UNIVERSITAT DE BARCELONA

Microalgae as a new source of chitosans

Derek Latil de Ros

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Derek Latil de Ros

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ABSTRACT

Chitosans are a family of linear polysaccharides generally obtained from the chemical deacetylation of chitin derived from crustacean shells that have been reported to have a wide range of bioactivities. However, the development of products taking advantage of these bioactivities has lagged behind expectations. The main reason is the poor reproducibility of the biological effects of chitosans due to a harsh chemical deacetylation process that impairs the structure of the polysaccharides inducing variability. Another reason is the immunologic responses that these polymers may produce in the presence of traces of crustacean proteins. It is therefore necessary to gather chitosans from other non-animal sources using a more environmentally friendly and reproducible process. Publications have commented that some microalgae species might contain chitin and even chitosan in their cell walls. Hence, in order to confirm these hypotheses, a screening method has been developed to detect each of these polymers specifically amongst the wide diversity of microalgae species of Greenaltech's library. Thanks to this screening method, natural chitins and chitosans have been found in several genera. The methodology has been validated with the extraction and physicochemical characterization of chitins and chitosans from *Chlorella*, something that, to our knowledge, had never been done before. At the same time, the enzymatic deacetylation process behind the natural production of chitosan in *Chlorella* has been studied emphasizing the identification of active chitin deacetylases. Finally, as part of an exploratory biofunctional study of microalgal chitosans, the antimicrobial, wound healing and nanocapsule-forming properties have been analyzed.

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I do not think anyone would say that writing a PhD thesis is an easy endeavor, certainly not me! I have required assistance in all sides; the research, the interpretation of the results, my personal life, work, etc., but thanks to the unconditional support I have received by deeply appreciated family, friends and colleagues, the writing of this thesis has been an enriching experience. I am very lucky to have had so many nice people by my side during this intense period of my life and all I feel now is an enormous gratitude.

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List of Abbreviations and Acronyms

- Arachidonic Acid (ARA)
- Acetylglucosamine (GlcNAc)
- Bold's Basal Media (BBM)
- Base pairs (bp)
- Chitin Deacetylase (CDA)
- Chitin Binding Protein (CBP)
- Chitosan Affinity Protein (CAP)
- Chitosanase (CsN)
- Chlorella Chitin Deacetylase (cCDA)
- Colony Forming Units (CFU)
- Complementary DNA (cDNA)
- Deoxyribonucleic acid (DNA)
- Degree of Acetylation (DA)
- Dimethylacetamide (DMA)
- Dimethylformamide (DMF)
- Dimethyl Sulfoxide (DMSO)
- Docosahexaenoic Acid (DHA)
- DOE Joint Genome Institute (JGI)
- Eicosapentaenoic Acid (EPA)
- Extracellular matrix (ECM)
- Enhanced Green Fluorescence Protein (eGFP)
- Epidermal Growth Factor (EGF)
- Expect Value (E)
- Fetal Calf Serum (FCS)
- Gene of Interest (GOI)
- Glucosamine (GlcN)
- High Performance/Precision Size-Exclusion Chromatography (HPSEC)
- Hydrophobic Interaction Chromatography (HIC).
- Homogenized and Lyophilized Biomass (HLB)
- Immunoaffinity Chromatography (IAC)

- Infra Red (IR)
- Lithium Chloride (LiCl)
- Luria Broth (LB)
- Mass spectrometer (MS)
- Milliamper (mA)
- Minimal Inhibitory Concentration (MIC)
- Minimal Bactericidal Concentration (MBC)
- Adjusted Minimal Bactericidal Concentration (aMBC)
- Minimal Fungicidal Concentration (MFC)
- Molar (M)
- Nanocapsules (NCs)
- Optical density (OD)
- Pattern of acetylation (PA)
- Phosphate Buffer Saline (PBS)
- Polidispersity Index (Ip)
- Polymerase Chain Reaction (PCR)
- Polyunsaturated Fatty Acid (PUFA)
- Proton Nuclear Magnetic Resonance (^{1}HNMR)
- Real time polymerase chain reaction (qPCR)
- Reverse transcription polymerase chain reaction (RT-PCR)
- Ribunocleic Acid (RNA)
- Room temperature (RT)
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
- Ultra Violet (UV)
- Weight-average molecular weight (Mw)
- Wild Type (WT)
- Weight / Volume (w/v)

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1 INTRODUCTION

The beginning of the 21st century marks a transition from an oil-based economy to a bio-based economy, from exploiting pollutant fossil finite resources to converting renewable biological resources into bio-based products ranging from food and feed to chemicals and energy[1,2]. Such a transformation is necessary to tackle the problems derived from the current unsustainable production and consumption patterns of our society. One of the main issues is climate change, which is a consequence of burning fossil fuels that release green house gases that otherwise would be trapped underneath the earth[3]. Another concern is resource scarcity; for instance, the global oil reserves are diminishing but the consumption continues to increase and some important producing countries have already reached their peak oil production[4]. There are many other benefits of a bio-based economy, such as increasing energy security by reducing dependence on the monopolized oil production or the generation of employment in rural settings[5].

In many fields, such as material sciences or energy, the transition to a bioeconomy to reduce oil consumption relies on a large and diverse group of biopolymers. While in the case of energy generation polymers are degraded to monomeric blocks to release the energy stored in them, in the case of renewable biomaterials, specific polymers are used based on their structural properties. Among the polymers used as biomaterials, there are a few that are attracting increasing attention because it is being discovered that they also possess functional bioactivities. The potential of such functional biopolymers

is evident, as they combine superior material properties with excellent biocompatibility and highly versatile biological activities that are applicable in medicine, agriculture, cosmetics, food sciences and many other different industries. Amongst the biofunctional polysacharides, chitin and chitosan are the most promising ones[6].

1.1 Chitins and Chitosans

Chitin and chitosan are described as a family of linear aminopolysaccharides consisting of varying amounts of β(1→4) linked residues of N-acetylated glucosamines (GlcNAc) and deacetylated glucosamines (GLcN) (Figure 1)[7,8]. What differentiates both polymers is the ability of chitosan to be dissolved in liquid acidic solutions (0.1M acetic acid), as a result of a higher percentage of GLcN units[9]. In general it is considered that when the polymer contains 60% GLcN residues or more it is chitosan[10].

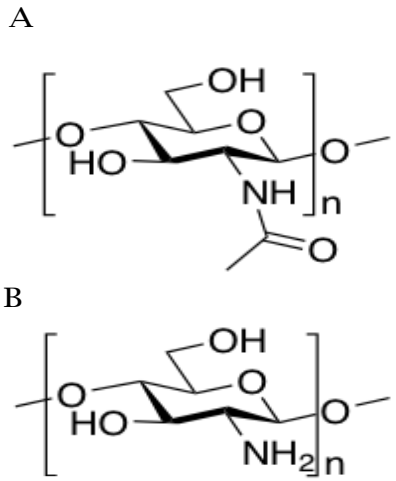

Figure 1 - Chitin and Chitosan structure.

A- Completelly acetylated chitin. B- Completely deacetylated chitosan

Chitin was first isolated from fungi by Braconnot in 1811[11]. These polysaccharides are widely distributed in nature, mainly as a structural component of the exoskeletons of arthropods (including insects and crustaceans), in the endoskeletons of mollusks such as squid and in the cell walls of fungi and diatoms[12]. Indeed, chitin is amongst the most abundant polymers in nature together with cellulose, hemicellulose, lignin and starch[7]. However, because of its insolubility in common solvents, chitin was considered intractable and thus for many years it stayed catalogued as a laboratory curiosity[11].

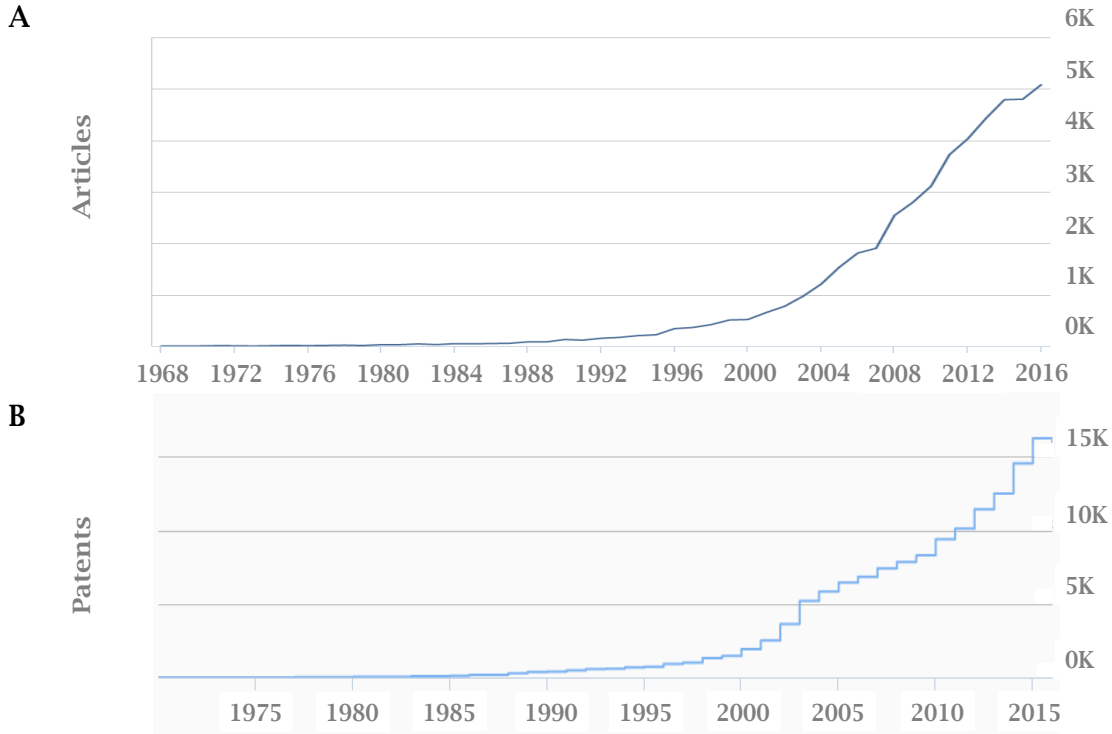

Figure 2 – Chitosan, a hot topic of research and innovation.

