheless, higher tip speed and friction velocity decreased both photosynthetic activity and growth to the value of the unstirred condition and even lower [69].

1.7 Harvesting

1.7.1 Centrifugation

This process contributes to 20-30% of the total biomass production cost [55]. The most common harvesting technique for *C. vulgaris* is centrifugation (5000 rpm, 15 min) [30, 70] because it is highly efficient (95% recovery), not time consuming, and treats large volumes. In addition, the morphology of *C. vulgaris* permits high centrifugal stress without damaging its structure during the process. Other techniques are also applied such as flocculation, flotation, and filtration or by combining two techniques to maximize recovery of the biomass.

1.7.2 Flocculation

During the exponential growth phase, the algal cells have high negative surface charge and are difficult to neutralize, and thus the cells remain dispersed. After reaching the stationary or the declining phase, the negative charge decreases allowing the cells to aggregate and to form lumps resulting in a process called auto-flocculation. This

phenomenon is associated with elevated pH due to CO_2, nitrate and phosphate assimilation [71]. Moreover, auto-flocculation can occur by interactions between algae and bacteria or excreted organic molecules or by simply cutting CO_2 supply; this method is less expensive but time-consuming. In general, culture of microalgae is very stable and auto-flocculation probability is negligible and sometimes misleading. In order to accelerate coagulation, it is necessary to increase the pH by adding a base. The most effective is sodium hydroxide, which induces more than 90% flocculation at pH 11 and requires less quantity (9 mg of NaOH per g of dry biomass) [71, 72]. But on an industrial scale, lime seems to be the most cost-efficient. This mechanism is associated to Mg^{2+} from hydrolysed $Mg(OH)_2$, which precipitates attracting with it the negatively charged microalgal cells. Chitosan is also an interesting flocculating agent [73], which showed maximum efficiency at pH 7 with 90% microalgal recovery. Further on, using bioflocculants like *Paenibacillus* sp. with the presence of a co-flocculant ($CaCl_2$) also showed an efficient flocculation (83%) at pH 11 [74]. Flocculation is sometimes considered as a pre-harvesting step in order to facilitate or complement other harvesting methods like centrifugation or filtration [75, 76].

1.7.3 Flotation

To our knowledge, there is very limited evidence of its feasibility, but this method consists of trapping the cells using a dispersed micro-air bubbles. Flotation can also occur naturally when the lipid content in microalgae increases. Cheng et al. [77] induced effective flotation on *C. vulgaris* by using dispersed ozone gas (0.05 $mg.g^{-1}$ biomass). Thus, unlike flocculation, this method does not require synthetic chemicals, but its economic viability is not yet known, especially on an industrial scale.

1.7.4 Filtration

This method involves continuous passing of the broth with the microalga across a filter on which algal cells will concentrate constantly until it reaches a certain thickness. Due to the small size of *C. vulgaris*, conventional filtration is not an adequate method to be applied. Instead, ultrafiltration or microfiltration are more efficient. Fouling generated by soluble compounds like exopolysaccharides of some microalgae such as *Porphyridium* is one of the major limitations during ultrafiltration process, but with *Chlorella* this phenomenon is

negligible, and thus its structure provides more important permeation flux without the need of an additional unit operation like swirling while filtering [78, 79]. Moreover, microfiltration and ultrafiltration are affected by different parameters such as filter type, transmembrane pressure, flow velocity, turbulent cross-flow and growth phase, and therefore a compromise that takes into consideration these parameters should be made. Furthermore, they can be accompanied by another harvesting technique (flotation or flocculation) that improves the process [75, 76, 80].

1.8 Primary composition

1.8.1 Proteins

Proteins are of central importance in the chemistry of microalgae. They are involved in capital roles such as growth, repair, and maintenance of the cell as well as serving as cellular motors, chemical messengers, regulators of cellular activities and defence against foreign invaders [44].

Total proteins content in mature *C. vulgaris* represents 42-58% of biomass dry weight [81-85], and varies according to growth conditions. Proteins have multiple roles, and almost 20% of total proteins are bound to the cell wall, more than 50% are internal and 30% migrate in and out of the cell [86]. Their molecular weight revealed by SDS-PAGE is comprised between 12 to 120 kDa, with the majority between 39-75 kDa after growing *C. vulgaris* under autotrophic or heterotrophic conditions. Nevertheless a higher intensity peak is observed for cells grown in autotrophic conditions [82, 87].

Protein nutritional quality is determined according to its amino acid profile [81, 88], and like the majority of microalgae, the amino acid profile of *C. vulgaris* compares favourably and even better to the standard profile for human nutrition proposed by WHO (World Health Organisation) and FAO (Food and Agricultural Organisation), because the cells of *C. vulgaris* can synthesise essential and non-essential amino acids (Table 1).

Amino acids	*C. vulgaris*[b]	*C. vulgaris*[a]	*C. vulgaris*[c]	Recommendation from FAO/WHO [b]	Eggs[b]	Soya[b]
Aspartic acid	9.30	10.94	9.80	N/A	11.00	1.30
Threonine	5.30	6.09	5.15	4.00	5.00	4.00
Serine	5.80	7.77	4.32	N/A	6.90	5.80
Glutamic acid	13.70	9.08	12.66	N/A	12.60	19.00
Glycine	6.30	8.60	6.07	N/A	4.20	4.50
Alanine	9.40	10.90	8.33	N/A	n.d	5.00
Cysteine	n.d	0.19	1.28	3.50	2.30	1.90
Valine	7.00	3.09	6.61	5.00	7.20	5.30
Methionine	1.30	0.65	1.24	N/A	3.20	1.30
Isoleucine	3.20	0.09	4.44	4.00	6.60	5.30
Leucine	9.5	7.49	9.38	7.00	7.00	7.70
Tyrosine	2.80	8.44	3.14	6.00	4.20	3.20
Phenylalanine	5.50	5.81	5.51	N/A	5.80	5.00
Histidine	2.00	1.25	1.97	N/A	2.40	2.60
Lysine	6.40	6.83	6.68	5.50	5.30	6.40
Arginine	6.90	7.38	6.22	N/A	6.20	7.40
Tryptophan	n.d	2.21	2.30	1.00	1.70	1.40
Ornithine	n.d	0.13	n.d	N/A	n.d	n.d
Proline	5.00	2.97	4.90	N/A	4.20	5.30

Table 1: Amino acid profile of *Chlorella vulgaris* compared to other resources.
n.d: not detected
N/A: not available
[a][83]
[b][89, 90]
[c] [91]

Protein extraction process is technically the same for all microalgae and is mainly conducted by solubilisation of proteins in alkaline solution (pH 10-12) with NaOH [83, 92, 93]. Further purification can follow by precipitating the solubilised proteins with trichloroacetic acid (25% TCA) [94, 95] or hydrochloric acid (0.1 N HCl) [96]. Quantification is carried out by elemental analysis, Kjeldahl, Lowry assay, Bradford assay or dye binding method. However, the first two analyses take into consideration total nitrogen present in the microalga, and multiplying it by the standard nitrogen to protein conversion factor (NTP) 6.25 may lead to overestimation or underestimation of the true protein quantity. Therefore, many studies calculated from an amino acid profile and recommended a new NTP lower then the standard 6.25 [97-101]. Nevertheless, a study conducted by Safi et al. [83] correlated the evaluation of the NTP to the rigidity of the cell wall by evaluating the NTP of five crude microalgae including *C. vulgaris* and their protein extract, and concluded that no

universal conversion factor could be recommended for multiple reasons such as cell wall rigidity, growth conditions, growth media and environmental uncertainty. Gonzalez-Lopez et al. [98] determined the NTP using a different technique that correlates protein content (Lowry assay) to total nitrogen content (Kjeldahl and elemental analysis) and also estimated that Kjeldahl method correlates better with Lowry assay. In addition, Servaites et al. [84] quantified proteins of 12 different microalgae including *C. vulgaris* by staining the protein isolate with Coomassie brilliant blue R-250 (CBB) on a paper and then eluting the remaining stained proteins in 1% sodium dodecyl sulphate (SDS) followed by measuring the absorbance at 600 nm. This method gave almost similar results compared to Dumas method. On the other hand, the colorimetric method of Lowry [102] was also considered as one of the most accurate methods to quantify proteins [103], but with time this method showed to only quantify hydro-soluble proteins [83, 88, 102-106], which represents the major part of teins. Lowry assay is more acceptable then Bradford assay because the latter does not react with all amino acids present in the extract and thus giving lower protein concentrations [94].

1.8.2 Lipids

Lipids are heterogeneous group of compounds that are defined, not by their structure, but rather by the fact that they are soluble in non-polar solvents and relatively insoluble in water [92]. During optimal growth conditions *C. vulgaris* can reach 5-40% lipids per dry weight of biomass [81], and are mainly composed of glycolipids, waxes, hydrocarbons, phospholipids, and small amounts of free fatty acids [15, 17]. These components are synthesised by the chloroplast and also located on the cell wall and on membranes of organelles (chloroplast and mitochondrion membranes). Nevertheless, during unfavourable growth conditions, lipids content (mainly composed of triacyglycerols) can reach 58% [8, 81, 107]. Unlike other lipids, triacylglycerols do not perform structural role but instead they accumulate as dense storage lipid droplets in the cytoplasm and in the inter-thylakoid space of the chloroplast [17].

Liu et al. [51] optimised a method that detects the accumulation of lipid droplets in *C. vulgaris* after each growth phase, by staining the cells with Nile red dye and then observing the accumulation of lipids with fluorescence microscope by emitting blue light that reveals lipid droplets, especially neutral lipids. This technique showed a correlation

between the quantity of neutral lipids accumulated and fluorescence intensity. However, according to Chen et al. [108] without cell disruption, this method could be ineffective due to the presence of a thick cell wall of some microalgae that can prevent complete access of the reagent inside the cell. Thus, cell disruption is a necessity to prevent wrong measurements and quantification.

The extraction process of total lipids from *C. vulgaris* is generally conducted by the method of Bligh and Dyer using a mixture of chloroform and methanol, or by hexane, or petroleum ether [31, 49, 51, 58, 109-111]. Quantification of total lipids is conducted gravimetrically after evaporating the extracting solvent, in addition column chromatography is applied in order to separate different lipid constituents followed by evaporating the solvent and then weighing the remaining lipid extract [112]. Indeed, these solvents are not be used on an industrial scale because they are harmful for the environment, toxic, highly flammable, and they can contaminate the extract [110]. Supercritical carbon dioxide (SC-CO_2) extraction has been identified as an alternative for a greener extraction since it gives pure extracts free of contamination. Moreover, in order to increase the yield of extraction, a co-solvent to SC-CO_2 such as ethanol can be used or a preliminary cell disruption technique can be performed [113].

The fatty acid profile changes according to each growth condition and is suitable for different applications. For instance, according to Yeh and Chang [63], the fatty acid profile of *C. vulgaris* grown under mixotrophic growth conditions can accumulate 60-68% saturated and monounsaturated fatty acids composed of palmitic acid C16:0, stearic acid C18:0 fatty acids, palmitoleic acid C16:1 and oleic acid C18:1 [31]. Such profile is more suitable for biodiesel production [114]. On the contrary, if it is grown under favourable growth conditions, its fatty acid profile is unsuitable for biodiesel [107] but more suitable for nutritional uses because it is more concentrated in polyunsaturated fatty acids such as linoleic acid C18:2, linolenic acid C18:3, and eicosapentaenoic acid C20:5 [108].

1.8.3 Carbohydrates

Carbohydrates represent a group of reducing sugars and polysaccharides such as starch and cellulose. Starch is the most abundant polysaccharide in *C. vulgaris*. It is generally located in the chloroplast and is composed of amylose and amylopectin, and together with sugars they serve as energy storage for the cells. Cellulose is a structural polysaccharide with high resistance, which is located on the cell wall of *C. vulgaris* as a protective fibrous barrier. In addition, one of the most important polysaccharides present in *C. vulgaris* is the β1→3 glucan [115], which has multiple health and nutritional benefits.

Total carbohydrates are generally quantified by the sulphuric-phenol method [116, 117], yielding simple sugars after hydrolysis at 110°C, then quantification of the latter by HPLC (especially HPIC). Starch quantification is much better using the enzymatic method compared to the acidic method [118, 119]. During nitrogen limitation, total carbohydrates can reach 12-55% dry weight.[120, 121]. Moreover, *Chlorella vulgaris* has a remarkably robust cell wall [122] , mainly composed of a chitosan like layer, cellulose, hemicellulose, proteins, lipids and minerals [123-125].
The sugar composition (Table 2) of the cell wall is a mixture of rhamnose, galactose, glucose, xylose, arabinose and mannose [126-130], rhamnose being the dominant sugar [128, 131, 132].

Neutral sugars	Percentage
Rhamnose	45-54
Arabinose	2-9
Xylose	7-19
Mannose	2-7
Galactose	14-26
Glucose	1-4

Table 2: Simple sugars composition of the cell wall [131].

1.8.4 Pigments

The most abundant pigment in *C. vulgaris* is chlorophyll, which can reach 1-2% dry weight and is situated in the thylakoids. *C. vulgaris* also contains important amounts of carotenoids (Table 3) that act as accessory pigments by catching light; β-carotene for

instance is associated to the lipid droplets in the chloroplast, and primary carotenoids are associated with chlorophyll in thylakoids where they trap light energy and transfer it into the photosystem. However, as in terrestrial plants, some pigments act as photo-protectors by protecting chlorophyll molecules from degradation and bleaching during strong exposure to radiation and oxygen [44].

Pigments	µg.g^{-1} (dw)	References
β-carotene	7-12000	[20, 65, 70, 133, 134]
Astaxanthin	550000	[134-136]
Cantaxanthin	362000	[133-135, 137]
Lutein		[20, 65]
	52-3830	[67, 70]
		[133]
		[134]
Chlorophyll-*a*		[65]
	250-9630	[20, 67]
		[68, 133]
Chlorophyll-*b*		[65]
	72-5770	[20, 67]
		[70, 133]
Pheophytin-*a*	2310-5640	[70]
Pheophytin-*b*	N/A	[70]
Violoxanthin		[65]
	10-37	[67]

Table 3: Potential pigments content in *C. vulgaris* under different growth conditions. N/A: not available

These pigments have multiple therapeutic properties, such as antioxidant activities [138], protective effect against retina degeneration [139, 140], regulating blood cholesterol, prevention from chronic diseases (cardiovascular and colon cancer) and fortifying the immune system [141, 142]. Pheophytins are biochemically similar to chlorophyll but lacking Mg^{++} ion, they can form after chlorophyll degradation during growth of microalgal cells or during harsh extraction conditions. In addition, these pigments are lipophilic and their extraction is generally associated to lipid extraction.

Many studies worked on optimizing the extraction process of pigments using solvents (dimethyl formamide, dichloromethane, acetone, hexane, ethanol), soxhlet, ultrasound-assisted extraction [70, 143-146], and pressurised liquid extraction (PLE) that

showed useful simultaneous extraction of carotenoids and chlorophyll, and also minimised the formation of pheophytins [70, 133] at high temperature (>110°C). Moreover, SC-CO_2 extraction was also carried out to enhance carotenoids recoveries, and the best conditions were 35 MPa and 40-55°C on crushed cells, and under these conditions the extract was golden and limpid unlike solvents extraction, thus by using SC-CO_2, higher selectivity can be achieved [133, 144]. This hypothesis is confirmed by Kitada et al. [20], using different optimum conditions (50 MPa and 80 °C) because the study was conducted on whole cells, thus stronger conditions were required. In addition, co-solvent such as 5% ethanol has been added as a booster to increase the extraction yield. Analyses and quantification of pigments are conducted by high performance liquid chromatography (HPLC) and spectrophotometry using specific equations [137] or by plotting the calibration curve for each pigment.

1.8.5 Minerals and Vitamins

Minerals are determined after incinerating the biomass and then analysis by atomic absorption spectrophotometry (Table 4). They play important functional roles in humans [44]. For instance, potassium cation is principal for human nutrition; it is associated with intracellular fluid balance, carbohydrate metabolism, protein synthesis and nerve impulses. In addition, it is used as chemical fertilizer in agriculture in the form of chloride (KCl), sulphate (K_2SO_4) or nitrate (KNO_3). Magnesium is important in maintaining normal and constant nervous activity and muscle contraction; hence magnesium deficiency in human organism can lead to depression and symptoms of suicidal behaviour. Zinc is an essential component of enzymes, which participate in many metabolic processes including synthesis of carbohydrates, lipids, proteins and it is also a cofactor of the superoxide dismutase enzyme, which is involved in the protection against oxidative processes and reducing the severity of strong diarrhea.

	Mineral content ($g.100g^{-1}$)		
Minerals	Maruyama et al. [91]	Tokusoglu and Unal [147]	Panahi et al. [148]
Microelements			
Na	N/A	1.35	N/A
K	1.13	0.05	2.15
Ca	0.16	0.59	0.27
Mg	0.36	0.34	0.44
P	N/A	1.76	0.96
Macroelements			
Cr	N/A	tr	tr
Cu	N/A	tr	0.19
Zn	N/A	tr	0.55
Mn	N/A	tr	0.40
Se	N/A	tr	N/A
I	N/A	N/A	0.13
Fe	0.20	0.26	0.68

Table 4: Minerals profile of *C. vulgaris*
tr: traces
N/A: not availbale

Vitamins are classified as water-soluble (C, B) and fat-soluble (A, D, E, K). *C. vulgaris* has an important vitamin profile (Table 5) that are key elements for cell growth and differentiation in human body (Vitamin A), and have antioxidant activity that acts as radical scavenger together with improving blood circulation and controlling muscle functions (vitamin E and C) [149]. Vitamin B complex occupies the largest number in living organisms and is major actor for enzymes activity in metabolism [150], promotes red blood cells growth, reduce the risk of pancreatic cancer, and maintain healthy skin, hair and muscles. Vitamins profile is sensitive to growth conditions, thus the best concentration was achieved after 24h autotrophic growth with 10% CO_2, but during heterotrophic conditions vitamins content was higher than autotrophic due to the presence of glucose in the medium and used as carbon source to produce organic compounds [87]. Another possible explanation for the high content of vitamins may be the alterations in the ultrastructure of the photosynthetic apparatus which were found to be associated with changes in cellular components [151].

	Content (mg.100g^{-1})		
Vitamins	Maruyama et al. [91]	Yeh et al. [114]	Panahi et al. [148]
B1 (Thiamine)	2.4	N/A	1.5
B2 (Riboflavin)	6.0	N/A	4.8
B3 (Niacin)	N/A	N/A	23.8
B5 (Pantothenic acid)	N/A	N/A	1.3
B6 (Pyridoxine)	1.0	N/A	1.7
B7 (Biotin)	N/A	N/A	191.6
B9 (Folic acid)	N/A	N/A	26.9
B12 (Cobalamin)	Tr	N/A	125.9
C (Ascorbic acid)	100.0	39.0	15.6
E (Tocopherol)	20.0	2787.0	N/A
A (Retinol)	N/A	13.2	N/A

Table 5: Vitamins profile of *C. vulgaris*
N/A: not available

1.9. Cell disruption techniques

Chlorella vulgaris has a resistant cell wall, which is a major barrier for digestibility and extraction process of all internal components. Breaking the cell wall is an important challenge and a costly unit operation. Multiple techniques have been carried out on *C. vulgaris* (Table 6). Cooling the system during mechanical cell breaking is always required because the high-energy input overheats the broken microalga and jeopardise the integrity of target components by damaging or oxidising them. The enzymatic treatment is a promising technique that requires a deep understanding of the ultrastructure and composition of the cell wall in order to select the appropriate enzyme and to reduce the enzyme concentration required to hydrolyse the cell wall. According to Lee et al. [109] and Zheng et al. [31] the best cell disruption techniques with 30% dry weight lipid recovery of *C. vulgaris* grown under autotrophic conditions were autoclaving, microwave, enzymatic and grinding with liquid nitrogen.

The success of cell disruption techniques is generally assessed by conducting microscopic observations or by comparing the extracted yield of a component before and after applying the cell disruption.

Cell disruption	Time	Experimental set-up	References
Acid treatment	25 min	Hot Ac_2O + H_2SO_4 (9:1, v:v)	[70]
Alkaline treatment	60 min	2 N NaOH	[83]
Autoclaving	5 min	125°C + 1.5 MPa	[109]
Bead milling	20 min	Beads: 0.4 - 0.6 mm Rotational speed 1500 rpm	[31]
	5 min	Beads: 0.1 mm, Rotational speed 2800 rpm	[109]
	2 min	Beads: 1 mm	[59]
Electroporation	N/A	Electric field: 3 kV/cm Electrode 2 cm	[73]
Enzymatic lysis	60 min	Snailase (5 mg. L^{-1}), 37°C	[31]
	10 h	Cellulase or Lysozyme (5 mg.L^{-1}), 55°C	
	N/A	4% Cellulase+1% others (w/v)	
		25 mM Sodium Phosphate buffer pH 7.0 0.5 M Mannitol	[152]
	10 h	4% Cellulase + 1% Macerozyme R10 + 1% Pectinase (w/v) pH 6.0 25 mM Phosphate buffer 0.6 M Sorbitol/Mannitol (1:1)	[93]
	24 h	Cellulase 0.5 mg.L 0.5 M Mannitol	[153]
French Press	N/A	138 MPa	[154]
	N/A	N/A	[78]
Manual grinding	1-10 min	With liquid nitrogen or quartz	[31]
	N/A	With dry ice	[155]
High pressure homogenizer	N/A	N/A	[156]
Microwaves	5 min	100°C, 2450 MHz	[31, 109]
	5 min	40-50°C, 2450 MHz	[110]
Osmotic shock	48 h	10% NaCl	[109]
	60 min	2 N NaOH	[83]
Ultra-sonication	6 min	10 W	[84]
	20 min	600 W	[31]
	5 min	10 kHz	[109]
	15-60 min	N/A	[50]

Table 6: Different cell disruption techniques carried out on *C. vulgaris*
N/A: not available

1.10 Applications and potential interests

1.10.1 Biofuel

Dependency on energy sources is growing faster especially with the exponential increase in demand, which is leading to more dramatic consequences for the environment. Third generation biofuel form algae or microalgae is considered as one of the alternatives to current biofuel crops such as soybean, corn, rapeseed and lignocellulosic feedstocks because it does not compete with food and does not require arable lands to grow [16]. However, biofuel from microalgae is promising on the long term because it is now accepted that the production cost is still high and cannot yet compete with conventional fuel. But it competes favourably with crops by their potential of producing 10-20 times more oil [157] within a shorter period of time. As mentioned previously, *C. vulgaris* has the potential to accumulate high amounts of lipids especially while growing it under mixotrophic conditions. Its fatty acid profile showed to be suitable for biodiesel production with an oxidative stability after transforming it to biodiesel, and has properties [158] that complies with the US Standard (ASTM 6751), European Standard (EN 14214), Brazilian National Petroleum Agency (ANP 255) and Australian Standard for biodiesel [159] and also compared favourably with (ASTM and EN) and Indian biodiesel standard [61]. After lipid extraction the remaining residue is rich in proteins, carbohydrates and minor amounts of lipids. Thus, Wang et al. [158] applied fast pyrolysis on *C. vulgaris* remnants using an atmospheric-pressure fluidised bed reactor at 500 °C and obtained bio-oil and biochar representing 94% of energy recovery from the remnant, without forgetting the small amount of biogas recovered. However, the quality of bio-oil was poor when intended to be catalytically upgraded to fuels due to high nitrogen presence (12.8% dry weight). Besides, *C. vulgaris* has high starch content and algal starch proved to be a good source for bioethanol production. Hirano et al. [160] extracted starch from *C. vulgaris* and achieved 65% ethanol-conversion rate after saccharification and fermentation with yeast. Hydrothermal liquefaction is another alternative route for biofuel production from microalgae. It involves the reaction of biomass in water at high temperature with or without the presence of a catalyst to obtain bio-crude [161]. The main advantage of this method is that it improved 10-15% the energetic value of *C. vulgaris* by acting on the whole biomass suggesting that oil is also derived from carbohydrates and proteins [162], and thus no need to stress the microalgae to increase lipid content. Hence, the best conditions applied on *C. vulgaris* in a batch reactor were 300-350 °C, with 150-200 bar in water or with

the presence of an organic acid or heterogeneous catalysts, and the results indicate that bio-oil formation follows the trend lipids > proteins > carbohydrates [161-163].

Nowadays, algal biofuel is suffering from several drawbacks jeopardising its commercialisation on an industrial scale due to high production cost that is far from being competitive with fossil fuel, and also questioning the sustainability of this production. Hence, different studies considered life cycle assessment analysis as an effective tool to identify the reasons leading to production deficit and exploring its environmental impact [164-171]. Therefore, it was agreed that the major costs come from infrastructure, production set-up, fertilizers, harvesting, drying the biomass, transportation, water footprints, cell disruption and oil extraction process. For instance, Lardon et al. [172] performed an analysis by taking into account all the energetic debt for 1 MJ biodiesel production from *C. vulgaris*. The only positive balance obtained was 0.57 MJ for wet oil extraction with low nitrogen for cell growth (Table 7), and all the other revealed negative balance. Hence, microalgal biofuel production still needs efficient improvement to reduce energy input needed in order to reach competitive prices with petroleum in the market, and more important to be an overall sustainable production.

Oil Extraction	Nitrogen for culture	Energy Production (MJ)	Cumulative Energy Demand (MJ)	Yield (MJ)
Dry	Sufficient	2.7	5.29	-2.59
Wet	Sufficient	3.84	3.99	-0.15
Dry	Low	1.57	2.32	-0.75
Wet	Low	2.23	1.66	0.57

Table 7: Cumulative Energy Demand and energy production associated with the production of 1 MJ of biodiesel from *C. vulgaris* [172].

1.10.2 Human nutrition

C. vulgaris is one of the few microalgae that can be found in the market as a food supplement or additive [5, 145], colorant (*C. vulgaris* after carotenogenesis) and food emulsion [119]. These products come in different forms such as capsules, tablets, extracts and powder [173, 174]. Nevertheless, despite all the healthy benefits that *C. vulgaris* and other microalgae can provide, and their remarkable richness in proteins, lipids, polysaccharides, pigments and vitamins, they are rather considered as nutraceuticals instead of food products due to the lack of clear common official legislations in terms of quality and requirements regarding microalgae [175, 176]. Moreover, *C. vulgaris* extract proved to have

preservative activity higher than those obtained synthetically, i.e., BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) [177].

1.10.3 Animal feed

It is estimated that about 30% of microalgal production is sold for animal feed purposes [178] due to the increasing demand for food with natural composition instead of synthesised ingredients. This has triggered intensive research into finding natural ingredients that improve the quality of animal food products [119]. Thus, while stressing *C. vulgaris*, it accumulates important amount of carotenoids and after feeding it to animals such as fish and poultry it showed interesting pigmentation potential for fish flesh and egg yolk in poultry, together with enhancing health and increasing life expectancy of animals [134, 155, 174, 178-181]. Moreover, *C. vulgaris* showed a protective effect against heavy metals and other harmful compounds (Lead, Cadmium, Naphtalene) by reducing significantly the oxidative stress induced by these harmful compounds, and increasing the antioxidant activity in the organisms of tested animals [182-184].

1.10.4 Wastewater treatment

Many studies demonstrated the remarkable potential of *C. vulgaris* in fixating up to 74% carbon dioxide when grown in a photobioreactor [185], and in absorbing 45-97% nitrogen, 28-96% phosphorus and reducing the chemical oxygen demand (COD) 61-86% from different type of wastewater such as textile, sewage, municipal, agricultural and recalcitrant [186-192]. Microalgae provide a pathway for the removal of vital nutrients (nitrogen and phosphorus), carbon dioxide, heavy metals and pathogens present in wastewaters and necessary for their growth. In addition, saving and requirements for chemical remediation and possible minimisation of fresh water use for biomass production are the main drivers for growing microalgae as part of a wastewater treatment process [46]. Thus, a faster growth rate accompanied with decreasing or eliminating water-contamination level are promising and advantageous process. Furthermore, performance of *C. vulgaris* in synthesised wastewater was improved when co-immobilised in alginate beads with microalgae growth-promoting bacteria, and removed 100% of ammonium (NH_4^+) during four consecutives cycles of 48 h, and 83% for phosphorus after one cycle of 48h [193]. Thus, *C. vulgaris* is considered as one of the best microalga for bioremediation of wastewater with

an impressive potential to completely remove ammonium and sometimes-modest potential for removal of phosphorus present in the medium [194].

1.10.5 Agrochemical applications

Blue-green algal extract excretes a great number of substances that influence plant growth and development [195]. These microorganisms have been reported to benefit plants by producing growth promoting regulators, vitamins, amino acids, polypeptides, antibacterial and antifungal substances that exert phytopathogen biocontrol and polymers, especially exopolysaccharides, that improve plant growth and productivity [196].

The bio-fertilization effect using algae extract are recommended for increasing the growth parameters of many plants [197, 198]. This is due to the biochemical profile of algae extract rich in nitrogenase, nitrate reductase, and minerals, which are essential nutrients for plant growth. The effect of the aqueous extract of *C. vulgaris* as foliar feeding on nutrients status, growth, and yield of wheat plant (*Triticum aestivum* L. var. Giz 69) has been investigated [199]. Thus, this study found that a concentration of 50% (v/v) algae extract as one time foliar spray (25 days after sowing) increased growth yield and weight gain 140% and 40% respectively. Moreover, another study showed the bio-fertilization impact of *C. vulgaris* on growth parameters and physiological response of *Lactuca sativa* germination seeds in culture medium containing microalga grown for 3, 6, 9, 12 and 15 days [200]. As a result, the addition of *C. vulgaris* to the culture medium or soil significantly increased fresh and dry weight of seedlings as well as pigments content. The best treatments were 2 and 3g dry alga kg^{-1} soil. All these studies were conducted on the liquid extract of *C. vulgaris* as bio-fertilizer for plant growth. Therefore, further studies should be carried out to estimate costs on a large scale of the algae cell extract as foliar fertilizer, compared to other commercial foliar fertilizers present in the market.

1.11 Algorefinery concept

The concept of biorefinery has been inspired from the petroleum refinery concept. It reflects a platform that integrates a process to fractionate the components of a biomass [201, 202] to produce multiple products, and thus biorefinery takes advantage of the various components in the biomass in order to improve the value derived from each component and also generating its own power, which maximises profitability and preserve the environment.

Hence, *C. vulgaris* with all its potential and richness in proteins, carbohydrates, lipids, pigments, minerals and vitamins described previously deserves to be completely refined (Fig 4) without forgetting that every operation unit should take into account the next stage and preserve the integrity of all components of interest in the downstream process.

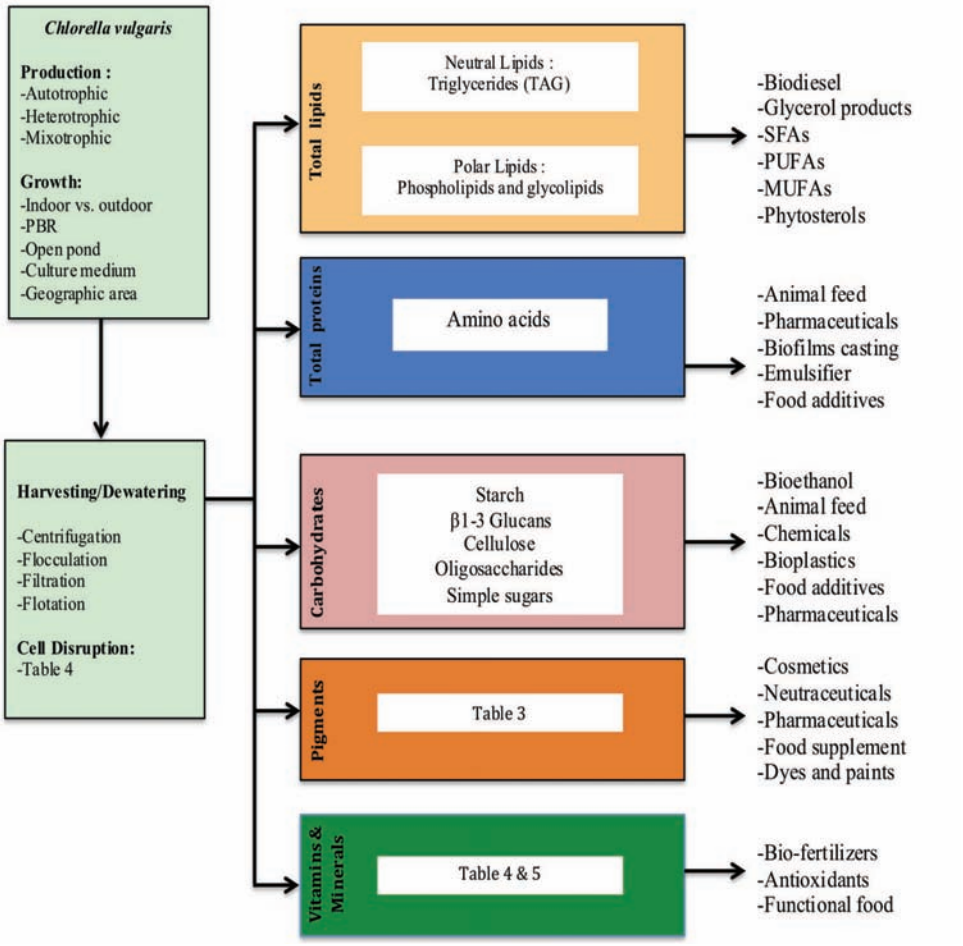

Figure 4: Algorefinery concept from production to valorisation

1.12 Conclusion

This review reflects a broader image about potential interest of *Chlorella vulgaris*, and gives an insight about the technological advancements already conducted. *Chlorella vulgaris* can easily be cultured with inexpensive nutrient regime and has faster growth rate as compared to terrestrial energy crops and high biomass productivity. However, production-

processing cost remains too high to compete in the market. Indeed, this is the major problem facing the microalgal industry nowadays, but it should be recognised that a lot of improvements have been achieved during the last decade and expectations are estimating that the nearest future of microalgal industry will be strongly competitive on different levels in the market. The remarkable values of *Chlorella vulgaris* sets the groundwork to additional research for futuristic applications where it will be represented as a strong candidate for tomorrow's bio-industry.

Acknowledgments
The authors would like to thank "Agence Nationale de Recherche (ANR)" for the financial support, and Laboratoire de Chimie Agro-industrielle (LCA) for providing all the necessary tools and requirements to dress this review.

1.13 Considerations on the algorefinery

The concept of biorefinery has been inspired from the petroleum refinery concept. It reflects a platform that integrates a process to fractionate the components of a biomass to produce multiple products, and thus biorefinery takes advantage of the various components in the biomass in order to improve the value derived from each component and also generating its own power, which maximises profitability and preserve the environment.

The concept requires a sequence of unit operations starting from pre-treating the biomass by a chemical depolymerisation of the microalgal cell wall polysaccharides or by a mechanical disruption of the cell wall integrity. This allows an easier intracellular access of the extraction solvent, and contributes in enhancing the recovery yield of the dedicated biomolecules.

On the one side, depolymerisation via hydrolysis of glycosidic bonds is ubiquitous in both nature and industry. However, to become a viable process for microalgae, hydrolysis must be carried out both energy-efficient and under mild conditions to preserve the chemical integrity of all the constituents or at least their functional properties in order to ensure broader commercial possibilities.

On the other side, and because of the reasons exposed above, nowadays the most efficient methods are bead milling and high-pressure homogenization. Mechanical methods are often preferred due to the short residence time and **lower operating costs**. Nevertheless, these mechanical methods generate friction that overheats the medium, which should be constantly cooled all along the process to avoid denaturation of proteins or thermal degradation of lipids.

All these biomolecules can generate important profit (Table 1) compared to biodiesel. Proteins can be sold at about 0.75 €/kg for feed protein, and at 5 €/kg for food proteins. Carbohydrates are sold in the market at about 1 €/kg, and if antiviral properties are identified, the price can be extremely high. Lipids for biofuels generate the lowest profit (about 0.5 €/kg), which is an additional reason for not simply focusing on producing biofuels from microalgae. But instead, it will be more profitable if these biomolecules are valorised

for their fatty acids (especially the unsaturated ones, sold at more than 2 €/kg). Finally, the pigments are also a valuable resource and their price can largely fluctuate depending on the purity of the sample and the target market (cosmetics or fish food for instance).

Primary compounds	Approximate content (%)	Unitary price of compound (€/ton)	Value for a biofuel case (€/ton microalgae)	Value for a multimarket case (€/ton microalgae)
Lipids	50	300 for biofuel without State aid 2000 for chemicals	150	1000
(Food) proteins	20	5000	0	1000
Carbohydrates	30	1000	0	300
Pigments (e.g. astaxanthin)	1	5 000 000	0	50 000
		Total	**150**	**52 300**

Table 1: Approximate value generated for biofuel compared to the profit for multimarket in the ideal case

The process that we will study is a **"primary algorefinery"** that will deal with the primary components of microalgae (lipids, proteins, carbohydrates and pigments). A sequence of unit operations will be implemented in order to obtain separated enriched fractions of the biomolecules described previously. By "enriched fraction" we understand the degree of purity equivalent to the "technical grade" of commercial chemical compounds.

Once that the scope of our work has been defined, we are now going to present the different challenges relative to the possible options from which an algorefinery could be carried out.

Option 1: with humid biomass (Fig 1)

This process starts with breaking the cell wall in an aqueous medium containing between 2 and 25% of dry matter depending on the cell disruption method applied. After separation of the solid (e.g. filtration or centrifugation), it would be obtained an emulsified mixture mainly composed of:

- Reserve lipids (triglycerides)
- Hydro-soluble proteins
- Polysaccharides
- Pigments

whereas the solid would be mainly composed of structural biomolecules (polysaccharides, phospholipids and proteins) as well as reserve polysaccharides (insolubilized starch). Each

fraction should undergo additional unit operations to properly fractionate their composition into enriched fractions.

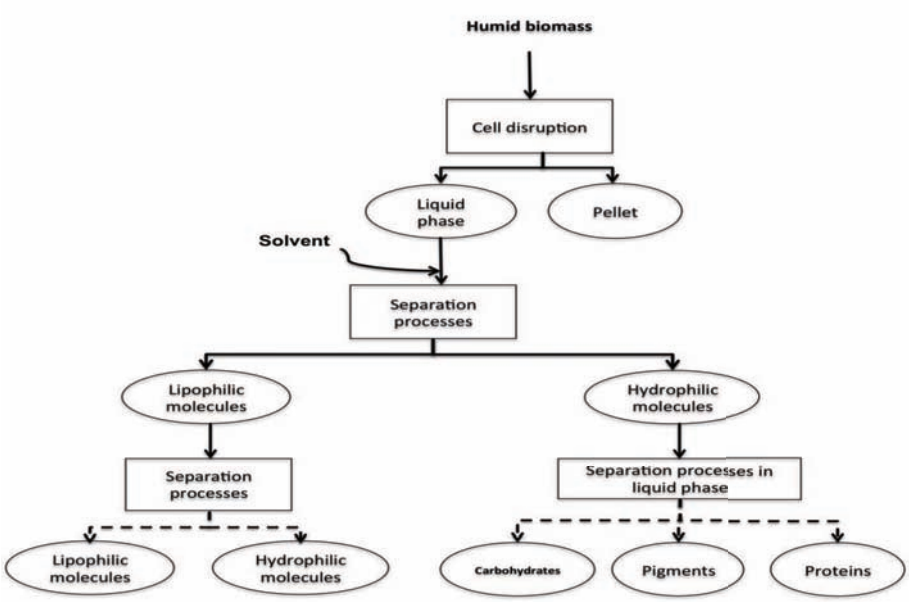

Figure 1: Process scheme for the first possible option, which starts from humid biomass

Option 2: with dry biomass (Fig 2)

After reducing the water content below 2%, an extraction with an organic solvent could be performed to recover in the liquid phase two types of lipids (reserve and structural) as well as liposoluble pigments. The solid fraction would be composed of defatted cells requiring further disruption to liberate the intracellular hydrophilic components. Contrarily to **option 1** the liquid fraction after cell wall lysis would not be emulsionated. The downstream process would be therefore easier to implement.

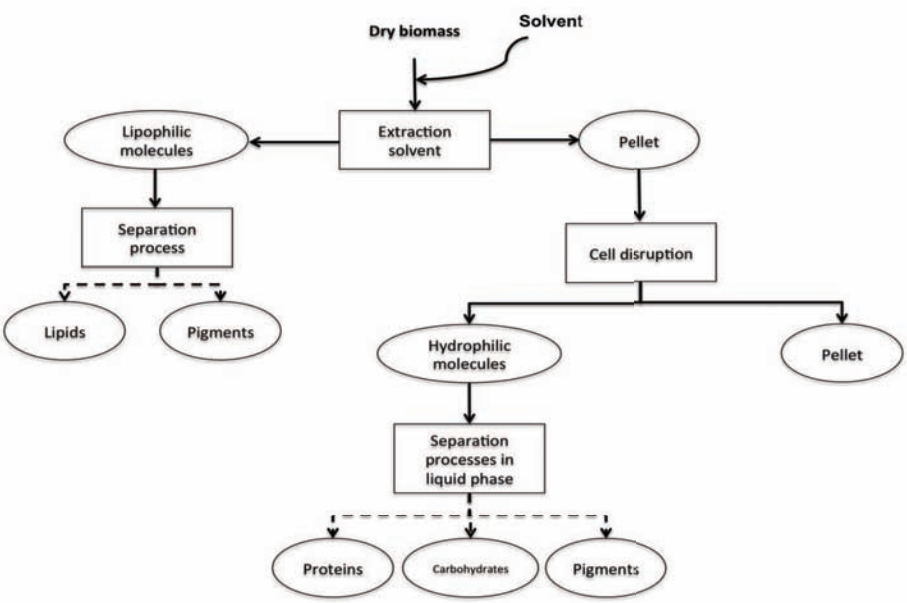

Figure 2: Process scheme for the second possible option, which starts from dry biomass

Globally we recognize that both options are feasible and possess distinctive advantages. The first process is more economic in terms of energy input (no drying), but the compositions of the solid and the emulsionated liquid are more complex and less selective, since they both contain hydrophilic and hydrophobic components. This is not the case for the second process in which the organic phase gathers both types of lipids (structural and reserve) in a single fraction, leaving all the hydrophilic biomolecules in the pellet. For these reasons, we decided to adopt **option 2** as the model process for the rest of the study. However, a major limitation for this process is the use of an organic solvent. This drawback could be solved if we use a green solvent such as supercritical carbon dioxide.

1.14 Chapter conclusion

All along this chapter we could demonstrate or set the following points:

- Microalgae are a complex family with a wide biochemical and physiological variety.
- *Chlorella vulgaris* is a species that has been largely exploited for biodiesel or for its nutritive value.
- This microalga has a rigid cell wall and will require a cell disruption to liberate its inner richness.
- *Chlorella vulgaris* is a reservoir of highly added value biomolecules; it is necessary to recover them in an "integrated process" in order to insure the economical sustainability for industrial production.
- Process 2, which implies a first defatting step to separate all the lipophilic molecules, was selected because *a priori* it will be easier to separate the different hydrophilic components in the downstream process.
- In order to improve the compliance of this process with the twelve principles of green chemistry, supercritical carbon dioxide was chosen as solvent for the extraction of lipids.

To summarize, our model process is the following (Fig 1):

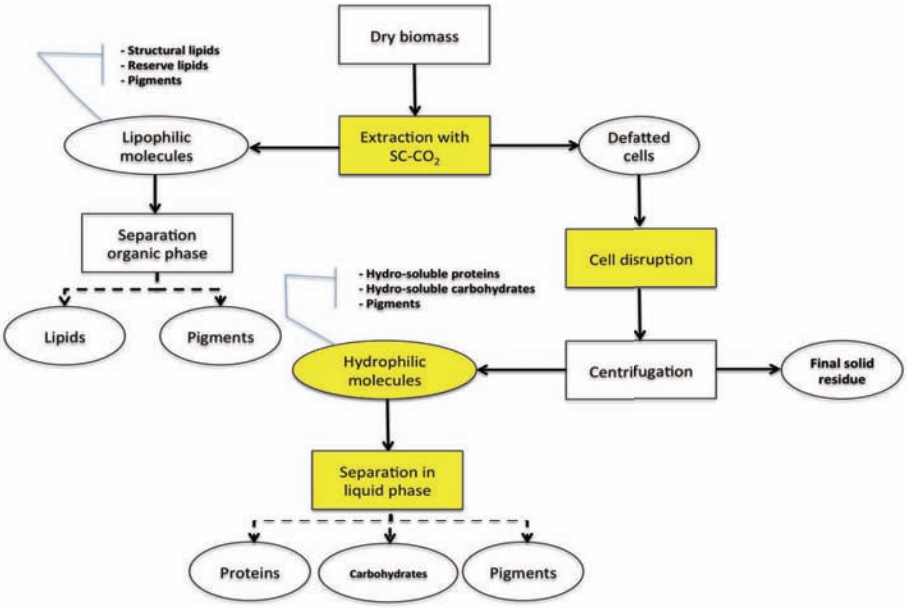

Figure 1: Process reflecting the main unit operation (in yellow) on which our work will be focused

In this figure we have highlighted (in yellow) the topics that could be studied within the 36 months of this thesis work. Therefore, it will be impossible to discuss and conclude on the economic and environmental sustainability of the whole process. The results generated will be however useful in the construction of an algorefinery scheme with the help of other researchers. The global task will take many years to be accomplished.

* * *

Before passing to the next chapter, it is necessary to point out that:

- The biomass was produced by the industry (Algosource Technology-ANR partner), which means we do not control the production process. For the same reason, we did not have the chance to work on stressed microalgae.
- The biomass was directly freezed prior to harvesting, and then sent to our laboratory. Therefore, we worked with the available pre-treated biomass.
- During the downstream process, the biomass was freeze-dried and then rehydrated with respect to the unit operations chain of the process selected. In other cases, the biomass was thawed and used directly afterwards.

References

[1] Ditfurth Hv. Im Anfang war der Wasserstoff. 2. Aufl. ed. Hamburg: Hoffmann und Campe; 1972.
[2] Brasier MD, Green OR, Jephcoat AP, Kleppe AK, Van Kranendonk MJ, Lindsay JF, et al. Questioning the evidence for Earth's oldest fossils. Nature. 2002;416:76-81.
[3] Dalton R. Microfossils: squaring up over ancient life. Nature. 2002;417:782-4.
[4] Venkataraman LV. *Spirulina platensis* (Arthrospira): Physiology, Cell Biology and Biotechnologym, edited by Avigad Vonshak. J Appl Phycol. 1997;9:295-6.
[5] Fradique M, Batista AP, Nunes MC, Gouveia L, Bandarra NM, Raymundo A. Incorporation of *Chlorella vulgaris* and *Spirulina maxima* biomass in pasta products. Part 1: Preparation and evaluation. J Sci Food Agric. 2010;90:1656-64.
[6] González-Fernández C, Sialve B, Bernet N, Steyer J-P. Impact of microalgae characteristics on their conversion to biofuel. Part I: Focus on cultivation and biofuel production. Biofuel Bioprod Bior. 2012;6:105-13.
[7] Tran NH, Bartlett JR, Kannangara GSK, Milev AS, Volk H, Wilson MA. Catalytic upgrading of biorefinery oil from micro-algae. Fuel. 2010;89:265-74.
[8] Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: A review. Renew Sust Energ Rev. 2010;14:217-32.
[9] Banerjee A, Sharma R, Chisti Y, Banerjee UC. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crc Cr Rev Biotechn. 2002;22:245-79.
[10] Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, et al. Microalgae: a green source of renewable H(2). Trends Biotechnol. 2000;18:506-11.
[11] Lorenz RT, Cysewski GR. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends biotechnol. 2000;18:160-7.
[12] Singh S, Kate BN, Banerjee UC. Bioactive compounds from cyanobacteria and microalgae: an overview. Crc Cr Rev Biotechn. 2005;25:73-95.
[13] Spolaore P, Joannis-Cassan C, Duran E, Isambert A. Commercial applications of microalgae. J Biosci Bioeng. 2006;101:87-96.
[14] Walker TL, Purton S, Becker DK, Collet C. Microalgae as bioreactors. Plant Cell Rep. 2005;24:629-41.
[15] Lee RE. Phycology. 4th ed. Cambridge, England ; New York: Cambridge University Press; 2008.
[16] Singh A, Nigam PS, Murphy JD. Renewable fuels from algae: an answer to debatable land based fuels. Bioresource Technol. 2011;102:10-6.
[17] Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, et al. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 2008;54:621-39.
[18] Beijerinck M. Kulturversuche mit Zoochlorellen, Lichenengonidien und anderen niederen Algen. Bot Zeitung. 1890.
[19] Burlew JS. Algal culture from laboratory to pilot plant: Carnegie Institution of Washington Publ.; 1953.
[20] Kitada K, Machmudah S, Sasaki M, Goto M, Nakashima Y, Kumamoto S, et al. Supercritical CO2extraction of pigment components with pharmaceutical importance from *Chlorella vulgaris*. J Chem Technol Biot. 2009;84:657-61.
[21] Morris HJ, Carrillo OV, Almarales Á, Bermúdez RC, Alonso ME, Borges L, et al. Protein hydrolysates from the alga *Chlorella vulgaris* 87/1 with potentialities in immunonutrition. Biotecnol Aplic. 2009;26:162-5.

[22] Justo GZ, Silva MR, Queiroz ML. Effects of the green algae *Chlorella vulgaris* on the response of the host hematopoietic system to intraperitoneal ehrlich ascites tumor transplantation in mice. Immunopharm Immunot. 2001;23:119-32.
[23] Konishi F, Tanaka K, Himeno K, Taniguchi K, Nomoto K. Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. Cancer immunol immun 1985;19:73-8.
[24] Morimoto T, Nagatsu A, Murakami N, Sakakibara J, Tokuda H, Nishino H, et al. Anti-tumour-promoting glyceroglycolipids from the green alga, *Chlorella vulgaris*. Phytochemistry. 1995;40:1433-7.
[25] Singh A, Singh SP, Bamezai R. Inhibitory potential of *Chlorella vulgaris* (E-25) on mouse skin papillomagenesis and xenobiotic detoxication system. Anticancer Res. 1999;19:1887-91.
[26] Yasukawa K, Akihisa T, Kanno H, Kaminaga T, Izumida M, Sakoh T, et al. Inhibitory effects of sterols isolated from *Chlorella vulgaris* on 12-0-tetradecanoylphorbol-13-acetate-induced inflammation and tumor promotion in mouse skin. Biol Pharmaceutical Bull. 1996;19:573-6.
[27] de Souza Queiroz J, Barbosa CM, da Rocha MC, Bincoletto C, Paredes-Gamero EJ, de Souza Queiroz ML. *Chlorella vulgaris* treatment ameliorates the suppressive effects of single and repeated stressors on hematopoiesis. Brain Behav Immun. 2013;29:39-50.
[28] Sano T, Kumamoto Y, Kamiya N, Okuda M, Tanaka Y. Effect of lipophilic extract of *Chlorella vulgaris* on alimentary hyperlipidemia in cholesterol-fed rats. Artery. 1988;15:217-24.
[29] Sano T, Tanaka Y. Effect of dried, powdered *Chlorella vulgaris* on experimental atherosclerosis and alimentary hypercholesterolemia in cholesterol-fed rabbits. Artery. 1987;14:76-84.
[30] Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process. Process Intensif. 2009;48:1146-51.
[31] Zheng H, Yin J, Gao Z, Huang H, Ji X, Dou C. Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl Biochem Biotechnol. 2011;164:1215-24.
[32] Chisti Y. Biodiesel from microalgae. Biotechnol Adv. 2007;25:294-306.
[33] Yamamoto M, Fujishita M, Hirata A, Kawano S. Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae). J Plant Res. 2004;117:257-64.
[34] Illman AM, Scragg AH, Shales SW. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme Microb Tech. 2000;27:631-5.
[35] Yamamoto M, Kurihara I, Kawano S. Late type of daughter cell wall synthesis in one of the Chlorellaceae, Parachlorella kessleri (Chlorophyta, Trebouxiophyceae). Planta. 2005;221:766-75.
[36] Yvonne N, Tomas K. Cell wall development, microfibril and pyrenoid structure in type strains of *Chlorella vulgaris*, *C. kessleri*, *C. sorokiniana* compared with *C. luteoviridis* (Trebouxiophyceae, Chlorophyta). Arch Hydrobiol 2000;100:95-105.
[37] Kapaun E, Reisser W. A chitin-like glycan in the cell wall of a *Chlorella* sp. (Chlorococcales, Chlorophyceae). Planta. 1995;197:577-82.

[38] Atkinson AW, Jr., Gunning BES, John PCL. Sporopollenin in the cell wall of *Chlorella* and other algae: Ultrastructure, chemistry, and incorporation of 14C-acetate, studied in synchronous cultures. Planta. 1972;107:1-32.
[39] Burczyk J, Hesse M. The ultrastructure of the outer cell wall-layer of *Chlorella* mutants with and without sporopollenin. Pl Syst Evol. 1981;138:121-37.
[40] Hagen C, Siegmund S, Braune W. Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation. Eur J Phycol. 2002;37:217-26.
[41] Biedlingmaier S, Wanner G, Schmidt A. A correlation between detergent tolerance and cell wall structure in green algae. Z. Naturforsch., C: Biosci.1987;42:245-250.
[42] Martínez F, Ascaso C, Orús MI. Morphometric and stereologic analysis of *Chlorella vulgaris* under heterotrophic growth conditions. Ann Bot. 1991;67:239-45.
[43] Kuchitsu K, Oh-hama T, Tsuzuki M, Miyachi S. Detection and characterization of acidic compartments (vacuoles) in *Chlorella vulgaris* 11h cells by 31P-in vivo NMR spectroscopy and cytochemical techniques. Arch Microbiol. 1987;148:83-7.
[44] Solomon EP, Berg LR, Martin DW. Biology. 5th ed. Fort Worth: Saunders College Pub.; 1999.
[45] Van den Hoek C, Mann D, Jahns H. Algae: An introduction to phycology: Cambridge University Press; 1995.
[46] Brennan L, Owende P. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Ener Rev. 2010;14:557-77.
[47] Přibyl P, Cepák V, Zachleder V. Production of lipids and formation and mobilization of lipid bodies in *Chlorella vulgaris*. J Appl Phycol. 2012:1-9.
[48] Lv JM, Cheng LH, Xu XH, Zhang L, Chen HL. Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. Bioresource Technol. 2010;101:6797-804.
[49] Pribyl P, Cepak V, Zachleder V. Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*. Appl Microbiol Biot. 2012;94:549-61.
[50] Widjaja A, Chien C-C, Ju Y-H. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem Eng. 2009;40:13-20.
[51] Liu ZY, Wang GC, Zhou BC. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technol. 2008;99:4717-22.
[52] Richmond A, Boussiba S, Vonshak A, Kopel R. A new tubular reactor for mass production of microalgae outdoors. J Appl Phycol. 1993;5:327-32.
[53] Qiang H, Richmond A. Productivity and photosynthetic efficiency of *Spirulina platensis* as affected by light intensity, algal density and rate of mixing in a flat plate photobioreactor. J Appl Phycol. 1996;8:139-45.
[54] Zhang K, Miyachi S, Kurano N. Evaluation of a vertical flat-plate photobioreactor for outdoor biomass production and carbon dioxide bio-fixation: effects of reactor dimensions, irradiation and cell concentration on the biomass productivity and irradiation utilization efficiency. Appl Microbiol Biot. 2001;55:428-33.
[55] Molina Grima E, Belarbi EH, Acién Fernández FG, Robles Medina A, Chisti Y. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv. 2003;20:491-515.
[56] Kojima E, Zhang K. Growth and hydrocarbon production of microalga *Botryococcus braunii* in bubble column photobioreactors. J Biosci Bioeng. 1999;87:811-5.

[57] Degen J, Uebele A, Retze A, Schmid-Staiger U, Trosch W. A novel airlift photobioreactor with baffles for improved light utilization through the flashing light effect. J Biotechnol. 2001;92:89-94.
[58] Lee Y-K. Microalgal mass culture systems and methods: Their limitation and potential. J Appl Phycol. 2001;13:307-15.
[59] Liang Y, Sarkany N, Cui Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett. 2009;31:1043-9.
[60] Ogawa T, Aiba S. Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and Scenedesmus acutus. Biotechnol Bioeng. 1981;23:1121-32.
[61] Mallick N, Mandal S, Singh AK, Bishai M, Dash A. Green microalga *Chlorella vulgaris* as a potential feedstock for biodiesel. J Chem Technol Biot. 2012;87:137-45.
[62] Patino R, Janssen M, von Stockar U. A study of the growth for the microalga *Chlorella vulgaris* by photo-bio-calorimetry and other on-line and off-line techniques. Biotechno Bioeng. 2007;96:757-67.
[63] Yeh KL, Chang JS. Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga *Chlorella vulgaris* ESP-31. Bioresource Technol. 2012;105:120-7.
[64] de-Bashan LE, Antoun H, Bashan Y. Cultivation factors and population size control the uptake of nitrogen by the microalgae *Chlorella vulgaris* when interacting with the microalgae growth-promoting bacterium *Azospirillum brasilense*. FEMS Microbiol Ecol. 2005;54:197-203.
[65] Gonzalez LE, Bashan Y. Increased growth of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*. Appl Environ Microb. 2000;66:1527-31.
[66] Munoz R, Guieysse B. Algal-bacterial processes for the treatment of hazardous contaminants: a review. Water Res. 2006;40:2799-815.
[67] de-Bashan LE, Bashan Y, Moreno M, Lebsky VK, Bustillos JJ. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. Can J Microbiol. 2002;48:514-21.
[68] Lebsky VK, Gonzalez-Bashan LE, Bashan Y. Ultrastructure of interaction in alginate beads between the microalga *Chlorella vulgaris* with its natural associative bacterium *Phyllobacterium myrsinacearum* and with the plant growth-promoting bacterium *Azospirillum brasilense*. Can J Microbiol. 2001;47:1-8.
[69] Leupold M, Hindersin S, Gust G, Kerner M, Hanelt D. Influence of mixing and shear stress on *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Chlamydomonas reinhardtii*. J Appl Phycol. 2012:1-11.
[70] Cha KH, Lee HJ, Koo SY, Song DG, Lee DU, Pan CH. Optimization of pressurized liquid extraction of carotenoids and chlorophylls from *Chlorella vulgaris*. J Agric Food Chem. 2010;58:793-7.
[71] Vandamme D, Foubert I, Fraeye I, Meesschaert B, Muylaert K. Flocculation of *Chlorella vulgaris* induced by high pH: role of magnesium and calcium and practical implications. Bioresource Technol. 2012;105:114-9.
[72] Wu Z, Zhu Y, Huang W, Zhang C, Li T, Zhang Y, et al. Evaluation of flocculation induced by pH increase for harvesting microalgae and reuse of flocculated medium. Bioresource Technol. 2012;110:496-502.
[73] Divakaran R, Sivasankara Pillai VN. Flocculation of algae using chitosan. J Appl Phycol. 2002;14:419-22.

[74] Oh H-M, Lee S, Park M-H, Kim H-S, Kim H-C, Yoon J-H, et al. Harvesting of *Chlorella vulgaris* using a bioflocculant from *Paenibacillus* sp. AM49. Biotechnol Lett. 2001;23:1229-34.
[75] Chang Y-R, Lee D-J. Coagulation–membrane filtration of *Chlorella vulgaris* at different growth phases. Dry Technol. 2012;30:1317-22.
[76] Lee D-J, Liao G-Y, Chang Y-R, Chang J-S. Coagulation-membrane filtration of *Chlorella vulgaris*. Bioresource Technol. 2012;108:184-9.
[77] Cheng YL, Juang YC, Liao GY, Ho SH, Yeh KL, Chen CY, et al. Dispersed ozone flotation of *Chlorella vulgaris*. Bioresource Technol. 2010;101:9092-6.
[78] Frappart M, Massé A, Jaffrin MY, Pruvost J, Jaouen P. Influence of hydrodynamics in tangential and dynamic ultrafiltration systems for microalgae separation. Desalination. 2011;265:279-83.
[79] Morineau-Thomas O, Jaouen P, Legentilhomme P. The role of exopolysaccharides in fouling phenomenon during ultrafiltration of microalgae (*Chlorella* sp. and *Porphyridium purpureum*): advantage of a swirling decaying flow. Bioproc Biosyst Eng. 2002;25:35-42.
[80] Hung MT, Liu JC. Microfiltration for separation of green algae from water. Colloid Surface B. 2006;51:157-64.
[81] Becker EW. Microalgae : biotechnology and microbiology. Cambridge ; New York: Cambridge University Press; 1994.
[82] Morris HJ, Almarales A, Carrillo O, Bermudez RC. Utilisation of *Chlorella vulgaris* cell biomass for the production of enzymatic protein hydrolysates. Bioresource Technol. 2008;99:7723-9.
[83] Safi C, Charton M, Pignolet O, Silvestre F, Vaca-Garcia C, Pontalier P-Y. Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. J Appl Phycol. 2012:1-7.
[84] Servaites JC, Faeth JL, Sidhu SS. A dye binding method for measurement of total protein in microalgae. Analyt Biochem. 2012;421:75-80.
[85] Seyfabadi J, Ramezanpour Z, Amini Khoeyi Z. Protein, fatty acid, and pigment content of *Chlorella vulgaris* under different light regimes. J Appl Phycol. 2011;23:721-6.
[86] Berliner MD. Proteins in *Chlorella vulgaris*. Microbios.1986;46:199-203
[87] Hanan MK. Comparative effects of autotrophic and heterotrophic growth on some vitamins, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile of *Chlorella vulgaris* Beijerinck. Afr J Biotechnol. 2011;10.
[88] Safi C, Charton M, Pignolet O, Pontalier P-Y, Vaca-Garcia C. Evaluation of the protein quality of *Porphyridium cruentum*. J Appl Phycol. 2012.
[89] FAO. A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish. Rome: FAO Fish Aquacult; 2008. p. 20.
[90] WHO. Protein and amino acid requirements in human nutrition. World Health Organisation, Geneva 2007. p. 284.
[91] Maruyama I, Nakao T, Shigeno I, Ando Y, Hirayama K. Application of unicellular algae *Chlorella vulgaris* for the mass-culture of marine rotifer *Brachionus*. Hydrobiologia. 1997;358:133-8.
[92] Bajguz A. Effect of brassinosteroids on nucleic acids and protein content in cultured cells of *Chlorella vulgaris*. Plant Physiol Bioch. 2000;38:209-15.
[93] Rausch T. The estimation of micro-algal protein content and its meaning to the evaluation of algal biomass I. Comparison of methods for extracting protein. Hydrobiologia. 1981;78:237-51.

[94] Barbarino E, Lourenço SO. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. J Appl Phycol. 2005;17:447-60.
[95] Oliveira R, Marques F, Azeredo J. Purification of polysaccharides from a biofilm matrix by selective precipitation of proteins. Biotechnol Tech. 1999;13:391-3.
[96] Chronakis IS, Galatanu AN, Nylander T, Lindman B. The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface. Colloid Surface A. 2000;173:181-92.
[97] Diniz GS. Gross Chemical Profile and Calculation of Nitrogen-to-Protein Conversion Factors for Five Tropical Seaweeds. Am J Plant Sci. 2011;02:287-96.
[98] Lopez CV, Garcia Mdel C, Fernandez FG, Bustos CS, Chisti Y, Sevilla JM. Protein measurements of microalgal and cyanobacterial biomass. Bioresource Technol. 2010;101:7587-91.
[99] Lourenço SO, Barbarino E, De-Paula JC, Pereira LOdS, Marquez UML. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. Phycol Res. 2002;50:233-41.
[100] Lourenço SO, Barbarino E, Lavín PL, Lanfer Marquez UM, Aidar E. Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. Eur J Phycol. 2004;39:17-32.
[101] Lourenço SO, Barbarino E, Marquez UML, Aidar E. Distribution of intracellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors. J Phycol. 1998;34:798-811.
[102] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193:265-75.
[103] Peterson GL. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal Biochem. 1979;100:201-20.
[104] Crossman DJ, Clements KD, Cooper GJS. Determination of protein for studies of marine herbivory: a comparison of methods. J Exp Mar Biol Ecol. 2000;244:45-65.
[105] Sriperm N, Pesti GM, Tillman PB. Evaluation of the fixed nitrogen-to-protein (N:P) conversion factor (6.25) versus ingredient specific N:P conversion factors in feedstuffs. J Sci Food Agric. 2011;91:1182-6.
[106] Yeoh H-H, Truong V-D. Protein Contents, Amino Acid Compositions and Nitrogen-to-Protein Conversion Factors for Cassava Roots. J Sci Food Agric. 1996;70:51-4.
[107] Stephenson AL, Dennis JS, Howe CJ, Scott SA, Smith AG. Influence of nitrogen-limitation regime on the production by *Chlorella vulgaris* of lipids for biodiesel feedstocks. Biofuels. 2009;1:47-58.
[108] Chen W, Sommerfeld M, Hu Q. Microwave-assisted nile red method for in vivo quantification of neutral lipids in microalgae. Bioresource Technol. 2011;102:135-41.
[109] Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM. Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technol. 2010;101 Suppl 1:S75-7.
[110] Mercer P, Armenta RE. Developments in oil extraction from microalgae. Eur J Lipid Sci Tech. 2011;113:539-47.
[111] Phukan MM, Chutia RS, Konwar BK, Kataki R. Microalgae *Chlorella* as a potential bio-energy feedstock. Appl Energy. 2011;88:3307-12.
[112] Olmstead IL, Hill DR, Dias DA, Jayasinghe NS, Callahan DL, Kentish SE, et al. A quantitative analysis of microalgal lipids for optimization of biodiesel and omega-3 production. Biotechnol Bioeng. 2013.

[113] Dejoye C, Vian MA, Lumia G, Bouscarle C, Charton F, Chemat F. Combined extraction processes of lipid from *Chlorella vulgaris* microalgae: microwave prior to supercritical carbon dioxide extraction. Int Journal Mol Sci. 2011;12:9332-41.
[114] Yeh KL, Chang JS. Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: implications for biofuels. Biotechnol J. 2011;6:1358-66.
[115] Lordan S, Ross RP, Stanton C. Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. Mar Drugs. 2011;9:1056-100.
[116] DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem. 1956;28:350-6.
[117] Shi Y, Sheng J, Yang F, Hu Q. Purification and identification of polysaccharide derived from *Chlorella pyrenoidosa*. Food Chem. 2007;103:101-5.
[118] Dragone G, Fernandes BD, Abreu AP, Vicente AA, Teixeira JA. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Appl Energy. 2011;88:3331-5.
[119] Fernandes B, Dragone G, Abreu A, Geada P, Teixeira J, Vicente A. Starch determination in *Chlorella vulgaris*—a comparison between acid and enzymatic methods. J Appl Phycol. 2012;24:1203-8.
[120] Branyikova I, Marsalkova B, Doucha J, Branyik T, Bisova K, Zachleder V, et al. Microalgae--novel highly efficient starch producers. Biotechnol Bioeng. 2011;108:766-76.
[121] Choix FJ, de-Bashan LE, Bashan Y. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: I. Autotrophic conditions. Enzyme Microb Technol. 2012;51:294-9.
[122] Janczyk P, Franke H, Souffrant WB. Nutritional value of *Chlorella vulgaris*: Effects of ultrasonication and electroporation on digestibility in rats. Anim Feed Sci Tech. 2007;132:163-9.
[123] Abo-Shady AM, Mohamed YA, Lasheen T. Chemical composition of the cell wall in some green algae species. Biol Plant. 1993;35:629-32.
[124] Griffiths DA, Griffiths DJ. The fine structure of autotrophic and heterotrophic cells of *Chlorella vulgaris* (Emerson strain). Plant Cell Physiol. 1969;10:11-9.
[125] Northcote DH, Goulding KJ, Horne RW. The chemical composition and structure of the cell wall of *Chlorella pyrenoidosa*. Biochemical J. 1958;70:391-7.
[126] Takeda H. Classification of *Chlorella* strains by means of the sugar components of the cell wall. Biochem Syst Ecol. 1988;16:367-71.
[127] Takeda H. Classification of *Chlorella* strains by cell wall sugar composition. Phytochemistry. 1988;27:3823-6.
[128] Takeda H. Sugar composition of the cell wall and the taxonomy of *Chlorella* (chlorophyceae)1. J Phycol. 1991;27:224-32.
[129] Takeda H. Chemical composition of cell walls as a taxonomical marker. J Plant Res. 1993;106:195-200.
[130] Takeda H, Hirokawa T. Studies on the cell wall of *Chlorella* V. Comparison of the cell wall chemical compositions in strains of *Chlorella ellipsoidea*. Plant Cell Physiol. 1984;25:287-95.
[131] Blumreisinger M, Meindl D, Loos E. Cell wall composition of chlorococcal algae. Phytochemistry. 1983;22:1603-4.
[132] Ogawa K, Ikeda Y, Kondo S. A new trisaccharide, α-d-glucopyranuronosyl-(1→3)- α -l-rhamnopyranosyl-(1→2)- α -l-rhamnopyranose from *Chlorella vulgaris*. Carbohydr Res. 1999;321:128-31.

[133] Cha KH, Kang SW, Kim CY, Um BH, Na YR, Pan CH. Effect of pressurized liquids on extraction of antioxidants from *Chlorella vulgaris*. J Agric Food Chem. 2010;58:4756-61.
[134] Gouveia L, Veloso V, Reis A, Fernandes H, Novais J, Empis J. *Chlorella vulgaris* used to Colour Egg Yolk. J Sci Food Agric. 1996;70:167-72.
[135] Mendes RL, Fernandes HL, Coelho J, Reis EC, Cabral JMS, Novais JM, et al. Supercritical CO2 extraction of carotenoids and other lipids from *Chlorella vulgaris*. Food Chem. 1995;53:99-103.
[136] Mendes RL, Nobre BP, Cardoso MT, Pereira AP, Palavra AF. Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. Inorg Chim Acta. 2003;356:328-34.
[137] Ritchie RJ. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res. 2006;89:27-41.
[138] Gouveia L, Raymundo A, Batista AP, Sousa I, Empis J. *Chlorella vulgaris* and *Haematococcus pluvialis* biomass as colouring and antioxidant in food emulsions. Eur Food Res Technol. 2005;222:362-7.
[139] Fernandez-Sevilla JM, Fernandez FG, Grima EM. Obtaining lutein-rich extract from microalgal biomass at preparative scale. Methods Mol Biol. 2012;892:307-14.
[140] Granado F, Olmedilla B, Blanco I. Nutritional and clinical relevance of lutein in human health. Brit J Nutr. 2003;90:487-502.
[141] Cha KH, Koo SY, Lee DU. Antiproliferative effects of carotenoids extracted from *Chlorella ellipsoidea* and *Chlorella vulgaris* on human colon cancer cells. J Agric Food Chem. 2008;56:10521-6.
[142] Tanaka K, Konishi F, Himeno K, Taniguchi K, Nomoto K. Augmentation of antitumor resistance by a strain of unicellular green algae, *Chlorella vulgaris*. Cancer Immunol Immun. 1984;17:90-4.
[143] Görs M, Schumann R, Hepperle D, Karsten U. Quality analysis of commercial *Chlorella* products used as dietary supplement in human nutrition. J Appl Phycol. 2009;22:265-76.
[144] Kong W, Liu N, Zhang J, Yang Q, Hua S, Song H, et al. Optimization of ultrasound-assisted extraction parameters of chlorophyll from *Chlorella vulgaris* residue after lipid separation using response surface methodology. J Food Sci Technol. 2012:1-8.
[145] Li H-B, Jiang Y, Chen F. Isolation and Purification of Lutein from the Microalga *Chlorella vulgaris* by Extraction after Saponification. Journal of Agricultural and Food Chem. 2002;50:1070-2.
[146] Maxwell DP, Falk S, Huner N. Photosystem II Excitation Pressure and Development of Resistance to Photoinhibition (I. Light-Harvesting Complex II Abundance and Zeaxanthin Content in *Chlorella vulgaris*). Plant Physiol. 1995;107:687-94.
[147] Tokuşoglu Ö, Unal MK. Biomass Nutrient Profiles of Three Microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrisis galbana*. J Food Sci. 2003;68:1144-8.
[148] Panahi Y, Pishgoo B, Jalalian HR, Mohammadi E, Taghipour HR, Sahebkar A. Investigation of the effects of *Chlorella vulgaris* as an adjunctive therapy for dyslipidemia: Results of a randomised open-label clinical trial. Nutr Diet. 2012;69:13-9.
[149] Becerra G, Menolasina S, Salvador A. Supercritical Fluid Extraction and Supercritical Fluid Chromatography of Vitamin E in Pharmaceutical Preparations. J High Res Chromatog. 1999;22:300-2.
[150] Brown MR, Jeffrey SW, Volkman JK, Dunstan GA. Nutritional properties of microalgae for mariculture. Aquaculture. 1997;151:315-31.

[151] Ochiai S, Hase E. Studies on chlorophyll formation in *Chlorella protothecoides* I. Enhancing effects of light and added δ-aminolevulinic acid, and suppressive effect of glucose on chlorophyll formation. Plant Cell Physiol. 1970;11:663-73.
[152] Honjoh K-i, Suga K, Shinohara F. Preparation of protoplasts from *chlorella vulgaris* k-73122 and cell wall regeneration of protoplasts from *C. vulgaris* k-73122 and c-27. J Fac Agri Kyushu U. 2003;47:257-66.
[153] Berliner MD. Protoplast induction in *Chlorella vulgaris*. Plant Sci Lett. 1977;9:201-4.
[154] Wilson KE, Huner NPA. The role of growth rate, redox-state of the plastoquinone pool and the trans-thylakoid;pH in photoacclimation of *Chlorella vulgaris* to growth irradiance and temperature. Planta. 2000;212:93-102.
[155] Gouveia L, Nobre BP, Marcelo FM, Mrejen S, Cardoso MT, Palavra AF, et al. Functional food oil coloured by pigments extracted from microalgae with supercritical CO_2. Food Chem. 2007;101:717-23.
[156] Yamada S, Nakamura T, Tanaka Y, Isogai Y, Nishio T, Oku T. Characterization and Amino Acid Sequences of Cytochromes c(6) from Two Strains of the Green Alga *Chlorella vulgaris*. Biosci Biotech Bioch. 2000;64:628-32.
[157] Demirbas MF. Biofuels from algae for sustainable development. Appl Energy. 2011;88:3473-80.
[158] Wang K, Brown RC, Homsy S, Martinez L, Sidhu SS. Fast pyrolysis of microalgae remnants in a fluidized bed reactor for bio-oil and biochar production. Bioresource Technol. 2013;127:494-9.
[159] Francisco ÉC, Neves DB, Jacob-Lopes E, Franco TT. Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. J Chem Technol Biot. 2010;85:395-403.
[160] Hirano A, Ueda R, Hirayama S, Ogushi Y. CO2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. Energy. 1997;22:137-42.
[161] Ross AB, Biller P, Kubacki ML, Li H, Lea-Langton A, Jones JM. Hydrothermal processing of microalgae using alkali and organic acids. Fuel. 2010;89:2234-43.
[162] Biller P, Ross AB. Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. Bioresource Technol. 2011;102:215-25.
[163] Biller P, Riley R, Ross AB. Catalytic hydrothermal processing of microalgae: decomposition and upgrading of lipids. Bioresource Technol. 2011;102:4841-8.
[164] Clarens AF, Resurreccion EP, White MA, Colosi LM. Environmental life cycle comparison of algae to other bioenergy feedstocks. Envir Science Tech. 2010;44:1813-9.
[165] Collet P, Hélias A, Lardon L, Ras M, Goy R-A, Steyer J-P. Life-cycle assessment of microalgae culture coupled to biogas production. Bioresource Technol. 2011;102:207-14.
[166] Jorquera O, Kiperstok A, Sales EA, Embiruçu M, Ghirardi ML. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresource Technol. 2010;101:1406-13.
[167] Lam MK, Lee KT. Microalgae biofuels: A critical review of issues, problems and the way forward. Biotechnol Adv. 2012;30:673-90.
[168] Sander K, Murthy G. Life cycle analysis of algae biodiesel. Int J Life Cycle Assess. 2010;15:704-14.
[169] Singh A, Olsen SI. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. Appl Energy. 2011;88:3548-55.

[170] Stephenson AL, Kazamia E, Dennis JS, Howe CJ, Scott SA, Smith AG. Life-cycle assessment of potential algal biodiesel production in the united kingdom: A comparison of raceways and air-lift tubular bioreactors. Energy Fuels. 2010;24:4062-77.
[171] Yang J, Xu M, Zhang X, Hu Q, Sommerfeld M, Chen Y. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresource Technol. 2011;102:159-65.
[172] Lardon L, Hélias A, Sialve B, Steyer J-P, Bernard O. Life-cycle assessment of biodiesel production from microalgae. Envir Science Tech. 2009;43:6475-81.
[173] Liang S, Liu X, Chen F, Chen Z. Current microalgal health food R & D activities in China. In: Ang P, Jr., editor. Asian Pacific Phycology in the 21st Century: Prospects and Challenges: Springer Netherlands; 2004. p. 45-8.
[174] Yamaguchi K. Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. J Appl Phycol. 1996;8:487-502.
[175] Grobbelaar JU. Quality control and assurance: crucial for the sustainability of the applied phycology industry. J Appl Phycol. 2003;15:209-15.
[176] Gulati OP, Berry Ottaway P. Legislation relating to nutraceuticals in the European Union with a particular focus on botanical-sourced products. Toxicology. 2006;221:75-87.
[177] Rodriguez-Garcia I, Guil-Guerrero JL. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food Chem. 2008;108:1023-6.
[178] Becker EW. Micro-algae as a source of protein. Biotechnol Adv. 2007;25:207-10.
[179] Chacón-Lee TL, González-Mariño GE. Microalgae for "Healthy" Foods-Possibilities and Challenges. Compr Rev Food Sci S. 2010;9:655-75.
[180] Gouveia L, Choubert G, Pereira N, Santinha J, Empis J, Gomes E. Pigmentation of gilthead seabream, Sparus aurata (L. 1875), using *Chlorella vulgaris* (Chlorophyta, Volvocales) microalga. Aquac Res. 2002;33:987-93.
[181] Gouveia L, Gomes E, Empis J. Potential use of a microalga (*Chlorella vulgaris*) in the pigmentation of rainbow trout (*Oncorhynchus mykiss*) muscle. Z Lebensm Unters Forch. 1996;202:75-9.
[182] Shim JY, Shin HS, Han JG, Park HS, Lim BL, Chung KW, et al. Protective effects of *Chlorella vulgaris* on liver toxicity in cadmium-administered rats. J Med Food. 2008;11:479-85.
[183] Vijayavel K, Anbuselvam C, Balasubramanian MP. Antioxidant effect of the marine algae *Chlorella vulgaris* against naphthalene-induced oxidative stress in the albino rats. Mol Cell Biochem. 2007;303:39-44.
[184] Yun H, Kim I, Kwon S-H, Kang J-S, Om A-S. Protective effect of *Chlorella vulgaris* against lead-induced oxidative stress in rat brains. J Health Sci. 2011;57:245-54.
[185] Keffer JE, Kleinheinz GT. Use of *Chlorella vulgaris* for CO_2 mitigation in a photobioreactor. J Industrial Microbiol Biot. 2002;29:275-80.
[186] Aslan S, Kapdan IK. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecological Engineering. 2006;28:64-70.
[187] Feng Y, Li C, Zhang D. Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. Bioresource Technol. 2011;102:101-5.
[188] Lau PS, Tam NFY, Wong YS. Wastewater nutrients removal by *Chlorella Vulgaris*: optimization through acclimation. Environ Technol. 1996;17:183-9.

[189] Lim SL, Chu WL, Phang SM. Use of *Chlorella vulgaris* for bioremediation of textile wastewater. Bioresource Technol. 2010;101:7314-22.
[190] Silva-Benavides A, Torzillo G. Nitrogen and phosphorus removal through laboratory batch cultures of microalga *Chlorella vulgaris* and cyanobacterium *Planktothrix isothrix* grown as monoalgal and as co-cultures. J Appl Phycol. 2012;24:267-76.
[191] Valderrama LT, Del Campo CM, Rodriguez CM, de- Bashan LE, Bashan Y. Treatment of recalcitrant wastewater from ethanol and citric acid production using the microalga *Chlorella vulgaris* and the macrophyte *Lemna minuscula*. Water Res. 2002;36:4185-92.
[192] Yun Y-S, Lee SB, Park JM, Lee C-I, Yang J-W. Carbon dioxide fixation by algal cultivation using wastewater nutrients. J Chem Technol Biot. 1997;69:451-5.
[193] de-Bashan LE, Moreno M, Hernandez JP, Bashan Y. Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae *Chlorella vulgaris* coimmobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*. Water Res. 2002;36:2941-8.
[194] González LE, Cañizares RO, Baena S. Efficiency of ammonia and phosphorus removal from a colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. Bioresource Technol. 1997;60:259-62.
[195] Ordog V. Beneficial effects of microalgae and cyanobacteria in plant/soil-systems, with special regard to their auxin-and cytokinin-like activity. International workshop and training course on microalgal biology and biotechnology Mosonmagyarovar, Hungary, lune1999. p. 13-26.
[196] de Mulé MCZ, de Caire GZ, de Cano MS, Palma RM, Colombo K. Effect of cyanobacterial inoculation and fertilizers on rice seedlings and postharvest soil structure. Commun Soil Sci Plan. 1999;30:97-107.
[197] Adam M. The promotive effect of the cyanobacterium *Nostoc muscorum* on the growth of some crop plants. Acta Microbiol Pol. 1999;48:163-71.
[198] Saffan E. Allelopathic effects of cyanobacterial exudates on some metabolic activities of *Cynara cardunculus* seeds during germination. Egypt J Biotechnol. 2001;10:157-78.
[199] Shaaban M. Green microalgae water extracts as foliar feeding to wheat plants. Pak J Biol Sci. 2001;4:628-32.
[200] Faheed F, Abd el Fattah Z. Effect of *Chlorella vulgaris* as bio-fertilizer on growth parameters and metabolic aspects of lettuce plant. J Agric Soc Sci. 2008;4:165-9.
[201] Naik SN, Goud VV, Rout PK, Dalai AK. Production of first and second generation biofuels: A comprehensive review. Renew Sust Energ Rev. 2010;14:578-97.
[202] Singh J, Gu S. Commercialization potential of microalgae for biofuels production. Renew Sust Energ Rev. 2010;14:2596-610.

Chapter 2: Recovery of the lipophilic fraction

2.1 Introduction

It has been mentioned previously that our biomass was not stressed and therefore grown under normal growth conditions. This implies that the microalga did not accumulate important amounts of reserve lipids (triglycerides). Hence, the major part of the available lipids in our microalga are polar (phospholipids), and are mainly structural lipids located on the cell wall and the intracellular membranes (chloroplast, mitochondria, thylakoids). The extraction of these components outside the rigid cell wall of *Chlorella vulgaris* would require a specific treatment in order to facilitate their extraction and increase the recovery yield.

It is generally agreed that one of the unit operations that adds almost 30% additional cost input on the total production cost is the extraction unit operation of that includes the step of cell disruption [1]. Thus, the chapter is composed of one publication accepted in *Journal of Applied Phycology*, and which inspects whether it is possible to bypass the cell disruption unit operation before conducing supercritical CO_2 extraction of the lipophilic fraction. Therefore, it analyses different aspect of extraction before and after complete disruption by bead milling or with and without the presence of an entrainer.

2.2 Extraction of lipids and pigments of *Chlorella vulgaris* by supercritical carbon dioxide: influence of bead milling on extraction performance

Carl SAFI [1,2], Bachar ZEBIB [1,2], Christine FRANCES [3,4], Séverine CAMY [3,4], Pierre-Yves PONTALIER [1,2], Carlos VACA-GARCIA [1,2,5]

[1] Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), 31030 Toulouse, FRANCE
[2] INRA, UMR 1010 CAI, F-31030 Toulouse, France
[3] Université de Toulouse; INP, UPS; LGC (Laboratoire de Génie Chimique); B.P. 84234, 4, Allée Emile Monso, F-31432 Cedex 4, France
[4] CNRS; LGC; F-31432 Cedex 4, France
[5] King Abdulaziz University, Jeddah, Saudi Arabia

E-mail : carl.safi@ensiacet.fr

This publication has been accepted in ***Journal of Applied Phycology***

Abstract

The influence of bead milling on the extraction of lipids and pigments by supercritical carbon dioxide was investigated in this study. Different operating parameters for the 3 h process were first tested on raw *Chlorella vulgaris*; 600 bar was the optimum pressure at 60°C with a 30 g.min^{-1} carbon dioxide flow rate. Under these operating conditions, 10% of total lipid containing chlorophyll and carotenoids with 1.61 and 1.72 mg/g dry weight of microalga respectively has been recovered. Microscopic observation was used to assess cell wall breakage through bead milling, which produced positive results in terms of increasing the yield of the biomolecules of interest. Thus, under the same operating conditions, the yield of total lipid extract, chlorophyll and carotenoids increased significantly. Moreover, the addition of a polar co-solvent to the raw microalga had considerable effect on the final extract. Overall, the addition of 5% w/w ethanol to raw microalga increased the total extract yield by 27%, and bead milling increased the total extract yield by 16%. Chlorophyll and carotenoids were also significantly affected by the addition of ethanol, with an 81% and 65% increase with the raw microalga, and 61% and 52% increase using bead milling, respectively.

Keywords Lipids, chlorophyll, carotenoids, bead milling, supercritical carbon dioxide and co-solvent.

2.2.1 Introduction

Microalgae represent considerable feedstock diversity in terms of isolation of natural biomolecules of significant commercial interest [2] for the pharmaceutical [3], cosmetics [4], animal nutrition and aquaculture [5] and bioenergy [6-9] industries. Thus, they reflect a biomass composed of multiple added value components. Over the last decade, many different industries have become increasingly interested in natural products that are beneficial for human health and environmentally friendly, and, microalgae are potential candidates that could contribute to satisfying this growing demand.

Chlorella vulgaris, a green microscopic microalga with a rigid cell wall [10], is an important species with an interesting composition that has attracted the attention of scientists over the last century. It is rich in chlorophyll and proteins, and if it is grown under specific conditions, it can accumulate large amounts of lipids [11-13], and valuable carotenoids such as astaxanthin, β-carotene and cantaxanthin [14]. Like all microalgae, there are two types of lipids in *Chlorella vulgaris*, neutral and polar. Phospholipids and glycolipids are polar lipids that are present on the cell wall as well as on the membranes of internal organelles such as the chloroplasts and the mitochondria. Conversely, neutral lipids such as triacylglycerol are in the form of lipid droplets in the chloroplast matrix, and can also be present in the cytoplasm if the microalga is grown under nitrogen starvation and other harsh conditions. Chlorophyll and primary carotenoids are concentrated in the thylakoids, but some carotenoids such as β-carotene exist inside lipid droplets. These biomolecules are of great nutritional interest because they are known to have antioxidant activities, can reduce the risk of cardiovascular diseases, and have antitumor activities [15] and other health benefits [16].