The figure shows the evolution in the number of publications per year in the last 50 years. A- Scientific articles per year containing the word chitosan (title, abstract or keyword. mainly articles, conference papers and reviews. Source: Scopus database). B- Patents published containing the word chitosan (granted patents and patent applications. Source: lens.org database)

In order to obtain chitosan, the deacetylation of chitin is required, a process that is very uncommon in nature. Only some fungi of the Zygomycota, Basidiomycota and Ascomycota phyla have been discovered to be capable of naturally producing chitosans[13]. As a result, the chitosan that can be found in the market all come from the chemical deacetylation of chitin. The deacetylated units have free amino groups that, at slightly acidic conditions, convey positive charges to the polymers, making them the only known polycationic polysaccharides[11,13]. Therefore, chitosans interact with polyanionic biomolecules such as proteins and nucleic acids and polyanionic phospholipidic membranes and sulfated polysaccharides like the human glycosaminoglycans at cell surfaces. As a consequence of these interactions, chitosan have been reported to have many bioactivities like: antimicrobial, wound healing, anti-oxidative, anti-inflammatory, mucoadhesive, immunostimulatory and ion chelating properties, amongst many others[10].

These bioactivities make chitosans the most advanced and promising biofunctional polymers, something that is drawing considerable attention from research and innovation standpoints (Figure 2).

1.1.1 Physicochemical Characteristics

Chitin and chitosan must not be regarded as a single polymer but as a family of polymers. For this reason not all chitins or chitosans can be used for every application. Depending on the particular characteristics of each chitosan it might have different properties, hence, a good characterization is paramount[10]. In fact, an incorrect characterization is one of the reasons why chitosan has historically faced reproducibility issues. Even today, commercially available chitosans are provided with very limited information about their traits[14]. The most important characteristics that have been directly associated with the functionalities of chitin and chitosan are the degree of acetylation, the molecular weight, the polydispersity and the cristallinty[10] (Figure 3).

The degree of acetylation (DA) is the percentage of GlcNAc monomers in the polymer. In the case of chitin this parameter ranges from 100 to 40% and for chitosan it goes from 40 to 0%[10] (Figure 3-A). It has an impact on the extent of moisture absorption, charge distribution, intrinsic viscosity and chitosan solubility in aqueous solutions[15]. The DA% can be determined by several methods such as ^{1}H-NMR spectroscopy, FTIR spectroscopy, UV spectrophotometry, elemental analysis, ^{13}C solid state NMR or titration methods, among others[15].

The weight average molecular weight (Mw) is an indication of the chain length of the polymer and it is given in kilodaltons (kDAs)[13] (Figure 3-C). Chitosan can be in the form of short oligomers or in the form o large polymers of over 1000 kDA, depending on the production methods and the source. This parameter is known to highly affect the physicochemical properties of chitosans, especially the solubility, the viscosity and the biocompatibility[13]. For this reason it is very important to determine the Mw in each batch of polymer produced. The most used techniques in the determination of the Mw are light scattering spectrophotometry and the measurement of the intrinsic viscosity[9]. The Mw of chitosan can be reduced by chemical, physical or enzymatic methods[13].

In case light scattering is used to determine the Mw, another parameter that can also be obtained is the polidispersity index (Ip). This value is a measure of the width of the molar mass distribution of the polymer[15] (Figure 3-C). Therefore, it is also an indication of the quality of the polymers; the more heterogeneous they are, the lower the efficiency and reproducibility. In general it is considered that an Ip value below 2 is an indication of low polydispersity and good quality[9].

The properties of chitosan also depend on its structure. There are three crystalline forms of chitins according to the different orientations of the polymer microfibrils: α-chitin (anti-parallel), β-chitin (parallel chains) and γ–chitin (every third microfibril has the opposite direction)[16] (Figure 3-D). These conformations are generally determined by X-ray diffraction but they can also be determined by FTIR spectroscopy[17]. Crystallinity is maximal for both fully acetylatd chitins (i.e. 0% deacetylated) and fully deacetylated chitosans (i.e. 100% deacetylated)[10]. Cristallinity is relevant because it affects the solubility and the ease of deacetylation of the polymers. It has also been reported that the origin of chitins also affects its cristallinity[10].

Another parameter that recently has started to be taken into account is the pattern of acetylation (PA). This pattern can either be random, non-random or in the form of blocks (Figure 3-B). The PA has a significant impact on the physicochemical properties (charge density), the digestibility of the polymer (enzyme recognition) and also on the solubility properties[13,18]. The PA is related to the method of preparation; the heterogenous process to deacetylate chitin yields block polymers and the homogeneous process yields non-random polymers. Only enzymatic deacetylation can produce non-random chitosans. Having non-random chitosans is essential to obtain chitosan oligomers with low dispersity[13]. The PA is generally determined by ^{13}C-NMR-spectroscopy[18]. Finally, other parameters that are also relevant, especially when chitosan is used for human consumption, are the determination of the ash, heavy metal, endotoxin and protein content and moisture[10].

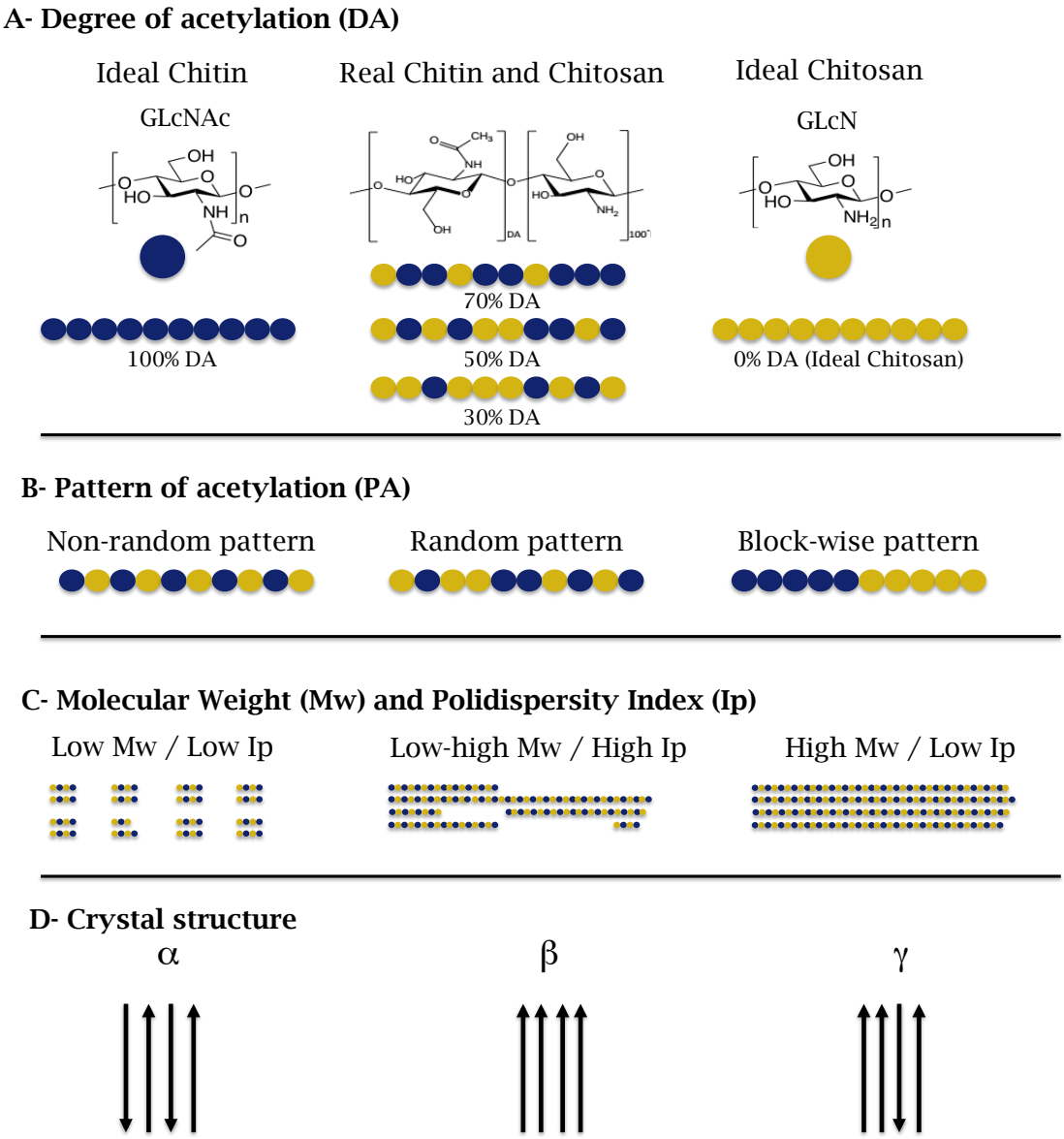

Figure 3 - Physicochemical characteristics of chitin and chitosan

1.1.2 Sources and production methods

1.1.2.1 Crustaceans

Chitin is present in many organisms. However, when it comes to the isolation of chitins at industrial levels, the raw material is usually crustacean shells obtained as a waste product from the food industry[8]. The extraction process involves the dissolution of the high calcium carbonate contents in acid. Also an alkaline extraction step to eliminate the proteins and a depigmention step are carried out[7,10].

The production of chitosans is done commercially mainly by chemically deacetylating chitins of crustacean origin. This process is based on hot alkali treatments to hydrolyze the acetamide groups of chitin. The concentration of sodium or potassium hydroxide ranges from very high (50%) to moderately high (13%)[10]. Also the temperatures can vary from 25ºC to 100ºC. In general, two major different methods to deacetylate chitins are known; the homogeneous method is the one using milder conditions and yielding better quality chitosans. However, the harsher heterogeneous method is more commonly used industrially[10].

Whichever of the aforementioned methods to deacetylate chitins are followed, high alkali effluents are generated making it an environmentally unfriendly process. Moreover, the structure of some of the polysaccharides gets impaired under such conditions, resulting in an increase in the polydispersity of weights and inconsistent levels of deacetylation[13]. Furthermore, the pattern of acetylation is not controllable and thus is completely random or block wise[18]. As a result of the variable physicochemical characteristics the bioactivity of chitosan is poorly reproducible[13]. At the same time, the crustacean origin is a disadvantage for the biomedical, pharmaceutical and cosmetics industries, mainly because of the risks of immunological responses that crustacean proteins tightly bound to the cationic polymers may cause[13,19]. Additionally, chitosans of crustacean origin has some other issues such as seasonal and geographical limited supply and variations[13].