Demand from the food industry for additives natural in origin, and with characteristics contributing to increased health benefits, is growing every year. In addition, legislation has imposed further quality enhancement on products destined for human consumption, while systematically restricting the use of conventional methods with potentially harmful consequences on human health. Thus, obtaining a product free from contaminants and solvents is extremely important to maintain the added value of the final product. In this respect, supercritical CO_2 extraction is a processing technique that respects the requirements imposed by the legislation as well as the environment, and improves the

quality of the final product by providing an additional argument for commercializing a healthy product without the expected side effects. Hence, several studies have reported results of lipid fraction extraction using the supercritical CO_2 process, by focusing on different aspects and the ultimate parameters that would maximize yield [17].

The literature covers a range of studies that have used supercritical carbon dioxide, in order to determine the best parameters for extracting valuable biomolecules such as lipids and pigments from microalgae. Among the latter should be noted two studies that aimed to show the beneficial effect of cell crushing before supercritical extraction [8, 14], and succeeded in at least doubling the extraction yield. The article of Crampon et al. (2011) presents an overview of compounds of interest obtained from supercritical CO_2 extraction of microalgae. The present study proposes the use of ethanol as co-solvent in supercritical extraction, to avoid additional energy input in terms of a supplementary unit operation of cell disruption.

2.2.2 Materials and methods

2.2.2.1 Microalga and materials

Sueoka culture medium was used for growing *C. vulgaris* (strain SAG 211-19) in batch mode in an indoor tubular Air-Lift PhotoBioReactor (PBR, 10 L) at 25°C, inoculated from a prior culture in a flat panel Air-Lift PBR (1 L). Culture homogenization was achieved by sterile air injection at the bottom of the PBR. The pH and temperature were recorded using a pH/temperature probe (Mettler Toledo SG 3253 sensor) monitored using LabVIEW acquisition software. The pH was maintained at 7.5 with CO_2 bubbling. The microalgae were harvested by centrifugation during the exponential growth phase and supplied as frozen paste from Alpha Biotech (Asserac, France). The harvested biomass contained 20.0% dry matter; total lipids represented 15.2% of dry matter (obtained by Bligh and Dyer method), chlorophyll 1.8% of dry matter (UV-Vis spectroscopic analysis) and carotenoids 1.3% of dry matter (UV-Vis spectroscopic analysis).

2.2.2.2 Mechanical cell disruption

Cells were treated in a stirred bead mill (LABSTAR-NETZCH). Disruption was conducted using 0.3-0.5 mm Y_2O_3-stabilized ZrO_2 grinding beads. Milling time for both

trials was 1-60 min with a 1/13 solid water ratio (w/v). The process was performed in batch mode. The initial cell suspension was placed in a pre-dispersion tank, and stirred at 350 rpm in order to avoid cell sedimentation and ensure a good homogeneity of the solid concentration. During the experiments, the suspension was continuously pumped from the tank to the mill inlet using a peristaltic pump at a flow rate of about 30L/h, and sent back again into the dispersion tank through a cartridge to keep the beads inside the chamber. Stirring speed of the cell suspension and the beads within the grinding chamber was 2500 rpm. The bead mill contained an integrated cooling system to prevent overheating, and thus after 1 h milling the temperature did not exceed 33°C. At the end, the broken cells were recovered for further processing.

2.2.2.3 Freeze-drying

The frozen paste of raw microalga and cells treated by bead milling were introduced directly into a Fisher Bioblock Scientific Alpha 2-4 LD Plus device (Illkirch, France). The pressure was reduced to 0.010 bar, the temperature further decreased to -80°C and freeze-drying was conducted under vacuum for 48 h to give a completely dry biomass. After freeze-drying, the mean diameter of particles, measured using a Mastersizer 2000 granulometer (Malvern Instruments Ltd.) was around 250 µm. Freeze-dried aggregates were then slightly crushed with a laboratory knife grinder to give a final size of 200 µm.

2.2.2.4 Supercritical carbon dioxide pilot

The experimental set-up used for supercritical extraction was an SFE100 from Separex Chimie fine (France). It was composed of a 25 mL tubular extractor (internal diameter 2 cm, height 8 cm) which could be operated up to 1000 bar and 200°C. One separator was connected to the extractor outlet, and the pressure in the extractor was adjusted by a backpressure regulator. At the beginning of the experiment, the extractor was filled with powdered freeze-dried microalgae (6 g) and CO_2 introduced at the bottom. The sample was left for 20 min at the desired operating temperature and pressure and CO_2 then introduced at a constant flow-rate. Ethanol can be used as a co-solvent and mixed with CO_2 at the extractor inlet and can also be used as a washing co-solvent. In this case, it is mixed with the extract (CO_2 and solutes) at the extractor outlet. This procedure gives efficient solute recovery in the separator, and extraction time was set at 180 min for all samples. The extracts were collected

in the separator and then stored in the dark at 4°C to prevent degradation of samples awaiting analyses. The extractor and the separator were cleaned after each run. Extraction yield is calculated after isolation of lipid extract using:

$$Y\ (\%) = \frac{m\ extract\ (g)}{m\ dry\ microalgae\ (g)}\ x\ 100$$

As supercritical CO_2 is a non-polar solvent, the extract is assumed to contain only neutral lipids and pigments, and in view of the very low amount of pigments, the global yield is thus assumed to be the neutral lipid yield. When ethanol is used as co-solvent, the polarity of the mixture is increased and therefore polar lipids are presumed to be extracted at the same time, in which case, the global yield is assumed to be the total lipid yield.

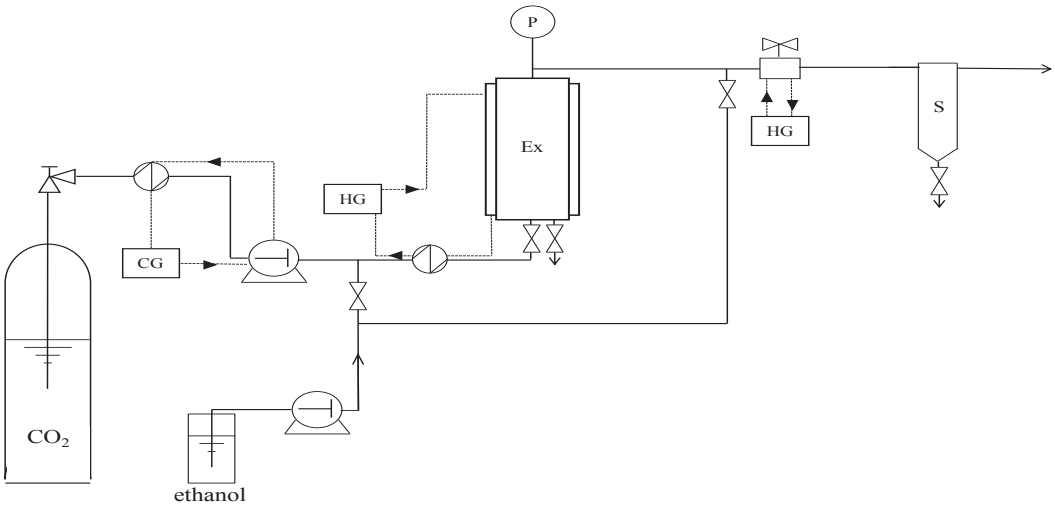

Figure 2: Description of the supercritical dioxide pilot

2.2.2.5 Pigments analysis

200 µL of aqueous extract were mixed with 1300 µL acetone and then incubated in the dark for 1h at 45°C. The samples were then centrifuged at 10000 *g* for 10 min at 20°C. The organic phase containing the pigments was then recovered and analysed using the following equations [18]:

Total chlorophyll (µg/mL) = $24.1209\ A_{632} + 11.2884 A_{649} + 3.7620 A_{665} + 5.8338 A_{696}$
Total carotenoids (µg /mL) = $4.74\ A_{466}$

2.2.2.6 Confocal microscopy

Cells were observed using an SP2-AOBS confocal laser-scanning microscope from Leica microsystems (Nanterre-France). Fluorochrome calcofluor white that binds to the cell wall was added to the samples. With an excitation wavelength of 488 nm, the cell walls appear light blue and at 633 nm the internal parts of the cells are red.

2.2.3 Results and discussion

2.2.3.1 Supercritical extraction of raw *C. vulgaris*

The present study focuses on using supercritical CO_2 on *C. vulgaris,* to assess the influence of operating parameters and pre-treatment of the cell by bead milling on the recovery of lipids and pigments. Supercritical extraction was carried out on raw cells, and cells treated by bead milling, and both were extracted using only CO_2 and CO_2 with ethanol as a co-solvent. To our knowledge, these three aspects have been tested separately on *C. vulgaris* but not in a single study, and the main objective of this work was to analyse whether bead milling can be used to break down the cell wall efficiently before submitting *C. vulgaris* to supercritical carbon dioxide extraction.

Firstly, the operating conditions for supercritical extraction were determined. By changing its density, pressure and temperature define the extraction power of supercritical carbon dioxide. While an increase of pressure directly improves solvent power by increasing CO_2 density, the effect of temperature is not equally predictable. Indeed, temperature is influencing solvent density as solute vapour pressure. Thus, the solubility of solute in CO_2 may be positively or negatively influenced by a temperature increase, depending on the pressure. In the present study, the pressure selected is high (more than 350 bar) and at the same time, the operating temperature set at 60°C, is expected to have a positive influence on the extraction yield [14]. The extraction kinetics of lipids and pigments (carotenoids and chlorophyll) obtained at 600 bar and 60°C, are shown on Figure 2.

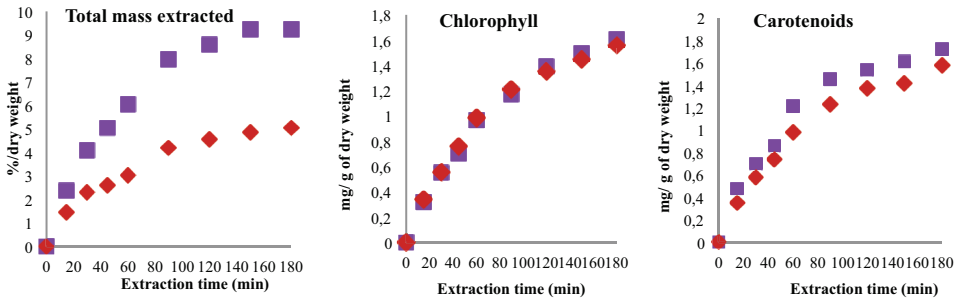

Figure 2: Influence of pressure on the extraction process of total lipids and pigments. ◆ Raw microalga (350 bar, 30 g.min^{-1} CO_2 flow rate, 60°C), ■ Raw microalga (600 bar, 30 g.min^{-1} CO_2 flow rate, 60°C).

The shape of the curves is typical of supercritical extraction processes, comprising a first linear part where extraction is limited by apparent solubility of solute(s) into $scCO_2$ (constant rate of extraction) and a second part where the rate of extraction is diminishing progressively mostly limited by internal mass transfer of the solute out of the cell. Considering the slope of the linear part of the curve (very restricted here), it can be assumed that the lipids are loosely linked to the structure of the cell. After 180 min of extraction, the recovery yield is 9%w/w, which means that, as expected, total lipids (15.2% w/w) are not being recovered. Moreover, without specific pre-treatment of the raw material, it is well known that, because of the cell structure [19], a part of the lipids remains inaccessible to the solvent. The shape of the curves corresponding to the extraction kinetics of pigments, are very similar to those for lipid extraction. As expected from the literature, a part of lipophilic carotenoids are extracted, although the yield is quite low (0.17% w/w compared to 1.3% w/w obtained using Soxhlet extraction), and a very similar result is obtained for chlorophyll (0.18%w/w compared to 1.8% w/w). Although it is well known that the latter compound is not soluble in $scCO_2$ its extraction from microalgae has already been reported at high pressure [20]. In addition, it can be observed that at 600 bar the extraction yield obtained after 180 min increased by 46% compared to that at 350 bar. At the latter pressure, the plateau is not reached after 180 min of extraction, which is still in progress. The gradient of the linear part is lower, which is consistent with lower lipid solubility at this pressure. The significant improvement in the total yield observed at high pressure, may be because of modifications of the algal wall due to high-pressure extraction of some structural components. The carotenoid yield is slightly improved at 600 bar (8%), and the pressure has no significant influence on the chlorophyll extraction yield.

2.2.3.2 Pre-treatment by bead milling

One of the important characteristics of *C. vulgaris* is the rigidity of its cell wall; it is composed of cellulose, hemicellulose, glucosamine, proteins, lipids and ash [21, 22]. Thus, breaking this cell wall allows solvent accessibility to the intracellular compartments generally leading to an increase in the total extract yield. Microscopic observations revealed complete disruption of the cell wall as shown on Figure 3, where it can be seen that the cells have completely lost their globular shape after bead milling for 1 h.

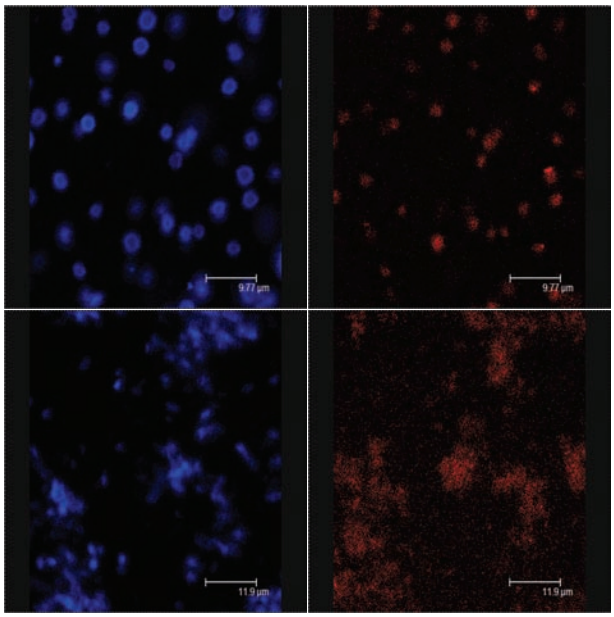

Figure 3: Confocal microscopic observations before bead milling (upper pictures), and after bead milling (lower pictures). The pictures on the left with a 488 nm excitation wavelength show the cell walls in light blue. The pictures on the right at 633 nm show the internal parts of the cells in red.

Microalga treated by bead milling have then been extracted using pure $scCO_2$ under the same operating conditions as for raw microalga, and the extraction kinetics for lipids and pigments are shown on Figure 4. Although final global yields are almost the same (slightly higher than 10%), the extraction kinetics is clearly improved by bead milling. At 600 bar, the maximum yield is obtained after 90 min although 180 min are necessary for raw material, and the same behaviour is observed for pigments. This result confirms the hypothesis concerning the efficiency of cell disruption with bead milling, because solutes become easily accessible to the solvent and diffusion limitations are alleviated. Pigment recovery is also

significantly affected by bead milling, meaning that this unit operation allows the solvent to access the phospholipid bilayer of the chloroplast where the pigments are mainly located in the thylakoids. Moreover, the global extraction kinetics observed at 350 and 600 bar are, in the case of bead milling, very similar, supporting the hypothesis of extraction being limited by internal diffusion within the cell. Indeed, when the cells are broken open, the lipids are more easily accessible to the solvent, in which case the influence of pressure concerns solute solubility only. Results obtained after bead milling, also confirm conclusions from other studies using different cell disruption methods to crush the *C. vulgaris* cell wall before conducting the extraction by supercritical carbon dioxide [14, 23, 24].

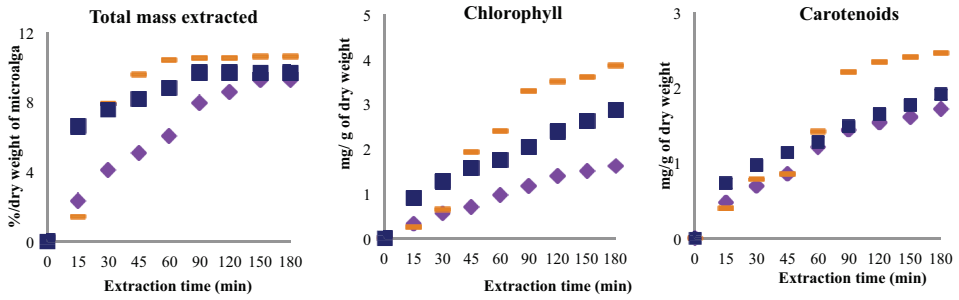

Figure 4: Assessing the effect of bead milling on the extraction of the biomolecules: - Bead milling (600 bar, 30 g.min^{-1} CO_2 flow rate, 60°C), ■ Bead milling (350 bar, 30 g.min^{-1} CO_2 flow rate, 60°C), ◆ Raw microalga (600 bar, 30 g.min^{-1} CO_2 flow rate, 60°C).

2.2.3.3 Extraction with ethanol as a co-solvent

It should be mentioned that the *C. vulgaris* was grown under normal growth conditions, and was thus expected to have a low lipid content mainly composed of polar lipids. Therefore, given the relatively high polarity of the lipid fraction, it seems pertinent to consider the addition of ethanol as a co-solvent to enhance the solubility of these biomolecules (Fig 5) as well as that of pigments.

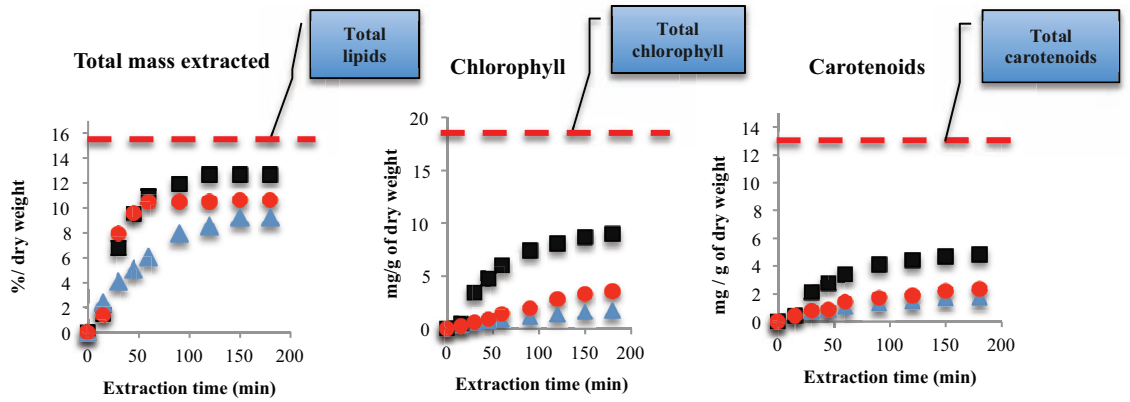

Figure 6: Comparison the effect of different treatments on the extraction of the biomolecules: ■ Crude microalga (600 bar, 30 g.min^{-1} CO_2 flow rate, 5% ethanol, 60°C), ◆ Crude microalga (600 bar, 30 g.min^{-1} CO_2 flow rate, 60°C), ○ Bead milling (600 bar, 30 g.min^{-1} CO_2 flow rate, 60°C).

Thus, by using the same operating conditions as previously, the addition of 5% w/w ethanol to $scCO_2$ when treating raw microalga, increased the total extract yield by 27% and 16% compared to the experiment conducted on raw microalga (without ethanol) and disrupted cells (by bead milling) respectively. Chlorophyll and carotenoids were also significantly affected by the addition of the co-solvent on the raw microalga, with 81% and 65% enhancement respectively compared to the experiment using raw microalga without ethanol, and by 61% and 52% respectively compared to cells disrupted by bead milling. Furthermore, the concentration of both pigments in the final extract changed according to the treatment applied, and in this respect the increasing concentration of chlorophyll in the extract followed the trend: no pre-treatment (18%) < bead milling (33%) < co-solvent (78%). Similarly, the increasing concentration of carotenoids followed the same trend but with lower concentrations: no pre-treatment (18%) < bead milling (22%) < co-solvent (37%). The effectiveness of adding a co-solvent was also covered in other studies. For instance, these results parallel those by Kitada et al. (2010) on *C. vulgaris*, where the effect of co-solvent on the solubility of a carotenoid was explained by the presence of highly polar alcohol with carbon dioxide, that modifies the characteristics of the solvent leading to an enhancement of pigment recovery. In addition, although use of supercritical carbon dioxide without a co-solvent leads to lower extraction yields, the selectivity for carotenoids such as lutein can be slightly improved.

2.2.4 Conclusion

According to the results described above, it can be deduced that high pressure inflicts some damage to the cell wall allowing supercritical carbon dioxide to reach the intracellular space as well as the intra-organelle matrix where the target biomolecules are located. Bead milling is a very effective cell disruption technique that completely breaks down the cells, but requires high-energy input that must be considered in the case of future process development. Optimisation of milling conditions is thus necessary to minimize production costs. Supercritical carbon dioxide is an interesting and selective extraction process, which is still considered costly compared to conventional methods. However, the degree of selectivity is an extremely important factor since it is a key element to bypass multiple unit purification operations that would decrease the final production cost, and simultaneously increase the added value of the final product, and compensate for its high production cost. Moreover, regarding the cleanliness of the final product, which is highly important, the presence of ethanol in trace quantities presents no problems as to its implementation in nutritional or pharmaceutical applications.

The study also gathered additional information regarding the necessity for conducting a preliminary cell disruption operation before supercritical carbon dioxide extraction. It was shown that the addition of 5%w/w ethanol into the supercritical device allowed better recovery of the lipid extract, chlorophyll and carotenoids, compared to the use of preliminary bead milling of *C. vulgaris*. These results were obtained at 600 bar, with a flow rate 30 $g.min^{-1}$, at 60°C, with a 3 h extraction time. However, optimization of extraction conditions may lead to shorter extraction times, and such a perspective will have to be considered with regard to the energy required by the entire purification process. Thus it would appear worthwhile to conduct additional studies, concerning optimization and implementation of the coupling of both bead milling and supercritical technology on a large scale, in order to achieve reasonable production costs in the near future.

Acknowledgments
This study was supported by the French National Research Agency (ANR) in the framework of the "Algoraffinerie" project. The authors would like to thank Alpha Biotech for gratefully providing the biomass. The authors are also grateful to the Laboratoire de Genie Chimique (LGC) for providing the facilities and the machines required to conduct this study.

2.3 Chapter Conclusion

Taking into account the set-up parameters fixed (pressure, CO_2 flow rate, temperature) for the extractor, and despite the efficiency of bead milling to break the cell wall of *Chlorella vulgaris*, it is possible to bypass the unit operation of cell disruption before conducting $scCO_2$ extraction by the addition of ethanol on raw microalga. Results showed that with the presence of ethanol, almost 90% of lipids were recovered. The lipid extract also included the lipophilic pigments that were better recovered compared to the extractions conducted after bead milling.

The next chapter will focus on proteins extraction with regard to cell disruption and the morphological role of the microalgal cells. Therefore, in order to compare different cell wall and morphological characteristics, different *Chlorella vulgaris* and other renowned microalgae were implemented in some studies. Taking into account the structure and the ultrastructure of the species should be helpful for better understanding the release of proteins.

References

[1] Kim J, Yoo G, Lee H, Lim J, Kim K, Kim CW, et al. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnol Adv. 2013;31:862-76.
[2] Spolaore P, Joannis-Cassan C, Duran E, Isambert A. Commercial applications of microalgae. J Biosci Bioeng. 2006;101:87-96.
[3] Kitada K, Machmudah S, Sasaki M, Goto M, Nakashima Y, Kumamoto S, et al. Supercritical CO_2 extraction of pigment components with pharmaceutical importance from *Chlorella vulgaris*. J Chem Technol Biot. 2009;84:657-61.
[4] Raposo MF, de Morais RM, Bernardo de Morais AM. Bioactivity and applications of sulphated polysaccharides from marine microalgae. Mar Drugs. 2013;11:233-52.
[5] Becker EW. Micro-algae as a source of protein. Biotechnol Adv. 2007;25:207-10.
[6] Chisti Y. Biodiesel from microalgae. Biotechnol Adv. 2007;25:294-306.
[7] Demirbas MF. Biofuels from algae for sustainable development. Appl Energy. 2011;88:3473-80.
[8] Gouveia L. Microalgae as a feedstock for biofuels. Springer briefs in microbiology,. Heidelberg ; New York: Springer; 2011 p. 69.
[9] Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N. Biofuels from microalgae. Biotechnol Progr. 2008;24:815-20.
[10] Yvonne N, Tomas K. Cell wall development, microfibril and pyrenoid structure in type strains of *Chlorella vulgaris*, *C. kessleri*, *C. sorokiniana* compared with C. luteoviridis (Trebouxiophyceae, Chlorophyta). Arch Hydrobiol 2000;100:95-105.
[11] Mallick N, Mandal S, Singh AK, Bishai M, Dash A. Green microalga *Chlorella vulgaris* as a potential feedstock for biodiesel. J Chem Technol Biot. 2012;87:137-45.
[12] Mizuno Y, Sato A, Watanabe K, Hirata A, Takeshita T, Ota S, et al. Sequential accumulation of starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species. Bioresource Technol. 2012;129C:150-5.
[13] Widjaja A, Chien C-C, Ju Y-H. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem E. 2009;40:13-20.
[14] Mendes RL, Fernandes HL, Coelho J, Reis EC, Cabral JMS, Novais JM, et al. Supercritical CO2 extraction of carotenoids and other lipids from *Chlorella vulgaris*. Food Chem. 1995;53:99-103.
[15] Konishi F, Tanaka K, Himeno K, Taniguchi K, Nomoto K. Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. Cancer Immunol Immun. 1985;19:73-8.
[16] Lordan S, Ross RP, Stanton C. Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. Mar Drugs. 2011;9:1056-100.
[17] Mendes RL, Nobre BP, Cardoso MT, Pereira AP, Palavra AF. Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. Inorg Chim Acta. 2003;356:328-34.
[18] Ritchie RJ. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res. 2006;89:27-41.
[19] Kapaun E, Reisser W. A chitin-like glycan in the cell wall of a *Chlorella* sp. (Chlorococcales, Chlorophyceae). Planta. 1995;197:577-82.
[20] Crampon C, Boutin O, Badens E. Supercritical Carbon Dioxide Extraction of Molecules of Interest from Microalgae and Seaweeds. Ind Eng Chem Res. 2011;50:8941-53.

[21] Abo-Shady AM, Mohamed YA, Lasheen T. Chemical composition of the cell wall in some green algae species. Biol Plant. 1993;35:629-32.
[22] Payne MF, Rippingale RJ. Evaluation of diets for culture of the calanoid copepod Gladioferens imparipes. Aquaculture. 2000;187:85-96.
[23] Dejoye C, Vian MA, Lumia G, Bouscarle C, Charton F, Chemat F. Combined extraction processes of lipid from *Chlorella vulgaris* microalgae: microwave prior to supercritical carbon dioxide extraction. Int J Mol Sci. 2011;12:9332-41.
[24] Gouveia L, Nobre BP, Marcelo FM, Mrejen S, Cardoso MT, Palavra AF, et al. Functional food oil coloured by pigments extracted from microalgae with supercritical CO2. Food Chem. 2007;101:717-23.

Chapter 3: Recovery of proteins: understanding the morphological role of the cells

3.1 Introduction

One of the specificities of microalgae is their cell wall characteristics, which undergo structural modifications according to the growth conditions and also undergo additional changes during different growth periods, and play an important role in regulating the passage of biomolecules through. This matter took much of our attention in this chapter due to the important role of the cell walls on permitting the intracellular and the intra-organelles access to the solvent for the recovery of biomolecules of interest. Therefore, the main objective of this chapter is to highlight the role of different cell wall structures of five different microalgae on the quantification of proteins, and on the recovery of biomolecules in the aqueous phase before and after applying a treatment that basically targets the cell wall. The microalgal species selected for this chapter represents a specimen of morphological diversity among microalgae. The species are: *Arthrospira platensis*, *Chlorella vulgaris*, *Haematococcus pluvialis*, *Nannochloropsis oculata* and *Porphyridium cruentum*.

The work described in this chapter is exposed in the form of four publications. The first publication already published in *Journal of Applied Phycology* reflects the work on determining the role of the cell wall on evaluating the nitrogen to protein conversion factor for each microalga mentioned previously, and whether it is possible to recommend a global conversion factor for the quantification of microalgal proteins. It should be mentioned that the same batch for each microalga was used during our work, and therefore the nitrogen to protein conversion factors obtained in this publication were considered for the next publications included in this chapter.

The second publication submitted to *Algal Research* intervenes on conducting a chemical treatment or a mechanical treatment on the cell walls of the five microalgae, and looks on differentiating the amino acid profile especially by evaluating the fraction of essential and non-essential amino acids that would bring a clearer insight on showing whether or not the same proteins are released by means of cell wall treatment method.

The third publication submitted to *Algal Research*, deals with inspecting the extractability of proteins in water by conducting different cell disruption methods on microalgae, in order to evaluate the role of the cell walls as well as the internal organelles on the release of proteins in water.

The fourth publication submitted to *Bioresource Technology* allows us to have a better understanding of the mechanisms involved in the biomolecules recovery during the process.

3.2 Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors

Carl Safi [1,2], Michaël Charton [1,2], Olivier Pignolet [1,2], Françoise Silvestre [1,2], Carlos Vaca-Garcia [1,2], Pierre-Yves Pontalier [1,2]

[1] Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), 31030 Toulouse, France
[2] INRA, UMR 1010 CAI, F-31030 Toulouse, France

E-mail : carl.safi@ensiacet.fr

This publication has been published in
Journal of Applied Phycology

Abstract

Additional evidence about the influence of the cell wall physical and chemical characteristics on protein extractability was determined by calculating the conversion factors of five different microalgae known to have different cell wall composition, and their protein extracts. The con- version factors obtained for crude rigid cell walled *Chlorella vulgaris*, *Nannochloropsis oculata* and *Haematococcus pluvialis* were 6.35, 6.28 and 6.25, respectively, but for their protein extracts the values were lower with 5.96, 5.86 and 5.63. On the other hand, conversion factor obtained for fragile cell walled microalgae *Porphyridium cruentum* and *Arthrospira platensis* was 6.35 for the former and 6.27 for the latter, with no significant difference for their protein extract with 6.34 for the former and 6.21 for the latter. In addition, the highest hydro-soluble protein percentage recovered from total protein was for *P. cruentum* 80.3 % and *A. platensis* 69.5 % but lower for *C. vulgaris* with 43.3 %, *N. oculata* with 33.3 % and *H. pluvialis* with 27.5 %. The study spotted the light on the influence of the cell wall on evaluating the conversion factor and protein extractability. In addition, it showed the necessity of finding the conversion factor everytime accurate protein quantification is required, and proved that there is not a universal conversion factor that can be recommended.

Keywords Amino acid profile, cell wall, conversion factor, nitrogen, protein extract.

3.2.1 Introduction

Microalgae have been consumed long time ago by the Aztecs and other Mesoamericans who used this biomass as an important food source [1]. Nowadays, in Japan for instance, *Chlorella vulgaris* is added to food such as noodles and pasta [2] to improve the nutritional quality of the meal.

Microalgae are gaining interest due to their capacity to accumulate important amounts of multiple components (proteins, lipids, carbohydrates and pigments) compared to any other sources, and therefore protein content is considered as one of the cardinal components determining their nutritional value. For instance, *Arthrospira* (*Spirulina*) *maxima* can accumulate proteins up to 71 % dry weight [3]. Thus, analysing and quantifying the protein content are key factors that should be thoroughly investigated. A capital point is to calculate precisely the nitrogen-to-protein conversion factor (NTP). While the standard value of 6.25 is used, Kjeldahl or elemental analysis may lead to an overestimation or underestimation of the protein quantity. Moreover, these two methods take into account the totality of the nitrogen present in the biomass from which 59–98 % [4-7] of total nitrogen belongs to protein and the rest comes from pigments, nucleic acids and other inorganic components. It is true that the colorimetric method of Lowry [8] is an accurate method for protein quantification [5, 9] and it does not require a conversion factor. Nevertheless, this method determines only the hydro- soluble proteins [10, 11] and not the total protein content. In addition, the extraction of proteins can be diminished by the cell wall barrier, which can prevent the solubilisation of all the intracellular proteins affecting thus the value of the nitrogen-to-protein conversion factor. Therefore, the impact of the cell wall characteristics on protein extractability should be taken into account and analysed in order to prevent an incorrect estimation of the protein content.

Multiple studies have focused on finding a method to recommend the right conversion factor; for instance, Gonzalez Lopez et al. (2010) focused on obtaining the conversion factor of five microalgae after breaking the cell wall, and then finding a correlation between protein content and total nitrogen content (elemental analysis or Kjeldahl). As a result, among five micro- algae a new mean conversion factor was estimated to 4.44 (elemental analysis) and 5.95 (Kjeldahl). Another study [12] determined the conversion factor for 19 tropical seaweeds harvested directly from the beach; and in a second

study [7], 12 marine microalgae were analysed under different growth phases and a mean value of 4.58 was found. The following study assesses the impact of the cell wall on the protein extractability and the evaluation of the NTP for five microalgae intensively grown worldwide and having wide taxonomic diversity.

3.2.2 Materials and methods

The microalgae used are: the cyanobacterium *Arthrospira platensis* (strain PCC 8005), two Chlorophyceae *Chlorella vulgaris* (strain SAG 211-19), *Haematococcus pluvialis* (unknown strain), one Rhodophyta *Porphyridium cruentum* (strain UTEX 161) and the Eustigmatophyceae *Nannochloropsis oculata* (unknown strain). Each microalga was cultivated in a different culture medium: Hemerick medium for *P. cruentum*, Sueoka medium for *C. vulgaris*, Basal medium for *H. pluvialis*, Conway medium for *N. oculata*, and Zarrouk medium for *A. platensis*. All were grown in batch mode in an indoor tubular air-lift photobioreactor (PBR, 10 L) at 25 °C [13] after inoculation from a prior culture in a flat panel air-lift PBR (1 L). Culture mixing was by sterile air injection at the bottom of the PBR. The pH and temperature were recorded by a pH/temperature probe and pH was regulated at 7.5 with CO_2. The algae were harvested during the exponential growth phase and concentrated by centrifugation, and then supplied as a frozen paste from Alpha Biotech (Asserac, France). The biomass contained 20 % dry weight. The frozen paste of crude microalgae was freeze-dried in a Fisher Bioblock Scientific Alpha 2–4 LD Plus device (Illkirch, France).

3.2.2.1 Protein extraction

Stock solutions were prepared with approximately 500 mL of ultrapure water and some drops of 2 N NaOH to adjust the solution to pH 12. A sample of 1 g of freeze-dried biomass was added to 50 mL of stock solution. The mixture was heated to 40 °C with stirring for 1 h followed by centrifugation at 5,000×g for 10 min. Samples were taken for analysis by the colorimetric method of Lowry et al. (1951), elemental analysis and amino acid analysis.

3.2.2.2 Lowry method

A calibration curve was prepared using a concentration range of bovine serum albumin from 0 to 1.500 $\mu g.mL^{-1}$. In order to measure the protein content, 0.2 mL of each

standard or samples containing the crude protein extract were withdrawn and then 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for exactly 10 min. After incubation, 100 µL of Folin Ciocalteu reagent (1 N) were added and again vortexed and incubated for exactly 30 min. The absorbance was then measured at 750 nm [8].

3.2.2.3 Elemental analysis

Total nitrogen of the freeze-dried biomass was evaluated using a PerkinElmer 2400 series II elemental analyser. Samples of 2 mg were placed in tin capsules and then heated at 925 °C using pure oxygen as the combustion gas and pure helium as the carrier gas, then evaluating the nitrogen percentage and converting it into protein percentage by using the conversion factors calculated for each microalga in this study.

3.2.2.4 Amino acid analysis and NTP calculation

The determination of the amino acid composition of the biomass was performed according to a widely used standard method [14]. The samples were hydro- lysed with 6 N hydrochloric acid at 103 °C for 24 h. Then, the hydrolysed material was adjusted to pH 2.2 with 6 N NaOH and stabilised with a pH 2.2 citrate buffer solution. The final solution was then filtered with 0.45 µm PTFE membrane to remove any residual solids remaining in the solution. The analysis was performed by using an amino acid analyser Biochrom Ltd 32 + (Cambridge, UK) equipped with a high pressure PEEK "column + pre-column" (size, 200 × 4.6 mm) packed with Ultropac cation exchange resin containing sodium. The separation of amino acids is carried out by elution with loading buffers (flow rate 25 $mL.h^{-1}$) at different pH. After reaction with ninhydrin (flow rate 35 $mL.h^{-1}$), amino acids are detected with a UV detector at a wavelength of 570 nm, with the exception of proline, for which detection occurs at 440 nm. Calculation of NTP was carried out according to the method (k_A) of Mossé (1990) and Sriperm et al. (2011). It should be mentioned that ammonia was added to compensate the value of some less resistant amino acids that disappeared after the strong acid hydrolysis. In addition, the strong hydrolysis of the peptide bonds generates one molecule of water from each amino acid and therefore during the quantification of total amino acid one molecule of water was subtracted from each amino acid in order to get the total amino acid residue, which represents the exact quantity of all amino acids [4, 7].

3.2.2.5 Confocal laser scanning microscopy

Cells were observed with an SP2-AOBS confocal laser- scanning microscope (Leica). The fluorochrome calcofluor white that binds to the β→1–4 linkages in the cell wall polysaccharides was added to the samples. Excited at 488 nm, the cells are identified coloured in light blue.

3.2.2.6 Statistical analysis

Three experiments were conducted separately on all micro- algae and their protein extract. Statistical analyses were carried out on Microsoft Excel. Measurements of three replicates for each sample were reproducible for ±5 % of the respective mean values.

3.2.3 Results

An amino acid analyser was used to obtain the amino acid profile of the crude microalgae (Table 1) and their protein extract (Table 2). The protein primary composition was reconstituted in order to find the conversion factor (Table 3) that takes into account only the protein nitrogen. The highest conversion factor evaluated for the crude biomass was 6.35 for *C. vulgaris* and *P. cruentum* and the lowest was 6.25 for *H. pluvialis*. If we compare the NTP value of the crude micro- algae and the protein extract, we observe that there is no significant difference for *P. cruentum* and *A. platensis*. However, a significant difference was measured for the other three species, which correspond to green microalgae (Table 3).

Crude microalgae amino acid composition					
Amino acids	***P. cruentum***	***A. platensis***	***C. vulgaris***	***N. oculata***	***H. pluvialis***
Aspartic acid	11.21 ± 0.45	11.82 ± 0.11	10.09 ± 0.02	9.14 ± 0.05	8.85 ± 0.10
Threonine	6.25 ± 0.25	6.16 ± 0.10	5.62 ± 0.01	5.91 ± 0.03	5.21 ± 0.06
Serine	8.11 ± 0.29	6.85 ± 0.02	7.17± 0.04	6.52 ± 0.01	6.63 ± 0.05
Glutamic acid	8.17 ± 0.29	10.50 ± 0.09	8.37 ± 0.01	10.30 ± 0.02	9.47 ± 0.11
Glycine	6.86 ± 0.28	7.76 ± 0.06	7.93 ± 0.01	9.00 ± 0.01	9.05 ± 0.09
Alanine	6.67 ± 3.67	9.91 ± 0.08	10.05 ± 0.03	10.92 ± 0.01	11.28 ± 0.12
Cysteine	0.33 ± 0.01	0.18 ± 0.02	0.18 ± 0.01	0.19 ± 0.01	0.22 ± 0.01
Valine	2.50 ± 0.10	2.86 ± 0.02	2.85± 0.01	3.29 ± 0.02	3.32 ± 0.04
Methionine	2.78 ± 0.11	1.72 ± 0.02	0.60 ± 0.01	1.50 ± 0.01	0.64 ± 0.01
Isoleucine	5.25 ± 0.24	0.12 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	4.53 ± 0.04
Leucine	5.83 ± 0.21	7.02 ± 0.02	6.91 ± 0.02	8.11 ± 0.05	8.09 ± 0.10
Tyrosine	4.43 ± 0.18	4.83 ± 0.05	7.78 ± 0.01	3.40 ± 0.02	2.80 ± 0.04
Phenylalanine	5.00 ± 0.20	4.82 ± 0.04	5.36 ± 0.01	5.05 ± 0.01	4.92 ± 0.07
Histidine	1.11 ± 0.04	0.90 ± 0.01	1.16 ± 0.01	0.94 ± 0.01	0.90 ± 0.01
Lysine	5.50 ± 0.21	5.10 ± 0.62	6.30 ± 0.07	5.70 ± 0.01	5.72 ± 0.08
Arginine	7.78 ± 0.29	7.69 ± 0.07	6.81 ± 0.03	5.93 ± 0.02	6.10 ± 0.08
Tryptophan	1.39 ± 0.05	1.22 ± 0.01	2.04 ± 0.01	1.24 ± 0.01	1.72 ± 0.02
Ornithine	0.27 ± 0.01	0.16 ± 0.09	0.12± 0.01	0.16 ± 0.01	0.07 ± 0.01
Proline	2.53 ± 0.17	1.95 ± 0.05	2.74 ± 0.07	4.20 ± 0.07	2.94 ± 1.15
Ammonia	8.02 ± 0.30	8.41 ± 0.09	7.82 ± 0.02	8.38 ± 0.08	7.52 ± 0.08

Table 1: Results of total amino acids of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments ± SD (*n*=3).

Protein extract amino acid composition					
Amino acids	***P. cruentum***	***A. platensis***	***C. vulgaris***	***N. oculata***	***H. pluvialis***
Aspartic acid	10.71 ± 0.02	9.70 ± 0.02	6.81 ± 0.28	4.47 ± 0.05	6.54 ± 0.07
Threonine	4.45 ± 0.01	5.54 ± 0.04	4.16 ± 0.19	3.18 ± 0.04	3.70 ± 0.02
Serine	7.49 ± 0.02	7.25 ± 0.03	5.73 ± 0.27	3.16 ± 0.06	5.66 ± 0.01
Glutamic acid	9.05 ± 0.01	11.65 ± 0.02	11.63 ± 0.51	22.60 ± 0.19	13.55 ± 0.01
Glycine	7.68 ± 0.01	8.42 ± 0.02	9.75 ± 0.42	8.79 ± 0.10	11.00 ± 0.01
Alanine	10.46 ± 0.02	10.94 ± 0.02	16.82 ± 0.75	14.02 ± 0.13	19.12 ± 0.03
Cysteine	0.27 ± 0.01	0.20 ± 0.01	0.24 ± 0.01	0.38 ± 0.01	0.40 ± 0.01
Valine	3.15± 0.01	3.31 ± 0.01	3.66 ± 0.15	2.36 ± 0.03	2.80 ± 0.01
Methionine	2.37± 0.01	1.71 ± 0.01	1.28 ± 0.04	1.23 ± 0.03	1.04 ± 0.01
Isoleucine	5.34± 0.02	0.12± 0.01	2.32 ± 1.87	1.08 ± 1.44	3.06 ± 0.01
Leucine	7.30 ± 0.01	8.02± 0.02	7.15 ± 0.33	4.11 ± 0.04	4.99 ± 0.03
Tyrosine	3.69 ± 0.01	4.33 ± 0.01	2.56 ± 0.10	2.15 ± 0.07	1.91 ± 0.02
Phenylalanine	4.12 ± 0.01	4.26 ± 0.01	2.74 ± 0.12	1.63 ± 0.02	2.63 ± 0.01
Histidine	0.79 ± 0.03	0.73 ± 0.01	0.53 ± 0.03	0.28 ± 0.01	0.43 ± 0.01
Lysine	5.60 ± 0.01	5.39 ± 0.01	6.16 ± 0.27	2.35 ± 0.01	3.48 ± 0.01
Arginine	6.63 ± 0.02	6.84 ± 0.01	5.85 ± 2.71	1.96 ± 0.09	6.88 ± 0.01
Tryptophan	0.72 ± 0.02	1.07 ± 0.01	0.49 ± 0.02	0.18 ± 0.01	0.31 ± 0.01
Ornithine	0.22 ± 0.01	0.23 ± 0.01	0.50 ± 0.02	0.55 ± 0.03	0.43 ± 0.01
Proline	1.87 ± 0.02	1.86 ± 0.08	2.11 ± 0.13	14.41 ± 0.33	1.61 ± 0.04
Ammonia	8.05 ± 0.04	8.39 ± 0.01	9.47 ± 0.44	11.09 ± 0.31	10.37 ± 0.06

Table 2: Results of total amino acids of the protein extract extracted at pH 12 and 40°C of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments ± SD (*n*=3).