1.1.2.2 Non-crustacean sources

In order to overcome the drawbacks of producing chitosan from chemically deacetylating chitin of crustacean origin, alternative producers of chitosan of of different origins have begun to appear. These producers take advantage of the chitin produced by the basidomycete *Agaricus Bisporus* (Kitozyme)[20]. Nevertheless, these companies still have to chemically deacetylate chitin if they want to convert it to chitosan[7]. Therefore, although the risk of allergic reactions is reduced with chitosan of a non-animal origin, the issues resulting from the chemical deacetylation process is not tackled.

A way to produce chitosan avoiding the chemical deacetylation process is to produce it naturally by enzymatic deacetylation. A selected group of fungal species, mainly from the zygomycetes class, is capable of doing so as a result

of the tandem action of chitin synthases (E.C. 2.4.1.16) and chitin deacetylases (EC 3.5.1.41)[13]. Fungal natural chitosan is more environmentally friendly, more consistent and do not cause immunogenic reactions. The only drawback is that sometimes these chitosans are complexed with glucans and other polysaccharides[7]. This promising technology still has not been established at industrial levels, although some companies planning on doing so like Mykodev Group have been created very recently[21].

Alternatively, it has been reported that some microalgae have considerably high quantities of glucosamine in their cell walls. Therefore, some authors have hypothesized that these microorganisms might also be capable of producing chitin[22–24]. Moreover, the release of the genome of the green microalgae *Chlorella variabilis NC64A* surprisingly showed that it contained 25 different putative chitin deacetylases. This has generated the suspicions that some microalgae might be capable of producing chitosan[25]. However, so far only the haptophyte *Phaeocystis* and some heterokontophytes of the Thalassiossirales order have been proven to be capable of producing α and β-chitin fibers, respectively[26,27]. No other chitins or chitosans have been obtained from photosynthetic organisms. Thus, further research is required to determine if microalgae can be another substitute of crustaceans for chitin and chitosan production.

1.2 Microalgae

1.2.1 General characteristics

Microalgae are microscopic, photosynthetic and unicellular or filamentous organisms that live typically in marine and freshwater ecosystems[28]. The word microalgae includes an enormous biodiversity as it is estimated that between 200,000 and several million species exist, compared with about 250,000 species of higher plants, although only a handful of them are cultivated in industrial quantities[29] The definition of microalgae includes both prokaryotic microalgae (Cyanophyta) and eukaryotic microalgae (formed by 9 divisions: Glaucophyta, Rhodophyta, Heterokontophyta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta and Chlorophyta)[30–32]. These groups are distinct in their evolutionary history and fundamentally different in their biology (Figure 4).

The origin of algae and plants is based on Cyanobacteria, the oldest photoautotrophs on earth[32]. Indeed, there is fossil evidence that cyanobacteria existed on earth 3.5 billion years ago and it is believed that it played a key role in the formation of the atmospheric oxygen required for all superior life to thrive[33]. Moreover, it is thought that cyanobacteria have evolved into today's chloroplasts of eukaryotic microalgae, macroalgae and plants[34–37]. Eukaryotic microalgae came from endosymbiotic events between unknown heterotrophic eukaryote and cyanobacteria between 1 and 1.5 billion years ago[35]. From these events, three primary microalgal eukaryotic lineages were created: Rhodophyta (red algae), Chlorophyta (green algae) and Glaucocystophyta (Figure 4). Afterwards, photosynthesis spread widely through secondary endosymbiotic events involving captures of either green or red microalgae[34,36,37]. The episodes involving Rhodophyta led to different eukaryotic microalgal lineages: Cryptomondas, Haptophytes, Heterokonts, Ciliates, Apicomplexa and Dinoflagellates (Figure 4). Some groups, such as apicomplexans, have already lost the ability to do photosynthesis, although they still retain cryptic plastids. In the case of ciliates, they are non-photosynthetic and have also lost the plastids[34,37]. In parallel, a secondary event involving Chlorophyta led to Euglenids and Chlorarachniophytes lineages after a secondary endosymbiosis (Figure 4). Moreover, from Chlorophytes land plants appeared later on[34–37]. In the Dynophyta division, tertiary endosymbiotic events involving the acquisition of plastids from cryptomonads, diatoms, haptophytes and green algae have also been reported[35,36].

Most microalgal species grow under an autotrophic metabolism in which only light, atmospheric CO_2 and inorganic nutrients are required for growth. This is the most common way of culturing microalgae because it is simple and sustainable, however self-shading issues leads to low density cultures and high harvesting costs[38]. A few microalgae species are also capable of growing under heterotrophic conditions (in the absence of light), replacing the fixation of CO_2 by the utilization of organic carbon sources dissolved in the culture media[39]. This culturing regime allows increasing the accumulation of biomass to obtain yields comparable to those of yeast in commercial fermentors[39]. A good compromise between autotrophic and heterotrophic growth regimes is mixotrophic culture, in which light, CO_2 and inorganic carbon sources are supplied to the culture[38]. Mixotrophy is accepted by a larger number of strains

and allows to take advantage of the high photosynthetic efficiency of microalgae while augmenting the productivity [38]. Apart from the carbon source and the light, there are many other requirements of nutrients (nitrogen, phosphate, sulfate, silicon etc.), micronutrients (iron, manganese, zinc, cobalt, copper, molybdenum, nickel, cadmium, selenium, etc.) and vitamins (biotin, thiamine, cobalamine) that vary between species[31,37,40]. Other critical factors are the light intensity, the pH, the temperature or the salinity[31,37,40]. In order to optimize culture conditions for the maximum growth or maximum product generation from a particular strain, all of these aspects need to be taken into account.

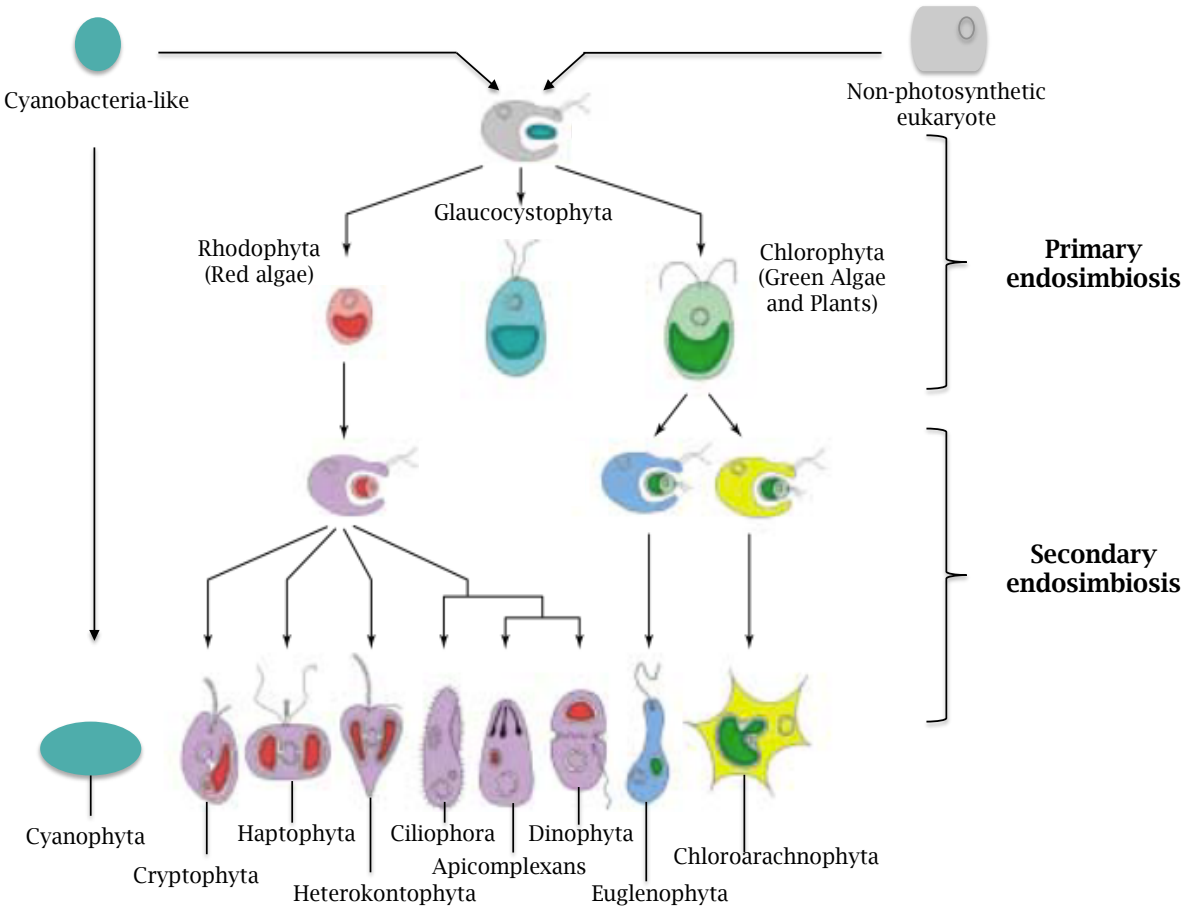

Figure 4 – The suggested view of plastid evolution leading to the appearance of eukaryotic microalgae, macroalgae and plants.

Adapted from Archibald et al. and Croft et al. [34,37]

Microalgal culturing takes place either in open ponds / raceways or in close photobioreactor systems. These two techniques were already in place in the 1950s but have been refined in the subsequent decades[41]. Open ponds or raceway systems are simple methods that allow culturing under autotrophic or mixotrophic regimes large amounts of biomass under the local environmental conditions by just applying mixture to recirculate. Most commercially exploited microalgae such as the Chlorophytes *Chlorella, Haematococcus* and *Dunaliella,* the Heterokontophyte *Nannochloropsis* or the Cyanophyte *Arthrospira*, are cultured following this method[40]. The main advantage of open ponds are the low construction and operation costs[39]. The disadvantages are that contamination may occur, especially if it is not a local, fast growing strain or a extremophile[39]. Moreover, open ponds are restricted to tropical and subtropical regions and low yields are obtained with respect to photobioreactors and fermentors[40]. Closed microalgae photobioreactors offer advantages in terms of avoiding contamination, increasing yields and completely controlling the growth conditions, allowing for the production of pharmaceutical goods[39]. They are transparent reactors that allow the light to penetrate so that microalgae inside can do the photosynthesis and are generally agitated with bubbles of CO_2. The issue with these closed systems is that they require a higher capital investment and improvements in productivity might not necessarily outweigh the costs[41]. In case the microalgae are grown under heterotrophic conditions they can be perfectly grown in any fermenter as no light is required[39,40].