The total nitrogen content of crude microalgae was determined by elemental analysis. The hydro-soluble proteins were quantified by the Lowry method. By using the NTP values from Table 3, the total proteins and the corresponding fraction of hydro-soluble proteins were accurately determined starting from the results of elemental analysis (Table 4). A small difference was observed between the essential and non-essential amino acids total percentage for *P. cruentum*, *A. platensis* and *C. vulgaris* and their protein extract, and an important difference was observed for *N. oculata* and *H. pluvialis* with a noticeable increase in non-essential amino acids percentage in the protein extract and a decrease in essential amino acids percentage (Table 5).

	NTP		
Microalgae	**Crude microalgae**	**Protein extract**	**% Relative difference**
P. cruentum	6.35 ± 0.03	6.34 ± 0.04	0.16
A. platensis	6.27 ± 0.02	6.21 ± 0.07	0.96
C. vulgaris	6.35 ± 0.07	5.96 ± 0.23	6.14
N. oculata	6.28 ± 0.06	5.86 ± 0.32	6.69
H. pluvialis	6.25 ± 0.07	5.63 ± 0.18	9.92

Table 3: Nitrogen-to-protein conversion factors for the crude microalgae and their protein isolate based on 3 replicates for 3 experiments ± SD (*n*=3).

Finally, the laser scanning confocal microscopic images presented in Fig. 1 showed that in the case of *P. cruentum* and *A. platensis* a total disruption of the cell wall occurred after the alkaline treatment, whereas *C. vulgaris*, *N. oculata* and *H. pluvialis* maintained their globular form indicating that at least a part of their cell wall was intact.

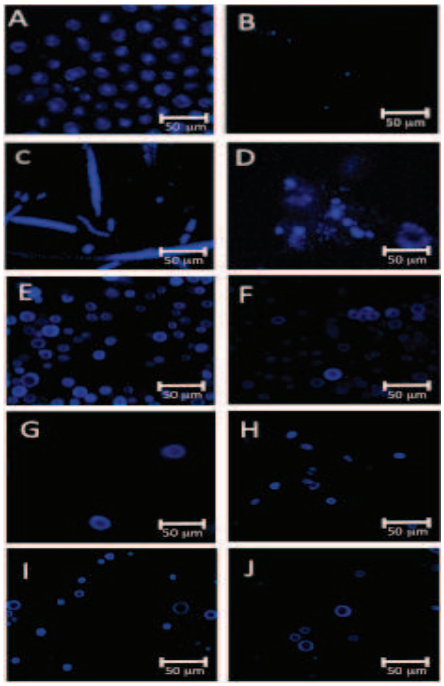

Figure 1. Laser scanning confocal microscopic observation before (left) and after (right) alkaline treatment for each microalga. A&B) *P. cruentum*, C&D) *A. platensis*, E&F) *C. vulgaris*, G&H) *N. oculata*, I&J) *H. pluvialis*.

3.2.4 Discussion

The present study contributes original individual NTP con- version factors for five current species of microalgae. Indeed, direct comparison of our results with literature values can only be done for *N. oculata* [7]; the report from Gonzalez Lopez et al. (2010) combines all the cyanobacteria and *P. cruentum* in a global result. To our knowledge, NTP data for *C. vulgaris* and *H. pluvialis* have never been reported. Moreover, according to Mossé (1990), there are three kinds of NTP conversion factors called k, k_A and k_P [15-17]. In this study, k_A was calculated for all species, and this kind of conversion factor is larger than kP and k. For this reason, there is a significant difference between the conversion factors calculated for *N. oculata* in this study (6.28) and the one calculated for the same microalga (4.87) in the Lourenço et al. (1998) study.

	Nea[a] (%)	**Total protein= Nea x NTP[b] (%)**	**Hydro-soluble protein= P_{Lowry}[c] (%)**	**Proportion of hydro-soluble protein in total protein= $\frac{P_{Lowry}}{Nea \times NTP} \times 100$ (%)**
P. cruentum	9.04 ± 0.69	57.33 ± 3.84	46.06 ± 0.97	80.34 ± 1.69
A. platensis	8.53 ± 0.20	53.51 ± 1.10	37.19 ± 2.67	69.50 ± 5.00
C. vulgaris	7.81 ± 0.18	49.59 ± 1.04	21.50 ± 0.34	43.35 ± 0.62
N. oculata	7.41 ± 0.39	46.55 ± 2.14	15.52 ± 0.42	33.34 ± 0.90
H. pluvialis	8.27 ± 0.07	51.73 ± 0.43	14.22± 0.69	27.48 ± 1.34

Table 4: Different protein contents in crude microalgae.
[a]Nea: Total nitrogen % (d.w) obtained by elemental analysis.
[b]NTP: Nitrogen-to-protein conversion factor from Table 3.
[c]P_{Lowry}: Hydro-soluble protein % (d.w) at pH 12 and 40°C calculated by Lowry method.

The NTP conversion factor of the crude microalgae and their protein extract depended on the type of microalgae. If we calculate the relative difference between these two values for a single species, we observe that there is almost no difference in the case of red algae and cyanobacteria, whereas it reached almost 10 % for green microalgae (Table 3). These differences can be correlated to the rigidity of the cell wall. Indeed, it is generally accepted that green microalgae possess a more rigid cell wall than red algae or cyanobacteria. More in detail, *P. cruentum* does not have a true cell wall but instead encapsulated by a layer of sulfurized polysaccharides [18-23]. *A. platensis* has a relatively fragile cell wall mainly composed of murein without cellulose [24, 25]. As far as the green microalgae, the chlorophycean *C. vulgaris* and the eustigmatophycean *N. oculata*, both have a cell wall mainly composed of cellulose and hemicelluloses [26]. The highest difference

perceived was for *H. pluvialis*, which has a thick trilaminar cell wall composed of cellulose and sporopollenin in the aplanospore stage [27-29]. The composition of its cell wall makes this microalga less permeable and extremely mechanically resistant [30].

Taking into account the standard deviation of the three samples considered for each microalga, we could affirm that, at 95 % of confidence level, all the values of total protein content shown in Table 4 are statistically equivalent. This fact is noteworthy, because when we consider the extracted hydro-soluble proteins (Table 4), we observe that their amounts decrease following the same correlation than the differences in NTP value: high extraction for the micro- algae with no real cell wall and very low extraction for the most rigid of all. Therefore, the hydro-soluble protein capable to be extracted under alkaline conditions from inside the cell is correlated to the cell wall characteristics and to the freeze-drying process that conserves well the samples but it makes the protein extraction more difficult for some algal species. In addition, the high values of hydro-soluble proteins extracted especially for *P. cruentum* and *A. platensis* could be explained by the possible presence of peptides and free amino acids because no precipitation was carried out [31, 32].

Further evidence was found in the variation in composition relative to essential and non-essential amino acids (Table 5) in the protein extract of the green microalgae. There was a significant drop in the percentage of essential amino acids ranging from 13.9 % for *C. vulgaris* to 49.1 % for *N. oculata* and an increase in non-essential amino acids from 6.7 % for the former to 25.2 % for the latter. On the contrary, only small changes in the composition of the proteins were detected for fragile cell wall microalgae, with a percentage difference ranging from 4.5 % for *A. platensis* to 4.8 % for the essential amino acids of *P. cruentum*, whereas non-essential amino acids difference ranged from 3.5 % for the former to 4.8 % for the latter.

	Crude microalgae			**Protein extract**		
	Essential	**Non-essential**	**Non identified**	**Essential**	**Non-essential**	**Non identified**
P. cruentum	46.81	44.89	8.29	44.58	47.15	8.27
A. platensis	41.76	49.67	8.57	39.87	51.50	8.62
C. vulgaris	41.02	51.03	7.94	35.32	54.70	9.97
N. oculata	41.00	50.46	8.53	20.88	67.47	11.64
H. pluvialis	43.91	48.50	7.58	29.01	60.17	10.81

Table 5: Percentage (d.w) of essential and non-essential amino acids for each microalga and its protein extract.

These correlations with the relative hardness of the cell wall can integrate a chemical dimension as well. Indeed, the sporopollenin contained in the most rigid cell wall (*H. pluvialis*) is known to be extremely resistant to chemical agents [30]. But in the case of cellulose-rich cell walls, such as *C. vulgaris* and *N. oculata*, the sodium hydroxide is able to penetrate the microcrystalline structure of cellulose to form alcoholates in a process similar to mercerisation. Sodium hydroxide can also dissolve the hemicelluloses attached to cellulose. The partial permeation of this kind of cell wall can therefore occur by alkaline action. Finally, *A. platensis* has a cell wall rich in amino sugars cross-linked with oligopeptide chains. The former are labile in alkaline conditions by deamidation of the N-acetylglucosamine and the latter are soluble in alkaline conditions. The cell wall becomes therefore very permeable allowing the alkaline extraction of proteins. In summary, the chemical action acts in synergy with the mechanical characteristics of the cell wall.

Extraction of proteins together with the evaluation of the conversion factor brought additional evidence that the cell wall of any microalga plays an important role in protein quantification. This means that not taking it into consideration may lead to wrong quantification of the protein con- tent. In addition, for microalgae, there is not a universal conversion factor that can be recommended for all species as demonstrated by comparison of our study with many studies which have been carried out on dozens of different microalgae. Therefore, every time accurate quantification of protein is needed, it will be required to evaluate the conversion factor. In addition, this study showed a correlation between the cell wall rigidity and/or the chemical structure and the differences in NTP conversion value. Microalgae with fragile cell wall did not show significant differences with their protein extract, which was the complete opposite for the microalgae having a rigid cell wall that showed noticeable difference on evaluating the conversion factor of their protein extract, and therefore, breaking or permeabilize the rigid cell wall of *C. vulgaris*, *N. oculata* and *H. pluvialis* is strongly required to prevent underestimation of the protein content after extraction. Logically, there is no need for cell wall breaking for *P. cruentum* since it does not have a cell wall and concerning *A. platensis* soft cell wall disruption technique is needed to make sure that its fragile cell wall will not hinder the extraction of any intracellular components.

Acknowledgments
The authors would like to thank Alpha Biotech for providing the biomass and also we are thankful to Dr Philippe Michaud, Dr Celine Laroche, Mr Jean-François Fabre and Mrs Cécile Pouzet for their support and expertise. This work was supported by the French National Research Agency (ANR) in the context of Algoraffinerie project.

3.3 Release of hydro-soluble microalgal proteins using mechanical and chemical treatments

Carl SAFI [1,2], Michael CHARTON [1,2], Alina Violeta URSU [3], Céline LAROCHE [3], Bachar ZEBIB [1,2], Pierre-Yves PONTALIER [1,2], Carlos VACA-GARCIA [1,2,4]

[1] Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), 31030 Toulouse, France
[2] INRA, UMR 1010 CAI, F-31030 Toulouse, France
[3] Université Blaise Pascal, Polytech, Institut Pascal UMR CNRS 6022, 24, Avenue des Landais, 63174 Aubière, France
[4] King Abdulaziz University, Jeddah, Saudi Arabia

E-mail : carl.safi@ensiacet.fr

This publication has been submitted and accepted in ***Algal Research***

Abstract

In order to release proteins in the aqueous phase, high-pressure homogenization and alkaline treatments were applied to rupture the cell walls of five intensively grown microalgae. Protein characterisation was carried out by analysing the amino acid profiles of both the crude microalgae and the protein extracts, obtained after both types of treatment. Results showed that the proportion of proteins released from microalgae following both treatments was, in descending order: *Porphyridium cruentum* > *Arthrospira platensis* > *Chlorella vulgaris* > *Nannochloropsis oculata* > *Haematococcus pluvialis*, reflecting the increasingly protective, cell walls. Nonetheless, mechanical treatment released more proteins from all the microalgae compared to chemical treatment. The highest yield was for the fragile cell walled *P. cruentum* with 88% hydro-soluble proteins from total proteins, and the lowest from the rigid cell walled *H. pluvialis* with 41%. The proportion of essential and non-essential amino acids in the extract was assessed and compared to the crude microalgae profile. It was higher after alkaline treatment and much higher after high-pressure homogenization. These results suggest that non-essential amino acids are more concentrated actually inside the cells and that different types of proteins are being released by these two treatments.

Keywords Microalgae, chemical treatment, mechanical treatment, proteins, amino acids profile.

3.3.1 Introduction

In the 9th century AD the Kanem Empire in Chad discovered the benefits of the cyanobacterium *Arthrospira platensis* and used it as food (called *dihé*) for human consumption [33]. Later on in the 14th century AD, the Aztecs harvested the same species from Lake Texcoco and used it to make a sort of cake called *tecuilatl*. They also used these microorganisms as fodder, fertilizers and remedies. Nowadays, additional species are being industrially and profitably marketed worldwide for the same purposes.

The microalgal industry has grown rapidly over the last decade. Primarily, this is due to the capacity of these micro-organisms to produce lipids suitable for the biodiesel industry, and to grow in a wide variety of geographical and environmental locations, thus precluding competition with arable lands as well as intensive deforestation. Therefore, the major part of microalgal studies has concentrated on enhancing this bioenergy production to the detriment of other high-value biomolecules, but forgetting ancient history and the other advantages of these species.

Today the microalgal bioenergy industry is struggling to find a place in the market due to its uncompetitive cost and its overall unsustainable production [34-38] sometimes leaving negative footprints on the environment, and public opinion.

Microalgae were originally considered as an important source of protein, a major fraction of their composition; on a dry weight basis the Cyanobacterium *Arthrospira platensis* is composed of 50-70% proteins [39, 40], the Chlorophycea *Chlorella vulgaris* 38-58% [41-43], the Eustigmatophyceae *Nannochloropsis oculata* 22-37% [44], the Chlorophycea *Haematococcus pluvialis* 45-50% [39], and the Rodophyta *Porphyridium cruentum* 8-56% [3, 45]. They have a profile composed of a set of essential and non essential amino acids [42], with relatively similar ratio between species and are generally unaffected by growth phase and light conditions [33]. To the best of our knowledge, studies on microalgal proteins have generally either concentrated on finding and proposing the nitrogen to protein conversion factor [5-7, 11, 42], in order to prevent incorrect estimations of microalgal total proteins content, or focused on determining the best method for protein quantification using colorimetric techniques [31, 46, 47]. However, for some species such as

the green microalgae *C. vulgaris*, *N. oculata* and *H. pluvialis*, maximising the recovery of proteins requires a unit operation leading to cell disruption to overcome the barrier of their rigid cell wall and release the intracellular biomolecules. Thus, many cell disruption methods were used to break the cell wall of these microalgae, such as bead milling, ultrasonication, microwave, enzymatic treatment and high-pressure homogenization [28, 48-51]. Conversely, fragile cell walled microalgae like *P. cruentum* and *A. platensis* require milder techniques to enhance recovery.

The main objective of this study is to evaluate the effect of two different cell disruption techniques on aqueous phase proteins extractability, in five microalgae having different cell wall characteristics, while simultaneously evaluating and comparing the profile of amino-acids after subsequent to these two cell disruption methods.

3.3.2 Materials and methods

3.3.2.1 Microalgae and materials

The selected microalgae were supplied as frozen paste from Alpha Biotech (Asserac, France): the Cyanobacteria *Arthrospira platensis* (strain PCC 8005), two different Chlorophyceae *Chlorella vulgaris* (strain SAG 211-19), and *Haematococcus pluvialis* (unknown strain), one Rhodophyta *Porphyridium cruentum* (strain UTEX 161), and the Eustigmatophyceae *Nannochloropsis oculata* (unknown strain).

Each microalga was cultivated in a different culture media; Hemerick media was used for *P. cruentum*, Sueoka media for *C. vulgaris*, Basal media for *H. pluvialis*, Conway media for *N. oculata* and Zarrouk media for *A. platensis*. All grown in batch mode in an indoor tubular Air-Lift PhotoBioReactor (PBR, 10 L) at 25°C inoculated from a prior culture in a flat panel Air-Lift PBR (1 L). Culture homogenization was achieved by sterile air injection at the bottom of the PBR. The pH and temperature were recorded by a pH/temperature probe (Mettler Toledo SG 3253 sensor) monitored by the acquisition software LabVIEW. The pH was regulated at 7.5 with CO_2 bubbling. Microalgae were harvested during the exponential growth phase, concentrated by centrifugation, and the biomass which contained 20% dry weight, was then frozen.

3.3.2.2 High-pressure cell disruptor

A "TS Haiva series, 2.2-kW" homogenizer from Constant Systems Limited (Northants, UK), was used. For each experiment, a biomass concentration of 2% dry weight (0.5 g of freeze-dried cells dispersed in 25 mL distilled water) was passed through the machine twice at a pressure of 2700 bar.

3.3.2.3 Alkaline treatment

Mother solutions were prepared with approximately 500 mL of ultrapure water and some drops of 2 N NaOH to adjust the solution to pH 12. A sample of 1 g of freeze-dried biomass was added to 50 mL of mother solution and the mixture was heated at 40°C with stirring for 1 h. Separation of the solid-liquid mixture was conducted by centrifugation at 5000*g* for 10 min. Samples of the supernatant were taken for analysis by the colorimetric method of Lowry, elemental analysis and amino acid analysis.

3.3.2.4 Lowry method

The procedure involves reaction of proteins with cupric sulphate and tartare in an alkaline solution, leading to the formation of tetradentate copper protein complexes. The addition of the Folin-Ciocalteu reagent leads to the oxidation of the peptide bonds by forming molybdenum blue with the copper ions. Therefore, a calibration curve was prepared using a concentration range of bovine standard albumin from 0 to 1500 μg mL^{-1}. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract were withdrawn and then 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for exactly 10 min at room temperature. After incubation, 100 μL of Folin-Ciocalteu Reagent (1 N) were added and again vortexed and incubated for exactly 30 min at room temperature. The blue colour solution absorbance was then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer, previously zeroed with a blank sample containing all the reagents minus the extract [8].

3.3.2.5 Elemental analysis

Total nitrogen was evaluated by LCC (Laboratoire de Chimie de Coordination,

Toulouse-France) using a PerkinElmer 2400 series II elemental analyser. Samples of 2 mg were placed in thin capsules and then heated at 925 °C using pure oxygen as the combustion gas, and pure helium as the carrier gas. The percentage nitrogen was evaluated and converted into protein percentage by using the conversion factors obtained for each microalga in another study [42].

3.3.2.6 Amino acid analysis

The biomass amino acid composition was determined using a well known standard method (Moore and Stein 1948). The samples were hydrolysed with 6 N hydrochloric acid at 103 °C for 24 h in an oven. Then, the hydrolysed material was adjusted to pH 2.2 with 6 N NaOH and stabilised with a pH 2.2 citrate buffer solution. The final solution was then filtered with 0.45 μm PTFE membrane to remove any residual solids remaining in the solution. The analysis was performed by using an amino acid analyser Biochrom Ltd 32+ (Cambridge, UK) equipped with a high pressure PEEK "column+pre-column" (size, 200 × 4.6 mm) packed with Ultropac cation exchange resin containing sodium. The separation of amino acids was carried out by elution with loading buffers (flow rate 25 $mL.h^{-1}$) at different pH. After reaction with ninhydrin (flow rate 35 $mL.h^{-1}$), amino acids were detected with a UV detector at a 570 nm wavelength, except for proline, where detection was at 440 nm. Ammonia was added to compensate for the value of some less resistant amino acids, broken down by the strong acid hydrolysis.

3.3.2.7 Statistical analysis

Three experiments were conducted separately on every microalgae. Measurements of three replicates for each sample were repeatable at maximum ±5% of the respective mean values.

3.3.3 Results

The total protein content of crude microalgae was determined from the value of total nitrogen obtained through elemental analysis, and the conversion factor found for each crude microalga in a separate study (Safi et al. 2012b). In all cases, the total protein content was high and consistent with the literature values, ranging from 49 to 58% dry weight (Table 1). The fraction of hydro-soluble proteins released in water after both cell disruption techniques

was presented in Figure 1, after quantification by the Lowry method in cases where the mechanical method recovered more proteins compared to the alkaline treatment.

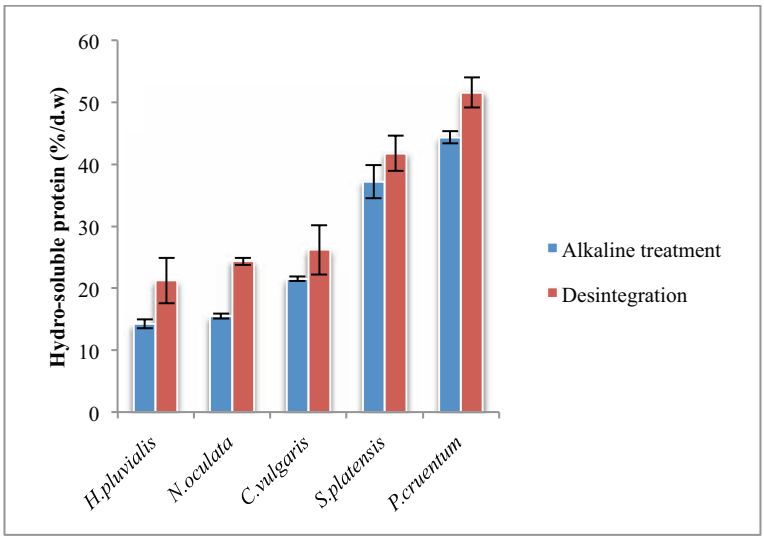

Figure 3 Protein % d.w of biomass in water after cell disruption of five microalgae by a mechanical and chemical method. Results are based on 3 replicates for 3 experiments ± SD (*n*=9).

The hydro-soluble protein fraction of total proteins present in the microalgae was also evaluated, and all these results are shown in Table1.

				Alkaline treatment		High-pressure cell disruption	
Microalgae	**N_{EA}[a] (%)**	**NTP[b]**	**P_{TOTAL} (%)**	**P_{Lowry}[c] (%)**	**$\frac{PLowry}{PTOTAL}\times100$ (%)**	**P_{Lowry}[c] (%)**	**$\frac{PLowry}{PTOTAL}\times100$ (%)**
P. cruentum	9.18 ± 0.61	6.35	58.29 ± 3.78	44.34 ± 0.97	76.07 ± 1.48	51.60 ± 2.45	88.52 ± 1.17
A. platensis	8.76 ± 0.16	6.27	54.92 ± 1.10	37.19 ± 2.67	67.72 ± 1.64	41.75 ± 2.82	76.02 ± 0.75
C. vulgaris	7.98 ± 0.16	6.35	50.67 ± 1.02	21.50 ± 0.34	42.43 ± 0.52	26.18 ± 3.99	51.68 ± 2.03
N. oculata	7.83 ± 0.31	6.28	49.17 ± 2.13	15.52 ± 0.42	31.56 ± 1.06	24.34 ± 0.58	49.50 ± 1.51
H. pluvialis	8.30 ± 0.04	6.25	51.87 ± 0.43	14.23 ± 0.69	27.43 ± 0.49	21.23 ± 3.66	40.93 ± 1.97

Table 1: Proportion of hydro-soluble protein in total protein for different microalgae
[a]Nea: Total nitrogen % (d.w) obtained by elemental analysis.
[b]NTP: Nitrogen-to-protein conversion factors of Safi et al. (2012b) for each microalga.
[c]P_{Lowry}: Hydro-soluble protein % (d.w) at pH 12 and 40°C and by high-pressure cell disruption calculated by Lowry method.
P_{TOTAL}: Total protein in microalgae = N_{EA} x NTP[b]

The amino acid profile was first determined for the crude microalgae with Aspartic acid being the highest member for *P. cruentum*, *A. platensis* and *C. vulgaris* and Alanine for *N. oculata* and *H. pluvialis* (Table 2). In addition, this profile was evaluated after alkaline

treatment, and here Aspartic acid was the highest for *P. cruentum*, Alanine for *A. platensis*, *C. vulgaris* and *H. pluvialis*, Proline for *N. oculata* (Table 3). However, with high-pressure cell disruption Proline was the highest for *P. cruentum*, *A. platensis* and *N. oculata* and Alanine for *C. vulgaris* and *H. pluvialis* (Table 4). Furthermore, the percentages of essential and non-essential amino acids before and after both cell disruption treatments, were also evaluated and are shown in Table 5. The proportion of non-essential amino acids was higher than that of essential amino acids for all the microalgae after both treatments. Nevertheless, it can be seen (Table 5) that the proportion of non-essential amino acids was much higher with high-pressure homogenization than with the alkaline treatment.

3.3.4 Discussion

This study used two different cell wall treatments on five different microalgae followed by a quantification of the proteins [8] released in the aqueous phase, and then assessed the amino acid profile of these proteins for each treatment. The characteristics of the microalgal cell walls play an important role in the release of these biomolecules. Nonetheless, regardless of cell wall characteristics we have shown that at the 95% confidence level using three replicates for each microalga, all the latter have statistically equivalent protein values (Table 1). It should however be noted that the total nitrogen estimation includes other obtained by elemental analysis was essential to calculate total proteins in the microalgae using the conversion factors adapted by the previous study of Safi et al. (2012b). Therefore, regardless of the specificities of their cell walls and taking into consideration three replicates for each microalga, we could affirm that at 95% of confidence level all the microalgae have statistically equivalent proteins value (Table 1). It should be noted that estimation of total nitrogen includes other nitrogenous compounds, such as intracellular inorganic materials [7] pigments, nucleic acid, glucosamine and amines that can account to about 10% of total nitrogen content in microalgae [39, 52]. After conducting both cell wall treatments, the highest content of hydro-soluble proteins in the extract was from *P. cruentum*, which has a pseudo-cell wall composed of exopolysaccharide mucilages [20-22] making it very fragile and offering very little resistance to any treatment. Conversely, the lowest microalgae protein content in this study was obtained from *H. pluvialis*, known for its cell wall composed of cellulose and sporopollenin, which is remarkably resistant to chemical and mechanical treatment [28, 42]. Moreover, if we observe the decrease in protein recovery, we can see that this mirrors the increasing rigidity of the cell walls (Table 1) in all the microalgae.

Nonetheless, compared to alkaline treatment, mechanical treatment gave more aqueous phase protein recovery for all the microalgae, with the lowest increase reserved for the fragile cell walled microalgae; 11% and 14% calculated for *A. platensis* and *P. cruentum* respectively. Indeed, both of these offer very little resistance to cell disruption treatment, and this small increase in protein recovery suggests more effective disruption of protein aggregates by high-pressure homogenization, leading to better solubilisation of hydro-soluble proteins in the aqueous phase. Similarly, a higher increase in protein recovery for the rigid cell walled microalgae was also detected, with 18%, 33% and 36% for *C. vulgaris*, *H. pluvialis* and *N. oculata* respectively. Here, the mechanical treatment applied in this study, is more effective at breaking the cell walls and protein aggregates, allowing more protein to be solubilised.

Furthermore, the alkaline treatment does have an effect on protein recovery, because the chemical action acts in synergy with the mechanical characteristics of the cell wall (Safi et al. 2012b). Similarly, as mentioned earlier, the sporopollenin contained in the most rigid cell wall (*H. pluvialis*) is known to be extremely resistant to chemical agents [30]. But for cellulose-rich cell walls, such as in *C. vulgaris* and *N. oculata*, the sodium hydroxide is able to penetrate the cellulose microcrystalline structure to form alcoholates in a process similar to mercerisation, and can also dissolve the hemicelluloses attached to the cellulose. Partial permeation of this kind of cell wall can therefore occur by alkaline action, favouring solubilisation of cell wall proteins but making it difficult to recover cytoplasmic and chloroplastic proteins. Finally, *A. platensis* is a gram-negative cyanobacteria with a thin cell wall rich in amino sugars cross-linked with oligopeptide chains. Under alkaline conditions, the former are labile by deamidation of the *N*-acetylglucosamine while the latter are soluble. Therefore the cell wall becomes highly permeable allowing alkaline extraction of proteins by penetration of the cytoplasmic and chloroplastic space, enhancing protein recovery.

The proteins' amino acid profile was also evaluated by analysing the crude microalgae (Table 2), the alkaline treatment protein extracts (Table 3) and the high-pressure homogenization extracts (Table 4). The proportion of essential and non-essential amino acids was also evaluated (Table 5), and showed that the percentage of non-essential amino acids derived from both treatments was higher than essential amino acids. This suggests that non-essential amino acids are more concentrated inside the cell wall barrier, and also that it is not the same proteins being released in the aqueous phase when comparing both treatments.

However, compared to the alkaline treatment, high-pressure homogenization increased the percentage of the non-essential amino acids for the fragile cell walled species from 20% to 26% for *A. platensis* and *P. cruentum* respectively. Similarly, for the rigid cell walled green species, they increased by 7%, 10% and 12% for *N. oculata*, *H. pluvialis* and *C. vulgaris* respectively. Moreover, for the latter species it is noteworthy that after alkaline treatment, the proportions of essential to non-essential amino acids was statistically the same compared with those for the crude fragile microalgae, and this was not the case after mechanical treatment of the same species. However, from the literature, few studies have distinguished between cell wall and intracellular amino acids of microalgae. It has been reported for instance, that after isolating and purifying the cell wall of *C. vulgaris* from the cytoplasmic medium, this contained peptides rather then proteins, although the amino acid profile was limited to their detection without quantifying the proportions [53].

Freeze-dried microalgae amino acid composition					
Amino acids	***P. cruentum***	***A. platensis***	***C. vulgaris***	***N. oculata***	***H. pluvialis***
Aspartic acid	12.41 ± 0.45	13.10 ± 0.11	11.20 ± 0.02	10.13 ± 0.05	9.76 ± 0.10
Threonine	6.91 ± 0.25	6.83 ± 0.10	6.24 ± 0.01	6.55 ± 0.03	5.75 ± 0.06
Serine	8.98 ± 0.29	7.59 ± 0.02	7.97± 0.04	7.23 ± 0.01	7.31 ± 0.05
Glutamic acid	9.04 ± 0.29	11.64 ± 0.09	9.30 ± 0.01	11.41 ± 0.02	10.44 ± 0.11
Glycine	7.59 ± 0.28	8.60 ± 0.06	8.81 ± 0.01	9.97 ± 0.01	9.98 ± 0.09
Alanine	7.39 ± 3.67	10.99 ± 0.08	11.17 ± 0.03	12.11 ± 0.01	12.44 ± 0.12
Cysteine	0.37 ± 0.01	0.20 ± 0.02	0.20 ± 0.01	0.22 ± 0.01	0.25 ± 0.01
Valine	2.76 ± 0.10	3.17 ± 0.02	3.17 ± 0.01	3.65 ± 0.02	3.67 ± 0.04
Methionine	3.08 ± 0.11	1.91 ± 0.02	0.66 ± 0.01	1.66 ± 0.01	0.71 ± 0.01
Isoleucine	5.81 ± 0.24	0.13 ± 0.01	0.09 ± 0.01	0.13 ± 0.01	4.99 ± 0.04
Leucine	6.46 ± 0.21	7.79 ± 0.02	7.68 ± 0.02	8.99 ± 0.05	8.92 ± 0.10
Tyrosine	4.90 ± 0.18	5.35 ± 0.05	8.63 ± 0.01	3.76 ± 0.02	3.08 ± 0.04
Phenylalanine	5.54 ± 0.20	5.34 ± 0.04	5.96 ± 0.01	5.59 ± 0.01	5.42 ± 0.07
Histidine	1.22 ± 0.04	1.00 ± 0.01	1.29 ± 0.01	1.04 ± 0.01	0.99 ± 0.01
Lysine	6.09 ± 0.21	5.65 ± 0.62	6.99 ± 0.07	6.32 ± 0.01	6.31 ± 0.08
Arginine	8.62 ± 0.29	8.52 ± 0.07	7.57 ± 0.03	6.58 ± 0.02	6.73 ± 0.08
Proline	2.80 ± 0.17	2.16 ± 0.05	3.04 ± 0.07	4.65 ± 0.07	3.24 ± 1.15

Table 2: Results of total amino acids of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments ± SD (*n*=9).

Protein extract amino acid composition after alkaline treatment					
Amino acids	***P. cruentum***	***A. platensis***	***C. vulgaris***	***N. oculata***	***H. pluvialis***
Aspartic acid	11.65 ± 0.02	11.27 ± 0.02	7.52 ± 0.34	5.02 ± 0.04	7.30 ± 0.09
Threonine	5.88 ± 0.01	6.05 ± 0.05	4.60 ± 0.23	3.57 ± 0.05	4.92 ± 0.02
Serine	8.15 ± 0.03	7.92 ± 0.03	7.42 ± 0.33	4.38 ± 0.09	6.32 ± 0.02
Glutamic acid	9.85 ± 0.01	12.71 ± 0.02	12.85 ± 0.63	25.42 ± 0.30	15.13 ± 0.03
Glycine	8.35 ± 0.01	9.19 ± 0.02	10.77 ± 0.52	9.89 ± 0.14	12.28 ± 0.02
Alanine	11.38 ± 0.02	11.95 ± 0.02	18.58 ± 0.92	15.77 ± 0.20	21.34 ± 0.04
Cysteine	0.30 ± 0.01	0.22 ± 0.01	0.27 ± 0.02	0.42 ± 0.01	0.45 ± 0.01
Valine	3.43 ± 0.01	3.62 ± 0.01	4.04 ± 0.20	2.66 ± 0.04	3.13 ± 0.01
Methionine	2.58 ± 0.01	1.86 ± 0.01	1.42 ± 0.06	1.38 ± 0.03	1.16 ± 0.01
Isoleucine	5.81 ± 0.02	0.14 ± 0.01	2.60 ± 0.07	1.21 ± 0.01	3.42 ± 0.02
Leucine	7.94 ± 0.01	8.75 ± 0.02	7.90 ± 0.40	4.62 ± 0.06	5.58 ± 0.03
Tyrosine	4.00 ± 0.01	4.74 ± 0.01	2.83 ± 0.13	2.42 ± 0.09	2.13 ± 0.02
Phenylalanine	4.50 ± 0.01	4.65 ± 0.01	3.03 ± 0.16	1.84 ± 0.03	2.94 ± 0.01
Histidine	0.86 ± 0.03	0.81 ± 0.01	0.59 ± 0.03	0.32 ± 0.01	0.48 ± 0.01
Lysine	6.08 ± 0.01	6.88 ± 0.01	6.81 ± 0.33	2.64 ± 0.10	3.88 ± 0.01
Arginine	7.21 ± 0.03	7.46 ± 0.01	6.46 ± 2.96	2.20 ± 0.11	7.68 ± 0.01
Proline	2.03 ± 0.02	2.03 ± 0.09	2.33 ± 0.14	16.21 ± 0.44	1.80 ± 0.04

Table 3: Results of total amino acids of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments ± SD (*n*=9).

Amino acid composition in the protein extract after high-pressure cell disruption					
Amino acids	***P. cruentum***	***A. platensis***	***C. vulgaris***	***N. oculata***	***H. pluvialis***
Aspartic acid	12.15 ± 0.14	8.67 ± 0.02	7.47 ± 0.01	7.27 ± 0.34	8.26 ± 0.10
Threonine	3.39 ± 0.03	4.66 ± 0.25	5.13 ± 0.02	4.45 ± 0.26	4.36 ± 0.09
Serine	5.57 ± 0.07	5.00 ± 0.03	5.46 ± 0.01	4.43 ± 0.05	5.59 ± 0.12
Glutamic acid	9.76 ± 0.51	13.85 ± 0.05	10.23 ± 0.06	11.51 ± 0.12	11.41 ± 0.11
Glycine	6.72 ± 0.09	7.67 ± 0.01	10.07 ± 0.05	9.15 ± 0.13	10.62 ± 0.09
Alanine	11.69 ± 0.02	10.10 ± 0.04	16.93 ± 0.11	10.28 ± 0.04	17.05 ± 0.14
Cysteine	1.21 ± 0.05	0.42 ± 0.01	0.39 ± 0.01	0.74 ± 0.20	0.87 ± 0.03
Valine	7.06 ± 0.20	5.63 ± 0.02	6.19 ± 0.05	5.36 ± 0.02	5.33 ± 0.10
Methionine	4.34 ± 0.10	1.09 ± 0.01	1.05 ± 0.02	1.43 ± 0.11	0.92 ± 0.41
Isoleucine	3.91 ± 0.06	4.14 ± 0.04	3.19 ± 0.03	3.05 ± 0.26	2.52 ± 0.33
Leucine	5.59 ± 0.06	7.13 ± 0.01	7.03 ± 0.04	7.14 ± 0.07	5.10 ± 0.17
Tyrosine	1.74 ± 0.07	2.27 ± 0.03	2.31 ± 0.01	1.09 ± 0.10	1.35 ± 0.02
Phenylalanine	2.01 ± 0.07	2.90 ± 0.04	2.74 ± 0.03	3.07 ± 0.08	2.31 ± 0.02
Histidine	0.92 ± 0.02	1.15 ± 0.01	0.98 ± 0.02	1.48 ± 0.03	0.96 ± 0.01
Lysine	3.13 ± 0.04	3.12 ± 0.04	4.95 ± 0.04	3.37 ± 0.18	3.61 ± 0.04
Arginine	4.26 ± 0.02	4.06 ± 0.03	3.89 ± 0.03	3.20 ± 0.27	5.92 ± 0.05
Proline	16.52 ± 0.80	18.11 ± 0.17	11.98 ± 0.15	22.93 ± 0.47	13.79 ± 0.19

Table 4: Results of total amino acids of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments ± SD (*n*=9).

	No treatment		**Alkaline treatment**		**High-pressure homogenization**	
Microalgae	**Essential AA**	**Non-essential AA**	**Essential AA**	**Non-essential AA**	**Essential AA**	**Non-essential AA**
P. cruentum	50.29	49.71	48.48	51.28	30.36	69.64
S. platensis	44.94	55.06	43.53	56.22	29.83	70.17
C. vulgaris	44.07	55.93	39.02	60.43	31.26	68.74
N. oculata	43.30	56.70	33.48	65.90	29.36	70.63
H. pluvialis	46.52	53.48	32.37	67.14	25.13	74.87

Table 5: Proportion of amino acids before and after treatment for five microalgae.

In conclusion, it has been noticed that after both treatments, essential and non-essential amino acids were present but in different ratios, suggesting that the quality and quantity of proteins in the extract depends on the effectiveness of the cell disruption method, and also on the structural morphology of each microalgal cell wall. Therefore, mechanical treatment is more effective than chemical treatment due to its capacity to disrupt the cell

walls and protein aggregates. And the logical next step will be to conduct high performance liquid chromatography in order to identify the type of proteins released after cell disruption.

At present, the FAO and WHO recommend microalgal proteins for human consumption because they contain all the necessary amino acids, however, the reported presence of toxins in microalgae [34], re-opens the debate on this biomass as a supplementary food product. Notwithstanding, microalgal technology is still in its infancy and has a promising future in tomorrow's food industry, although additional clarification is required to include microalgae in the daily food intake.

Acknowledgments
This study was supported by the French National Research Agency (ANR) in the framework of "the Algoraffinerie" project. The authors would like to thank Alpha Biotech for providing the biomass and are also thankful to Pr. Philippe Michaud, Pr. Gholamreza Djelveh and Dr. Sébastien Jubeau for their support.

3.4 Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods

Carl SAFI [1,2], Alina Violeta URSU [3], Céline LAROCHE [3], Bachar ZEBIB [1,2], Othmane MERAH[1,2], Pierre-Yves PONTALIER [1,2], Carlos VACA-GARCIA [1,2,4]

[1] Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), F-31030 Toulouse, France
[2] INRA, UMR 1010 CAI, F-31030 Toulouse, France
[3] Université Blaise Pascal, Polytech, Institut Pascal UMR CNRS 6022, 24, Avenue des Landais, 63174 Aubière, France
[4] King Abdulaziz University, Jeddah, Saudi Arabia

E-mail : carl.safi@ensiacet.fr

This publication has been submitted and accepted in ***Algal Research***

Abstract

The microalgal structure has been considered in this study to evaluate the release of proteins in aqueous medium from five microalgae after conducting different cell disruption techniques: manual grinding, ultrasonication, alkaline treatment, and high-pressure treatment. After conducting cell disruption, proteins concentration in water was determined for all the microalgae and results were discussed with regard to their cell wall structure. It was found that the aqueous medium containing most proteins concentration followed the order: high-pressure cell disruption > chemical treatment > ultrasonication > manual grinding. Fragile cell-walled microalgae were most attacked according to the following order: *Haematococcus pluvialis* < *Nanochloropsis oculata* < *Chlorella vulgaris* < *Porphyridium cruentum* ≤ *Arthrospira platensis*.

Keywords: Cell disruption, microalgae, proteins extraction, aqueous medium, cell wall structure

3.4.1 Introduction

Microalgae were first exploited for their capacity to accumulate proteins and through time, interest in this biomass took a new course especially during the last two decades with increasing demand for sustainable energy. This biomass proved to be an important source of lipids suitable for biodiesel production. Hence, the majority of studies were concentrated on lipids extraction for energetic purposes, neglecting in way the potential of microalgae to produce proteins and other highly added value components. However, up till now all studies and estimations confirmed that production of biodiesel from microalgae cost remains high [55, 56] and far from being competitive with fossil fuel. Researchers are since then turning towards valorising other components present in the microalgae such as proteins, pigments, dyes, sugars and other valuable components.