1.2.2 Products from microalgae

Microalgae have been exploited by humanity for thousands of years. The first reported use by humans is when Chinese introduced *Nostoc* and other edible cyanobacteria as an emergency food source around two thousand years ago[29,31]. Nevertheless, microalgae mass cultivation did not start until the 1960's when several *Chlorella* cultivation facilities were established in Japan, using circular ponds to produce biomass sold as "health food"[42]. Indeed, it is for human nutrition or for aquaculture that these photosynthetic microorganisms are mostly cultured today[28]. The biochemical composition of microalgae varies widely between species and depends on environmental parameters. However, in general it can be said that most microalgal species exhibit high protein

content (30-70%) and are capable of synthesizing all amino acids, providing the essential ones to humans and animals upon consumption[29]. Other compounds that are of particular interest and highly valued for nutrition include polyunsaturated fatty acids (PUFAs) of the omega-3 and omega-6 families, chlorophyls, carotenoids, phytosterols, tocopherols, bioactive polysaccharides, fibers, and all essential vitamins[41,43–46].

Microalgal overall digestibility is high, which is why there is no limitation to using dried whole microalgae in foods or feeds[29]. Only in some cases like *Chlorella,* the cell wall is not digestible and in consequence the cells must be disrupted to make the intracellular compounds bioavailable for human and other non-ruminants[47]. For human nutrition the whole or partially disrupted microalgae is sold as a nutritional supplement or mixed in foods, beverages or tablets claiming a wide range of positive effects such as the alleviation of disorders like: hypertension, malnutrition, constipation, gastric ulcers, hyperlipidemia and elevated serum glucose level, preventing the appearance of cancer, cholesterol, atherosclerosis and having antioxidant, immunostimulatory and free radical scavenging properties[46]. The most utilized genera are *Arthrospira (Spirulina)*, *Chlorella, Haematococcus* and *Dunaliella,*[28,29,46]. Microalgae powders containing the whole cell are also incorporated in animal feeding, especially in fisheries. In fact, 30% of the current world microalgal production is used in animal feeding[25]. Microalgae are used to provide more balanced nutrition and improve animal growth. The main genera used in animal nutrition are the Chlorophytes *Chlorella* and *Tetraselmis,* the Haptophytes *Isochrysis* and *Pavlova* and the Heterokontophytes *Phaeodactylum, Chaetoceros*, *Nannochloropsis, Skeletonema* and *Thalassiosira*[28,47,48].

The microalgal biomass, besides being sold as whole biomass for nutrition, contains valuable compounds that are extracted and successfully marketed. Carotenoids are accessory pigments in the photosynthetic apparatus of microalgae. They may also accumulate as secondary metabolites under stress conditions. Their antioxidant potential is the main reason of their exploitation[44]. Although there are over 400 known carotenoids only a few such as β-carotene, astaxanthin and lutein are commercialized from microalgae. The Chlorophytes *Dunaliella* and *Haematococcus* are the two most cultured genera

for the commercialization of microalgal carotenoids[28,43]. Additionally, microalgae have been shown to produce long chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA, 20:5 ω 3) (the Heterokontophytes *Nannochloropsis, Phaeodactylum* and *Nitzschia* or the Haptophytes *Isochrysis, Diacronema*), docosahexaenoic acid (DHA, 22:6 ω 3) (the Dianophytes *Crypthecodinium* and *Schizochytrium*), γ -linolenic acid (GLA, 18:3 ω 6) (the Cianophytes *Arthrospira*) and arachidonic acid (AA, 20:4 ω 6) (the Rhodophyte *Porphyridium*). These long chain PUFAs play a structural role in microalgae membrane and serve as precursors of eicosanoids. Enhancing LC-PUFAs levels in human diet has been demonstrated to be beneficial for human heath[43,46]. It is estimated that only a healthy human can generate EPA and DHA but to a very limited extent, for this reason it is important to include these fatty acids in daily diet[44,46].

Although not yet a commercially available product, the use of microalgae for biofuel production has been a hot topic in research in the last years, especially during the years of high petroleum prices, and has attracted considerable public and private funding. Microalgae produce high amounts of oils that could be used as a potential replacement for fossil fuels if it can be produced at competitive prices[30,41,49]. Additionally, microalgae biomass contains carbohydrates that can be fermented to make bioethanol or other valuable chemical building blocks[50]. Although microalgal biofuels are not commercially available yet, all the research performed has allowed to create the concept of biorefineries. The idea behind it is to integrate several processes and industries to obtain as many possible different products from the same biomass in order to make the production of food, energy and chemical commodities from microalgae affordable. It is called biorefinery because it mimics the successful petroleum refinery model[48,51].

As it can be observed in Figure 5, practically none of the products that are currently obtained from microalgae most of the products that can be obtained from microalgae come from the cell wall. Indeed, in most cases the cell wall is discarded or included with the rest of microalgal products to avoid the separation step claiming that it is a good source of dietary fiber. Nevertheless, a much higher value can be obtained from the microalgal cell wall by extracting some of the polysaccharides it contains. In this way new products

can be added to the microalgal biorefinery to make the microalgal cultivation more profitable. For instance, as it has been indicated in subsection 1.1.2.2, there are a few research articles suggesting that there could be chitins and chitosans in the cell wall of some microalagae.

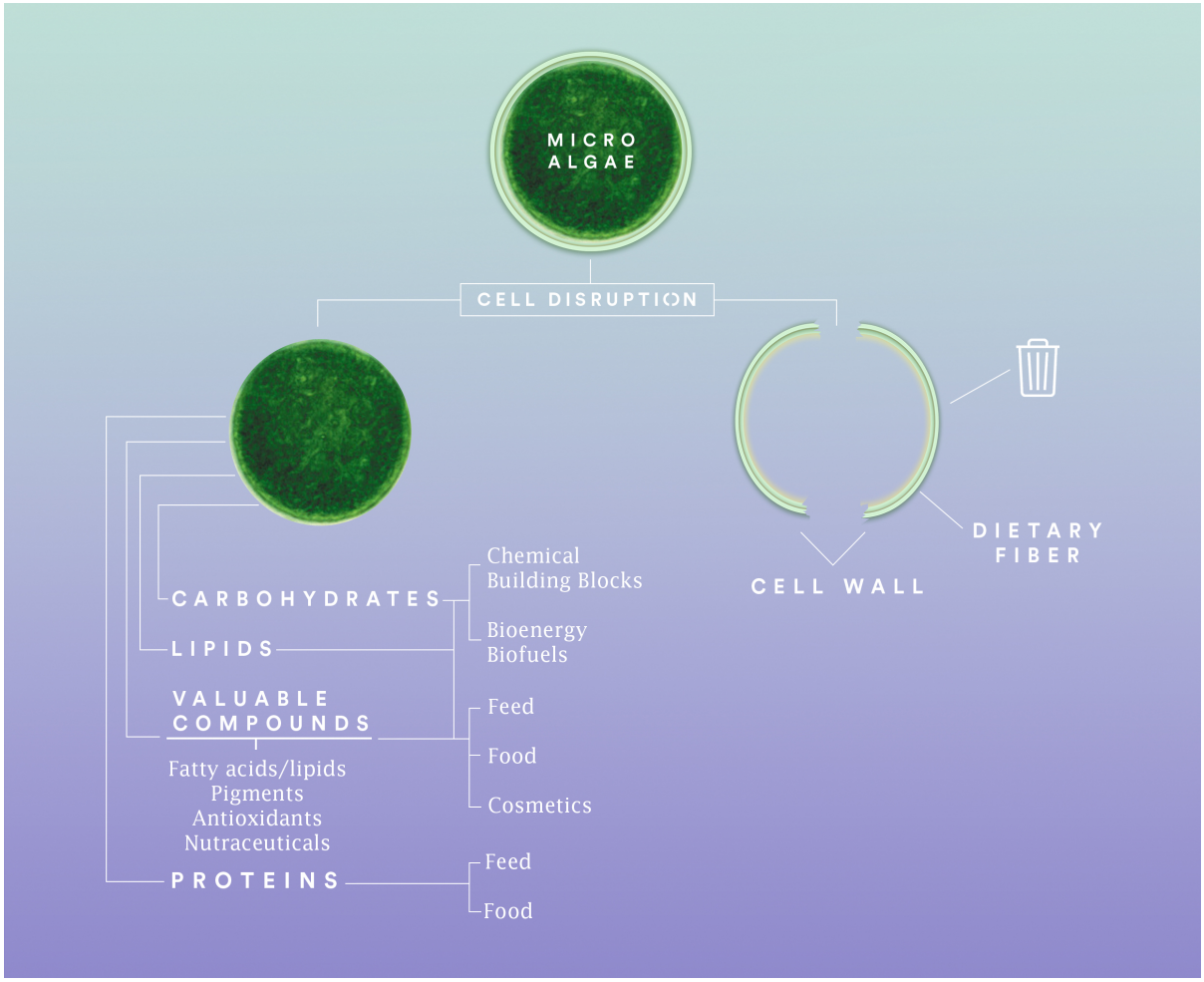

Figure 5 - Compounds that can be obtained from microalgae and their uses.

The image shows how currently all valuable products are extracted from the cell while the cell wall in most cases is discarded or used as dietary fiber in the best case.

1.3 Objectives

On the one hand, there is a need for a more environmentally friendly process to reproducibly obtain chitins and chitosans from non-animal sources. On the other hand, the microalgal biorefinary model requires more products to be successful and the cell wall is currently underexploited. Both needs could be fulfilled if the reports indicating the presence of chitins and chitosans in some microalgal cell walls were confirmed and microalgae could become a new source of these polymers. Therefore, this project aims to:

i. Design a method to screen microalgae for the presence of chitins and chitosans.

ii. Validate this screening method using standard techniques of chitin and chitosan research.

iii. Design a method to extract the chitins and chitosans detected.

iv. Characterize the polymers with standard techniques used in chitin and chitosan research.

v. Explore the molecular mechanisms behind the natural process of chitin and chitosan production.

vi. Explore the bioactivities and possible applications of these new polymers.

1.4 Greenaltech and the Nano3bio

This PhD Thesis project has been carried out at Greenaltech, S.L., a private company devoted to the commercial exploitation of microalgae to serve the needs of the health and wellbeing markets. Greenaltech possesses the necessary equipment to carry out the project within its molecular biology and organic chemistry laboratories. Moreover, Greenaltech has a pilot plant to produce any required biomass within the scope of the project in bioreactors and, what is most important; it owns a varied microalgae library that can be screened for the presence of chitins and chitosans. This library harbors a collection of approximately 150 different microalgal strains belonging to the 5 main phyla (Rodophyta, Chlorophyta, Cyanophyta, Heterokontophyta and Dinophyta) and also the less common phyla Chryptophyta. Around 60% of these species were isolated and the rest were obtained from public libraries. The research presented here has been performed as part of the European FP7 project Nano3Bio, of which Greenaltech is a member. Nano3Bio's main goals are to obtain biotechnologically produced chitosans and to explore their applicability as raw materials for medicine, cosmetics, agriculture and many other fields.

2 SCREENING MICROALGAE FOR THE PRESENCE OF CHITINS AND CHITOSANS

2.1 Background and aims

The existence of glucosamines (GlcN) in the walls of certain microalgal species has been previously described, however, it alone does not ensure the presence of chitins and chitosans. On one hand, chitin is a linear polysaccharide composed of β(1→4) linked units of acetylglucosamines (GlcNAc). On the other hand, chitosan is a linear polysaccharide obtained by extensive deacetylation of chitin, hence, it is composed of two kinds of β(1→4) linked units: GlcNAc and GlcN (Figure 1)[52]. Moreover, the two mentioned polymers are not the only structures in which glucosamines can be found in a cell. In general, aminosugars can be components of glycoproteins, proteoglycans or other polymers such as hyaluronic acid or chitin-glucan compounds[24,53]. Therefore, chitins and chitosans entail a complex structure than cannot be inferred based on the presence of glucosamines only.

Some researchers have tried to demonstrate that the glucosamines found in the cell wall of certain microalgae are indeed forming part of the polymeric structure of chitins and chitosans. Crystallographic, chemical and enzymatic techniques have been used to demonstrate that Heterokontophytes of the

Thalassiosira and *Cyclotella* genera, both members of the Thalassiossirales order, are capable of secreting long thin β–chitin fibers[54–56]. β-chitin shows parallel packing and can be found in other organisms like worms or squid. This contrasts with α-chitin, which has an anti-parallel packing and is chitin's most common structure as it can be found in arthropods, crustaceans and fungi[57]. A highly crystalline version of this structure has been found to be ejected by the Haptophyte *Phaeocysitis,* from which it was extracted and investigated by FTIR spectroscopy, electron microscopy, X-ray diffraction and electron diffraction analysis.[26,58]

The Chlorophyta *Chlorella* is another genus that has accumulated some evidence of the presence of chitins and chitosans in its cell wall, although in this case only a very limited number of inconclusive publications are available. The presence of GlcN is a well-known characteristic of the cell walls of this genus[23,53,59–64]. Some authors refer to it as a component of glycoproteins embedded in the structure of the cell wall[23,59]. Other authors indicate that GlcN is polymerized to form chitin based upon extrapolations from the presence of GlcN[23], something that cannot be directly assumed, as previously commented. Infrared spectroscopy, X-ray diffraction and chitinases that degrade the cell wall of *Chlorella*, were used to identify a glycosaminoglycan that was described as a chitin-like glycan[22,24]. The secretion of chitin fibers by *Chlorella* infected by Chloroviruses has also been documented, although the production of the polymer can be attributed to the chitin synthases from the viral genome[65–67]. Finally, the presence of a chitosan-like polymer in the cell wall of *Chlorella* has also been suggested. Two studies reached this conclusion based on digestions with chitosanases[24,68]. However, in those assays chitosanases are mixed with other enzymes, so, although the addition of chitosanases improves the digestion of the cell walls, its action alone was not tested. Moreover, chitosanases are able to digest very small oligomers, and thus its activity is not an indication of the presence of chitosan polymers. In another study, chitosans are detected based on colorimetric reactions[69]. In this case what is questionable is the specificity towards chitosans of these reactions. In all, there are no studies in which these polymers have been extracted and characterized, so it has never been really confirmed that the glucosamines found in the cell wall of *Chlorella* are arranged in the form of chitins or chitosans.

The increasing availability of genomic information is further supporting the hypothesis that microalgae may be contributors to the chitin and chitosan reservoir. The availability of genomic sequences from the Heterokintophytes *Thalassiosira pseudonana*[70] and *Phaeodactylum tricornutum*[71] has revealed that all genes necessary for the synthesis of chitin are present in both species, despite the fact that *P. tricornutum* does not produce chitin fibers. Similarly, homologous genes to chitin synthases have been discovered in *Skeletonema costatum, Chaetoceros socialis* and *Lithodesmium undulatum,* suggesting the possible presence of chitins[27]. The genome of *Chlorella Variabilis NC64A* was published in 2010 and it revealed the existence of several gene sequences identified as homologous to genes responsible for chitosan synthesis and degradation[25]. The hypothesis to explain the presence of these sequences in *Chlorella* is horizontal transfer from virus[25]. In case these enzymes were active and produced deacetylated chitins, *Chlorella* would be the sole photosynthetic organism known to produce chitosans naturally.

The aim of this chapter is to confirm the previous studies that suggest that there are chitins and chitosans in some *Chlorella* cell walls. Moreover, the presence of these polymers in diatoms is also studied, putting special interest on the identification of chitosans. Furthermore, taking advantage of the diversity of species in Greenaltech's proprietary microalgae library, a wide variety of other species with no previous background about the production of chitins and chitosans are also analyzed.

The development of a specific but yet flexible high-throughput screening technique that allows the detection of chitins and chitosans in diverse microalgal biomasses is the key to achieve the above stated goals. The results from this screening are validated with more thorough techniques such as a glycosidic linkage analysis of the microalgal cell walls. Further validations of the screening are also performed by extracting chitin and chitosan-containing fractions and characterizing them with standard techniques used for this purpose such as proton nuclear magnetic resonance (^{1}H-NMR), Fourier transform infrared spectroscopy (FTIR), hydrolysis using chitinases and chitosanases or high-performance size exclusion liquid chromatography (HPSEC).

2.2 Results

2.2.1 Screening through the microalgal diversity for the presence of chitins and chitosans

As it has been previously mentioned, it is known that *Thalassiosira* and *Cyclotella* are capable of producing chitins. The hypothesis that there could be other microalgal chitin producers, especially *Chlorella*, and also the hypothesis that some of them might be able to naturally convert these chitins into chitosans, is what this chapter is aiming to confirm. In order to do so, a method to easily, rapidly and reproducibly detect the presence of chitins and chitosans in a wide diversity of microalgae was developed.

2.2.1.1 Design of a high-throughput method to detect chitins and chitosans in microalgae

A technique was designed with the main goal of selectively detecting chitins and chitosans in microalgal biomass. Differentiating between the two polymers was very important due to the fact that there are some indications that microalgae might be capable of naturally producing chitosans; something that is very rare in nature and to our knowledge had never before been detected in other organisms besides a few fungi of the Zygomycota, Basidiomycota and Ascomycota phyla[13].

The fact that it is not common to find organisms producing both polymers made it difficult to determine a way of performing the desired assay. On the one hand, widely used tools to detect both polymers such as calcofluor white or eosin Y offer no specificity[72]. On the other hand, no lectins and no antibodies with specificity to chitosans are available[72]. Fortunately, Dr. Moerschbacher from the University of Münster suggested using chitin binding proteins (CBPs) and chitosan affinity proteins (CAPs), which had recently been developed in his laboratory (Personal communications)[72,73]. While CBP was the chitin-binding module of a Chitinase from *Bacillus licheniformis* DSM13, CAP was a Chitosanase (CSN) from *Bacillus sp.* MN with no hydrolytic activity, as it had been inactivated by site directed mutagenesis[72,73]. Both proteins were expressed in *E. coli* fused to the enhanced green fluorescent protein (eGFP) that could be detected using fluorescence microplate readers or fluorescence

microscopy[72,73]. The high specificity of CBPs for chitins and CAPs for chitosans had been demonstrated[73]

Therefore, a method to directly detect chitins and chitosans in microalgae was developed based of previous assays in which chitins and chitosans had been specifically detected with CAPs and CBPs[72,73]. Briefly, as Figure 6 shows, the first step of the technique consisted in the cultivation, harvesting and freezing of microalgal biomass. In order to detect both secreted chitins/chitosans and those embedded in the cell wall, each strain was analyzed with and without previously being disrupted. Then, the biomasses were incubated with CAPs, CBPs or nothing (as a control). Finally, the signal given by the eGFP protein fused to the CAPs and CBPs that was attached to the targeted polymer was measured in a fluorescent microplate reader. The method can be found in more detail in subsection 2.4.2.

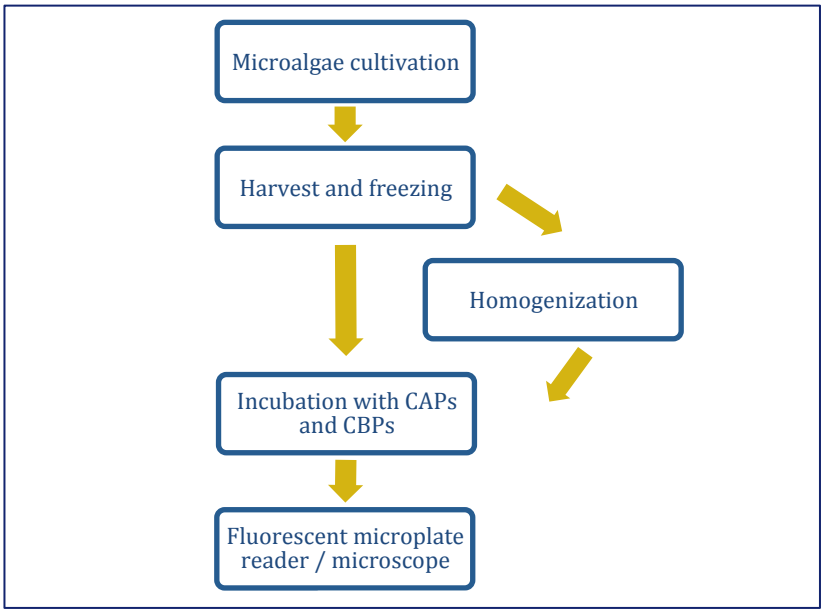

Figure 6 - High-throughput chitin and chitosan detection method for microalgae

2.2.1.2 Screening microalgae to detect chitins and chitosans

Thirteen species from Greenaltech's proprietary microalgae library were chosen with the goal of screening a large diversity of genera in a single analysis. Among the selected microalgae there were four Heterokontophytes from the genera *Thalassiosira, Chaetoceros, Phaeodactylum* and *Nannochloropsis.* Additionally, 8 Chlorophytes from the genera *Chlorococcum,*

Dunaliella, Scenedesmus, Chlorella (2), *Haematococcus, Bracteacoccus* and *Chlamydomonas* were tested. Also, one Haptophyte from the genus *Isochrysis* was selected. These species were chosen based on the premise of diversity but also taking into account the ease of growth. Amongst these, the biomass from *Thalassiosira pseudonana* was used as positive control for chitin production in microalgae as specific problems could arise due to the autoflorescence of microalgal pigments and it was known that this specie was capable of producing chitin fibers[27]. Also, the biomass from *Chlamydomonas reinhardtii* was used as a negative control since it is a well-studied model strain with no signs of chitin or chitosan production, not even at the genome level[74]. Finally, the zygomycete *Mucor Circinelloides* was also included as a positive control for chitin and chitosan production, as it is one of the few organisms known to naturally produce chitosans[75]. Each species was tested for the presence of chitins and chitosans according to the protocol described in subsection 2.4.2.