Extracting the totality of a specific component is often prevented by the intrinsic rigidity of cell wall of microalgae. To overcome this barrier, a preliminary operation unit of cell disruption is required to permit complete access to the internal components and facilitate the extraction process. Hence, many cell disruption techniques have been tested to break the cell wall of microalgae such as bead milling [50, 57], ultrasonication [49, 58, 59], microwave radiation [51], enzymatic treatment (Fleurence 1999), cell homogenizer [28] and high-pressure cell disruption [60] to recover different components. The efficiency of cell disruption was usually evaluated by extracting a single component especially lipids before and after applying the treatment or by microscopic observation. To our knowledge, studies on microalgal proteins were focused on evaluating the nitrogen to protein conversion factor [5-7, 42, 45] or finding the best method to analyse proteins and differentiate between soluble and non-soluble proteins [31] or analysing the behaviour of proteins at the air/water interface [40]. Therefore, the present study focuses on evaluating the effect of different cell disruption techniques on protein extractability in water of five different microalgae having different cell wall ultrastructures. Namely, the Cyanobacteria *Arthrospira platensis*, which has a relatively fragile cell wall, composed mainly of murein and no cellulose [24, 25]. Then the Chlorophycea *Chlorella vulgaris* and the Eustigmatophyceae *Nanochloropsis oculata*, which have a cell wall mainly composed of cellulose and hemicelluloses [26]. Another Chlorophycea *Haematococcus pluvialis* has a thick trilaminar cell wall composed of cellulose and sporopollenin [27-29]. The composition of its cell wall, similar to that of

spores, makes this microalga less permeable and extremely mechanically resistant [30]. The Rodophyta *Porphyridium cruentum* lacks a true cell wall but instead it is encapsulated by a layer of sulfurized polysaccharides [18-23]. In addition, the microalgae selected in this study have a cytoplasm containing soluble proteins, and they all have a chloroplast except for *A. platensis* which instead has thylakoids bundles circling the peripheral part of the cytoplasm with their associated structures, the phycobilisomes (containing the phycobiliproteins) present on the surface of the thylakoids like in the chloroplast of *P. cruentum* [24]. Furthermore, the chloroplast also contains soluble proteins and a central pyrenoid, which is a non-membrane, bound organelle composed of RubisCO.

Proteins released in the aqueous medium were evaluated and commented in accordance with the cell wall ultrastructure of each microalga together with the effect of each cell disruption technique used in this study.

3.4.2 Materials and methods

3.4.2.1 Microalgae

The microalgae selected are: the Cyanobacteria *Arthrospira platensis* (strain PCC 8005), two different Chlorophyceae *Chlorella vulgaris* (strain SAG 211-19), and *Haematococcus pluvialis* (unknown strain), one Rhodophyta *Porphyridium cruentum* (strain UTEX 161), and the Eustigmatophyceae *Nanochloropsis oculata* (unknown strain).
Each microalga was cultivated in a different culture medium, and therefore Hemerick medium was used for *P. cruentum*, Sueoka medium for *C. vulgaris*, Basal medium for *H. pluvialis*, Conway medium for *N. oculata* and Zarrouk medium for *A. platensis*. All grown in batch mode in an indoor tubular Air-Lift PhotoBioReactor (PBR, 10 L) at 25°C [13] inoculated from a prior culture in a flat panel Air-Lift PBR (1 L). Culture homogenization was achieved by sterile air injection at the bottom of the PBR. The pH and temperature were recorded by a pH/temperature probe (Mettler Toledo SG 3253 sensor) monitored by the acquisition software LabVIEW. The pH was regulated at 7.5 with CO_2 bubbling. Microalgae were harvested during the exponential growth phase and concentrated by centrifugation, and then supplied as frozen paste from Alpha Biotech (Asserac, France). The biomass contained 20-24% dry weight.

3.4.2.2 Reagents

Lowry kit (prepared mixture of Lowry reagent and BSA standards and 2 N Folin-Ciocalteu reagents) from Thermo Scientific. NaOH granules and HCl 37% were purchased from Sigma Aldrich and used as received.

3.4.2.3 Microalgae pre-treatment

3.4.2.3.1 Freeze-drying

The frozen paste of crude microalga (about 70 grams) was directly introduced to a Fisher Bioblock Scientific Alpha 2-4 LD Plus device (Illkirch, France). The pressure was reduced to 0.010 bar and the temperature was further decreased to -80°C and freeze-drying was conducted under vacuum for 48 h. Dry biomass was stored under anhydrous conditions. Before any disruption treatment, the cells were vigorously rehydrated in distilled water to insure a good homogeneity of the sample.

3.4.2.4 Microlagae treatments

3.4.2.4.1 Blank

Cells (0.5 g) were dispersed for 2 h in 25 mL distilled water and the supernatant was recovered by centrifugation at 10000*g* for 10 min at 20°C for protein analysis. This treatment was considered as a blank to compare with the other extraction treatments.

3.4.2.5 High-pressure cell disruptor

The "TS Haiva series, 2.2-kW" disrupter from Constant Systems Limited (Northants, UK), was used with two passages at the pressure of 2700 bar, with a biomass concentration of 2% dry weight. (0.5 g of dry cells dispersed in 25 mL distilled water).

3.4.2.6 Ultrasonication

This treatment was carried out using a VC-750HV (20 kHz, probe 13 mm) ultrasonic processor, where 0.5 g of dry cells were dispersed in 25 mL distilled water. Total treatment time was 30 min in cycles of 5 seconds of ultrasonication and 15 seconds of resting time in order to prevent overheating the sample.

3.4.2.7 Manual grinding

Dry microalgae were manually ground using a mortar for 5 min, and then 0.5 g were dispersed in 25 mL distilled water for 2 h. Samples were taken for protein analysis.

3.4.2.8 Chemical treatment

Mother solutions were prepared with approximately 500 mL of distilled water and some drops of 2 N NaOH to adjust the solution to pH 12 for maximum protein solubility. A sample of 0.5 g of freeze-dried biomass was added to 25 mL of mother solution. The mixture was then stirred for 2 h at 40°C. The separation of the supernatant from the pellet was conducted by centrifugation at 10000*g* for 10 min at 20°C. The supernatant was then adjusted to pH 3 with 0.1 M HCl in order to precipitate the proteins. The protein isolate was collected after centrifugation at 10000*g* for 10 min at 20°C and the pellet was neutralised with 0.01 M NaOH [40]. Samples were taken for protein analysis.

3.4.2.9 Lowry method

After every disruption treatment, the liquid/solid separation was conducted by centrifugation at 10000*g* for 10 min at room 20°C and the supernatant was analysed by the Lowry method. A calibration curve was prepared using a concentration range of bovine standard albumin from 0 to 1500 $\mu g\ mL^{-1}$. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract were withdrawn and then 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for exactly 10 min. After incubation, 100 mL of Folin-Ciocalteu Reagent (1 N) were added and again vortexed and incubated for exactly 30 min. The blue colour solution was then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer after being zeroed with blank sample containing all the chemicals without the extract [8].

3.4.2.10 Elemental analysis

Total nitrogen was evaluated by using a PerkinElmer 2400 series II elemental analyser. Microalgal samples of 2 mg were placed in thin capsules and then heated at 925°C using pure oxygen as the combustion gas and pure helium as the carrier gas, and then evaluating the nitrogen percentage. For all the previous analyses, three experiments were

conducted separately on all the microalgae. Measurements of three replicates for each sample were reproducible within ± 5% of the respective mean values.

3.4.2.11 Confocal laser scanning microscopy

Cells were observed with an SP2-AOBS confocal laser-scanning microscope from Leica microsystems (Nanterre-France). The fluorochrome calcofluor white that binds to the cell wall was added to the samples. Excited at 488 nm, the cells are identified coloured in light blue.

3.4.3 Results

The total proteins content of crude microalgae was determined by obtaining total nitrogen through elemental analysis and converting it into protein percentage by using the conversion factor found for each crude microalga in the study conducted by Safi et al. (2012b). In all cases, the total protein content was high, ranging from 46 to 57%w (Table 1).

Microalga	Total nitrogen (%.dw^{-1})	Total proteins (%.dw^{-1})
H. pluvialis	8.27±0.08	51.73±0.43
N. oculata	7.41±0.40	46.55±2.14
C. vulgaris	7.81±0.18	49.59±1.04
A. platensis	8.53±0.20	53.51±1.10
P. cruentum	9.04±0.70	57.33±3.84

Table 1: Protein and nitrogen content for each microalga based on three replicates for three experiments ±SD (*n*=9).

The fraction of hydro-soluble proteins released in water after each cell disruption technique was presented in Fig 1 after being quantified by the Lowry method. The fraction of hydro-soluble proteins from total proteins present in the microalgae was also evaluated and all these results are gathered in Table 2.

Microalga	Water	Manual grinding	Ultrasonication	Chemical	Disintegration
H. pluvialis	6.46±0.23	7.43±0.06	8.47±0.04	15.78±0.11	41.04±3.66
N. oculata	8.09±0.05	9.75±0.03	13.53±0.07	31.09±2.02	52.28±0.58
C. vulgaris	9.71±0.48	8.97±0.09	18.15±0.03	33.20±0.02	52.78±0.58
A. platensis	19.01±0.06	35.05±1.16	47.13±0.96	53.36±0.22	78.02±2.82
P. cruentum	24.83±0.32	49.48±0.67	67.01±0.90	73.50±1.19	90.00±2.45

Table 2: Fraction of hydro-soluble proteins from total proteins released in the aqueous phase after each cell disruption based on three replicates for three experiments ±SD (*n*=9). It was calculated according to the following equation:
***Proportion of hydro-soluble protein in total protein = $\frac{\mathrm{PLowry}}{\mathrm{Nea} \times \mathrm{NTP}} \times 100$ (%)**
***Nea: Total nitrogen % (d.w) obtained by elemental analysis.**
***NTP: Nitrogen-to-protein conversion factor from (Safi et al. 2012b).**
***P_{Lowry}: Hydro-soluble protein % (d.w) calculated by Lowry method after each cell disruption method applied.**

Five cell disintegration techniques were compared, with a probe done with distilled water in order to evaluate the effect of water on protein exiting by diffusion through the membranes and walls. The recovery yield ranges from 6.5%w with *H. Pluvialis* to 25%w with *P. cruentum*. The latter is considered as fragile and the former as resistant. Among the tested techniques, high-pressure cell disintegration was the best technique for all the microalgae with a recovery yield from 41% to 90%. Moreover, the lowest protein concentration for all microalgae was obtained by water treatment and manual grinding especially for rigid cell walled microalgae. A relative difference was noticed in the concentration of proteins released between the microalgae with fragile and rigid cell walls. *P. cruentum* released the most compared to *A. platensis*. After ultrasonication a minor increase in protein concentration is noticeable for the green microalgae especially for *C. vulgaris*, and a more important increase was observed for the *A. Platensis* and *P. cruentum*. Furthermore, the chemical treatment showed an important increase of protein concentration released in water for all microalgae without forgetting that statistically, *N. oculata* and *C. vulgaris* released the same protein concentration (Fig 1).

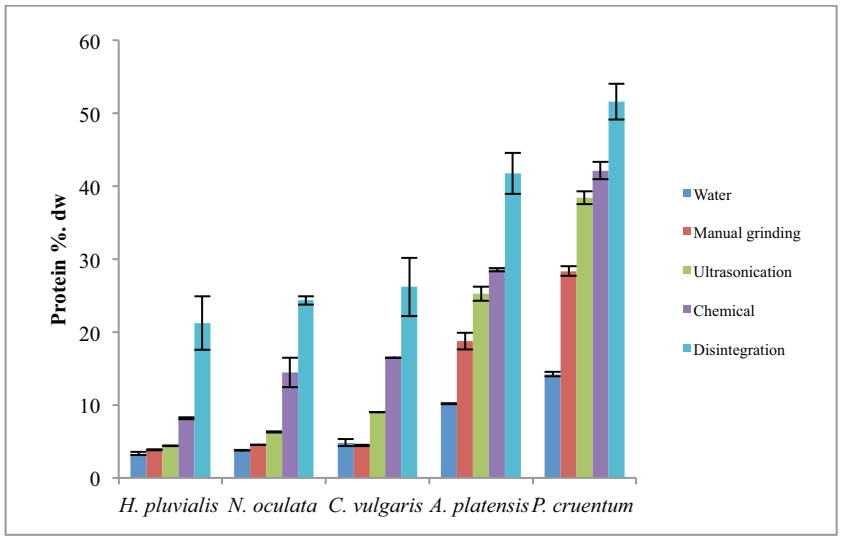

Figure 1: Percentage of proteins released in water after each cell disruption technique per dry weight of biomass based on three replicates for three experiments ±SD (*n*=9).

In order to better interpret these results, microscopic observation was carried out. The laser scanning confocal microscopic images presented in Fig 2 showed that in the cases of *P. cruentum* and *A. platensis* a total disruption of the cell wall occurred after high pressure cell disruption, whereas for *C. vulgaris*, *N. oculata* and *H. pluvialis*, the majority of cells were completely disrupted, but few cells maintained their globular form.

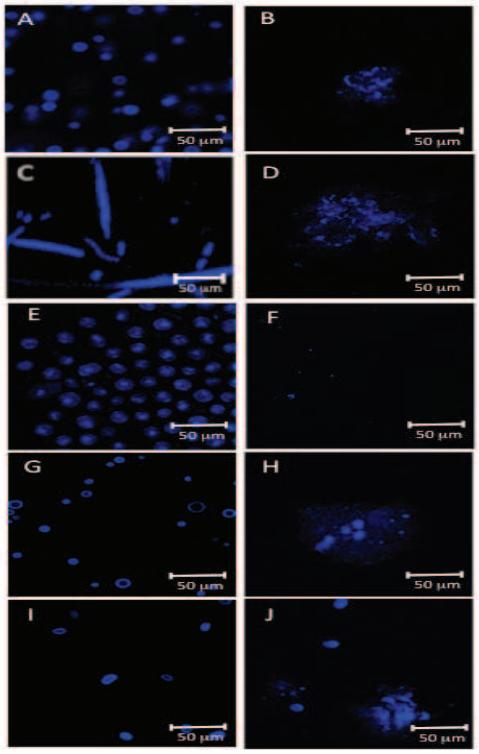

Figure 2: Confocal laser scanning microscopy of five microalgae before (right) and after (left) high-pressure cell disintegration. A and B *C. vulgaris*, C and D *A. platensis*, E and F *P. cruentum*, G and H *H. pluvialis*, I and J *N. oculata*.

3.4.4 Discussion

The goal of the present study was to highlight the release of proteins in the aqueous medium after different cell disruption techniques by attributing the results not only of the mechanical rigidity of the cell wall of each microalga but also to its chemical structure. Indeed, having a deep insight on the ultrastructure is necessary in order to evaluate the release of components after any treatment conducted on the cells. This approach has been considered in a study conducted by Jubeau et al. (2012) in order to selectively extract intracellular components such as proteins and phycoerythrin after cell disruption of *P. cruentum*. Thus, the release of proteins in the aqueous medium depends on the cell disruption technique used as well as the ultrastructure of every species.

Osmosis is the net movement of solvent (water) molecules through a partially permeable membrane into a region of higher solute concentration. Water usually travels through the membrane, the vacuole, the chloroplast, the mitochondria by diffusing across the

phospholipid bilayer *via* water channels (aquaporins), which are proteins embedded in the cell membrane that regulate the flow of water. Hence, water treatment is not considered as a cell disruption technique, but it was carried out in this study as a reference for the other techniques conducted in water. Surprisingly, the dispersion of cells in water released up to 19-25% of total proteins per dry weight (Table 2) from *A. platensis* and *P. cruentum*, and colouring the water in light blue for the former and light red for the latter. This indicates that water not only did not face resistance from the cell walls of both microalgae but also succeeded to penetrate the intra-thylakoids space of *A. platensis* and permeated the chloroplast of *P. cruentum* to slightly dissolve the phycobilisomes present on the thylakoids membranes. On the contrary, osmosis phenomenon was not strongly effective for the green microalgae, which are known to have rigid cell walls that kept resisting water to permeate the structure leading thus to only 6-10% proteins recovery (Table 2).

Taking into account the standard deviation of three samples considered for the green microalgae (*C. vulgaris, N. oculata and H. pluvialis*), we could affirm that at 95% of confidence level, all the values of released proteins after water treatment and manual grinding shown in Fig 1 are statistically equivalent indicating again the resistance of their cell walls after manual grinding. This was not the case for the *A. platensis* and *P. Cruentum* due to the stronger coloration of water in phycobilisomes, accompanied with increase in proteins concentration designating that the internal structure of both microalgae is being more altered, and simultaneously facilitating the penetration of water to dissolve more proteins.

Ultrasonication produces cavitation in cells and facilitates cell disintegration; it did not make any significant change for *H. pluvialis*, but showed minor effect on the cell wall of *N. oculata* and *C. vulgaris* by possibly giving difficult access for water to extract cytoplasmic proteins without altering the structure of their chloroplast. Concentration of proteins and coloration kept increasing for the fragile cell-walled microalgae by releasing 47-68% of protein from total proteins per dry weight.

Chemical treatment was a key treatment that showed significant increases in proteins concentration compared to the previous treatments. *P. cruentum* lacks a well-defined cell wall and since protein solubility is dependent of pH, the high pH easily solubilised proteins without any resistance from its pseudo-cell wall. But in the case of the green

microalgae, the sodium hydroxide is able to perform a process similar to mercerisation, by penetrating the microcrystalline structure of the cellulosic cell walls of the green microalgae. The alkaline solution can also easily dissolve the hemicelluloses attached to cellulose as it has been demonstrated during the refining of lignocellulosic substrates (straw, bran, wood). In addition, it indicates that this treatment gave more access to their cytoplasmic proteins and at 95% of confidence level, chemical treatment recovered the same concentration of proteins from *N. oculata* and *C. vulgaris* (Fig 1). However, the sporopollenin contained in the most rigid cell wall (*H. pluvialis*) is known to be extremely resistant to chemical agents [30]. *A. platensis* has a cell wall rich in amino sugars cross-linked with oligopeptide chains. The former are labile in alkaline conditions by deamidation of the *N*-acetylglucosamine and the latter are soluble in alkaline conditions. The cell wall becomes therefore permeable allowing the alkaline extraction of proteins (Safi et al. 2012b). Hence, all these results demonstrate that the chemical action acts in synergy with the mechanical characteristics of the cell wall.

High-pressure cell disruption was the most efficient technique for all microalgae; at 95% confidence level the concentration of proteins was statistically the same for the green microalgae with evidence that the majority of the cells were broken while some of them remained intact (Fig 2). The chloroplast of these species was also partially damaged as it is revealed by the coloration in light green (chlorophyll) of the aqueous extract. Indeed, chlorophyll is a hydrophobic pigment; its presence in the aqueous phase indicates the formation of micellar structures and signals an alteration of the chloroplast. The other indication is that some cell debris containing the green pigment were extremely reduced in size and did not precipitate in the pellet after centrifugation at 10000*g* leading to a greenish colour of the supernatant as it occurred in a previous work by Gerde et al. (2012). Hence, after two passages, water had access to cytoplasmic proteins and partially infiltrated the chloroplast to recover almost half of proteins from total proteins present inside the rigid cell-walled microalgae (Table 2) signalling again the resistance of their cell wall. On the contrary, as expected according their fragile cell wall (Table 2), *A. platensis* and *P. cruentum* did not show much resistance, and the protein concentration yielded 78% for the former and 90% for the latter complemented with an important coloration of the aqueous extract for both microalgae and a pellet having lost its red coloration for *P. cruentum*. This explanation was also supported by microscopic observation showing that their structure was completely altered (Fig.2).

The same order of rigidity was obtained in the study of Safi et al. (2012b) that took into account the values of the conversion factors before and after proteins extraction and then attributed them to the rigidity of the cell walls. This result shows that to compare the efficiency of cell disruption technology, it is more accurate to use fragile cell like *P. cruentum*.

The present study brings additional insight on understanding the recovery of proteins after different cell disruption. Hence, among all the techniques used the cell disruptor was the most efficient but not enough to recover more than 50% of the proteins for the green microalgae indicating that more passages are required to completely disrupt their ultrastructure, and thus more energy input will be necessary. The process would also require a life cycle assessment to evaluate the cost input and its environmental impact.

Acknowledgments
This study was supported by the French National Research Agency (ANR) in the framework of "Algoraffinerie" project. The authors would like to thank Alpha Biotech for providing the biomass and are also thankful to Pr. Philippe Michaud, Pr. Gholamreza Djelveh and Dr. Sébastien Jubeau for their support.

3.5 Understanding the effect of cell disruption methods on the diffusion of *Chlorella vulgaris* proteins and pigments in the aqueous phase

Carl SAFI [1,2], Christine FRANCES [3,4], Alina Violeta URSU [5], Céline LAROCHE [5], Cécile POUZET [6], Carlos VACA-GARCIA [1,2,7], Pierre-Yves PONTALIER [1,2]

[1] Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), 31030 Toulouse, France
[2] INRA, UMR 1010 CAI, F-31030 Toulouse, France
[3] Université de Toulouse; INP, UPS; LGC (Laboratoire de Génie Chimique); B.P. 84234, 4, Allée Emile Monso, F-31432 Cedex 4, France
[4] CNRS; LGC; F-31432 Cedex 4, France
[5] Université Blaise Pascal, Polytech, Institut Pascal UMR CNRS 6022, 24, Avenue des Landais, 63174 Aubière, France
[6] Fédération de Recherche 3450, Plateforme Imagerie, Pôle de Biotechnologie Végétale, F-31320 Castanet-Tolosan, France
[7] King Abdulaziz University, Jeddah, Saudi Arabia

E-mail : carl.safi@ensiacet.fr

This publication has been submitted to ***Bioresource Technology***

Abstract

The following study brought additional insight on understanding the diffusion behaviour of proteins and pigments of *Chlorella vulgaris* in the aqueous medium, after testing different cell disruption methods. Results were revealed by microscopic observations, by quantifying the concentration of the biomolecules of interest, and by calculating their diffusion coefficient. Hence, microscopic observations showed intact cells after applying chemical hydrolysis and ultrasonication. However, the majority of cells lost their globular shape after bead milling and high-pressure homogenization. Additionally, the concentration of proteins increased by following the order: ultrasonication < chemical hydrolysis < high-pressure homogenization < bead milling. On the other hand, their diffusion followed a different order: chemical hydrolysis > bead milling > ultrasonication > high-pressure homogenization. Pigments were not detected in the aqueous phase after chemical hydrolysis, but for the mechanical treatments their concentration and their diffusion followed the similar order like proteins.

Hence, based on these results, diffusivity of these biomolecules is not directly related to the increase of their concentration in the aqueous phase. This suggests that even if cells were completely broken, the diffusivity can follow the phenomenon of hindered biomolecules diffusion, which implies that somehow cells were not completely disrupted.

Keywords: Cell wall, ultrastructure, release kinetics, aqueous medium, cell disruption

3.5.1 Introduction

Several years of intensive research for biofuel production from microalgae and the main obstacle remained the same; the overall production cost is too high to be competitive in the market and is unsustainable according to many life cycle assessment (LCA) [34-38, 61-64]. Thus, the last few years the question for the possibility of finding a solution to reduce the production cost became preponderant. Nowadays, it is agreed that microalgae would bring more benefits if they were completely valorised for their multiple highly added value components in the framework of a biorefinery, which will possibly inverse the slope from deficit to profit. However, the selection of the species is a major criterion, and one of the microalga that took much attention during the last century is *Chlorella vulgaris*.

It is a unicellular microscopic species with a mean diameter ranging from 2 to 10 mm [65, 66]. It is easy to grow, multiplies rapidly, resistant to harsh conditions and invaders, and accumulates a variety of highly added components. The high protein content was the main signal to increase interest on this microalga as an unconventional protein source. The comparison of its protein content to reference food protein recommended by World Health Organisation [67] and Food and Agriculture Organization [68] is favourable and even better since its protein content is higher then some reference food protein and also contains an interesting set of essential and non-essential amino acids [3, 42]. Proteins are located in the different parts of the cells; they represent part of the cell wall as well as the cytoplasm, the chloroplast and all the other organelles inside the barrier of the cell wall. Additionally, when *C. vulgaris* is grown under favourable conditions, it is capable to accumulate 1-2% chlorophyll of its dry weight, which gives it the dense green colour masking the colour of less concentrated pigments such as astaxanthin and other carotenoids. These pigments are located in the thylakoids (chlorophyll and some carotenoids) of the chloroplast and some (β-carotene) are associated to the lipid droplets synthesised in the chloroplast [69].

C. vulgaris has a rigid cell wall, mainly composed of cellulose, hemicellulose, β1-3 glucan, glucosamine, proteins, lipids and ash [70-72]. As in terrestrial plants, the most common skeletal polysaccharide is cellulose, but during maturation the cell wall gradually increases in thickness reaching 17-21 nm [73], where a microfibrillar layer is detected representing a chitosan-like layer [74, 75], which brings additional rigidity to its cell wall.

The thickness and composition are not constant because they can change based on different growth and environmental conditions [76-78].

Many studies considered breaking the cell wall of *C. vulgaris* by using different methods such as bead milling [50, 79], ultrasonication [51, 80], lysing buffer [81], high-pressure homogenization [82], microwaves [83] and enzymatic treatment [84, 85] in order to liberate the internal components especially lipids to transform them into biodiesel. However, a deep understanding on the interaction of the cell wall with the cell disruption technique leading to the release of the internal components has not been evoked yet. To our knowledge the attribution of the ultrastructure of the cells to the release of a specific component was never been deeply considered except in the study of [60] where this approach was considered for the release of proteins and phycoerythrin from *Porphyridium cruentum* after applying high-pressure cell disruption.

The main purpose of this study is to understand the diffusion behaviour of proteins and pigments (chlorophylls and carotenoids) in the aqueous phase after applying different cell disruption methods. Pigments quantification was used as marker for chloroplast alteration in order to explore the effect of each cell disruption technique on the integrity of the cell wall as well as the chloroplast of *C. vulgaris*. The techniques used are chemical hydrolysis, ultrasonication, bead milling and high-pressure homogenization.

3.5.2 Materials and methods

3.5.2.1 Microalga and materials

Sueoka culture medium was used for growing *C. vulgaris* (strain SAG 211-19) grown in batch mode in an indoor tubular Air-Lift PhotoBioReactor (PBR, 10 L) at 25°C, inoculated from a prior culture in a flat panel Air-Lift PBR (1 L). Culture homogenization was achieved by sterile air injection at the bottom of the PBR. The pH and temperature were recorded by a pH/temperature probe (Mettler Toledo SG 3253 sensor) monitored by the acquisition software LabVIEW. The pH was regulated at 7.5 with CO_2 bubbling. The microalga was harvested by centrifugation during the exponential growth phase and supplied as frozen paste from Alpha Biotech (Asserac, France). The biomass contained 28% dry matter constituted of 55% proteins, 2% Chlorophyll and 1% carotenoids.

Glucanex was purchased from Sigma Aldrich. Chemicals were purchased from different distributor: Methanol 99.9%, HCl 37%, NaOH beads, H_2SO_4 97% and sodium phosphate buffer (pH 6.5) from Sigma Aldrich, Lowry kit (prepared mixture of Lowry reagent and BSA standards and 2 N Folin-Ciocalteu reagent) from Thermo Scientific.

3.5.2.2 Chemical hydrolysis

Mother solutions were prepared with approximately 500 mL of distilled water and some drops of 2 N NaOH were added to adjust the solution to pH 12 for maximum protein solubility. A sample of 0.5 g of freeze-dried biomass was added to 25 mL of mother solution. The mixture was then stirred for 2 h at 40°C. The separation of the supernatant from the pellet was conducted by centrifugation at 10000*g* for 10 min at 20°C. The supernatant was then adjusted to pH 3 with 0.1 M HCl in order to precipitate the proteins. The protein isolate was collected after centrifugation at 10000*g* for 10 min at 20°C and the pellet was neutralised with 0.01 M NaOH. Samples were taken for protein and pigments analysis.

3.5.2.3 Bead milling

Cells were treated in a stirred bead mill (LABSTAR-NETZCH). Disruption was conducted using 0.3-0.5 mm Y_2O_3-stabilized ZrO_2 grinding beads. Milling time for both trials was 1-60 min with a 1/13 solid water ratio (w/v). The process was performed in a batch mode. The initial cell suspension was put into a pre-dispersion tank, stirred at 350 rpm in order to avoid cell sedimentation and ensure a good homogeneity of the solid concentration. During the runs, the suspension was continuously pumped from the tank to the mill inlet thanks to a peristaltic pump at a flow rate of about 30L/h and sent back again into the dispersion tank through a cartridge maintaining the beads inside the chamber. Agitation speed of the cell suspension and the beads within the grinding chamber was 2500 rpm. The bead mill contained an integrated cooling system to prevent overheating and thus after 1 h milling the temperature did not exceed 33°C. At the end of the runs, the broken cells were recovered for further processing.

3.5.2.4 Ultrasonication

This treatment has been carried out using a VC-750HV (20 kHz, probe 13 mm) ultrasonic processor, where 0.5 g of dry cells were dispersed in 25 mL distilled water. Ultrasonication time was 30 min with 5 s of ultrasonication and 15 s of resting time to prevent overheating the sample. The separation was conducted by centrifugation at 10000*g* for 10 min at 20°C and the supernatant was analysed for proteins.

3.5.2.5 High-pressure homogenization

The "TS Haiva series, 2.2-kW" disrupter from Constant Systems Limited, Northants, UK, was used. The operating parameter is the pressure (2700 bars), cell concentration (2% dry weight) and number of passages (two passages). Before treatment the cells were well mixed in distilled water to insure a good homogeneity of the sample. All the tests were performed in triplicate. After disruption, samples were centrifuged at 10000*g* for 10 min at 20 °C. The separation was conducted by centrifugation at 10000*g* for 10 min at 20°C and the supernatant was analysed for proteins.

3.5.2.6 Lowry method

The procedure involves reaction of proteins with cupric sulphate and tartare in an alkaline solution, leading to the formation of tetradentate copper protein complexes. The addition of the Folin-Ciocalteu reagent leads to the oxidation of the peptide bonds by forming molybdenum blue with the copper ions. Therefore, a calibration curve was prepared using a concentration range of bovine standard albumin from 0 to 1500 mg mL^{-1}. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract were withdrawn and then 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for exactly 10 min. After incubation, 100 mL of Folin-Ciocalteu Reagent (1 N) were added and again vortexed and incubated for exactly 30 min. The blue colour solution is then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer after being zeroed with blank sample containing all the chemicals without the extract [8].

3.5.2.7 Pigments analysis

Conducted by using the equations of. Thus, 200 mL of aqueous extract were mixed with 1300 mL pure methanol and then incubated in dark for 1h at 45°C. Further on, the samples were centrifuged at 10000 *g* for 10 min at 20°C. The organic phase containing the pigments was recovered and analysed using the following equations [86]:

Total chlorophyll μg/mL = (19,3443 x A652) + (4,3481 x A665)

Total carotenoids μg/mL = 4 x A480

3.5.2.8 Diffusivity

In order to understand the behavior of the biomolecules after cell disruption, it is possible to evaluate the experimental diffusivities. Diffusion of neutral macromolecules in dilute solution is well described by an expression that employs the frictional coefficient of the molecule, such as Stokes-Einstein equation. However for biological macromolecules, such a simple equation cannot be used, because they show a strongly non-ideal behavior, in diluted solutions [87]. Nevertheless, the experimental results are not so far from the models and give for lysozyme a value of about 1.1 10^{-10} m^2/s [88]. In the present work, it is difficult to define a standard value because our analysis was carried out on total proteins.

Hence a global diffusion coefficient was calculated using the following equation: $\frac{\Delta C}{\Delta t} = D\frac{\Delta C^*}{\Delta x}$ where:

Δt is laps time (s)
ΔC is the concentration difference obtained with regard to time (Δt) for a considered solute (kg/m^3)
ΔC^* is the gradient concentration between the concentration at equilibrium in the liquid phase, and the concentration at the instant t (kg/m^3)
Δx is the length of the co-called boundary layer (m)
D is the diffusivity of the macromolecule (m^2/s)
Definition of the boundary layer thickness is rather difficult in this case since because of the change in the cell size and of the hydrodynamic conditions. Its calculation used the classic equation in the case of laminar flow: $\Delta x = \frac{5.L}{\sqrt{Re}}$ [89]

Where L is the diameter of the particle (m) and Re the Reynolds number. Since the hydrodynamic conditions are different for all the apparatus and the size of the cell can change with time, and therefore an available standard condition has been defined for all the conditions with a Reynolds number of 0.2 and a particle size of 10^{-6}m. Hence, the comparison of the value between all the extraction procedures will be difficult especially when the mixing of the solution is intense and when the cell size is highly reduced (as in bead milling). The diffusion was calculated for protein and, when possible, for chlorophyll and carotenoids.

3.5.2.9 Confocal microscopy

Cells were observed with an SP2-AOBS confocal laser-scanning microscope from Leica microsystems (Nanterre-France). The fluorochrome calcofluor white that binds to the cell wall was added to the samples. Excited at 488 nm, the cell walls are identified coloured in light blue and at 633 nm where the internal part of the cells are coloured in red.

3.5.3 Results and discussion

The cell wall is a complex entity with unique characteristics related to the growth phase of a given microalga species; it differs in thickness, rigidity and constituents. A microalga cannot long exist unless its body is firmly covered and its organelles possess the collective mechanical strength of the cell walls in order to insure a defence mechanism as well as controlling the intracellular and extracellular transport of the biomolecules. The multiple variations observed in microalgae cell walls, ultrastructures and compositions distinguish them from each other. Thus, *C. vulgaris* is basically distinguished by its rigid cell wall, its high chlorophyll and protein concentration. Therefore, conducting a treatment on its cell wall is necessary to increase assimilation and bioavailability of the intracellular biomolecules into the extraction solvent. Nonetheless, the unit operation of cell disruption cannot be applied without considering the integrity of the biomolecules of interest in the downstream process. Hence, all the techniques applied on *C. vulgaris* in this study considered the temperature to preserve the integrity the biomolecules.

Microscopic observations represent a qualitative approach for the success of different cell disruption techniques. Thus, before treatment cell wall appeared ring like shape

at 488 nm colored in blue and surrounding the internal part excited at 633 nm and appears colored in red at this wavelength (Fig 1). Their diameter ranged from 3-7 μm, which corresponds to the findings in the literature [65, 66, 73].

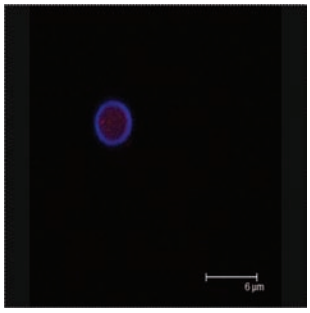

Figure 1: Isolated cell of *Chlorella vulgaris* before applying a cell disruption treatment.

3.5.3.1 Chemical hydrolysis

It is estimated that 20% of *C. vulgaris* proteins are bound to the cell wall [90], and overall proteins of this species have molecular weights ranging from 12-120 kDa [91, 92]. During chemical hydrolysis, the concentration of hydro-soluble proteins in the aqueous phase increased in terms of stirring time and reached 26 ± 0.8 % on a dry weight basis after 24 h. This suggests that the alkaline solution slowly weakened the cell wall of *C. vulgaris* by partially penetrating its structure, and by firstly recovering the proteins bound to the cell wall. Further on, it hardly recovered some small sized cytoplasmic proteins that managed to pass through the pores of the weakened membrane that hindered in the same time the diffusion of larger size proteins. Furthermore, the lack of pigments in the aqueous phase implies that the alkaline solution was not capable to penetrate the phospholipid bilayer of the chloroplast in which pigments such as chlorophyll and carotenoids are embedded inside the thylakoids. Moreover, the analysis of their diffusivity in the aqueous phase reinforced the previous approach toward proteins. Hence, by following the evolution of proteins diffusivity in terms of time, a first set of proteins diffuses rapidly (after 10 s) in the aqueous phase. However, few minutes afterwards, the proteins diffusion became very slow, with a very low value (4.10^{-9} m^2/s) for the diffusion coefficient that is lower than the diffusion coefficient of proteins in water [87, 88]. A decreasing of the diffusion coefficient is also discussed when the protein concentration increase in the extract, which was not the case. Therefore, a plausible explanation must be with regard to the decreasing of the gradient concentration. Hence, it appears that the extraction leads to the solubilization of surface proteins, which

diffuse rapidly in the solution. When the pool of accessible proteins decreases, the recovery rate decreases simultaneously as presented in figure 2. Through this assessment, it can be supposed that sodium hydroxide does not completely hydrolyze the cell wall of *C. vulgaris*, which explains the low recovery yield as well as the lack of pigments in the solution. Such values usually describe hindered proteins diffusion inside a pore, and confirm that no cell disruption occurred due to the resistance of the rigid cell wall. Moreover, microscopic observations at different wavelengths showed intact cells that maintained their globular shape after chemical hydrolysis (Fig 2). This observation supports the assessment mentioned previously for this method.

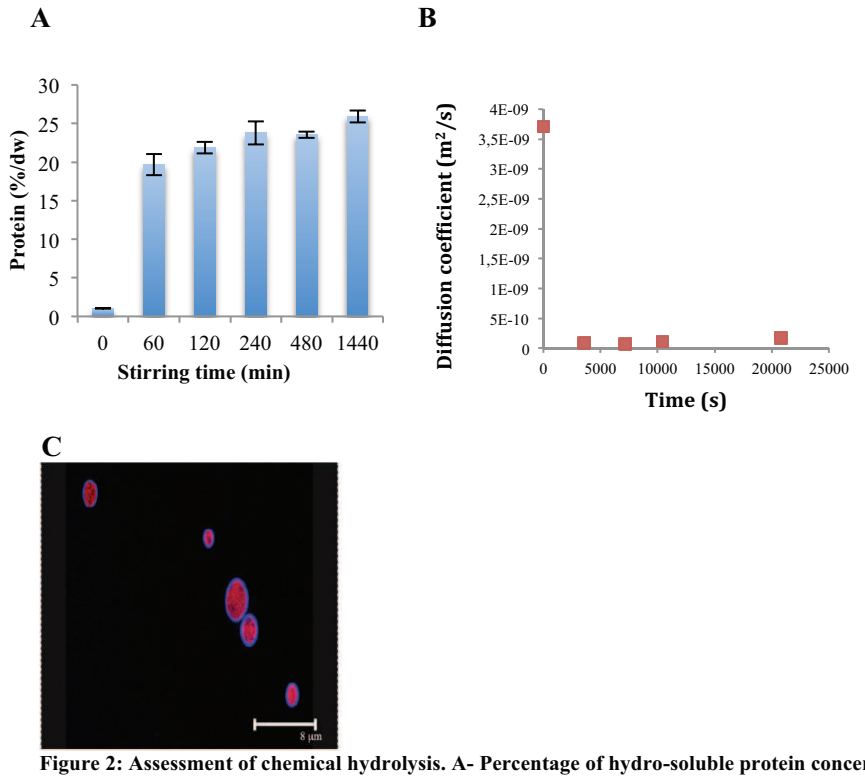

Figure 2: Assessment of chemical hydrolysis. A- Percentage of hydro-soluble protein concentration per dry weight of biomass. B- evolution of proteins diffusion coefficients in terms of extraction time. C- Microscopic observation of the cells after treatment.

3.5.3.2 Ultrasonication

The functionality of ultrasonication dwells in creating cavitation on the cell wall. In another term, it occurs when vapor bubbles of a liquid form in an area where pressure of the liquid is lower than its vapor pressure. These bubbles grow when pressure is negative and compress under positive pressure, which causes a violent collapse of the bubbles. If it occurs

close to cell walls, possible damage can occur and the intracellular components are released [83]. Nonetheless, some points such as the characteristics of the cell wall of the species (thickness, composition, rigidity) prohibit or contribute to the effectiveness of this technology, and therefore after 30 min of ultrasonication (Fig 3), the concentration of hydro-soluble proteins in the aqueous phase followed the same trend compared to the chemical hydrolysis but with lower quantity of proteins liberated (9% per dry weight after 25 min). Moreover, the presence of pigments indicated that the treatment produced small cavities on the cell wall as well as on the chloroplast allowing some proteins to penetrate through the membrane of the cells. Furthermore, taking into account the diffusivity of all the biomolecules, proteins exit rapidly and then the diffusion coefficient to a value of 2.10^{-9} m^2/s. This suggests, that this behavior is linked to the diffusion of soluble proteins after cell wall disruption. After 500s of treatment, the pigments diffusion coefficients increase, indicating an alteration of the membrane of the chloroplast. Diffusion of both molecules is rapid for hydrophobic molecules, similar to the value obtained for proteins. Usually, these molecules are linked to the proteins in a complexes, that are much more hydrophilic and increase the diffusion rate. Diffusion coefficient of these complexes was evaluated in the cell membrane at about 3.10^{-14} m^2/s but with very faster transfer from grana to stroma in a few seconds [93].

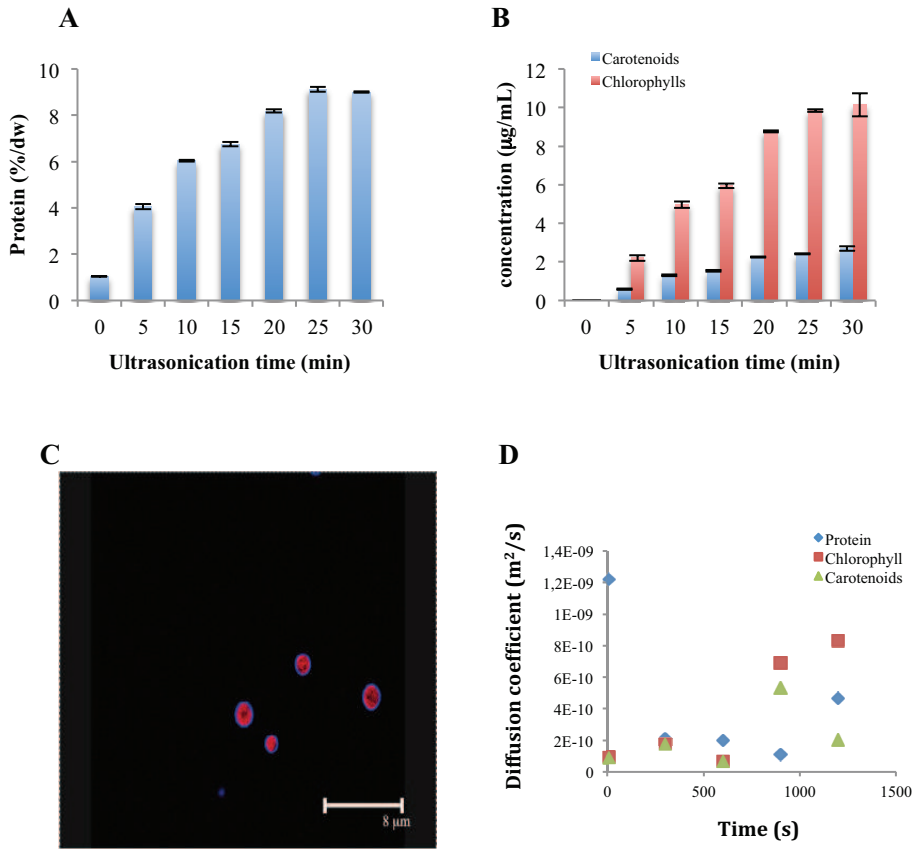

Figure 3: Assessment of ultrasonication. A- Percentage of hydro-soluble protein concentration per dry weight of biomass. B- Quantification of pigments released in the aqueous phase. C- Microscopic observation of the cells. D- evolution of proteins and pigments diffusion coefficients in terms of extraction time.

Our quantitative assessment of the results supported by microscopic observations indicated that ultrasonication was not a reliable method to increase recovery of the biomolecules of interest. This matches the assessment of other studies that used this technology for cell disruption on *C. vulgaris* [50, 94, 95] to recover lipids for bioenergy purposes. These studies concluded that this technology was poorly effective to increase the lipid recovery yield and to break the cell wall. On the other hand, ultrasonication was effective on other species having different cell wall characteristics such as *Spirulina platensis* [58].