Results of the screening are presented in Figures 7 and 8 as a ratio between the fluorescence signal after incubation with CAPs/CBPs and the auto-fluorescence of each biomass. The exact values are presented on a Tables 19 and 20, in Appendix 1. Regarding the detection of chitins, it can be seen that the genera *Chlorella* and *Scenedesmus,* both belonging to the phylum Chlorophyta had the highest signal. These two strains were followed by the Heterokontophyta *Thalassiosira,* which is known to produce chitins. Afterwards, the Chlorophytes *Bracteacoccus* and *Haematococcus* also showed values considerably higher than the rest, including the positive control, *Mucor circinelloides*. Hence, according to the screening, in decreasing order of probability, *Scenedesmus, Chlorella, Bracteacoccus* and *Haematococcus* were pointed as possible new producers of chitins. It is also important to highlight that, as expected, the negative control *C. reinhardtii* was one of the strains with a lower fluorescence / auto-fluorescence ratio after the incubation with the CBPs. What was not expected was the fact that *Mucor,* a positive control for chitin production, had similar values to *Chlamydomonas.*

Concerning the presence of chitosans, the screening method indicated that Chlorophytes of the genera *Chlorella* and *Scenedesmus,* had the highest amount of chitosans of all the samples tested. A significant signal was also detected in the Heterokontophyta *Thalassiosira*. It must be kept in mind that

the production of chitosans in *Thalassiosira,* unlike the production of chitins, had never been reported. Surprisingly, *Chlorella* and *Thalassiosira* had higher signals than the positive control *M. circinelloides* and *Scenedesmus* showed quite similar values. In decreasing order of probability, the screening method indicated that *Chlorella, Thalassiosira* and *Scenedesmus* could be new natural producers of chitosans. As, expected, the negative control, *Chlamydomonas,* had the lowest fluorescence ratio after the incubation with the CAPs.

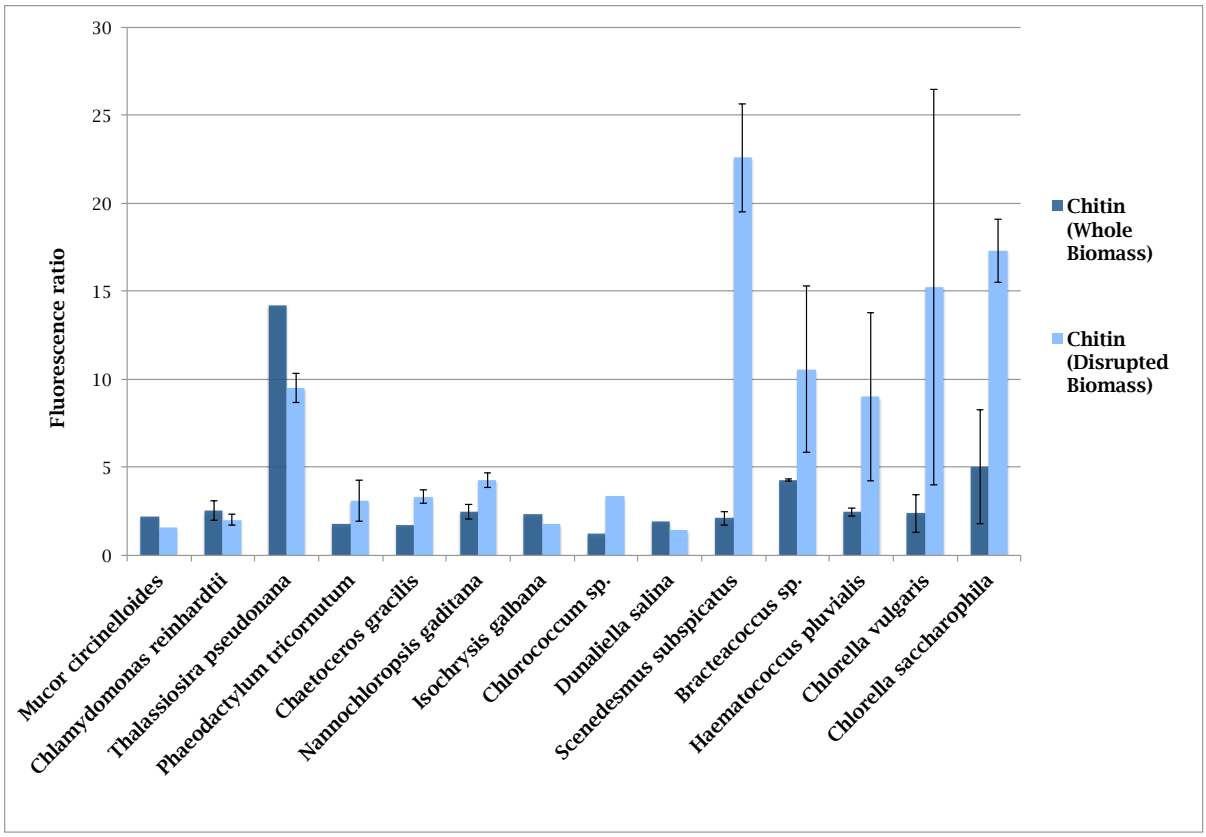

Figure 7 - Screening microalgae for the presence of chitins with CBPs.

Values are a ratio between the fluorescence signal after incubation with CBPs and the auto-fluorescence of each biomass. The error bar represents the standard deviation between two biological replicates. In some cases no biological replicates could be performed, therefore there is no error bar. For each biological replicate, three technical replicates were done.

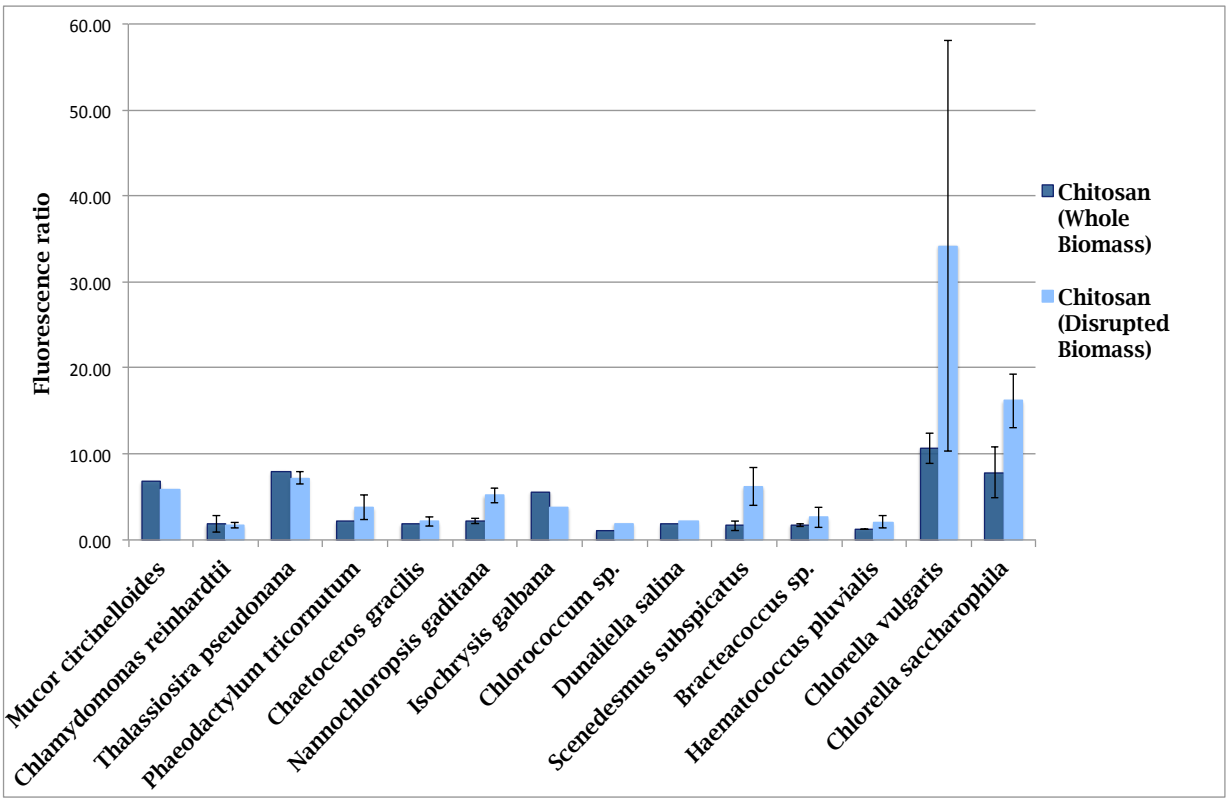

Figure 8 - Screening microalgae for the presence of chitosans with CAPs.

Values are a ratio between the fluorescence signal after incubation with CAPs and the auto-fluorescence of each biomass. The error bar represents the standard deviation between two biological replicates. In some cases no biological replicates could be performed, therefore there is no error bar. For each biological replicate, three technical replicates were done.

In all, the results of the first screening of microalgae using CAPs and CBPs for the presence of chitins and chitosans were highly promising, especially because they indicated that some microalgae could be capable of directly producing natural chitosans. Nevertheless, the positive results and the screening method still needed to be validated using standard methods to characterize chitins and chitosans.

2.2.2 Confirmation of the presence of chitosans in microalgae

There are many organisms that are capable of naturally producing chitin. The chitin of some crustaceans is chemically deacetylated to produce chitosan, a polymer that is more valuable because it is water-soluble and thus can be used in more applications. Notwithstanding, there is a small group of microorganisms capable of performing the deacetylation reaction naturally by enzymatic means, yielding naturally produced chitosans. In subsection 2.2, a screening was performed looking for chitosans in 13 different microalgal species of a wide phylogenetic variety. Results indicated that some of the species tested, particularly *Chlorella* and maybe *Scenedesmus* and *Thalassiosira,* seem to be capable of producing chitosans naturally. Therefore, in order to validate the screening and confirm that these microalgae belong to the selected group of microorganisms capable of enzymatically producing chitosans, standard techniques to characterize chitosans were applied to study the selected microalgal biomasses. Before performing the characterization of the biomass, taking advantage of the fact that chitosans should be easily separated from the rest of components by solubilization in acidic conditions, a fraction enriched in chitosans was prepared.

2.2.2.1 Preparation of a microalgal fraction enriched in chitosans

A process to try to obtain a fraction rich in chitosan from microalgae was developed based on already available information about the extraction of this polymer from fungi[76]. Concisely, first the cells were lysed and afterwards, the lysed biomass was incubated in a basic solution to eliminate the proteins. Then, chitosans were separated from the rest of cell wall components taking advantage of their solubility in acidic media. Afterwards, chitosans were precipitated and recovered by increasing the pH of the solution. Finally, the fraction obtained was washed and dried. At the same time, the fraction that was insoluble in acid was also washed to eliminate salts that could interfere in its analysis. At the end of the process, two fractions were recovered; the first one was the fraction that was soluble in the acidic solution and should contain any present chitosans. The second fraction contained the remaining material from the cell wall that was not soluble in acidic conditions, water, ethanol, and/or acetone. In this fraction chitin should be found if there was any.

Further details of the process can be found in the scheme presented in Figure 9 and in subsection 2.4.3.

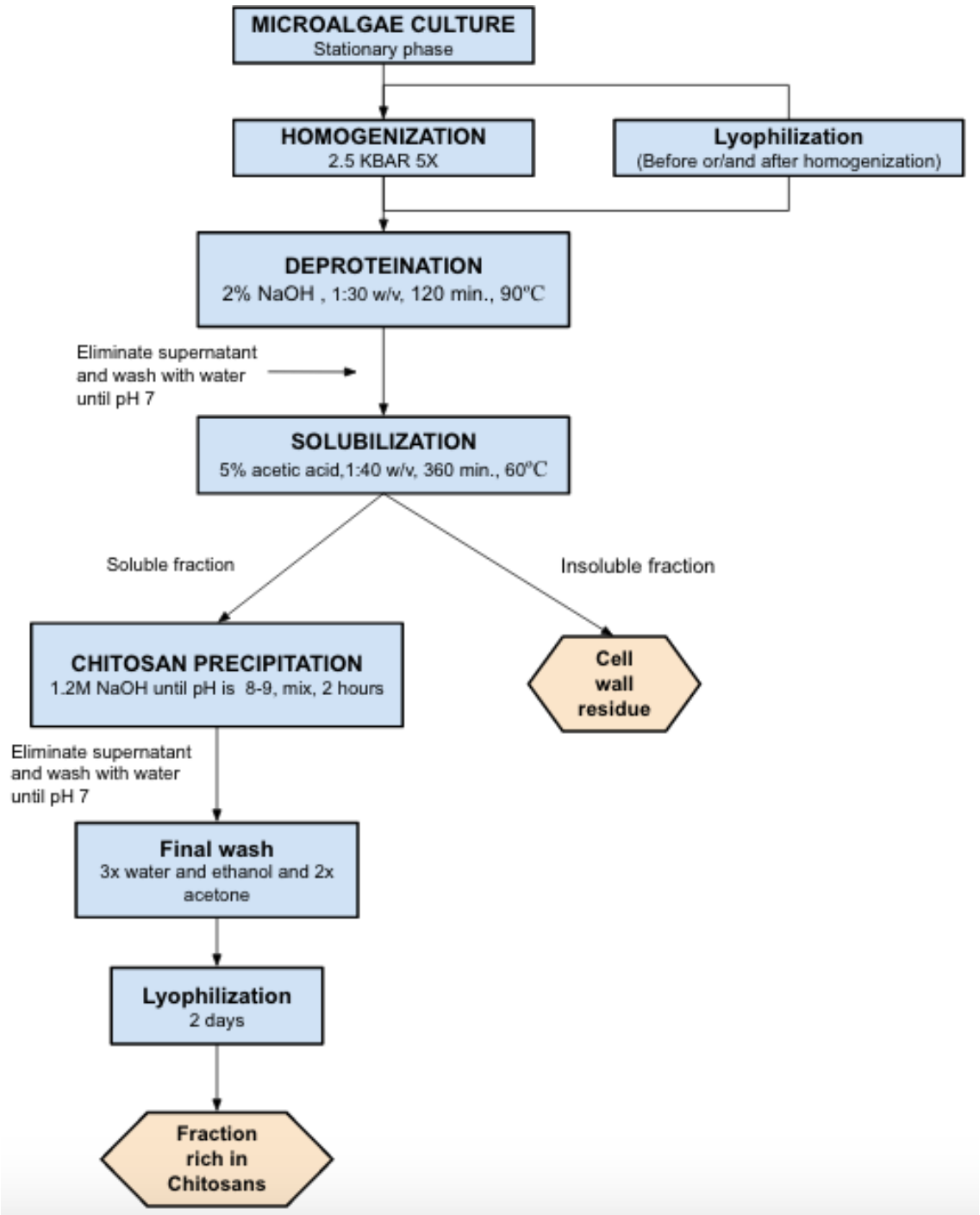

Figure 9 – Schematic representation of the microalgal fraction rich in chitosans

Several biomasses went through the process of extraction of chitosans. Taking into account the information accumulated so far, *Chlorella* was the genus with more chances of containing chitosans in its cell wall. Firstly, there were a few publications commenting this possibility after analyzing the *Chlorella* cell wall[24,68,69]. Secondly, many different putative chitin deacetylases in the genome of *Chlorella vulgaris NC64A* were detected after its genome was made available[25]. Thirdly, in the results obtained with the CAPs assay in subsection 2.2.1 the two species with the largest signal belonged to the *Chlorella* genus. Based on these assumptions, thirteen different *Chlorella* strains from the library of Greenaltech were chosen for this assay in order to increase the possibilities of finding chitosans and determining if there is a phylogenetic variety in the production of chitosans in the same specie. The two species with the largest signal for chitosans in the CAPs assay were included: *Chlorella sacharophila (211/9A)* and *Chlorella vulgaris (CS-41).* Another *Chlorella vulgaris* (*H1993*) was also selected together with six *Chlorella sp.* strains locally collected by Greenaltech *(GAT-1, GAT-2, GAT-3, GAT-4, GAT-7,* and *GAT-10),* one *Chlorella sorokiniana (2805) and* one *Chlorella zoofingensis (211/14).* These strains were grown autotrophically with CO_2 supplementation. Additionally, the strain *Chlorella variabilis NC64A, which* was sequenced in 2010 revealing the presence of chitosan related putative enzymes, many of them being chitin deacetylases[25], was chosen for this assay. This strain is also interesting because it is grown mixotrophically in the presence of glucose, something that could have an inicidence in the production of chitonsa. All of the above commented strains were cultured in lab scale photobioreactors under controlled conditions in Greenaltech. Finally, one *Chlorella* grown in open-ponds (*OP)* was analyzed to assess the effects of a non-sterile environment, taking into account that chitosans could be a defense mechanism of microalgae.

Apart from studying *Chlorella*, the *Scenedesmus subspicatus (AC139)* that had given the bests results in the CBPs assay and also had a considerably high signal for chitosans when using CAPs was also included in the test. Also, two other *Scenedesmus sp.* strains *(GAT-8* and *GAT-9)* collected by Greenaltech were analyzed. Additionally, *Thalassiosira* was included in the screening because it is known to produce chitins and it was the second genus with the largest signal in the screening with CAPs. In this case, however, a different

strain than the one analyzed using CAPs and CBPs was studied: *Thalassiosira weissflogii (CCMP1336).* Dr. Chris Bowler from the CNRS kindly provided the biomass of this strain as part of a collaboration within the Nano3Bio because, according to the literature, this specific strain is capable of producing chitins[27] . Also, although not the highest producers of chitosans or chitins according to the CAPs/CBPs assay, *I. galbana (LB2307)* and *H. pluvialis (K0084)* were also included in the assay because these strains are of industrial interest. Finally, *Chlamydomonas reinhardtii (CC124)* was included as a negative control. All of these strains, apart from *Thalassiosira*, were cultured at Greenaltech under controlled conditions.

2.2.2.2 Analysis of the fraction possibly containing chitosans

As it can be seen in Table 1, a fraction possibly containing chitosans was obtained from all of the 20 species studied, with considerable variation in the yield. The table indicates the proportion of the chitosan-containing fraction in the pellet of broken cells (Homogenized and Liophilized Biomass, HLB). A very small amount of chitosan fraction was obtained in the negative control *C. reinhardtii* but surprisingly *C. sorokiniana 2805* had an even smaller fraction. The same was the case for *S. subspicatus AC139* and *H. pluvialis K0084.* In the case of the *C. sorokiniana* these results do not coincide with previous literature indicating that it has a high glucosamine content in its cell wall[23]. In fact, according to the same publication, *C. sorokiniana* should accumulate a larger fraction than *C. sacharophila,* which in this case has one of the largest fractions. An explanation could be that the glucosamine commented in the publication was not present in the form of chitosans, or that the glucosamine content in the cell wall can largely vary between strains and growth conditions[23]. Also, the results obtained for *S. subspicatus* do not coincide with the high fluorescent signals measured in the assay with CAPs. The small amount of material recovered from these samples could be caused by a loss of material during the extraction process.

In all other cases the chitosan-containing fraction was significantly larger in comparison to the negative control. As it was for the CAPs assay, all species with a larger chitosan-containing fraction belong to the *Chlorella* genus. *Chlorella sp.* (GAT-7) clearly had the largest chitosan-containing fraction, as it

was 10.92% of the total HLB weight. *C. vulgaris H1993, C. vulgaris CS-41 and C. saccharophila 211/9A,* all with a chitosan-containing fraction between 2 and 2.5% of the HLB weight were the other *Chlorella* with significantly high values. It is important to highlight that two of these *Chlorella* strains (*H1993* and *CS-41)* were the ones with the best fluorescence ratio in the CAPS assay.