3.5.3.3 Bead milling

As expected, bead milling proved to be effective to inflict heavy damages to the cell wall of *C. vulgaris*. It can be noticed in figure 4 that the concentration of proteins and pigments started to increase after 5 min, and kept increasing in concordance to bead milling time by reaching a maximum recovery at 40 min (96% proteins from total proteins), which implies that water had access to the different intracellular organelles and recovered the majority of proteins. In addition, the strong concentration of pigments especially chlorophyll in the aqueous phase signals a strong alteration of the chloroplast allowing the release of the intra-thylakoids pigments. Moreover, microscopic observations revealed some broken cells after 5 min of bead milling and total disruption was observed after 30 min where debris of cell wall are noticeable colored in blue and the interior fragments colored in red lost their blue cover. Furthermore, the results indicate that the biomolecules of interest are rapidly diffusing out of the cells, which signal that cell wall together with the intracellular membranes are disrupted for some cells. Nonetheless, the diffusion coefficient remains low since it might be hindered by the media organization. This also supposes that the concentration gradient is low, and it increases with respect to the increasing number of disrupted cells. Indeed, chlorophyll and carotenoids are hydrophobic pigments; their presence in the aqueous phase indicates the formation of micellar structures and it points to an alteration of the chloroplast. The other indication is that some cell debris containing the green pigment were extremely reduced in size and did not precipitate in the pellet after centrifugation at 10000 *g* leading to a greenish colour of the supernatant as it occurred in a previous work [59].

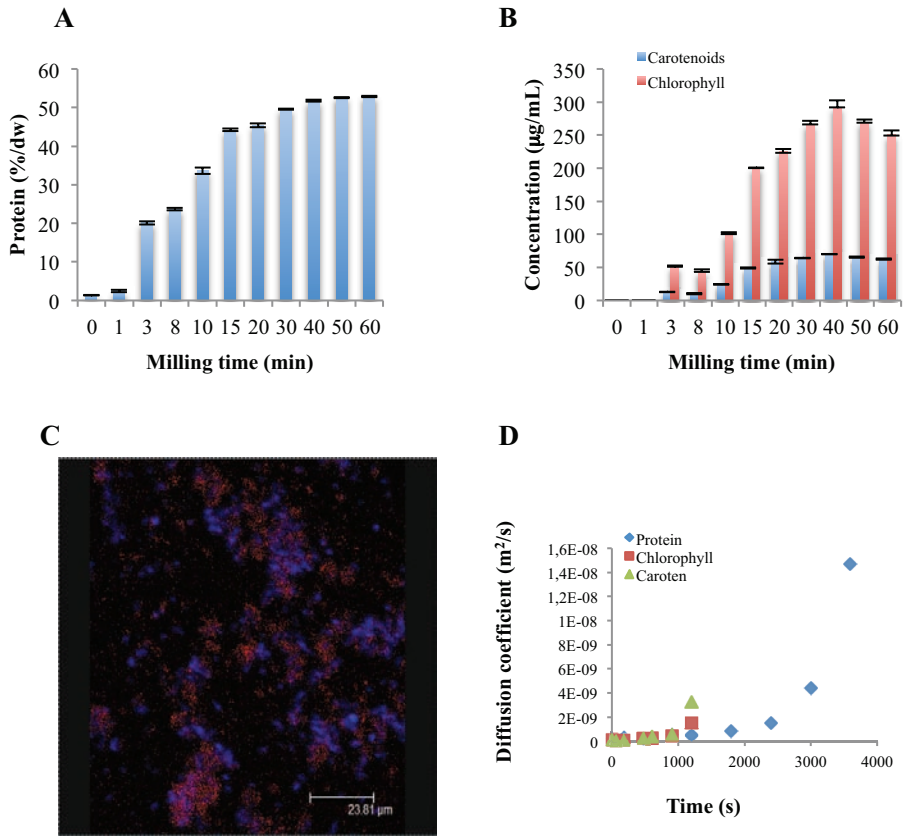

Figure 4 : Assessment of bead-milling. A- Percentage of hydro-soluble protein concentration per dry weight of biomass. B- Quantification of pigments released in the aqueous phase. C- Microscopic observation of the cells after 30 min of bead milling. D- Evolution of proteins and pigments diffusion coefficients in terms of extraction time.

This method remains highly efficient especially for microalgae having a rigid cell wall like *Chlorella vulgaris*, due to the high contact surface between the microalgal cells and the beads. The setup parameters such as beads diameter and composition, agitation speed, and bead milling time also play a key role for the efficiency of cell disruption. Nevertheless, despite its efficiency, this method has a major drawback for microalgae research since it is considered a highly energetic method that increases the final production cost of the process.

3.5.3.4 High-pressure homogenization

High-pressure cell disruption is also a reliable method for cell disruption [96, 97]. It acts according to high pressure applied on the piston that violently and rapidly smashes the cells on the top of the feeding chamber. Hence, after two passages, water had access to

cytoplasmic proteins and infiltrated the chloroplast to recover 66% of proteins from total proteins present inside the rigid cell wall (Fig 5). The presence of chlorophyll in the aqueous phase was 12 fold lower than bead milling, which suggests that the intensity of the chloroplast alteration was lower compared to bead milling. In addition, microscopic observations revealed that the majority of the cells were broken while some of them remained intact, which brings additional insight that explain the lower concentration of proteins and pigments in the aqueous phase (Fig 5).

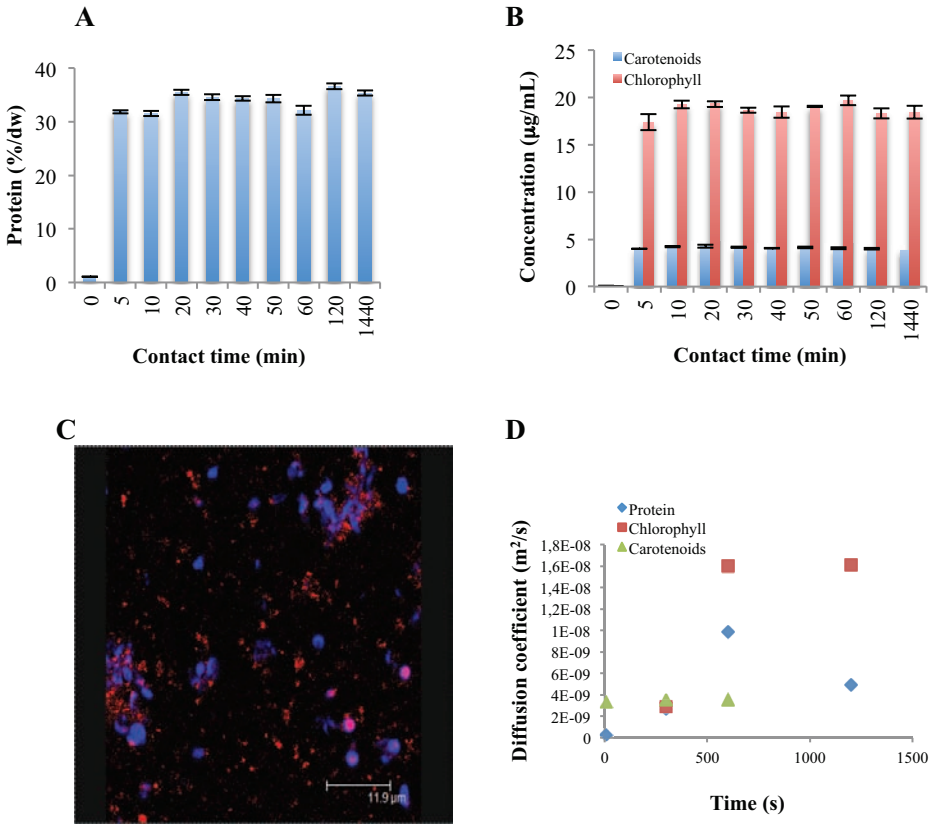

Figure 5: Assessment of high-pressure homogenization. A- Percentage of hydro-soluble protein concentration per dry weight of biomass. B- Quantification of pigments released in the aqueous phase. C- Microscopic observation of the cells after cell disruption treatment. D- evolution of proteins and pigments diffusion coefficients in terms of extraction time.

Contrarily to all the methods assessed previously, it can be noticed that the results obtained after high-pressure homogenization showed that the mechanical action of this method rapidly (300 s) increased the diffusion of the proteins and the chlorophyll out of the cells while the diffusion of the carotenoids was not significantly changed in terms of contact time. Within few minutes, the biomolecules were found in the aqueous phase and their concentration was

almost unchanged with regard to contact time and the diffusion becomes zero afterwards. It appears that the cells are rapidly destroyed allowing a faster recovery of the components and metabolites, and therefore the molecules transfer is not hindered by any structures. Furthermore, the high values of the diffusivities coefficients indicate that the initial hypothesis for their calculation is not available anymore. Particularly, the hydrodynamic conditions for the calculation of the boundary layer are not the same during high-pressure homogenization or chemical extraction, and the size of the particles are different in size compared to the initial cell. In the case of extraction, mass transfer limits the recovery rate while it is not the case anymore during high-pressure homogenization. Therefore, it seems that with this method, the diffusion of the biomolecules acts by means of velocity of the cell disruption method, allowing a faster diffusion in the aqueous phase compared to all the other methods tested in this study.

3.5.4 Conclusion

Our study pointed out that despite the efficiency of a cell disruption method on breaking the cell wall and maximizing the recovery of intracellular biomolecules, the diffusion of the latters does not follow the same trend. Hence, the results showed that chemical hydrolysis leads to a sharp decrease of the diffusion coefficient, but the mechanical disruption methods allow a higher recovery yield, and also improve the diffusion efficiency. Therefore, the transfer of the biomolecules is not any more limited by the membrane. Nevertheless, mass transfer seems to occur according to hindered internal diffusion, as if the cell lyses were not complete. From these results, it seems that even if the mechanical action allows an efficient cell disruption, it is not enough to have a free diffusion of the molecules out of the cells.

While focusing on the extraction part of the process it is worthwhile to take into account the ultrastructure of the cells and the diffusion kinetics of the biomolecules in order to understand more closely the behavior of these biomolecules by means of different cell disruption conditions. Therefore, additional studies are required to understand more in depth the diffusion phenomenon and additional studies should be conducted to understand the morphological changes of the cells after being submitted to a cell disruption technique,

which can bring additional insights and explanations on the release kinetics of the intracellular components.

Acknowledgments
This work was supported by the French National Research Agency (ANR) in the framework of Algoraffinerie project. The authors are grateful to thank Alpha Biotech for providing the biomass.

3.6 Chapter conclusion

This chapter spotted the light on the diversity of the cell walls of microalgae as well as the role they are capable to play on releasing proteins with respect to cell disruption methods. Therefore, it has been noticed that a correlation exist between the cell wall characteristics and the quantification of proteins by means of nitrogen to protein conversion factors, which suggests that each time accurate protein quantification is required it would be necessary to calculate the conversion factor. Thus, it is not coherent to recommend a universal nitrogen to protein conversion factor for all microalgae except for global description of the recovery yield.

The amino acids profile reflects the quality of proteins and the operation unit of cell disruption has its benefits on releasing intracellular biomolecules, and can also have multiple faces. Hence, a mechanical and a chemical treatment were applied in order to follow up on the quality of proteins released in the aqueous medium with respect to the essential and non-essential amino acids fractions. Thus, it was concluded that the difference in quality of proteins released after each treatment was increasing following the rigidity of the species. Which suggests that the quality of proteins is strongly affected by the method of cell disruption applied.

While extracting intracellular biomolecules such as proteins, the ultrastructure should be also considered to understand their release. Nonetheless, different cell disruption methods were applied and for the first time the ultrastructures of five different microalgal species was strongly considered in order to show that beside the cell wall characteristics the internal organelles also play a role and express some resistance against releasing proteins in the aqueous phase.

It was also noticed that among all the methods tested, bead milling released the highest concentration of proteins and pigments in the aqueous phase. However, the diffusion of both biomolecules was 6 fold slower compared to high-pressure homogenization that released lower concentrations of both biomolecules. This suggests that the diffusion velocity of the biomolecules of interest is not directly related to the effectiveness of the method of cell disruption applied.

Nevertheless, the next chapter covers another problematic. It deals with the fractionation of the aqueous phase by a continuous process in order to separate proteins, sugars and starches in different fractions.

References

[1] Venkataraman LV. *Spirulina platensis* (*Arthrospira*): Physiology, Cell Biology and Biotechnologym, edited by Avigad Vonshak. J Appl Phycol. 1997;9:295-6.
[2] Fradique M, Batista AP, Nunes MC, Gouveia L, Bandarra NM, Raymundo A. Incorporation of *Chlorella vulgaris* and *Spirulina maxima* biomass in pasta products. Part 1: Preparation and evaluation. J Sci Food Agric. 2010;90:1656-64.
[3] Becker EW. Microalgae : biotechnology and microbiology. Cambridge ; New York: Cambridge University Press; 1994.
[4] Fujihara S, Kasuga A, Aoyagi Y. Nitrogen-to-Protein Conversion Factors for Common Vegetables in Japan. J Food Sci. 2001;66:412-5.
[5] Lopez CV, Garcia Mdel C, Fernandez FG, Bustos CS, Chisti Y, Sevilla JM. Protein measurements of microalgal and cyanobacterial biomass. Bioresource Technol. 2010;101:7587-91.
[6] Lourenço SO, Barbarino E, Lavín PL, Lanfer Marquez UM, Aidar E. Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. Eur J Phycol. 2004;39:17-32.
[7] Lourenço SO, Barbarino E, Marquez UML, Aidar E. Distribution of intracellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors. J Phycol. 1998;34:798-811.
[8] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193:265-75.
[9] Peterson GL. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal Biochem. 1979;100:201-20.
[10] Crossman DJ, Clements KD, Cooper GJS. Determination of protein for studies of marine herbivory: a comparison of methods. J Exp Mar Biol Ecol. 2000;244:45-65.
[11] Diniz GS. Gross Chemical Profile and Calculation of Nitrogen-to-Protein Conversion Factors for Five Tropical Seaweeds. Am J Plant Sci. 2011;02:287-96.
[12] Lourenço SO, Barbarino E, De-Paula JC, Pereira LOdS, Marquez UML. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. Phycol Res. 2002;50:233-41.
[13] Loubiere K, Pruvost J, Aloui F, Legrand J. Investigations in an external-loop airlift photobioreactor with annular light chambers and swirling flow. Chem Eng Res Des. 2011;89:164-71.
[14] Moore S, Stein WH. Photometric ninhydrin method for use in the chromatography of amino acids. J Biol Chem. 1948;176:367-88.
[15] Mosse J. Nitrogen-to-protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. J Agr Food Chem. 1990;38:18-24.
[16] Sriperm N, Pesti GM, Tillman PB. Evaluation of the fixed nitrogen-to-protein (N:P) conversion factor (6.25) versus ingredient specific N:P conversion factors in feedstuffs. J Sci Food Agric. 2011;91:1182-6.
[17] Yeoh H-H, Truong V-D. Protein Contents, Amino Acid Compositions and Nitrogen-to-Protein Conversion Factors for Cassava Roots. J Sci Food Agr. 1996;70:51-4.
[18] Adda M, Merchuk JC, Arad S. Effect of nitrate on growth and production of cell-wall polysaccharide by the unicellular red alga *Porphyridium*. Biomass. 1986;10:131-40.
[19] Arad S, Adda M, Cohen E. The potential of production of sulfated polysaccharides from *Porphyridium*. Plant Soil. 1985;89:117-27.
[20] Arad SM, Friedman OD, Rotem A. Effect of nitrogen on polysaccharide production in a *Porphyridium* sp. Appl Environ Microb. 1988;54:2411-4.

[21] Geresh S, Arad S. The extracellular polysaccharides of the red microalgae: Chemistry and rheology. Bioresource Technol. 1991;38:195-201.
[22] Geresh S, Mamontov A, Weinstein J. Sulfation of extracellular polysaccharides of red microalgae: preparation, characterization and properties. J Biochem Bioph Meth. 2002;50:179-87.
[23] Sobczuk TM, Camacho FG, Grima EM, Chisti Y. Effects of agitation on the microalgae *Phaeodactylum tricornutum* and *Porphyridium cruentum*. Bioproc Biosyst Eng. 2006;28:243-50.
[24] Lee RE. Phycology. 4th ed. Cambridge, England ; New York: Cambridge University Press; 2008.
[25] Lu HK, Hsieh CC, Hsu JJ, Yang YK, Chou HN. Preventive effects of *Spirulina platensis* on skeletal muscle damage under exercise-induced oxidative stress. Eur J Appl Physiol. 2006;98:220-6.
[26] Payne MF, Rippingale RJ. Evaluation of diets for culture of the calanoid copepod *Gladioferens imparipes*. Aquaculture. 2000;187:85-96.
[27] Aflalo C, Meshulam Y, Zarka A, Boussiba S. On the relative efficiency of two- vs. one-stage production of astaxanthin by the green alga *Haematococcus pluvialis*. Biotechnol Bioeng. 2007;98:300-5.
[28] Mendes-Pinto MM, Raposo MFJ, Bowen J, Young AJ, Morais R. Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability. J Appl Phycol. 2001;13:19-24.
[29] Montsant A, Zarka A, Boussiba S. Presence of a nonhydrolyzable biopolymer in the cell wall of vegetative cells and astaxanthin-rich cysts of *Haematococcus pluvialis* (Chlorophyceae). Mar Biotechnol. 2001;3:515-21.
[30] Hagen C, Siegmund S, Braune W. Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation. Eur J Phycol. 2002;37:217-26.
[31] Barbarino E, Lourenço SO. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. J Appl Phycol. 2005;17:447-60.
[32] Nguyen RT, Harvey HR. A rapid micro-scale method for the extraction and analysis of protein in marine samples. Mar Chem. 1994;45:1-14.
[33] Barsanti L, Gualtieri P. Algae: Anatomy, Biochemistry, and Biotechnology: Taylor & Francis Group; 2006.
[34] Jorquera O, Kiperstok A, Sales EA, Embiruçu M, Ghirardi ML. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresource Technol. 2010;101:1406-13.
[35] Lam MK, Lee KT. Microalgae biofuels: A critical review of issues, problems and the way forward. Biotechnol Adv. 2012;30:673-90.
[36] Singh A, Olsen SI. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. Appl Energy. 2011;88:3548-55.
[37] Stephenson AL, Kazamia E, Dennis JS, Howe CJ, Scott SA, Smith AG. Life-Cycle Assessment of potential algal biodiesel production in the United Kingdom: A comparison of raceways and air-lift tubular bioreactors. Energy Fuels. 2010;24:4062-77.
[38] Yang J, Xu M, Zhang X, Hu Q, Sommerfeld M, Chen Y. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresource Technol. 2011;102:159-65.

[39] Batista AP, Raymundo A, Sousa I, Empis J. Rheological characterization of coloured oil-in-water food emulsions with lutein and phycocyanin added to the oil and aqueous phases. Food Hydrocolloid. 2006;20:44-52.
[40] Chronakis IS, Galatanu AN, Nylander T, Lindman B. The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface. Colloid Surface A. 2000;173:181-92.
[41] Batista AP, Gouveia L, Bandarra NM, Franco JM, Raymundo A. Comparison of microalgal biomass profiles as novel functional ingredient for food products. Algal Research. 2013.
[42] Safi C, Charton M, Pignolet O, Silvestre F, Vaca-Garcia C, Pontalier P-Y. Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. J Appl Phycol. 2012:1-7.
[43] Tokuşoglu Ö, Unal MK. Biomass Nutrient Profiles of Three Microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrisis galbana*. J Food Sci. 2003;68:1144-8.
[44] Rebolloso-Fuentes MM, Navarro-Perez A, Garcia-Camacho F, Ramos-Miras JJ, Guil-Guerrero JL. Biomass nutrient profiles of the microalga *Nannochloropsis*. J Agric Food Chem. 2001;49:2966-72.
[45] Safi C, Charton M, Pignolet O, Pontalier P-Y, Vaca-Garcia C. Evaluation of the protein quality of *Porphyridium cruentum*. J Appl Phycol. 2012.
[46] Servaites JC, Faeth JL, Sidhu SS. A dye binding method for measurement of total protein in microalgae. Anal Biochem. 2012;421:75-80.
[47] Slocombe SP, Ross M, Thomas N, McNeill S, Stanley MS. A rapid and general method for measurement of protein in micro-algal biomass. Bioresource Technol. 2013;129:51-7.
[48] Fleurence J. The enzymatic degradation of algal cell walls: a useful approach for improving protein accessibility? J Appl Phycol. 1999;11:313-4.
[49] Gouveia L, Oliveira AC. Microalgae as a raw material for biofuels production. J Ind Microbiol Biot. 2009;36:269-74.
[50] Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM. Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technol. 2010;101 Suppl 1:S75-7.
[51] Zheng H, Yin J, Gao Z, Huang H, Ji X, Dou C. Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl Biochem Biotech. 2011;164:1215-24.
[52] Becker W. Microalgae in Human and Animal Nutrition. Handbook of Microalgal Culture: Blackwell Publishing Ltd; 2007. p. 312-51.
[53] Punnett T, Derrenbacker EC. The amino acid composition of algal cell walls. J Gen Microbiol. 1966;44:105-14.
[54] Heussner AH, Mazija L, Fastner J, Dietrich DR. Toxin content and cytotoxicity of algal dietary supplements. Toxicol Appl Pharm. 2012;265:263-71.
[55] Chisti Y. Biodiesel from microalgae. Biotechnol Adv. 2007;25:294-306.
[56] Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioeng. 2009;102:100-12.
[57] Doucha J, Livansky K. Influence of processing parameters on disintegration of *Chlorella* cells in various types of homogenizers. Appl Microbiology Biot. 2008;81:431-40.

[58] Furuki T, Maeda S, Imajo S, Hiroi T, Amaya T, Hirokawa T, et al. Rapid and selective extraction of phycocyanin from *Spirulina platensis* with ultrasonic cell disruption. J Appl Phycol. 2003;15:319-24.
[59] Gerde JA, Montalbo-Lomboy M, Yao L, Grewell D, Wang T. Evaluation of microalgae cell disruption by ultrasonic treatment. Bioresource Technol. 2012;125:175-81.
[60] Jubeau S, Marchal L, Pruvost J, Jaouen P, Legrand J, Fleurence J. High pressure disruption: a two-step treatment for selective extraction of intracellular components from the microalga *Porphyridium cruentum*. J Appl Phycol. 2012.
[61] Clarens AF, Resurreccion EP, White MA, Colosi LM. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ Sci Technol. 2010;44:1813-9.
[62] Collet P, Hélias A, Lardon L, Ras M, Goy R-A, Steyer J-P. Life-cycle assessment of microalgae culture coupled to biogas production. Bioresource Technol. 2011;102:207-14.
[63] Lardon L, Hélias A, Sialve B, Steyer J-P, Bernard O. Life-cycle assessment of biodiesel production from microalgae. Environ Sci Technol. 2009;43:6475-81.
[64] Sander K, Murthy G. Life cycle analysis of algae biodiesel. Int J Life Cycle Assess. 2010;15:704-14.
[65] Illman AM, Scragg AH, Shales SW. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme Microb Tech. 2000;27:631-5.
[66] Yamamoto M, Fujishita M, Hirata A, Kawano S. Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae). J Plant Res. 2004;117:257-64.
[67] WHO. Protein and amino acid requirements in human nutrition. World Health Organisation, Geneva2007. p. 284.
[68] FAO. A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish. Rome: FAO Fish Aquacult; 2008. p. 20.
[69] Solomon EP, Berg LR, Martin DW. Biology. 5th ed. Fort Worth: Saunders College Pub.; 1999.
[70] Abo-Shady AM, Mohamed YA, Lasheen T. Chemical composition of the cell wall in some green algae species. Biol Plant. 1993;35:629-32.
[71] Blumreisinger M, Meindl D, Loos E. Cell wall composition of chlorococcal algae. Phytochemistry. 1983;22:1603-4.
[72] Griffiths DA, Griffiths DJ. The fine structure of autotrophic and heterotrophic cells of *Chlorella vulgaris* (Emerson strain). Plant Cell Physiol. 1969;10:11-9.
[73] Yamamoto M, Kurihara I, Kawano S. Late type of daughter cell wall synthesis in one of the Chlorellaceae, *Parachlorella kessleri* (Chlorophyta, Trebouxiophyceae). Planta. 2005;221:766-75.
[74] Kapaun E, Reisser W. A chitin-like glycan in the cell wall of a *Chlorella* sp. (Chlorococcales, Chlorophyceae). Planta. 1995;197:577-82.
[75] Yvonne N, Tomas K. Cell wall development, microfibril and pyrenoid structure in type strains of *Chlorella vulgaris*, C. kessleri, C. sorokiniana compared with C. luteoviridis (Trebouxiophyceae, Chlorophyta). Arch Hydrobiol 2000;100:95-105.
[76] Aach HG, Bartsch S, Feyen V. Studies on *Chlorella* protoplasts. Planta. 1978;139:257-60.
[77] Berliner MD. Protoplast induction in *Chlorella vulgaris*. Plant Sci Lett. 1977;9:201-4.
[78] Liu S, Liu C, Huang X, Chai Y, Cong B. Optimization of parameters for isolation of protoplasts from the antarctic sea ice alga *Chlamydomonas* sp. ICE-L. J Appl Phycol. 2006;18:783-6.

[79] Liang Y, Sarkany N, Cui Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett. 2009;31:1043-9.
[80] Araujo GS, Matos LJ, Fernandes JO, Cartaxo SJ, Goncalves LR, Fernandes FA, et al. Extraction of lipids from microalgae by ultrasound application: prospection of the optimal extraction method. Ultrason Sonochem. 2013;20:95-8.
[81] Packeiser H, Lim C, Balagurunathan B, Wu J, Zhao H. An extremely simple and effective colony PCR procedure for bacteria, yeasts, and microalgae. Appl Biochem Biotech. 2013;169:695-700.
[82] Yamada S, Nakamura T, Tanaka Y, Isogai Y, Nishio T, Oku T. Characterization and amino acid sequences of cytochromes c(6) from two strains of the green alga *Chlorella vulgaris*. Biosci Biotechnol Biochem. 2000;64:628-32.
[83] Mercer P, Armenta RE. Developments in oil extraction from microalgae. Eur J Lipid Sci Techn. 2011;113:539-47.
[84] Gerken HG, Donohoe B, Knoshaug EP. Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. Planta. 2013;237:239-53.
[85] Yamada T, Sakaguchi K. Electron microscopic studies of *Chlorella ellipsoidea* protoplast formation. J Gen Microbiol. 1982;128:1319-27.
[86] Ritchie RJ. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res. 2006;89:27-41.
[87] Kim Y-Cb, Myerson A. Diffusivity of protein in aqueous solutions. Korean J Chem Eng. 1996;13:288-93.
[88] Brune D, Kim S. Predicting protein diffusion coefficients. Proceedings of the National Academy of Sciences. 1993;90:3835-9.
[89] Schlichting H, Gersten K. Boundary-layer theory. 8th rev. and enl. ed. Berlin ; New York: Springer; 2000.
[90] Berliner MD. Proteins in *Chlorella vulgaris*. Cambridge, UK: Faculty Press; 1986.
[91] Hanan MK. Comparative effects of autotrophic and heterotrophic growth on some vitamins, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile of *Chlorella vulgaris* Beijerinck. Afr J Biotechnol. 2011;10.
[92] Morris HJ, Carrillo OV, Almarales Á, Bermúdez RC, Alonso ME, Borges L, et al. Protein hydrolysates from the alga *Chlorella vulgaris* 87/1 with potentialities in immunonutrition. Biotecnol Aplic. 2009;26:162-5.
[93] Kirchhoff H, Haferkamp S, Allen JF, Epstein DB, Mullineaux CW. Protein diffusion and macromolecular crowding in thylakoid membranes. Plant Physiol. 2008;146:1571-8.
[94] Halim R, Harun R, Danquah MK, Webley PA. Microalgal cell disruption for biofuel development. Appl Energy. 2012;91:116-21.
[95] Widjaja A, Chien C-C, Ju Y-H. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem E. 2009;40:13-20.
[96] Komaki H, Yamashita M, Niwa Y, Tanaka Y, Kamiya N, Ando Y, et al. The effect of processing of *Chlorella vulgaris*: K-5 on in vitro and in vivo digestibility in rats. Anim Feed Sci Tech. 1998;70:363-6.
[97] Samarasinghe N, Fernando S, Lacey R, Faulkner WB. Algal cell rupture using high pressure homogenization as a prelude to oil extraction. Renew Energ. 2012;48:300-8.

Chapter 4: Fractionation of the aqueous phase of *Tetraselmis suecica*

4.1 Introduction

Extraction and fractionation of microalgal lipids for bioenergy production dominated the major part of the studies conducted during the last decade. This area of studies has recently hurt a rigid wall of what we stressed on previously, which is the high cost and the unsustainable overall production. Around the world, many industries concentrating their work on biodiesel from microalgae bankrupted or shifted their work toward the so-called Algorefinery in order to compensate their losses by valorising the numerous highly added value biomolecules present in microalgae, and therefore maximizing the value of the biomass and possible transformation of the deficit to profit. Hence, the algorefinery concept emerged recently and it consists on refining the biomass in order to obtain bioenergy and bioproducts for food and other applications.

To our knowledge, there are no published studies regarding this concept on microalgae. In this respect, we decided to break the monotonous and repetitive focus on the area of biodiesel production and showed some interest to other scientific possibilities that lie within microalgae family. In chapter 2, we have showed the possibility to extract lipids at high yield. Thus, fractionation can be restricted to aqueous separation between polysaccharides and proteins and pigments.

The main goal of this chapter is to add some building blocks to the Algorefinery concept in terms of a continuous fractionation process of an aqueous phase containing different biomolecules after applying a cell disruption unit operation. Nonetheless, the species was specifically chosen in order to find an intermediate microalga among all the species that we already studies in our work. Therefore, *Tetraselmis suecica* was the best compromise for many reasons; it lacks an exopolysaccharides layer that would increase the risk of fouling phenomenon, it has intermediate cell wall rigidity and it was grown under normal growth conditions, which limits heavy accumulation of lipids.

The chapter is composed of a single publication mirroring the concept defined previously. The process consists of breaking the cell wall of the microalga selected by high-pressure homogenization without overheating the medium in order to preserve the integrity of the biomolecules and followed afterwards by a centrifugal recovery of the supernatant containing the biomolecules (starches, sugars, proteins and pigments). Further on, the supernatant was ultrafiltrated through a two-stage process in order to fractionate the supernatant. All the unit operations of the process were carried out with respect to green chemistry principles by avoiding the use of harmful and toxic solvents.

4.2 Two-stage ultrafiltration process for separating multiple components of *Tetraselmis suecica* after cell disruption

Carl SAFI [1,2,3], Dylan Zhe LIU [3], Benjamin Hao Jie YAP [3], Gregory John Oliver MARTIN [3], Carlos VACA-GARCIA [1,2,4], Pierre-Yves PONTALIER [1,2]

[1] Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), 31030 Toulouse, France
[2] INRA, UMR 1010 CAI, F-31030 Toulouse, France
[3] Department of Chemical and Biomolecular Engineering, University of Melbourne, Parkville, Victoria 3010, Australia
[4] King Abdulaziz University, Jeddah, Saudi Arabia

E-mail : carl.safi@ensiacet.fr

This publication has been submitted to ***Separation and Purification Technology***

Abstract

A two-stage ultrafiltration process was applied on the aqueous phase of *Tetraselmis suecica* after breaking its cell wall via high-pressure homogenization. Microscopic observations revealed that the cells were completely disrupted from 600 bar and fragmentation of the cells was also noticeable after 800 bar. In addition, the highest concentration of all the molecules of interest in the aqueous phase was observed at 1000 bar and the temperature was 46°C while preserving the integrity of the molecules of interest in the downstream process. After centrifugation the aqueous phase was submitted to ultrafiltration through two consecutive membranes with different molecular weight cut-offs. Complete retention of starch was possible with a 100 kDa membrane and separation of sugars from proteins with a 10 kDa membrane on the remaining mixture. After testing the process with model solutions, the transmembrane pressure selected was 30 psi succeeding to retain starch and pigments during the first part of the process, and proteins during the second part. A linear correlation between the flow rate and the pressure were observed in both parts of the process.

Keywords High-pressure homogenization, proteins, pigments, sugars, starch, ultrafiltration.

4.2.1 Introduction

Microalgae are considered as a promising feedstock for biofuel production due to their ability to convert carbon dioxide into carbon rich-lipids [1]. They grow rapidly and do not need arable land. However, the feasibility of this new technology has not yet been exploited on an industrial scale due to its currently uncompetitive high production cost and its overall unsustainable production [2-6]. The potential for large scale commercial exploitation of these micro organisms is possible if they are completely valorised in the framework of a biorefinery [1, 7].

The majority of research into microalgal biotechnology has been focused on the production and accumulation of lipids [8-10], methods for extraction [11-14], analysis [9, 15] and transformation of lipids to biofuel [16, 17]. Some studies have also considered isolating other principal microalgal components such as proteins by solubilisation in alkaline solution followed by a precipitation with acid [18, 19], or polysaccharides by precipitation with ethanol [20-22].

In order to avoid using solvents and chemicals, alternative techniques to separate components by ultrafiltration already exist and can be scaled-up to an industrial level [23, 24]. For microalgae this technique has so far been used mainly for harvesting the cells [25-27], but its use in separating microalgal biomass components in an integrated process is yet to be established. A few studies to date have investigated this technique on microalgae to purify a single component such as the polysaccharides of *Porphyridium cruentum* [28], *Spirulina platensis* and *Chlorella pyrenoidosa* [21], or to examine the role of exopolysacharides of *Chlorella* sp. and *Porphyridium purpureum* in the fouling of ultrafiltration membranes [29]. However, there is a current lack of literature on the separation of multiple components of microalgal biomass.

The microalga implemented in this study is *Tetraselmis suecica*, which is an ovaloid unicellular green flagellated specie of 9-13 mm in length and 7-8 mm in width [30]. Its biochemical composition contains a variety of potentially valuable components. In particular, its protein content can be high (up to 44% dry weight) and has a balanced amino acid profile that includes both essential and non-essential amino acids [31-33]. Carbohydrates represent 8-57% dry weight, [30, 34, 35] with starch being the dominant component when

accumulated under nitrogen starvation and low irradiance [36]. Glucose is the predominant intracellular monosaccharide, followed by galactose, xylose, rhamnose, mannose and arabinose that are present in the polysaccharide components of the cell wall [31, 33, 35]. Lipids can represent between 7-30% of its dry weight with a fatty acid composition suitable for biodiesel production [37-39]. Like all microalgae, its composition varies according to the growth conditions, which will affect the accumulation of the components of interest.

The following study investigates the effectiveness of two-stage ultrafiltration process for separating internal cell components of *T. suecica* disrupted by high-pressure homogenization. Two membranes with different molecular weight cut-offs are used to separate starch from proteins and sugars in the first step and then proteins from sugars in the second step. The process was first tested on model solutions containing starch, proteins and sugars, and then applied to *T. suecica*.

4.2.2 Methods

All chemicals and biomolecules including soluble starch ($C_{12}H_{22}O_{11}$) and milk proteins (12-250 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. The Lowry assay kit was purchased from Fisher Scientific.

4.2.2.1. Microalga

Tetraselmis suecica (strain CS 187) was grown in outdoor photobioreactors in medium with a modified 'f-medium' nutrients and trace elements [40]. This medium consisted of 200 $mg.L^{-1}$ $NaNO_3$, 25 $mg.L^{-1}$ KH_2PO_4, 9.0 $mg.L^{-1}$ iron (III) citrate, 9.0 $mg.L^{-1}$ citric acid, 0.360 $mg.L^{-1}$ $MnCl_2{\cdot}4H_2O$, 0.044 $mg.L^{-1}$ $ZnSO_4{\cdot}7H_2O$, 0.022 $mg.L^{-1}$ $CoCl_2{\cdot}6H_2O$, 0.020 $mg.L^{-1}$ $CuSO_4{\cdot}5H_2O$, 0.008 $mg.L^{-1}$ $Na_2MoO_4{\cdot}2H_2O$ and trace levels of vitamins B12, biotin, and thiamine. Mixing in the photobioreactors was achieved by compressed air aeration. Temperature and illumination intensity were dependent on local weather conditions in Melbourne, Victoria, Australia.

Suspensions of *Tetraselmis suecica* used in this study were made by mixing frozen microalgal paste (containing 5.5% dry weight) in distilled water to a concentration of

approximately 17 $g.L^{-1}$ dry weight. Aggregates in suspension were dispersed by stirring up to an hour prior to homogenization.

4.2.2.2. High-pressure homogenization

A GEA Panda2K NS1001L high-pressure homogenizer (GEA Niro Soavi, Parma, Italy) with a cell disruption valve (Re+ valve) attached was used for cell disruption. *T. suecica* cells were suspended in distilled water at 17 $g.L^{-1}$ and then passed through the homogenizer at different pressures ranging from 200 to 1000 bar. Temperature of the homogenized suspension was monitored to avoid the denaturation of components in the medium. The aqueous extracts (supernatants) were recovered after centrifugation at 10000 *g* for 10 minutes at 21°C for subsequent process and analyses.

4.2.2.3. Model suspensions

Model suspension 1 was composed of 40% milk proteins (12-250 kDa), 35% starch and 25% sugars. The model suspension 2 was composed of 60% milk proteins (15-250 kDa) and 40% sugars. Both suspensions were vigorously stirred for 2 h to ensure maximum solubilisation in distilled water (ratio 1:5 w:v). However, both suspensions were not limpid indicating that the biomolecules were dispersed and not completely solubilised in water.

4.2.2.4. Ultrafiltration (UF)

Model suspensions and supernatant of homogenized aqueous phase of *Tetraselmis suecica* were fractionated by two-steps ultrafiltration using a Labscale^{TM} TFF system (Millipore, Billerica, MA). The TFF system includes a 500 mL acrylic reservoir with a system base containing a magnetic stirrer and diaphragm pump. It also includes two pressure gauges; the retentate gauge indicates the pressure of the fluid exiting the Pellicon XL 50 (cm^2) device. Two different Pellicon XL 50 (cm^2) polyethersulfone membranes cartridges were used with different molecular weight cut-offs (100 kDa and 10 kDa).

Two modes were tested for the model solutions, the recycling mode and the concentration mode. During the recycling mode both retentates and permeates were recycled in order to select the appropriate transmembrane pressure from 10 to 30 psi. Further on, during the concentration mode, the retentate was recycled while the permeate was recovered

until reaching two thirds of the initial injected volume. During this step, samples were taken from both phases for further analysis. During both modes, the feed solution for each step of the process was filtrated and then followed by the necessary analysis of the retentate and permeate. During both modes, the feeding solution is constantly stirred in the feeding chamber to ensure the complete solubilisation of the components in the extract. The flow rate was
evaluated according to the following equation:

$$\text{Flow rate } (kg.h^{-1}.m^{-2}) = \frac{\textbf{permeate mass recovered (kg)}}{\textbf{time (h) x membrane surface } (\mathbf{m^2})}$$

After each run, the membranes were cleaned according to the following procedure: flushing with distilled water, then cleaning with 0.1 M NaOH solution during 60 min and then rinsing with distilled water for 30 min at 20 psi.

4.2.2.5. Pigments analysis

200 µL of supernatant was mixed with 1300 µL pure methanol and then incubated in dark for 1h at 45°C. Further on, the samples were centrifuged at 10000 *g* for 10 min at 20°C. The organic phase (methanol) containing the pigments was recovered, and were determined using the equations proposed by Ritchie (2006).

(1) Total chlorophyll $\mu g.L^{-1} = (9.3443 \text{ x } A652) + (4.3481 \text{ x } A665)$
(2) Total carotenoids $\mu g.L^{-1} = 4 \text{ x } A480$

4.2.2.6. Sugar analysis

The procedure consists of adding 0.25 mL of the sample to 0.75 mL distilled water and 2 mL of DNS reagent. The mixture is vortexed then heated at 90°C for 5 minutes. Immediately after, 2 mL distilled water are added, and then the mixture is cooled at room temperature for 2-3 minutes after being vortexed. The color of the mixture should be dark red and measure by spectrophotometer (Varian Cary 3E UV visible spectrophotometer) at 570 nm after being zeroed with the blank solution, which consists of the same mixture only distilled water replaces the sample.

4.2.2.7. Starch-iodine assay

The analysis consists of mixing 0.25 mL of supernatant sample with 5 mL of iodine reagent. The mixture is then vortexed for 5 seconds then stranded for 2-5 minutes for the color to stabilize. Absorbance is measured at 620 nm against a blank of distilled water and iodine reagent.

4.2.2.8. Proteins analysis

4.2.2.8.1. Lowry assay

The procedure involves reaction of proteins with cupric sulphate and tartare in an alkaline solution, leading to the formation of tetradentate copper protein complexes. The addition of the Folin-Ciocalteu reagent leads to the oxidation of the peptide bonds by forming molybdenum blue with the copper ions. Therefore, a calibration curve was prepared using a concentration range of bovine standard albumin from 0 to 1500 $\mu g.mL^{-1}$. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract were withdrawn and then 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for exactly 10 min. After incubation, 100 μL of Folin-Ciocalteu reagent (1 N) were added and again vortexed and incubated for exactly 30 min. The blue colour solution is then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer after being zeroed with blank sample containing all the chemicals without the extract.

4.2.2.8.2. SDS-PAGE

The protein content of the supernatants, the permeates and the retentates were analysed by SDS–PAGE using a BioRad Criterion Cell electrophoresis unit (BioRad Laboratories, Richmond, CA). The SDS–PAGE was performed by diluting the samples four times with distilled water. First, 20 μL of diluted samples were mixed with 22 μL of BioRad Laemmli buffer containing 5% beta mercaptoethanol and placed in a boiling water bath for 5 min. Next aliquots (10 μL) of samples were loaded into 8–16% linear gradient precast Tris–HCl Criterion 18 well gels and run at 100 V for 130 min. Gels were stained with Biosafe Coomassie Blue (BioRad) and digitally scanned and quantified using a BioRad Gel Doc XR + Imager (BioRad Laboratories, Richmond, CA).

4.2.2.9. Optic microscopy

All observations were performed using an Olympus BX51 light microscope with a DP72 digital camera attachment (Olympus, Mt Waverly, VIC, Australia) under white light without dyes.

4.2.3 Results and discussion

4.2.3.1 High-pressure homogenization

High-pressure homogenization was used to disrupt *T. suecica* to allow recovery and subsequent fractionation of its internal components. The efficiency of cell disruption as a function of homogenization pressure was examined microscopically (Fig 1).

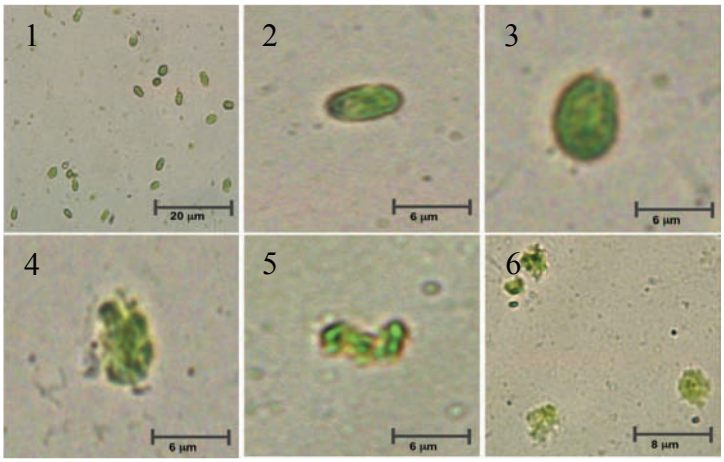

Figure 4: Microscopic observation before and after cell disruption of *T. suecica*. 1) Before disruption, 2) 200 bar, 3) 400 bar, 4) 600 bar, 5) 800 bar, 6) 1000 bar.

The cells were resistant at pressures up to 400 bar while the temperature increased only from 21 to 32°C. However, the efficiency of high-pressure homogenization started to be observed from 600 bar with broken cells losing their globular shape. Further on, besides being broken, it was observed that cells were also severely fragmenting after applying 800 or 1000 bar with a continuing increase in temperature up to 46°C. The complete disruption of the cell wall might be accompanied by possible alteration of the phospholipid bilayers of its internal organelles. In correspondence to the increase in cell rupture as a function of pressure, there was an expected increase of intracellular components (starch, sugars, proteins and pigments) remaining in supernatants of centrifuged lysates (Fig 2). The elevation of

temperature resulting from homogenization (32°C at 400 bar, 46°C at 1000 bar) may have also played a role in solubilising some components especially starch and protein. Without sufficient solubilisation resulting from heating of the medium the starch granules would remain in pelleted cell debris after centrifugation. Even at 1000 bar the temperature rise to 46 °C was below that required for protein denaturation and starch gelatinization. Homogenization at 1000 bar was effective at achieving cell disruption to release part of the intracellular components into the aqueous phase while not damaging the protein component. The increase in chlorophyll released as a function of homogenization pressure indicates that the chloroplast was broken allowing water to penetrate the inter-thylakoid space where the green pigment and some carotenoids are located. However, these pigments have hydrophobic nature, and their presence in the aqueous phase involves adsorption onto very small cell debris that did not decanted with the pellet after centrifugation or present inside small lipid droplets (emulsion), or even attached to amphiphilic structures (phospholipids).

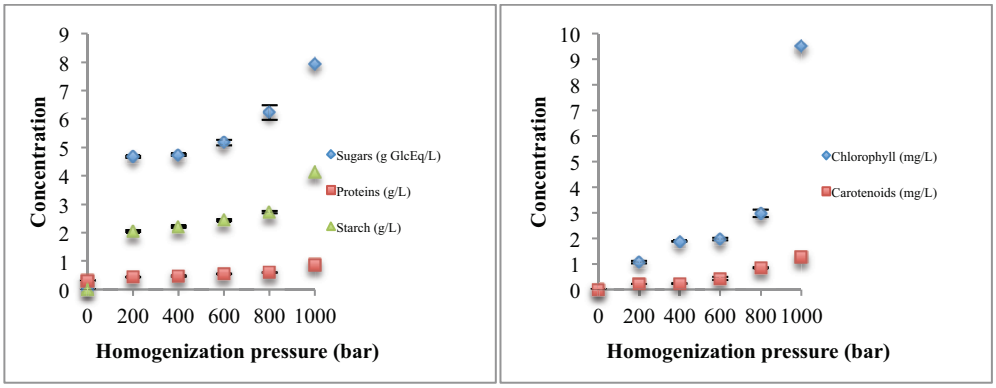

Figure 2: Concentration of the components present in the aqueous phase after cell disruption and before ultrafiltration. Results are the mean of three replicates for three experiments ± SD (n=9).

4.2.3.2 Ultrafiltration process

The aim of this part was to study the fractionation between a large polymer as starch, proteins and small sugars. As the size of starch is superior to 100 kDa, the first step of the ultrafiltration process employing a 100 kDa membrane is to retain starch while allowing proteins and sugars to pass into the permeate. Further on, according to Schwenzfeier et al. (2011) proteins size of *T. suecica* is between 15-50 kDa, and therefore the second step employs a 10 kDa membrane in order to retain proteins while allowing sugars to be concentrated in the permeate. The process was first conducted on the concentrated model

solutions in order to verify its feasibility on a highly concentrated suspension and to obtain the necessary parameters. Afterwards, the process was extrapolated on the microalgal extract obtained after breaking the cell wall of *T. suecica* by high-pressure homogenization. Nonetheless, after each trial the concentration of the different biomolecules was calculated for the retentates and the permeates to follow-up with the mass balance.

4.2.3.3 Model suspensions

A membrane of 100 kDa was used with model suspension 1 (proteins, starch and sugars), whereas model suspension 2 (proteins and sugars) was filtrated over a 10 kDa membrane. For both membranes, flow rate decreased with time during 30 min to reach a steady state condition. The flow stabilised at 50-70% of the initial value with the 100 kDa membrane, while only 10-20% for the 10 kDa membrane. For the first case, the decrease seems to be related to a polarisation concentration layer, and thus the formation of an asymptotic curve. For the second case, the decrease is lower maybe due to a less important influence of the polarisation layer. The difference between the compositions of both layers is the presence of starch, which can have gelling properties that may hinder the filtration. Nevertheless in both cases, the steady flow rate increases almost linearly with pressure indicating the lack of a gel layer.

Correlation between the flow rate and the different transmembrane pressures (TMP) was $R^2 = 0.88$ (Fig 3), which is relatively low and confirms the action of the large size polysaccharides [41] that get retained by the 100 kDa membrane and strongly contribute to the fouling phenomenon [29]. As the flow rate during 30 min was greatest at 30 psi with a flow rate of 47.83 $kg.h^{-1}.m^{-2}$, and the fouling was not more severe than at lower pressures, 30 psi was used for subsequent tests performed in concentration mode. In the latter tests, the concentration was managed until getting a volumetric concentration ratio of 2.32 ± 0.04 obtained after 30 min, with a final flow rate 42.8 ± 1.3 $kg.h^{-1}.m^{-2}$. A complete retention of starch was achieved, with no starch observed in the permeate.

Further on, a more linear relationship between flow rate and TMP was observed when operating the 10 kDa membrane in recycling mode using the second model solution that contains sugars and milk proteins (Fig 3) with no starch. The highest initial flow rate of 55.43 $kg.h^{-1}.m^{-2}$ was obtained at a TMP of 30 psi. This flow rate decreased to 50.39 $kg.h^{-1}.m^{-}$

^{2} after 30 min of processing. Concentration mode was again operated at 30 psi with a stable flow rate of 44.30 ± 1.2 $kg.h^{-1}.m^{-2}$ obtained during the 30 min of operation to reach a volumetric concentration ratio of 3.01 ± 0.05. The mass balance indicated that 4 to 5% of the sugars were detected in the membrane due to the high concentration of these components in the solution, but protein loss was negligible (< 1%) according to Lowry assay measurements of the permeate. Nearly complete retention of the protein was also verified by SDS-PAGE analysis of the permeates that showed only a very low intensity band of low molecular weight protein, close in size to the cut-off of the membrane (Fig 4). As for sugars, the operation in concentration mode for 30 min at 30 psi allowed 63% of the sugars to be transferred to the permeate.

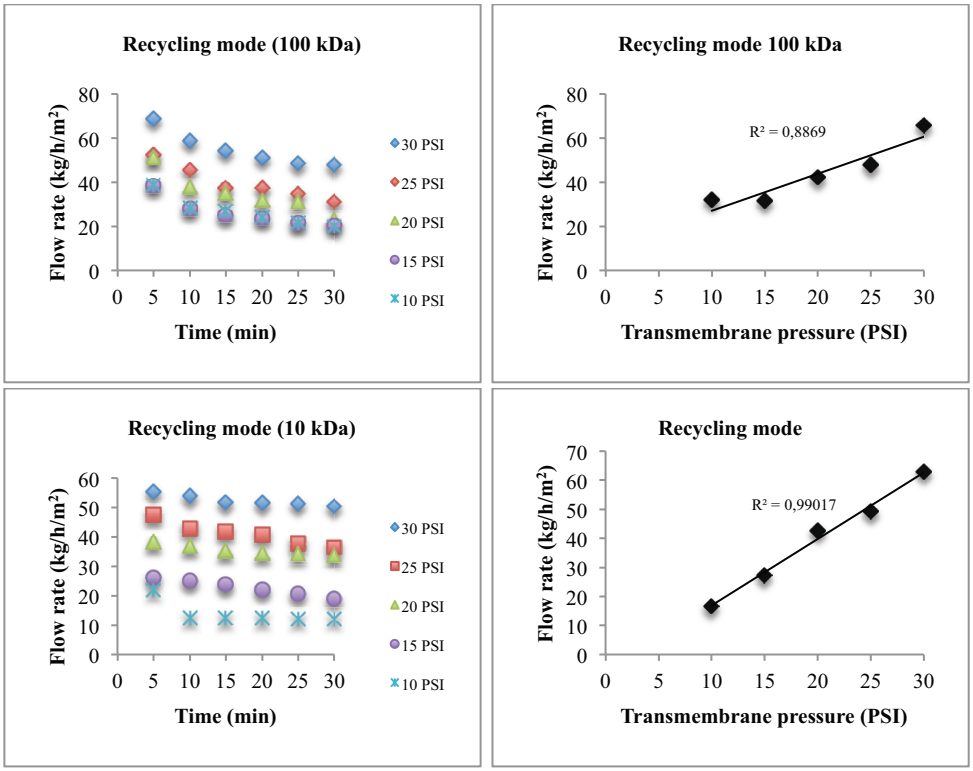

Figure 3: Recycling mode of both model solutions. Model 1 with 100 kDa and model 2 with 10 kDa. Results are based on the three replicates for three experiments ± SD (n=9).

Nearly complete retention of the protein was also verified by SDS-PAGE analysis of the permeates that showed only a very low intensity band of low molecular weight protein, close in size to the cut-off of the membrane (Fig 4). As for sugars, the operation in

concentration mode for 30 min at 30 psi allowed 63% of the sugars to be transferred to the permeate.

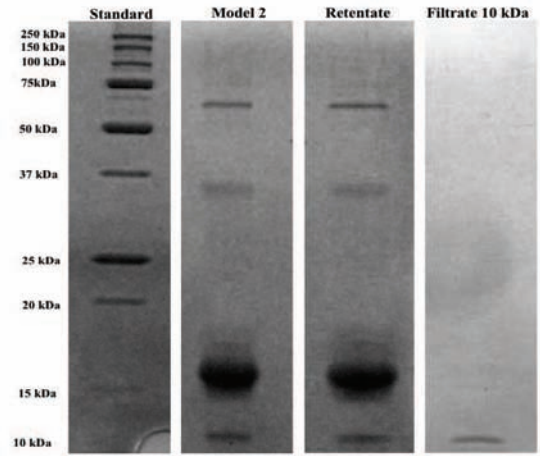

Figure 4: SDS-PAGE after ultrafiltration of the second model solution with 10 kDa membrane.

4.2.3.4 Aqueous phases before/after cell disruption of *T. suecica*

Having demonstrated the effectiveness of a two-stage filtration process for fractionating biomass components in model solutions, experiments were performed on actual lysates from microalgal material obtained at different homogenization pressures by conducting the concentration mode for 30 min. For lysates obtained at all homogenization pressures, neither starch nor pigments were detected in the Permeate 1, indicating complete retention of these components in Retentate 2 (Table 1). While the retention of the starch was expected given the size of the granule, the retention of the pigments could be explained by their presence in small lipid droplets or in very small cell debris remaining in the aqueous medium and both are larger than the cut off of the 100 kDa membrane. In addition, given the highly hydrophilic characteristics of the membrane (Polyethersulfone), it retains the former that are hydrophobic.

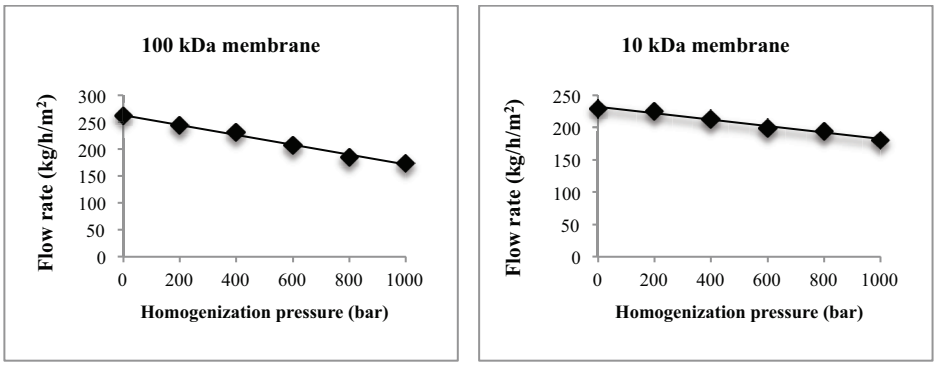

Figure 5: Correlation between the flow rate and the different pressures applied for cell disruption. Results are based on the three replicates for three experiments ± SD (n=9).

While maintaining the TMP constant at 30 psi the flow rate decreased when samples obtained at higher homogenization pressure were tested by the concentration mode during 30 min, with volumetric concentration ratio of 3.06 ± 0.04 (Fig 5). Despite of this fact and despite of the fact that the lysate obtained at 1000 bar homogenization pressure contains two-fold more proteins than the lysate obtained at 200 bar (Fig 2), the amount of protein in Permeate 1 increased only from 50 to 80% in the range. This suggests that the proteins are more aggregated at low homogenization pressure and are therefore retained more by the membrane. The fraction of sugars that was passed through the membrane increased from about 75% to 90% between 200 and 600 bar of homogenization pressure, and decreased to about 75% for lysates produced at 1000 bar.

Table 1: Composition of Permeate 1 after ultrafiltration with 100 kDa membrane of the aqueous phases after cell disruption of *T. suecica*. Results are the mean of three replicates for three experiments ± SD (n=9).

High-pressure homogenization (bar)	Sugars (g GlcEq.L^{-1})	Proteins (g.L^{-1})	Starch (g.L^{-1})	Chlorophyll (mg.L^{-1})	Carotenoids (mg.L^{-1})
0	n.d.	0.10±0.01	n.d.	n.d.	n.d.
200	3.45±0.04	0.23±0.01	n.d.	n.d.	n.d.
400	3.66±0.04	0.26±0.01	n.d.	n.d.	n.d.
600	4.55±0.01	0.33±0.03	n.d.	n.d.	n.d.
800	5.42±0.01	0.46±0.01	n.d.	n.d.	n.d.
1000	5.98±0.16	0.70±0.03	n.d.	n.d.	n.d.

***n.d. not detected**

At a TMP of 30 psi the highest final flow rate was 262 ± 2 $kg.h^{-1}.m^{-2}$ for the 100 kDa membrane. This flow decreased constantly as a function initial homogenization pressure, falling to reach 174 ± 2 $kg.h^{-1}.m^{-2}$ for lysates obtained at 1000 bar (Fig 5). A decline

in flow rate as a function of disurption pressure was similary observed for UF with the 10 kDa membrane. The maximum flow was 229 ± 2 $kg.h^{-1}.m^{-2}$ for samples homogenized at 200 bar, decreasing to 181 ± 1 $kg.h^{-1}.m^{-2}$ for lysates obtained at 1000 bar. In any cases, these fluxes are higher than those obtained with the model solution because of a lower initial concentration and also may be because of the presence of others compounds.

Permeate 1 was subsequently ultrafiltrated using a 10 kDa membrane until a volumetric concentration ratio of 2.57 ± 0.03. According to Lowry assay measurements and SDS-PAGE analysis of the permeates (Fig 6), no proteins were found in Permeate 2 but in Retentate 2 for all the samples obtained at different homogenization pressures.

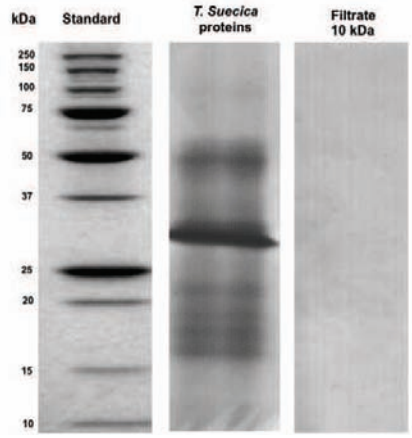

Figure 6: SDS-PAGE after ultrafiltration of the aqueous extract of the 1000 bar trial for cell disruption with 10 kDa membrane.

The permeation rate of the sugars is approximately 90% through the 10 kDa membrane regardless of the pressure used for cell rupture, and 65% of total sugars present in the supernatant were found in permeate 2. This indicates that at least 65% of saccharides having an aldehyde function have a size inferior to 10 kDa (Table 2). These results are consistent with the study conducted by Schwenzfeier et al. (2011), which showed that the proteins of *T. suecica* have a molecular weight range between 15 and 50 kDa. Most of the proteins were enzymes with multiple polypeptide chains, including Rubisco that has two subunits of 50 kDa and 15 kDa [33, 42]. Full retention of the proteins is therefore expected from ultrafiltration with a 10-kDa membrane. The results indicates that the separation between sugars and proteins is efficient. Nevertheless, under these conditions the sugar

recovery yield is about 50%, but should be increase by diafiltration with fresh water of the retentate.

Table 2: Concentration of the sugars in the Permeate 2 after ultrafiltration at 10 kDa. Results are the mean of three replicates for three experiments ± SD (n=9).

High-pressure homogenization (bar)	Sugars (g GlcEq.L^{-1})	Proteins ($g.L^{-1}$)	Starch ($g.L^{-1}$)	Chlorophyll ($mg.L^{-1}$)	Carotenoids ($mg.L^{-1}$)
0	n.d .	n.d.	n.d.	n.d.	n.d.
200	3.09±0.01	n.d.	n.d.	n.d.	n.d.
400	3.34±0.05	n.d.	n.d.	n.d.	n.d.
600	4.09±0.02	n.d.	n.d.	n.d.	n.d.
800	4.65±0.03	n.d.	n.d.	n.d.	n.d.
1000	5.18±0.05	n.d.	n.d.	n.d.	n.d.

*n.d. not detected

The global process on *T. suecica* was not jeopardised by major hurdles, starting from breaking the cell wall untill separating the components of interest by ultrafiltration (Fig 7). These results show it is possible to achieve good separation of intracellular microalgal biomass components using a two stage sequential UF process. This process could be applied to other microalgae, and could be used with various cell disruption techniques and membranes with different molecular weight cut-offs depending on the properties of the algae such as the cell strength and protein composition. For instance, *Chlorella vulgaris* proteins are mostly within a molecular weight range of 12 to 120 kDa [43] and *Haematococcus pluvialis* 10-100 kDa [44-46], and both have more resistant cell walls [47].

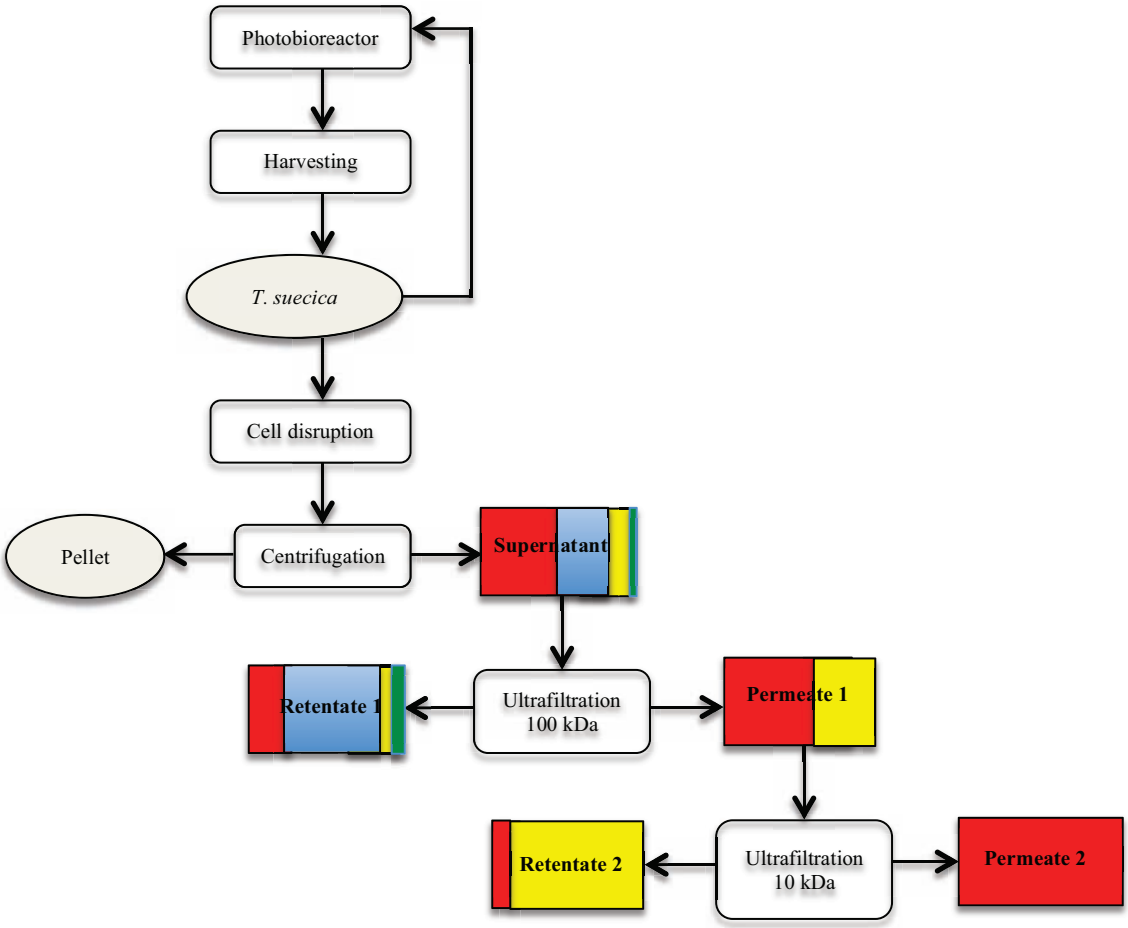

Figure 7: Overall process reflecting all the steps from growth to fractionation after homogenization at 1000 bar. Colours represent relative quantities: red is for sugars, blue for starch, yellow for proteins and green for pigments.

4.2.4 Conclusion

In this study multiple microalgal components were fractionated using an integrated process that does not require solvents or environmently harmful chemicals. The overall process was shown to be effective on *T. suecica*, resulting in three streams, enriched in pigments and starch, proteins, and sugars respectively. Indeed, additional work is required to optimise the process especially on finding better conditions to maximise the solubilisation of some components of interest without denaturing the rest in the downstream process. In addition, life cycle assessment of the process would be necessary to evaluate the energy input and to ensure the sustainability and feasibility of the process on an industrial scale.

Acknowledgments
This work was supported by the French national research agency (ANR) in the framework of "the Algoraffinerie" project. The authors are grateful to the "Direction de la Recherche de l'INP" for its additional financial support SMI-2013, and are also sincerely thankful for the Department of Chemical and Biomolecular Engineering of The University of Melbourne for providing the facilities and all the necessary tools to conduct this work and dress this study.

4.3 Chapter conclusion

The process integrated multiple goals, but attaining them will not be without consequences. Indeed the major objective of this study was the continuous fractionation of the aqueous phase, which was basically attained according to expectations. Another objective was to preserve the integrity of the biomolecules in the downstream process, which explains the reason cell disruption by means of high-pressure homogenization was stopped at 1000 bar to prevent overheating the medium that will denature proteins and pigments. Consequently, considering this approach together with implementing only water as the sole extraction solvent will reduce the complete recovery of the target molecules especially proteins and starches. In this sense, this new process brought additional building blocks for the Algorefinery concept without neglecting that it is possible to extrapolate it to other species but with minor changes especially by considering the modification of the membrane cut-off with regards to proteins size. Simultaneously the process opened some perspectives for further studies especially on finding better operational compromises to increase the concentration of the biomolecules in the aqueous medium, scaling up the process to an industrial level and conducting life cycle assessments in order to verify the feasibility and the sustainability of the process.

References

[1] Wijffels RH, Barbosa MJ. An Outlook on Microalgal Biofuels. Science. 2010;329:796-9.
[2] Lardon L, Hélias A, Sialve B, Steyer J-P, Bernard O. Life-cycle assessment of biodiesel production from microalgae. Environ Sci Technol. 2009;43:6475-81.
[3] Sander K, Murthy G. Life cycle analysis of algae biodiesel. Int J Life Cycle Assess. 2010;15:704-14.
[4] Singh A, Olsen SI. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. Appl Energy. 2011;88:3548-55.
[5] Stephenson AL, Kazamia E, Dennis JS, Howe CJ, Scott SA, Smith AG. Life-cycle assessment of potential algal biodiesel production in the United Kingdom: A comparison of raceways and air-lift tubular bioreactors. Energy Fuels. 2010;24:4062-77.
[6] Yang J, Xu M, Zhang X, Hu Q, Sommerfeld M, Chen Y. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresource Technol. 2011;102:159-65.
[7] Williams PJlB, Laurens LML. Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. Energ Environ Sci. 2010;3:554-90.
[8] Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process. 2009;48:1146-51.
[9] Rodríguez-Ruiz J, Belarbi E-H, Sánchez J, Alonso D. Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. Biotechnol Tech. 1998;12:689-91.
[10] Widjaja A, Chien C-C, Ju Y-H. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Insti Chem Eng. 2009;40:13-20.
[11] Araujo GS, Matos LJ, Fernandes JO, Cartaxo SJ, Goncalves LR, Fernandes FA. Extraction of lipids from microalgae by ultrasound application: prospection of the optimal extraction method. Ultrason Sonochem. 2013;20:95-8.
[12] Dejoye C, Vian MA, Lumia G, Bouscarle C, Charton F, Chemat F. Combined extraction processes of lipid from *Chlorella vulgaris* microalgae: microwave prior to supercritical carbon dioxide extraction. Int Journal Mol Sci. 2011;12:9332-41.
[13] Halim R, Danquah MK, Webley PA. Extraction of oil from microalgae for biodiesel production: A review. Biotechnol Adv. 2012;30:709-32.
[14] Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM. Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technol. 2010;101 Suppl 1:S75-7.
[15] Olmstead IL, Hill DR, Dias DA, Jayasinghe NS, Callahan DL, Kentish SE. A quantitative analysis of microalgal lipids for optimization of biodiesel and omega-3 production. Biotechnol Bioeng. 2013;100:2096-2104.
[16] Li X, Xu H, Wu Q. Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnol Bioeng. 2007;98:764-71.
[17] Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N. Biofuels from microalgae. Biotechnol Progr. 2008;24:815-20.
[18] Barbarino E, Lourenço SO. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. J Appl Phycol. 2005;17:447-60.

[19] Chronakis IS, Galatanu AN, Nylander T, Lindman B. The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface. Colloid Surface A. 2000;173:181-92.
[20] Gloaguen V, Ruiz G, Morvan H, Mouradi-Givernaud A, Maes E, Krausz P. The extracellular polysaccharide of *Porphyridium* sp.: an NMR study of lithium-resistant oligosaccharidic fragments. Carbohyd Res. 2004;339:97-103.
[21] Pugh N, Ross SA, ElSohly HN, ElSohly MA, Pasco DS. Isolation of three high molecular weight polysaccharide preparations with potent immunostimulatory activity from *Spirulina platensis*, *Aphanizomenon flos-aquae* and *Chlorella pyrenoidosa*. Planta Medic. 2001;67:737-42.
[22] Shi Y, Sheng J, Yang F, Hu Q. Purification and identification of polysaccharide derived from *Chlorella pyrenoidosa*. Food Chem. 2007;103:101-5.
[23] Akoum O, Jaffrin MY, Ding L-H. Concentration of total milk proteins by high shear ultrafiltration in a vibrating membrane module. J Membrane Sci. 2005;247:211-20.
[24] Susanto H, Arafat H, Janssen EML, Ulbricht M. Ultrafiltration of polysaccharide–protein mixtures: Elucidation of fouling mechanisms and fouling control by membrane surface modification. Sep Purif Technol. 2008;63:558-65.
[25] Frappart M, Massé A, Jaffrin MY, Pruvost J, Jaouen P. Influence of hydrodynamics in tangential and dynamic ultrafiltration systems for microalgae separation. Desalination. 2011;265:279-83.
[26] Rossignol N, Vandanjon L, Jaouen P, Quéméneur F. Membrane technology for the continuous separation microalgae/culture medium: compared performances of cross-flow microfiltration and ultrafiltration. Aquacult Eng. 1999;20:191-208.
[27] Zhang X, Hu Q, Sommerfeld M, Puruhito E, Chen Y. Harvesting algal biomass for biofuels using ultrafiltration membranes. Bioresource Technol. 2010;101:5297-304.
[28] Patel AK, Laroche C, Marcati A, Ursu AV, Jubeau S, Marchal L, et al. Separation and fractionation of exopolysaccharides from *Porphyridium cruentum*. Bioresource Technol. 2012.
[29] Morineau-Thomas O, Jaouen P, Legentilhomme P. The role of exopolysaccharides in fouling phenomenon during ultrafiltration of microalgae (*Chlorella* sp. and *Porphyridium purpureum*): advantage of a swirling decaying flow. Bioproc Biosyst Eng. 2002;25:35-42.
[30] Renaud SM, Thinh L-V, Parry DL. The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. Aquaculture. 1999;170:147-59.
[31] Brown MR. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. J Exp Mar Biol Ecol. 1991;145:79-99.
[32] Lourenço SO, Barbarino E, Marquez UML, Aidar E. Distribution of intracellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors. J Phycol. 1998;34:798-811.
[33] Schwenzfeier A, Wierenga PA, Gruppen H. Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp. Bioresource Technol. 2011;102:9121-7.
[34] D'Souza FML, Kelly GJ. Effects of a diet of a nitrogen-limited alga (*Tetraselmis suecica*) on growth, survival and biochemical composition of tiger prawn (*Penaeus semisulcatus*) larvae. Aquaculture. 2000;181:311-29.
[35] Whyte JNC. Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. Aquaculture. 1987;60:231-41.

[36] Yao C, Ai J, Cao X, Xue S, Zhang W. Enhancing starch production of a marine green microalga *Tetraselmis subcordiformis* through nutrient limitation. Bioresource Technol. 2012;118:438-44.
[37] Dunstan GA, Volkman JK, Jeffrey SW, Barrett SM. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 2. Lipid classes and fatty acids. J Exp Mar Biol Ecol. 1992;161:115-34.
[38] Fabregas J, Herrero C, Cabezas B, Abalde J. Mass culture and biochemical variability of the marine microalga *Tetraselmis suecica Kylin* (Butch) with high nutrient concentrations. Aquaculture. 1985;49:231-44.
[39] Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. J Exp Mar Biol Ecol. 1989;128:219-40.
[40] Guillard RR, Ryther JH. Studies of marine planktonic diatoms. *I. Cyclotella nana Hustedt*, and *Detonula confervacea* (cleve) Gran. Can J Microbiol. 1962;8:229-39.
[41] Eteshola E, Gottlieb M, Arad S. Dilute solution viscosity of red microalga exopolysaccharide. Chem Eng Sci. 1996;51:1487-94.
[42] Wang X, Kolattukudy PE. Isolation of a protein containing covalently linked large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Botryococcus braunii*. Plant Physiol. 1996;111:441-5.
[43] Morris HJ, Carrillo OV, Almarales Á, Bermúdez RC, Alonso ME, Borges L, et al. Protein hydrolysates from the alga *Chlorella vulgaris* 87/1 with potentialities in immunonutrition. Biotecnol Aplic. 2009;26:162-5.
[44] Kim J-D, Lee W-S, Kim B, Lee C-G. Proteomic analysis of protein expression patterns associated with astaxanthin accumulation by green alga *Haematococcus pluvialis* (chlorophyceae) under high light stress. J Microbiol Biotechn. 2006;16:1222-1228.
[45] Pelah D, Marton I, Wang W, Shoseyov O, Altman A, Cohen E. Accumulation and protection activity of protease-resistant heat-stable proteins in *Haematococcus pluvialis* during high light and nitrogen starvation. J Appl Phycol. 2004;16:153-6.
[46] Wang S-B, Hu Q, Sommerfeld M, Chen F. An optimized protocol for isolation of soluble proteins from microalgae for two-dimensional gel electrophoresis analysis. J Appl Phycol. 2003;15:485-96.
[47] Safi C, Charton M, Pignolet O, Silvestre F, Vaca-Garcia C, Pontalier P-Y. Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. J Appl Phycol. 2012:1-7.

General Conclusions

Besides bioenergy, the valorisation of the bio-products became of major importance, and it will be unfortunate to neglect them, due to their highly added value. Therefore, algae scientists worldwide are largely agreeing the concept of algorefinery for the following reasons:

- Overall, they are economically viable, and it is possible to transform the deficit haunting this technology into profit.
- They can target multiple areas in the market (bioplastics, cosmetics, pharmaceuticals, nutrition, aquaculture and animal feed).

The strategy of the "Algoraffinerie project" was to implement a primary biorefinery in order to extract and fractionate the major biomolecules in an integrated process. Hence, at Laboratoire de Chimie Agro-Industrielle (LCA), we showed major interest on recovering the lipophilic and the hydrophilic fraction by taking into account the important role the cell wall and the ultrastructure can play on different levels, and on the fractionation of the aqueous phase obtained after cell disruption. Our additional challenge at LCA was to respect during our work the principles of "green chemistry", by avoiding the use of harmful solvents during the extraction and fractionation processes.

Since multiple microalgae attracted our attention in the sequence of studies conducted, and since the characteristics of the cells changed according to the species, it was important to have a deep understanding on the composition of the cell walls, the ultrastructure and the intra/extracellular location of the biomolecules composing the cells. This morphological and structural diversity influenced the calculation of the nitrogen to protein conversion factor (NTP) for each species, especially for the rigid cell walled microalgae that showed significant difference between the NTPs obtained for the raw microalgae and their protein extract. Furthermore, taking into account the recovery of proteins after testing different cell disruption methods on the same species, it could be noticed that the internal organelles can also show some resistance toward cell disruption. Nonetheless, depending on the cell disruption methods, the quality of proteins is likely to be different according to their amino acid profile, which suggests that not the same proteins are released and also brings additional insight that the morphological characteristics of the cells would hinder the liberation of these biomolecules.

The previous set of studies gave us a broader idea on the role of the cell wall on the release of the proteins. However, it is interesting to look more in depth on their diffusion behaviour based on different cell disruption methods. Hence, while the efficiency and non-efficiency of some cell disruption to break the cell wall was expected, the diffusion behaviour was not unexpected since slow diffusion was detected for almost all the methods applied. This implies that the success of a cell disruption method does not necessarily allow a faster diffusion of the biomolecules of interest in the aqueous phase. Moreover, it is possible to bypass the unit operation of cell disruption to extract lipids and pigments by adding ethanol with supercritical carbon dioxide, and by setting the optimum parameters for the extractor. Our results showed that lipids recovery (ethanol + $scCO_2$) from raw *Chlorella vulgaris* was significantly higher compared to the extraction after bead milling.

The last part of our work consisted of a continuous fractionation process that also takes into account the integrity of the biomolecules in the downstream process. The process was first tested on model suspensions and then extrapolated to the aqueous phase of the microalga. Hence, following cell disruption by high-pressure homogenization, the aqueous phase of *Tetraselmis suecica* contained starches, proteins, sugars and pigments. The highest concentration of these biomolecules was after homogenization at 1000 bar. Thus, it is worthwhile fractionating it to obtain separate fractions. The fractionation process is based on a two-stage ultrafiltration using two different molecular weights cut off for the membranes. Therefore, the first stage completely retained starches and pigments with the 100 kDa. The remaining filtrate was only composed of proteins and sugars. Further on, the second stage of the process succeeded to completely retain proteins of the filtrate using a 10 kDa membrane, and sugars were the only biomolecules detected in the second filtrate.

The following points could summarize the strong force of our work:

- The principles of “green chemistry” were respected in terms of the extracting solvent used.
- It has been shown that is not relevant to recommend a universal nitrogen to protein conversion factor, since many factors including the cell wall of the species could affect its calculation.

- It has been pointed out that despite the renowned role of the cell wall on biomolecules recovery, the ultrastructure of some microalgae could also show a resistance against some mechanical and chemical agents.
- New insight was brought on understanding the diffusion behaviour of some biomolecules after applying a cell disruption method.
- The unit operation of cell disruption could be avoided before supercritical carbon dioxide extraction of lipids.
- A groundwork has been set for a fractionation process that could be scaled up to an industrial level.

Theoretically every process has its advantages and inconveniences; some parts of our work represent different drawbacks for the following reasons:

- The most efficient cell disruption methods tested in our work such as bead milling and high-pressure homogenization remain costly in terms of energy input.
- The technique of $scCO_2$ is still considered energetic.
- Despite the success of the fractionation process, the recovery of the biomolecules of interest in the aqueous phase was not total.

George Bernard Shaw (1925 winner of the Nobel Prize of literature) quoted: ***"Science never solves a problem without creating ten more"***. This quote is inspiring, it could be read and understood in different contradictory ways. To my personal point of view, it reflects the beauty and richness of science that dwell in solving problems, innovating, creating, proposing new hypothesis and opening future perspectives. Therefore, taking into account the numerous investigations carried out during this research, additional scientific aspects can be further investigated:

- A life cycle assessment will be necessary to estimate the cost and the sustainability of the fractionation process.
- It will be interesting to understand more closely the diffusion phenomenon of the biomolecules in the aqueous phase. For instance:
    - ❖ Tracking which type of proteins is diffusing as a function of time after applying a method of cell disruption.
    - ❖ Obtaining more sophisticated microscopic images that would follow up with the morphological modifications during the process of cell disruption

- For the rigid cell walled microalgae, it is a serious challenge to find a cell disruption method that does not require high-energy input.
- It is worthwhile conducting a supercritical carbon dioxide extraction on wet microalgae, which will save us the cost of drying the biomass before the extraction.
- Define the optimal conditions for the biomolecules fractionation by membrane. Particularly, it should be define if appropriate diafiltration condition could produce more refined (pure) fractions.

Scientific production

Carl SAFI (2011-2013)

Papers in peer-reviewed international scientific journals

Papers accepted and published

1. **SAFI C.,** CHARTON M., PIGNOLET O., PONTALIER PY., VACA-GARCIA C (2013) Evaluation of the protein quality of *Porphyridium cruentum*. **Journal of Applied Phycology** 25:497-501

2. **SAFI C.,** CHARTON M., PIGNOLET O., SILVESTRE F., VACA-GARCIA C., PONTALIER PY (2013) Influence of microalgae cell wall characteristics on protein extractability and determination of the nitrogen-to-protein conversion factors. **Journal of Applied Phycology** 25:523-529

Papers accepted for publication

3. **SAFI C.,** CHARTON M., URSU AV., LAROCHE C., ZEBIB B., PONTALIER PY., VACA-GARCIA C (2013). Release of hydro-soluble microalgal proteins using mechanical and chemical treatments. **Algal Research**. DOI: 10.1016/j.algal.2013.11.017.

4. **SAFI C.,** CAMY S., FRANCES C., PONTALIER PY., VACA-GARCIA C (2013) Extraction of lipids and pigments of *Chlorella vulgaris* by supercritical carbon dioxide: influence of bead milling on extraction performance. **Journal of Applied Phycology**. DOI: 10.1007/s10811-013-0212-3.

5. **SAFI C.,** URSU AV., LAROCHE C., ZEBIB B., MERAH O., PONTALIER PY., VACA-GARCIA C (2013). Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. **Algal Research**. DOI: 10.1016/j.algal.2013.12.004.

Papers submitted or in preparation

6. **SAFI C.,** ZEBIB B., MERAH O., PONTALIER PY., VACA-GARCIA C (2013). Morphology, Composition, Production, Processing and Applications of *Chlorella vulgaris*: A review. **Renewable and Sustainable Energy Reviews**. Under review.

7. **SAFI C.,** ZHE LIU D., HAO JIE YAP B., MARTIN GJO., VACA-GARCIA C., PONTALIER PY (2013). Two-stage ultrafiltration process for separating multiple components of *Tetraselmis suecica* after cell disruption. **Separation and Purification Technology**. Under review.

8. **SAFI C.,** FRANCES C., URSU AV., LAROCHE C., POUZET C., VACA-GARCIA C., PONTALIER PY (2013). Release kinetics of biomolecules of *Chlorella vulgaris* after different cell disruption. **Bioresource Technology**. Under review.

9. **SAFI C.,** PONTALIER PY., VACA-GARCIA C (2013) Algorefinery Challenges and Scopes. A review. **Biotechnology Advances**. In preparation.

10. **SAFI C.,** PONTALIER PY., VACA-GARCIA C (2013) Biomass Nutrient profiles of *Haematococcus pluvialis* grown under different growth conditions. **Food Chemistry**. In preparation.

Poster presentations

1. **SAFI C.**, LIU D., YAP B., MARTIN G., PONTALIER PY., VACA-GARCIA C. Two-stage ultrafiltration process for separating multiple components of *Tetraselmis suecica* after cell disruption. 7th International Algae Conference. Hamburg-Germany (December 2013).

2. **SAFI C.,** PIGNOLET O., PONTALIER PY., VACA-GARCIA C. ALGORAFFINERIE: Integrated process for the whole volarization of the microalgae *Chlorella vulgaris* and *Porphyridium cruentum* Algae, new resources for Industry? Alg'n' Chem - Montpellier-France (November 2011).

3. **SAFI C.,** Pignolet O., Ursu A., Mati N., Laroche C., Vacca-Garcia C., Djelveh G., Michaud P. at "ABO - Algal Biomass Summit Minneapolis-USA (October 2011).
   Title: Extraction and quantification of polysaccharides from the microalgae *Porphyridium cruentum* and *Chlorella vulgaris*.

4. **SAFI C.,** PIGNOLET O., PONTALIER PY., VACA-GARCIA C. Influence of the washing parameters in the removal of nutriment medium in harvested microalgae. 1st International conference on Algal Biomass, Biofuels and Bioproducts. Saint Louis-USA (July 2011).

Oral presentations

SAFI C., PONTALIER PY., VACA-GARCIA C. Algoraffinerie: an integrated process for cell disruption and fractionation of *Chlorella vulgaris*. 8th Asia-Pacific Conference on Algal Biotechnology – APCAB 2012. Adelaide-Australia (July 2012).

SAFI C., PIGNOLET O., PONTALIER PY., VACA-GARCIA C. Les microalgues: un enjeu industriel et économique pour le futur. AGRIMIP INNOVATION-Biotechnologies vertes et semences Toulouse-France (October 2011).

Awards and Grants

1. **Springer Prize** for best Oral Presentation at the 8th Asia-Pacific Conference on Algal Biotechnology – APCAB 2012" at **Adelaide-Australia** (July 2012).
2. **SMI-2013 (8000 €)** for international mobilisation to The University of Melbourne. **Toulouse-France** (January 2013).
3. **Tremplin Jeune Chercheur (1500 €)** from Agrimip Innovation at **Toulouse-France** (July 2011).

Scientific Dissemination

1- International TV interviews:
   *MTV-Lebanon (www.youtube.com/watch?v=GvpvmVt6PF4)
   *OTV-Lebanon (www.youtube.com/watch?v=qvhcqHxYH0o)
2- Interview at l'Orient Le Jour (French Daily newspaper)

Abstract

A primary algorefinery, concept that deals with the main components of microalgae (lipids, proteins, carbohydrates and pigments), has been studied. A sequence of unit operations has been implemented in order to obtain separated enriched fractions of these biomolecules by conserving their integrity in the downstream process. The study was mainly centred on *Chlorella vulgaris*, a species known for its rigid cell wall. Most of the lipophilic fraction (lipids and pigments) was recovered using supercritical carbon dioxide with ethanol as a co-solvent, without a preliminary unit operation of cell disruption. The hydrophilic fraction (proteins and polysaccharides) was recovered in the aqueous phase after bed milling as cell disruption method. Subsequently, the aqueous phase was fractionated into three fractions by means of a process of two-stage ultrafiltration. Thus, starches, pigments, proteins and sugars were successfully separated from each other. A life cycle assessment will be necessary to estimate the cost and the sustainability of the fractionation process.

Keywords: biorefinery, microalgae, fractionation, ultrafiltration, cell disruption, nitrogen-to-protein conversion factor, *Chlorella vulgaris*

Résumé

Le concept d'une algoraffinerie primaire traitant les principaux composants de microalgues (lipides, protéines, glucides et pigments) a été étudié. Une séquence d'opérations unitaires a été mise en œuvre afin d'obtenir des fractions enrichies de ces biomolécules tout en conservant leur integrité dans le procédé en aval. L'étude a été principalement centrée sur *Chlorella vulgaris*, une espèce connue pour sa paroi cellulaire rigide. La majorité de la fraction lipophile (lipides et pigments) a été récupérée en utilisant du dioxyde de carbone supercritique avec de l'éthanol en tant que co-solvant, sans opération unitaire de cassage cellulaire préalable. La fraction hydrophile (protéines et polysaccharides) a été récupérée dans la phase aqueuse après broyage à billes comme méthode de cassage cellulaire. Par la suite, la phase aqueuse a été séparée en trois fractions par un procédé d'ultrafiltration en deux étapes. Ainsi, les amidons, les pigments, les protéines et les sucres ont été séparés les uns des autres avec succès. Une analyse du cycle de vie sera nécessaire pour estimer le coût et la durabilité du procédé de fractionnement.

Mots clés: bioraffinerie, microalgues, fractionnement, ultrafiltration, cassage cellulaire, facteur de conversion azote-protéines, *Chlorella vulgaris*

Résumé général

Chapitre 1 : Etat de l'art

Le XX^{e} siècle a connu une augmentation exponentielle de la population mondiale et une révolution industrielle qui a consommé sans limite les ressources fournies par notre planète. On estime que la population mondiale dépassera les 9 milliards d'habitants d'ici la fin de ce siècle, et que l'espérance de vie atteindra 85 ans. En outre, l'utilisation des combustibles fossiles de manière incontrôlée devrait mener à leur épuisement complet en 2050. Cette dangereuse interférence anthropique avec le système climatique, démontrée définitivement par le 5ème rapport du GIEC en 2013, a déjà entraîné une élévation sans précédent de la température, qui a contribué au réchauffement climatique, à l'augmentation du niveau des océans, à l'augmentation des catastrophes naturelles, et à d'autres catastrophes qui témoignent de l'ampleur de ces changements. Or, notre planète semble incapable de tenir cette pression, et des mesures seront donc requises pour limiter ces modifications.

La valorisation des microalgues en raison de leur importante diversité et des nombreux avantages qu'elles recèlent pourrait faire partie de ces mesures. Ces deux dernières décennies, cette biomasse a attiré l'attention de nombreux chercheurs autour du monde pour sa capacité à accumuler des lipides pour la production de biodiesel. Les microalgues peuvent en outre se développer dans l'eau à la fois douce et marine ainsi que dans presque toutes les conditions environnementales. Ainsi, elles n'entrent pas en concurrence avec les terres agricoles et ne provoquent pas de conflit avec la production alimentaire. De plus comme les microalgues consomment le dioxyde de carbone, elles peuvent être cultivées près des cheminées industrielles. Ce serait un moyen de traitement des effluents tout en produisant de biocarburants potentiels. Pour toutes ces raisons, la majorité des études se sont concentrées sur l'optimisation des techniques d'extraction et de production des lipides pour les transformer en biodiesel, mais ont ainsi négligé indirectement l'importance des autres biomolécules de hautes valeurs ajoutées présentes dans les microalgues. Or, la production de lipides est confrontée depuis le début à un mur freinant ses perspectives de développement car toutes les analyses de cycle de vie présentent cette production comme coûteuse, loin d'être compétitive dans le marché et non-durable. S'il est vrai que les biocarburants de

troisième génération à partir de microalgues sont un sujet passionnant et une technologie innovante, il serait regrettable, compte tenu de tous les avantages de cette biomasse, de se concentrer uniquement sur les objectifs bioénergétiques et de négliger les autres biomolécules.

Les microalgues reflètent une ancienne histoire qui a laissé une empreinte datant de 3,4 milliards d'années. Les plus vieilles microalgues connues, appartenant au groupe des cyanobactéries, ont été trouvées fossilisées dans des roches d'Australie occidentale. Les biologistes évolutionnistes estiment que les algues pourraient être les ancêtres des plantes. Ainsi, à travers le temps les algues ont donné lieu à d'autres plantes marines et ont colonisé la terre pendant l'ère paléozoïque il y a 450 millions d'années. Des études ont confirmé que jusqu'à nos jours leur structure est restée inchangée. Mais bien qu'elles soient anciennes, les microalgues sont des formes complexes et organisées.

Comme tout autre phytoplancton, ces micro-organismes ont une valeur nutritionnelle importante. Les premiers à consommer cette biomasse en tant que source de nourriture étaient les Aztèques et d'autres populations mésoaméricaines. Aujourd'hui, ces organismes microscopiques sont consommés en tant que complément alimentaire (*Chlorella vulgaris* et *Spirulina platensis* par exemple) et leurs biomolécules sont utilisées dans les colorants, les produits pharmaceutiques, l'alimentation animale, l'aquaculture et la cosmétique. Durant ces deux dernières décennies, la transformation a pris une nouvelle direction avec des applications motivées par l'épuisement des réserves de pétrole fossile. Les puissances mondiales se sont vu forcées à trouver des stratégies globales pour diminuer les rejets de dioxyde de carbone et proposer des ressources renouvelables alternatives, et à intensifier les recherches sur les biocarburants de troisième génération. Néanmoins une autre approche peut être envisagée, combinant la récupération des lipides et d'autres biomolécules, c'est le concept d'algoraffinage.

Nos travaux entrent dans le cadre du projet Algoraffinerie financé par l'Agence nationale de la recherche (ANR) et porte des enjeux scientifiques pour casser l'approche classique et surtout le cercle limitant dans lequel la recherche sur les microalgues continue de se focaliser, pour la production des biocarburants. Ainsi, l'objectif principal de ce projet est de mettre en place une bioraffinerie de première génération tout en prenant en compte l'intégrité des biomolécules dans le procédé en aval.

L'esprit de ce manuscrit demeure au sein des multiples publications publiées ou soumises dans des périodiques internationaux à comité de lecture.

1.1 Enjeux et considérations

Le concept de bioraffinage a été inspiré du concept de la raffinerie pétrolière. Il se conçoit comme une plate-forme qui intègre les procédés de fractionnement des différents composants d'une biomasse. Ainsi une bioraffinerie valorise les divers composants de la biomasse, afin de maximiser sa rentabilité. Toutes ces biomolécules peuvent générer des profits importants par rapport au biodiesel. Les protéines peuvent être vendues à environ 0,75 €/kg pour l'alimentation animal, et à 5 €/kg pour la nutrition humaine. Les glucides sont vendus sur le marché à environ 1 €/kg, et si des propriétés antivirales sont identifiées, le prix peut être extrêmement élevé. Les lipides pour les biocarburants génèrent le bénéfice le plus faible (environ 0,5 €/kg), ce qui est une raison supplémentaire pour ne pas se concentrer uniquement sur la production de biocarburants à partir de microalgues. Mais à la place, il sera plus rentable si ces biomolécules sont valorisées pour leurs acides gras insaturés (surtout les plus courts, vendus à plus de 2 €/kg). Enfin, les pigments sont aussi une ressource précieuse et leur prix peut largement varier en fonction de la pureté de l'échantillon et le marché cible (cosmétiques ou alimentaires des poissons par exemple).

Le concept d'algoraffinage nécessite une séquence d'opérations unitaires commençant par le prétraitement de la biomasse, par exemple en dépolymérisant chimiquement les polysaccharides de la paroi cellulaire des microalgues ou en effectuant un cassage mécanique. Ceci facilite l'accès du solvant d'extraction aux zones intracellulaires, et contribue à améliorer le rendement de récupération des biomolécules.

D'un côté, la dépolymérisation par hydrolyse des liaisons glycosidiques est omniprésente dans la nature et l'industrie. Toutefois, pour devenir un processus viable, l'hydrolyse doit être effectuée à la fois dans des conditions douces pour préserver l'intégrité chimique de tous les constituants, ou du moins leurs propriétés fonctionnelles.

Actuellement, les méthodes les plus utilisées sont le broyage à billes et l'homogénéisation à haute pression. Ces méthodes mécaniques sont souvent préférées en raison de la courte durée de séjour et les coûts d'exploitation. Néanmoins, ces méthodes génèrent des frictions qui surchauffent le milieu, qui doit être constamment refroidi tout au long du processus afin d'éviter la dénaturation des protéines ou de la dégradation thermique des lipides.

Le processus que nous avons étudié est une « algoraffinerie primaire » qui portera sur les principaux composants des microalgues (lipides, protéines, polysaccharides et pigments). Une séquence d'opérations unitaires sera mise en œuvre afin d'obtenir séparément des fractions enrichies de ces biomolécules. Par "fraction enrichie" nous comprenons le degré de pureté équivalent à la qualité « technique » des composés chimiques commerciaux.

Une fois que la portée de notre travail a été définie, nous allons maintenant présenter les différents défis relatifs aux options possibles parmi lesquelles une algoraffinerie pourrait être effectuée.

Option numéro 1 :

Ce processus commence par la rupture de la paroi de la cellule dans un milieu aqueux contenant entre 2 et 25% de la matière sèche en fonction de la méthode de lyse de la cellule appliquée. Après séparation de la matière solide (par exemple, filtration ou centrifugation), il serait obtenu un mélange émulsionné composé principalement de :

- Lipides de réserve (triglycérides)
- Protéines hydrosolubles
- Polysaccharides
- Pigments

Le solide serait composé principalement de biomolécules structurelles non libérées et des molécules solubilisées restées bloquées dans la matrice. Chaque fraction liquide doit subir des opérations unitaires supplémentaires de purification afin de produire des fractions enrichies des différentes molécules d'intérêt.

Option numéro 2 :

Après réduction de la teneur en eau à moins de 2%, une extraction par un solvant organique peut être effectuée pour récupérer dans la phase liquide les deux types de lipides (réserves et structurelles) ainsi que des pigments liposolubles. La fraction solide résiduelle serait composée de cellules dégraissées nécessitant une extraction pour libérer les composants hydrophiles intracellulaires. Contrairement à l'option numéro 1, la fraction aqueuse obtenue après cassage cellulaire ne serait pas émulsionnée. Le processus en aval serait donc plus facile à mettre en œuvre.

Globalement, nous reconnaissons que les deux options sont possibles et possèdent des avantages distinctifs. Le premier procédé est plus économique en termes d'apport d'énergie (pas de séchage), mais les compositions de la matière solide et le liquide émulsionné sont plus complexes, car ils contiennent tous les deux des composants hydrophiles et hydrophobes. Ce n'est pas le cas pour le second procédé dans lequel on recueille dans la phase organique, les deux types de lipides (structurelles et réserves) en une seule fraction, laissant toutes les biomolécules hydrophiles dans le culot. Pour ces raisons, nous avons décidé d'adopter l'option numéro 2 (Figure 1) pour le reste de l'étude. Toutefois, une limitation importante de ce processus est l'utilisation d'un solvant organique. Cet inconvénient pourrait être résolu en utilisant un solvant vert tels que le dioxyde de carbone supercritique.

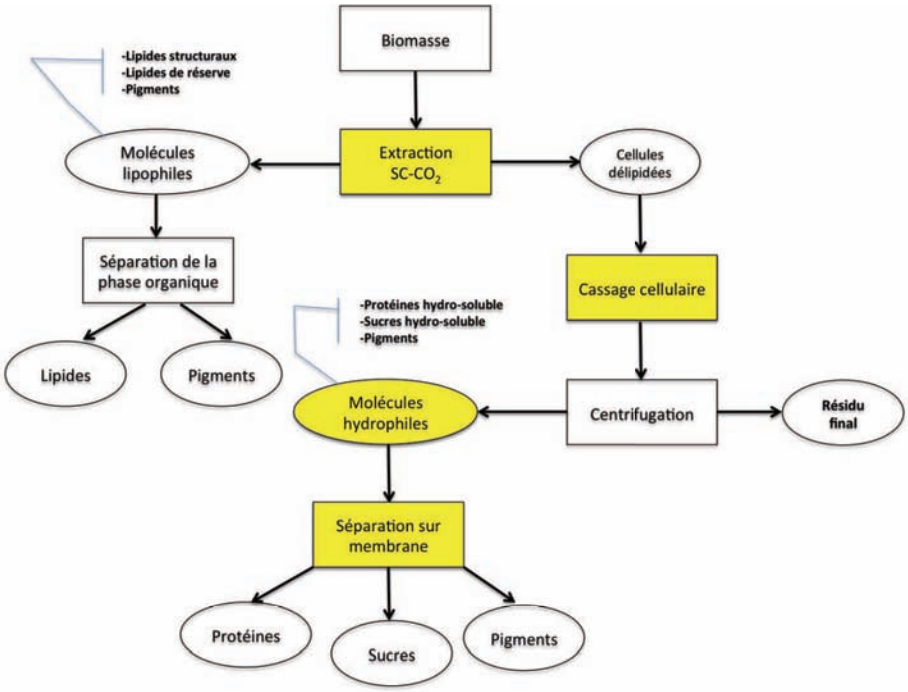

Figure 1 : Procédé de fractionnement sélectionné pour le reste de l'étude

Chapitre 2 : Récupération de la fraction lipophile

2.1 Publication # 2

La biomasse mise à notre disposition était cultivée dans des conditions de croissance normales. Cela implique que la microalgue n'a pas accumulé d'importantes quantités de lipides de réserve (triglycérides) comme elle le fait lorsqu'elle est soumise à des stress de croissance. Par conséquent, la majeure partie des lipides disponibles dans notre microalgue sont polaires (phospholipides), et sont principalement des lipides structuraux situés sur la paroi cellulaire et les membranes intracellulaires (chloroplastes, mitochondries, thylakoids). L'extraction de ces composants à l'extérieur de la paroi rigide de *Chlorella vulgaris* exigerait un traitement particulier afin de faciliter leur extraction et augmenter le rendement de récupération.

Il est généralement admis que l'une des opérations unitaires qui ajoute près de 30% supplémentaire sur le coût total de production est le cassage cellulaire. Ainsi, le chapitre est composé d'une publication soumise à ***Journal of Applied Phycology***, et qui examine s'il est possible d'éviter l'utilisation de cette opération unitaire avant l'extraction de la fraction lipophile au CO_2 supercritique. Par conséquent, différents aspects de l'extraction ont été évalués, avant et après cassage par broyage à billes, ou avec et sans la présence d'un co-solvant.

L'extraction des lipides et d'autres molécules lipophile à l'aide de méthodes respectant l'environnement et sans solvant est un grand défi pour l'industrie d'aujourd'hui. Une méthode verte prometteuse pour récupérer la fraction lipidique, y compris une partie des pigments, semble être le CO_2 supercritique. L'extraction des biomolécules à l'aide du CO_2 supercritique comme solvant, présente de nombreux avantages, dont le plus important est la non exposition du produit final à des solvants toxiques, qui apporte également une dimension supplémentaire pour la qualité du produit.

La littérature contient une série d'études qui déterminent les meilleurs paramètres pour extraire des lipides et des pigments à partir de microalgues. Deux études visaient à

démontrer les avantages de la rupture des cellules avant l'extraction par CO_2 supercritique. Par ce moyen, ils ont réussi à augmenter le rendement d'extraction d'un facteur 2. Notre étude propose l'utilisation de l'éthanol comme co-solvant afin d'éviter un apport énergétique élevé lié au cassage cellulaire.

L'extraction au CO_2 supercritique a été réalisée sur des lots de *Chlorella vulgaris* lyophilisés. Les essais ont été réalisés pendant 3 h de processus à 600 bar de pression, à 60°C et avec un débit de 30 $g.min^{-1}$ de dioxyde de carbone. Dans ces conditions le rendement d'extraction des lipides est de 67%, et les extraits contiennent 1,61 et 1,72 mg/g de chlorophylle et de caroténoïdes respectivement. Le broyage à bille, en brisant la paroi cellulaire permet d'augmenter les rendements d'extraction au CO_2 supercritique des biomolécules d'intérêt. Ainsi, dans les mêmes conditions, le rendement de l'extrait lipidique total, la chlorophylle et des caroténoïdes sont augmentés respectivement de 16%, 61% et 52%. Des essais d'extraction avec un co-solvant polaire ont également été effectués dans les mêmes conditions sur la microalgue brute non broyée. Les résultats montrent que l'addition de 5% d'éthanol sur la microalgue brute a augmenté de 27% le rendement total de l'extrait lipidique par rapport à l'essai sans broyage. La chlorophylle et les caroténoïdes ont également été affectés de façon significative par l'addition d'éthanol avec 81% et 65% d'augmentation par rapport à la microalgue brute. Cet effet est plus élevé que celui du broyage.

Selon les résultats décrits précédemment, il est possible d'en déduire qu'une pression de 600 bar permet au CO_2 supercritique d'atteindre l'espace intracellulaire, ainsi que la matrice intra-organites où les biomolécules cibles sont situées. Le débit de CO_2 est également un paramètre important qui devra être étudié afin d'optimiser les résultats obtenus. Le broyage à billes est une technique de cassage des cellules très efficaces qui permet d'augmenter le rendement de manière significative, mais comme elle nécessite de l'énergie qui augmente le coût de production, il ne semble pas pertinent de l'inclure dans le procédé.

L'ajout d'un co-solvant polaire, dans les conditions opératoires testées, augmente significativement le rendement et extrait des lipides et des pigments. Ce serait une technique alternative au broyage de cellules, avec un rendement plus élevé et un coût énergétique plus faible.

Les résultats obtenus montrent que l'extraction au CO_2 supercritique avec un solvant polaire est une technique d'extraction très sélective. Elle pourrait être utilisée comme une première étape d'un fractionnement sélectif, pour limiter le nombre d'étapes de purification dans le cas d'un procédé de bioraffinerie. Néanmoins, il est nécessaire de mieux définir les conditions optimales de fractionnement. En effet, le CO_2 supercritique est toujours considéré comme coûteux par rapport aux méthodes conventionnelles, et le degré de sélectivité est le paramètre clé à optimiser car il permet de réduire le nombre d'opération unitaire du procédé en aval. Ce sont des perspectives d'approfondissement de cette étude.

Chapitre 3 : Récupération de la fraction hydrophile : lumière sur le rôle morphologique des microalgues

Les microalgues représentent une grande biodiversité dépassant un million d'espèces. Elles appartiennent à différentes classes, et représentent une variété morphologique entre ces classes et dans les classes elles-mêmes. Ainsi, enquêter sur toutes les microalgues est une tâche complexe qui exige des décennies de recherche scientifique tout en sachant que la morphologie de ces espèces est susceptible de subir des modifications structurelles et ultra-structurelles en fonction des conditions de croissance et aussi au cours de leur croissance.

La paroi cellulaire joue un rôle important dans la régulation du transfert de biomolécules. Cette question représente une grande partie de ce chapitre en raison du rôle déterminant que les parois cellulaires jouent pour contrôler l'accès du solvant d'extraction des biomolécules intracellulaire d'intérêt. L'objectif principal de ce chapitre est de souligner le rôle des différentes structures de la paroi cellulaire de cinq microalgues différentes sur la quantification des protéines, et sur la récupération des biomolécules dans la phase aqueuse avant et après l'application d'un traitement de cassage qui cible essentiellement la paroi cellulaire. Les espèces de microalgues sélectionnées pour ce chapitre représentent un échantillon de la diversité morphologique des microalgues: *Arthrospira platensis*, *Chlorella vulgaris*, *Haematococcus pluvialis*, *Nannochloropsis oculata* et *Porphyridium cruentum*.

Les travaux décrits dans ce chapitre sont exposés sous forme de quatre publications. La première publication, déjà parue dans le ***Journal of Applied Phycology***, reflète le travail sur la définition du rôle de la paroi cellulaire lors de l'évaluation du facteur de conversion de l'azote en protéines des microalgues sélectionnées, et s'il est possible de recommander un facteur de conversion universel pour la quantification des protéines des microalgues. Il convient de mentionner que le même lot de chaque micro-algue a été utilisé au cours de notre travail, et donc les facteurs de conversion obtenus dans la présente publication ont été pris en considération pour les publications suivantes incluses dans ce chapitre.

La seconde publication (acceptée dans ***Algal Research***) intervient sur la réalisation d'un traitement chimique ou un traitement mécanique sur les parois cellulaires des cinq

microalgues, et s'intéresse à différencier le profil d'acides aminés obtenus notamment par l'évaluation de la fraction d'acides aminés essentiels et non essentiels qui clarifie si les mêmes protéines sont libérées en fonction de la nature du traitement appliqué sur la paroi cellulaire.

La troisième publication (acceptée dans ***Algal Research***), étudie la libération des protéines dans l'eau selon différentes méthodes de rupture cellulaires, afin d'évaluer le rôle des parois cellulaires ainsi que les organelles internes sur la libération de protéines dans de l'eau.

La quatrième publication (soumise à ***Bioresource Technology***) évalue la diffusivité des protéines et pigments dans le milieu aqueux suite au cassage cellulaire. Différentes méthodes de cassage ont été testées afin de comparer entre l'efficacité de la méthode et son impact sur la diffusivité.

Tous les traitements effectués sur les microalgues de ce chapitre ont été appliqués sans l'utilisation de produits chimiques nuisibles à l'environnement ou de solvants toxiques. Par conséquent, travailler dans ces conditions nous rapproche des principes de la chimie verte, qui est un de nos principaux objectifs qui se marie aussi avec les objectifs du "projet Algoraffinerie".

3.1 Publication # 3

L'analyse et la quantification de la teneur en protéines sont des facteurs clés qui méritent d'être examinés minutieusement. Il est vrai que la méthode colorimétrique de Lowry est une méthode précise pour la quantification des protéines et ne nécessite pas un facteur de conversion. Néanmoins, cette méthode ne détermine que les protéines hydrosolubles et non le contenu protéique total. En outre, l'efficacité de l'extraction des protéines risque d'être confrontée à la paroi cellulaire, ce qui peut empêcher la solubilisation de l'ensemble des protéines intracellulaires affectant ainsi la valeur du facteur de conversion. Par conséquent, l'impact des caractéristiques de la paroi cellulaire sur l'extractibilité des protéines doit être pris en compte et analysé afin d'éviter une mauvaise estimation de la teneur en protéines. Un point capital est de calculer précisément le facteur de conversion pour transformer l'azote en protéine. Ainsi, quand le facteur de conversion standard de 6,25

est utilisé, la méthode de Kjeldahl ou une analyse élémentaire conduisent à une surestimation ou sous-estimation de la quantité de protéines. En effet, ces deux méthodes prennent en compte la totalité de l'azote présent dans la biomasse à partir de laquelle 59 à 98% de l'azote total appartient aux protéines et le reste provient des pigments, des acides nucléiques et des minéraux.

Plusieurs études se sont focalisées à déterminer une méthode pour recommander le facteur de conversion; par exemple, une étude a porté sur l'obtention du facteur de conversion de cinq microalgues après la rupture de la paroi cellulaire, puis de trouver une corrélation entre la teneur en protéines et la teneur en azote total (Kjeldahl ou une analyse élémentaire). En conséquence, parmi les cinq microalgues un nouveau facteur de conversion a été estimé à 4,44 (analyse élémentaire) et 5,95 (Kjeldahl). Une autre étude a déterminé le facteur de conversion pour 19 algues tropicales récoltées directement de la plage et dans une seconde étude, 12 microalgues marines ont été analysées sous différentes phases de croissance et une valeur moyenne de 4,58 a été estimée. L'étude suivante évalue l'impact de la paroi cellulaire sur l'extractibilité des protéines et de l'évaluation du facteur de conversion pour cinq microalgues intensivement cultivées dans le monde et ayant une large diversité taxonomique.

Ces cinq microalgues différentes ayant des caractéristiques de parois cellulaires différentes, et leurs extraits protéiques. Les facteurs de conversion que nous avons obtenus grâce à la détermination du profil d'acides aminés pour les espèces brutes rigides comme *Chlorella vulgaris*, *Nannochloropsis oculata* et *Haematococcus pluvialis* étaient 6,35, 6,28 et 6,25, respectivement, mais pour leurs extraits protéiques les valeurs étaient de 5,96, 5,86 et 5,63. D'autre part, les facteurs de conversion obtenus pour les espèces brutes ayant une paroi fragile comme *Porphyridium cruentum* et *Arthrospira platensis* étaient 6,35 pour le premier et 6,27 pour le second, sans différence significative avec leur extrait protéique avec 6,34 pour le premier et 6,21 pour le second. En outre, le pourcentage de protéines hydrosoluble récupéré des protéines totales était de 80,3% pour *P. cruentum* et 69,5% pour *A. platensis*, mais inférieur pour *C. vulgaris* avec 43,3%, *N. oculata* avec 33,3% et *H. pluvialis* avec 27,5%.

En conclusion, l'extraction des protéines ainsi que l'évaluation du facteur de conversion apportent une preuve supplémentaire que la paroi cellulaire joue un rôle important dans la quantification des protéines. Cela implique que ne pas considérer cette approche pourra conduire à une fausse quantification de la teneur en protéines. En outre, pour les microalgues, il n'est pas possible de recommander un facteur de conversion universel pour toutes les espèces comme le montre notre étude et les nombreuses autres réalisées sur des dizaines de microalgues. Par conséquent, le facteur de conversion devra être évalué à chaque fois lorsqu'une quantification précise des protéines est nécessaire. De plus, cette étude a montré une corrélation entre la rigidité de la paroi cellulaire et/ou sa structure chimique et les différences de valeur des facteurs de conversion. Ce sera le cas par exemple lors de l'extraction de protéines à partir de microalgues ayant une paroi rigide. En effet alors que les microalgues avec une paroi cellulaire fragile n'ont pas montré de différences significatives avec leur extrait protéique, les microalgues ayant une paroi cellulaire rigide ont montré une différence notable de leur extrait protéique. Ces résultats montrent que pour ces derniers, l'extraction des protéines correspond à la sortie des protéines solubles qui ont une composition très différente des protéines structurales. Par conséquent, la rupture ou la perméabilisation de la paroi cellulaire de *C. vulgaris*, *N. oculata* et *H. pluvialis* est indispensable pour la récupération des protéines. Logiquement, il n'est pas nécessaire d'exercer une opération unitaire de cassage cellulaire pour *P. cruentum* car elle n'a pas une paroi cellulaire bien défini, et concernant *A. platensis* un cassage souple de la paroi cellulaire serait suffisant.

3.2 Publication # 4

L'objectif principal de cette étude est d'évaluer l'effet du cassage cellulaire sur l'extractabilité des protéines hydrosolubles de cinq microalgues ayant des caractéristiques de paroi cellulaire différentes. Deux techniques de cassage différentes ont été évaluées afin de caractériser le rôle de la paroi sur la libération des protéines.

Afin de relarguer les protéines dans la phase aqueuse, des traitements par homogénéisation haute pression ou sous conditions alcalines ont été appliquées pour fragiliser la paroi cellulaire de cinq microalgues. La caractérisation des protéines a été réalisée par l'analyse des profils d'acides aminés des microalgues brutes et de leurs extraits protéiques obtenus suivant les deux types de traitements. Les résultats ont montré que la

proportion de protéines libérées à partir de microalgues après deux traitements suivait l'ordre suivant: *Porphyridium cruentum* > *Arthrospira platensis* > *Chlorella vulgaris* > *Nannochloropsis oculata* > *Haematococcus pluvialis*, qui correspond à l'ordre de fragilité de la paroi cellulaire. De plus, le traitement mécanique a libéré plus de protéines pour toutes les microalgues par rapport au traitement chimique. Le rendement le plus élevé était pour *P. cruentum* ayant la paroi cellulaire la plus fragile avec 88% de protéines hydrosolubles des protéines totales, et le plus faible a été attribué à *H. pluvialis* ayant une paroi cellulaire très fragile avec 41% de protéines hydrosolubles. Le rapport entre les acides aminés essentiels et non essentiels, a été évaluée dans l'extrait et comparé au profil d'acides aminés des microalgues brutes. Le rapport de ce dernier est plus important après traitement alcalin et beaucoup plus élevé après rupture des cellules par homogénéisation haute pression. Ces résultats suggèrent que les acides aminés non essentiels sont plus concentrés à l'intérieur des cellules et confirment que ce ne sont pas les mêmes protéines qui sont libérées ou qui constituent la paroi.

3.3 Publication # 5

L'étude suivante évalue l'effet de cinq méthodes de destruction cellulaire sur l'extractibilité des protéines dans l'eau de cinq microalgues ayant différentes structures et ultrastructures. Ces cinq microalgues sont : La cyanobactérie *Arthrospira platensis*, qui contient une paroi cellulaire relativement fragile principalement composée de muréine. La Chlorophycée *Chlorella vulgaris* et l'Eustigmatophyceae *Nannochloropsis oculata*, qui ont une paroi cellulaire composée principalement de cellulose et d'hémicelluloses. La Chlorophycée *Haematococcus pluvialis* contenant une paroi cellulaire tridermique épaisse composée de cellulose et de sporopollénine. La composition de la paroi cellulaire, semblable à celui des spores, rend cette micro-algue moins perméable et très résistante aux traitements mécaniques et chimiques. La Rhodophycée *Porphyridium cruentum* est principalement composée d'une pseudo-paroi d'exopolysaccharides sulfurés. En outre, les microalgues sélectionnées dans cette étude ont un cytoplasme contenant des protéines solubles, et elles possèdent toutes un chloroplaste, sauf pour *A. platensis* qui est composée de faisceaux de thylakoïdes orbitant la périphérie du cytoplasme, les phycobilisomes (contenant les phycobiliprotéines) présents à la surface des thylakoïdes comme dans les chloroplastes de *P.*

cruentum. En outre, le chloroplaste contient également des protéines solubles et une pyrénoïde centrale composée de Rubisco.

La libération des protéines dans le milieu aqueux a été évaluée en lien avec la structure et l'ultrastructure des microalgues après avoir appliqué différentes techniques de cassages cellulaires : broyage manuel, ultrasons, traitement alcalin et homogénéisation haute pression. La concentration de protéines dans l'extrait a été déterminée pour toutes les microalgues et les résultats ont été examinés en tenant compte de la structure de la paroi cellulaire. L'augmentation de la concentration en protéines dans le milieu aqueux suivait l'ordre suivant: rupture des cellules à haute pression > traitement chimique > ultrasons > broyage manuel.

3.4 Publication # 6

L'étude suivante apporte un aperçu supplémentaire sur la compréhension de la diffusion des protéines et des pigments de *Chlorella vulgaris* dans le milieu aqueux, après avoir appliqué les différentes méthodes de cassage cellulaires. Les résultats sont obtenus par des observations microscopiques, par la quantification de la concentration des biomolécules d'intérêt, et par le calcul de leur coefficient de diffusion. Les observations microscopiques ont montré des cellules intactes après l'application de l'hydrolyse chimique et de l'ultrason. Cependant, la majorité des cellules ont perdu leur forme globulaire après le broyage à billes et l'homogénéisation à haute pression. En outre, la concentration en protéines augmente en suivant l'ordre: ultrasonication < hydrolyse chimique < homogénéisation à haute pression < broyage à billes. D'autre part, leur diffusion a suivi un ordre différent: l'hydrolyse chimique > broyage à billes > ultrasons > d'homogénéisation à haute pression. Les pigments n'ont pas été détectés dans la phase aqueuse après hydrolyse chimique, mais pour les traitements mécaniques leur concentration et leur diffusion ont suivi le même ordre que celui des protéines.

Ainsi, en se basant sur ces résultats, la diffusivité de ces biomolécules n'est pas directement corrélée à la concentration finale de l'extrait. Ces résultats montrent que les techniques chimiques testées libèrent uniquement les protéines de surface de la paroi, que la diffusion des protéines est donc libre, mais conduit à une faible extraction.

Les techniques de cassage de la paroi cellulaire qui permettent de maximiser la récupération des biomolécules intracellulaires, présentent des diffusions plus lentes. Il semble que même si l'action mécanique permet de détruire la cellule, elle n'est pas suffisante pour permettre une libre diffusion des molécules hors des cellules.

Chapitre 4 : Procédé de fractionnement du milieu aqueux

4.1 Publication # 7

Dans le deuxième chapitre, il a été démontré qu'il était possible de réaliser une extraction efficace des molécules organique (lipides et pigments) par CO_2 supercritique sur des microalgues brutes. Dans le contexte de l'étude de l'algoraffinerie, les étapes en avales auront pour principal objectif l'extraction et la purification de fractions enrichies en polysaccharides et protéines. En effet, ce sont les principales fractions restant dans la cellule après extraction au CO_2 supercritique.

Il n'existe que peu d'études réalisées sur cette séparation dans le domaine des microalgues, et généralement elles utilisent des procédés à membranes. Les travaux de ce chapitre se focalisent donc sur cette technique. L'objectif de l'étude (soumise à ***Separation and Purification Technology***) est de montrer qu'il est possible de fractionner ces molécules après broyage de la microalgue. La sélection de l'espèce de microalgue est basée surtout pour une question de disponibilité (stage en Australie) mais aussi, sur le fait de trouver une espèce ayant des caractéristiques intermédiaires à comparer avec toutes les espèces déjà étudiées dans ce manuscrit. Ainsi, *Tetraselmis suecica* était le meilleur compromis à notre disposition pour plusieurs raisons : le manque d'une couverture d'exo-polysaccharides qui risque d'augmenter le phénomène de colmatage ; la rigidité intermédiaire de la paroi cellulaire, et l'absence de lipides qui correspond à l'état des microalgues après extraction au CO_2 supercritique.

Dans un premier temps les conditions d'extraction ont été étudiées et différents extraits ont été produits. Un procédé d'ultrafiltration en deux étapes a été appliqué sur la phase aqueuse de *Tetraselmis suecica* après la rupture de sa paroi cellulaire par l'intermédiaire d'un homogénéisateur à haute pression. Mais avant d'appliquer le procédé de séparation sur la phase aqueuse de *T. suecica*, deux suspensions reconstituées contenant les biomolécules d'intérêt (amidons, sucres, protéines) étaient préparées afin de vérifier d'abord la faisabilité du procédé et d'obtenir les conditions nécessaires au fractionnement avec un extrait. Sachant que la taille des amidons est supérieure à 100 kDa, la première étape du

procédé d'ultrafiltration utilise une membrane de 100 kDa pour retenir l'amidon tout en permettant aux protéines et les sucres de passer dans le filtrat. De plus, selon la littérature la taille des protéines de *T. suecica* est comprise entre 15 à 50 kDa, et donc la seconde étape utilise une membrane de 10 kDa afin de retenir les protéines, tout en permettant de concentrer les sucres dans le filtrat. Les essais sur solution reconstituée ont permis de définir les conditions optimales de filtration. Par conséquent, le processus a été extrapolé sur la phase aqueuse de l'hydrolysat *T. suecica* avec une pression transmembranaire de 2 bars (30 psi) pendant 30 minutes après avoir effectuer une homogénéisation haute pression de 200 à 1000 bar pour casser la paroi cellulaire. Des observations microscopiques ont révélé des cellules complètement cassées à partir de 600 bar et la fragmentation des cellules était également perceptible après 800 bar. Mais, la concentration la plus élevée dans la phase aqueuse de toutes les biomolécules d'intérêt a été obtenue après 1000 bar à une température de 46°C. La filtration a été réalisée après centrifugation de l'extrait. Après chaque essai, la concentration des différentes biomolécules a été calculée pour les rétentats pour assurer le bilan matière. Les résultats ne montrent pas de pertes significatives des biomolécules à signaler entre les deux membranes. La rétention totale de l'amidon et des pigments a été possible avec une membrane de 100 kDa et les protéines ont été retenues avec une membrane de 10 kDa tout en permettant aux sucres d'être concentrés dans le filtrat.

Ces résultats montrent donc qu'il est envisageable de produire des fractions enrichies en utilisant deux étapes de filtration sur membranes. Néanmoins, les conditions doivent encore être optimisées afin d'augmenter la pureté des fractions obtenues, en utilisant une étape de diafiltration par exemple.

Conclusion générale

La valorisation des bioproduits des microalgues est devenue d'une importance capitale aussi importante que les biocarburants. Il serait une erreur de les négliger car ils présentent une haute valeur ajoutée, qui peut conditionner le développement du procédé de fractionnement. Cette vision contribue au développement de l'application du concept de bioraffinerie aux plantes.

La stratégie du projet Algoraffinerie est de mettre en œuvre une bioraffinerie de première génération afin d'extraire, fractionner et purifier les principales biomolécules dans un procédé intégré. Ainsi, au Laboratoire de Chimie Agro-Industrielle (LCA), nous nous sommes focalisés sur le rôle important que la paroi cellulaire et l'ultrastructure sont capables de jouer à différentes échelles. De plus, nous sommes aussi intéressé au fractionnement de la phase aqueuse obtenue après cassage cellulaire. Notre défi supplémentaire au LCA était de respecter au cours de nos travaux les principes de la « chimie verte », en évitant l'utilisation des solvants toxiques durant les procédés d'extraction et de fractionnement.

Au cours de nos recherches bibliographiques, plusieurs microalgues avaient attiré notre attention. Mais comme les caractéristiques morphologiques des cellules changent en fonction des espèces, nous avons essayé de caractériser la composition des parois cellulaires, des ultrastructures et la localisation intra/extracellulaire des biomolécules. Cette diversité morphologique nous a permis de déduire qu'il n'est pas pertinent de recommander un facteur de conversion (NTP) universel pour la quantification des protéines des microalgues à partir d'une analyse élémentaire, surtout celles qui possèdent une paroi rigide. Ces dernières ont montré une différence significative entre le NTP obtenu pour les microalgues brutes et leur extrait protéique. De plus, compte tenu de la récupération des protéines suite aux différentes méthodes de cassage cellulaire, il est possible de déduire que les organelles intracellulaires jouent également un rôle de résistance face aux méthodes de cassage. Néanmoins, selon la méthode de cassage, la qualité des protéines libérées est susceptible d'être différente en fonction du profile d'acides aminés. Ce qui laisse supposer que selon les conditions, ce ne sont pas les mêmes protéines qui sont récupérées. Les résultats montrent que les caractéristiques morphologiques des cellules entraveraient la libération de ces biomolécules. Il convient de mentionner que l'efficacité de toutes les méthodes de cassage a été évaluée par

des observations microscopiques confocales à balayage laser, et par la quantification des biomolécules d'intérêts avant et après l'application de la méthode.

La précédente série d'études nous a donné une idée plus claire sur le rôle de la paroi cellulaire sur la libération des protéines. Cependant, il serait intéressant de se pencher de manière approfondie sur la diffusion des biomolécules dans le milieu aqueux suite au cassage cellulaire. En effet alors que, l'efficacité des différentes méthodes de cassage était attendue, la cinétique de diffusion lente des ces biomolécules était surprenante. Ceci suggère qu'en dépit de l'efficacité d'une méthode de cassage, la diffusion des biomolécules n'est pas directement corrélée à l'efficacité de la méthode. De plus, les résultats indiquent qu'il est possible d'éviter l'opération unitaire de cassage avant d'extraire les lipides. L'étude montre qu'après avoir défini les paramètres d'extraction au pilote de CO_2 supercritique, l'extraction effectuée sur *Chlorella vulgaris* lyophilisée par le CO_2 supercritique et 5% d'éthanol avait permis d'obtenir un rendement significativement supérieur a celle obtenu après cassage par broyage à billes.

La dernière partie de nos travaux de recherche consistait à mettre en place un procédé de fractionnement continu, qui prend en compte l'intégrité des biomolécules. Premièrement, le procédé avait été testé sur des suspensions modèles contenant les mêmes biomolécules qui nous intéresse, et ensuite extrapolé sur le milieu aqueux de *Tetraselmis suecica* obtenu après cassage par homogénéisation haute pression. Différentes pression de cassage avaient été testées. La plus forte concentration de biomolécules libérées dans l'eau était obtenue à 1000 bar, et après centrifugation le surnageant contenait des protéines, des pigments, des amidons et des sucres.

Le fractionnement de cet extrait est basé sur l'ultrafiltration en deux étapes en utilisant deux membranes ayant des seuils de coupures différents. La première étape a entièrement retenu les amidons et les pigments avec une membrane de 100 kDa, tout en laissant passer les protéines et les sucres dans le filtrat. Les pigments, de nature hydrophobe, semblent être rejetés par la nature hydrophile de la membrane (Polyethersulfone), ce qui expliquerait leur forte rétention. La deuxième étape du procédé a retenu les protéines par une membrane de 10 kDa, tout en permettant aux sucres seuls d'être concentrés dans le deuxième filtrat.

Les points suivants pourraient résumer la force de notre travail :

- Les principes de la "chimie verte" ont été respecté en ce qui concerne le solvant d'extraction utilisé.
- Une quantification des protéines de microalgues précise nécessite le calcul du facteur de conversion. Il n'est donc pas pertinent de recommander un facteur de conversion universel pour les microalgues.
- Malgré le rôle connu de la paroi cellulaire sur la récupération des composants, l'ultrastructure de certaines microalgues pourrait également résister aux méthodes de cassage mécaniques ou chimiques.
- La vitesse de diffusion des biomolécules n'est pas nécessairement corrélée à l'efficacité de la méthode de cassage.
- Il est possible d'extraire de manière sélective les composés organiques (lipides et pigments) sans broyage des cellules.
- Il est possible de séparer l'amidon et les protéines contenus dans un extrait de microalgue par procédé sur membranes.

Théoriquement, chaque procédé a ses avantages et ses inconvénients; certaines parties de nos travaux représentent des inconvénients pour les raisons suivantes :

- Les méthodes de cassage les plus efficaces comme le broyage à billes et l'homogénéisation haute-pression restent coûteuses en termes d'apport énergétique.
- La technique du CO_2 supercritique est toujours considérée coûteuse.
- Malgré la faisabilité du procédé de fractionnement, le rendement de récupération des biomolécules extraites après cassage reste faible, surtout au niveau des protéines.

George Bernard Shaw (Lauréat du prix Nobel de littérature en 1925) a cité : **"La science ne résout jamais un problème sans en soulever dix autres"**. Cette citation peut être interprétée de différentes manières contradictoires. Mais de mon point de vue personnel, elle reflète la beauté et la richesse de la science qui résident dans la résolution des problèmes, l'innovation, la création, la proposition d'hypothèses et d'ouvrir de nouvelles perspectives. Par conséquent, tenant compte des nombreuses études menées au cours de nos recherches, les aspects scientifiques supplémentaires peuvent encore être étudiés :

- Une évaluation du cycle de vie sera nécessaire pour estimer le coût et la durabilité du procédé de fractionnement.
- Il sera intéressant de comprendre de plus prêt le phénomène de diffusion des biomolécules dans la phase aqueuse. Par exemple :
    - ❖ Etablir une méthode de suivi de diffusion des différents types de protéines en fonction du temps après cassage.
    - ❖ Obtenir des images plus précise des modifications morphologiques subies à la paroi cellulaire après cassage.
- Un grand défi sera de trouver une méthode de cassage pertinente et rapide, tout en évitant qu'elle soit coûteuse en terme d'énergie.
- Il sera utile de procéder à une extraction par le CO_2 supercritique sur des microalgues humides, afin d'économiser le coût de séchage de la biomasse avant l'extraction.
- Optimiser les conditions de fractionnement des biomolécules sur membrane. En particulier, il convient de définir des conditions appropriées de diafiltration pour obtenir des fractions plus raffinées.