The other species that produced a considerable fraction that may contain chitosans was *I. galbana LB2307.* This is surprising when looking at the CAPS assay because, although it shows a fluorescence ratio between two or three times larger than that of *Chlamydomonas,* the fluorescence ratio for *Chlorella* is much higher (around 10 times that of *Chlamydomonas)*, hence, a lower chitosan containing fraction was expected for *I. galbana*. The confirmation of the presence of chitosans in this species would be a very good outcome because it is cultured at industrial levels. Finally, a considerable acid soluble fraction was also obtained for *T. weïssflogii CCMP133;* characterizing it would allow confirming the presence of chitosans for the first time in this species that is only known to produce chitin.

2.2.2.2.1 Validation of the presence of chitosans by ^{1}H-NMR and determination of the degree of acetylation

There are plenty of techniques described in the literature to characterize chitosans that could be used to analyze the existence of these polymers in the microalgal fraction that is soluble in acetic acid. However, none of them have been defined as the standard. Some of the possible techniques previously cited in the literature are UV spectroscopy, IR spectroscopy, X-ray diffraction, ^{1}H-NMR, and ^{13}C-NMR, among many others[9,77–79]. After analyzing the possibilities, liquid phase ^{1}H-NMR was the method of choice because it has been demonstrated to be fast, precise, reproducible, rugged, robust and stable, unlike most of the other techniques that are time consuming and often show considerable discrepancies in the obtained degree of acetylation (DA) values[78]. In order to obtain precise measurements with some of the other techniques, an accurate weighting of chitosans is required. This means that moisture needs to be fully removed and the purity of the samples must be previously determined. In contrast, when using the ^{1}H-NMR method, the amount of chitosan analyzed does not need to be weighted so accurately and the impurity of the sample does not need to be determined as long as these impurities do not affect the

regular peaks of the chitosan spectra[78]. Moreover, the sample preparation is simple, milligrams are required and there is no need for a calibration curve with samples with known DA%[78].

The ^{1}H-NMR spectra obtained reveals that the polymer present in the acetic acid soluble fraction of some microalgae is indeed chitosan. Nine different *Chlorella* strains (*GAT7, H1993, CS-41, 211/9A, OP, GAT-3, GAT4, GAT-10* and *NC64A),* have been confirmed to have chitosans, as the spectrum is very similar to the spectrum of chitosan 75% DA from Sigma Aldrich (ref. C3646). All of these 9 samples show the expected typical peaks of a chitosan ^{1}H-NMR spectrum performed at 70ºC, such as the deacetylated monomer (H1-D), the acetylated monomer (H1-A), the signal for protons H-2, H-3, H-4, H-5, H-6, the H-2 peak of the deacetylated monomer (H-2D) and the peak of the acetyl group (H-Ac). The other peaks that are observed are the water peak (HOD), which is suppressed or at least reduced in most cases and the Trimethylsilylpropanoic acid (TMSP) peak that is used as the internal reference for the NMR. In the cases when some extra peaks are observed in the spectrum, these are considered to be contaminations. Another aspect to highlight is the fact that the samples analyzed (including Sigma Aldrich's chitosan) are contaminated with acetic acid, as the intensity of the H-Ac peak is higher than expected. For this reason, only peaks H-1(D) and H-1(A) were used to calculate the DA%.

The ^{1}H-NMR spectra obtained of all the samples tested can be observed individually in Appendix 3. All the characteristic peaks of chitosans can be seen in Figure 10, which is an example showing the spectrum of *211/9A* compared with the spectrum of the chitosan from Sigma Aldrich. There are minor differences in the two spectra, for instance a clear one can be observed around 4.3 ppm, but it is just the water peak (HOD) that was better suppressed in the case of the chitosan from Sigma Aldrich. By using equation I from the materials and methods section (2.4.4), the %DA of this particular microalgal chitosan is 25.8%. Using the same equation to calculate the DA of the chitosan from Sigma Aldrich, the value obtained was 22.9%, which corresponds with the manufacturers claims that the DA should be lower than 25%. As Table 1 shows, the DA% could be quantified for all 9 *Chlorella* strains with the ^{1}H-NMR spectrum, except from *GAT-4* because the HOD peak overlapped with the H-1(A) peak. Just by looking at the DA% values of the 9 positive strains that

range from 5 to 25%, it seems apparent that not all *Chlorella* produce the same chitosans.

According to the extractions and the ^{1}H-NMR spectra obtained, there are no chitosans in any of the other strains tested apart from the nine *Chlorella* commented above. Amongst the rest of strains assayed that apparently do not produce chitosans there are four *Chlorella* strains (*GAT-1, GAT-2, B32,* and *2805*). The case of *GAT-1* is special because a polymer formed by hexose monomers is observed in the spectra, but it cannot be classified as chitosan because most of the required peaks are lacking. Further research is required to identify it (Appendix 3). For the other three *Chlorella* strains without chitosan, nothing could be detected apart from the H-Ac peak, indicating that either all the chitosan was lost due to experimental errors or that there are no acid soluble polymers in the cell walls of these species. The same reasoning can be applied to the analysis performed on the acid soluble fractions obtained for the other non-*Chlorella* strains that could be analyzed with ^{1}H-NMR (*LB2307, CCMP1336, GAT-8 and GAT-9*). The enigma lies in deciphering what was it then that accumulated in the acid soluble fraction after the extraction process, sometimes in considerable quantities such as in the case of *I. galbana LB2307* or *T. weissflogii CCMP1336.* Finally, the amount of material accumulated by the strains with a smaller acid soluble fraction (*CC124, K0084, AC139* and *2805)* was not enough to perform the ^{1}H-NMR analysis and thus the presence of chitosans could not be detected (Table 1).

In all, the ^{1}H-NMR spectra obtained clearly validated the initial screening with CAPs in the sense that it confirmed that some *Chlorella* produce chitosans naturally. *Chlorella* had been the genus with the highest signal in the CAPs assay and the ^{1}H-NMR analysis showed that it was the only one of all strains screened that contained chitosans.

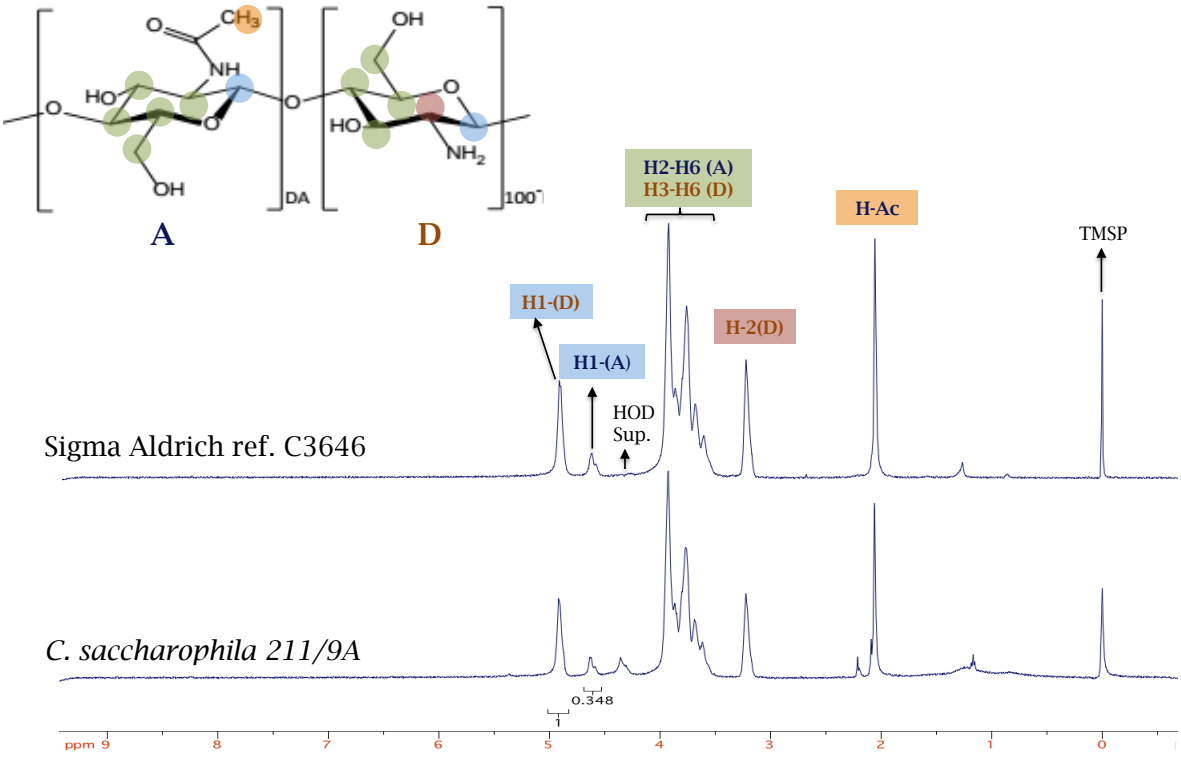

Figure 10 – ^{1}H-NMR spectrum of pure chitosans (Sigma Aldrich ref. C3646) (22.9% DA) compared to the spectrum of chitosans from *C. saccharophila 211/9A* (25.8% DA).

The different peaks are labeled and it can be seen how they correspond between the two spectra. The area values of peaks H1D (1) and H1A (0.348) are given because they are the ones required to determine the DA%.

2.2.2.2.2 Validation of the presence of chitosans by chitosanase and chitinase digestion

The ^{1}H-NMR spectra are almost definitive evidence that the CAPs screening method is capable of successfully predicting the presence of chitosans in microalgal biomass. Nevertheless, in order to ensure that the spectra obtained belonged to chitosans and not to a similar polymer, an irrefutable proof would be to digest the chitosan-rich fractions with chitosanases (CsN). Chitosanases (E.C.3.2.1.132) are enzymes that specifically hydrolyse the β–1–4 glycosidic bonds of chitosans (and not chitins) to reduce them to short chain oligomers that can be detected by LC-MS[80]. Hence, oligomers would only be present in the MS spectrum in case there are chitosans in the sample.

According to the results obtained from the analysis with ^{1}H-NMR, 9 different *Chlorella* strains are capable of producing chitosans. In order to confirm these results, chitosan fractions from the 3 best producers (*GAT-7*, *H1993* and *CS41)* were incubated with a chitosanase from *Bacillus sp. MN* (GenBank ac. No. AFD19011.1). As it can be seen in Figure 11, chitosam oligomers were obtained in the three cases. In fact, the spectra obtained were very similar between them, showing monomers only up to DP7 mostly conformed by deacetylated monomers. Clearly, the most common product after the digestion is a D-glucosamine oligomer of DP3. This confirms the previous results indicating that the chitosans from microalgae are highly deacetylated.

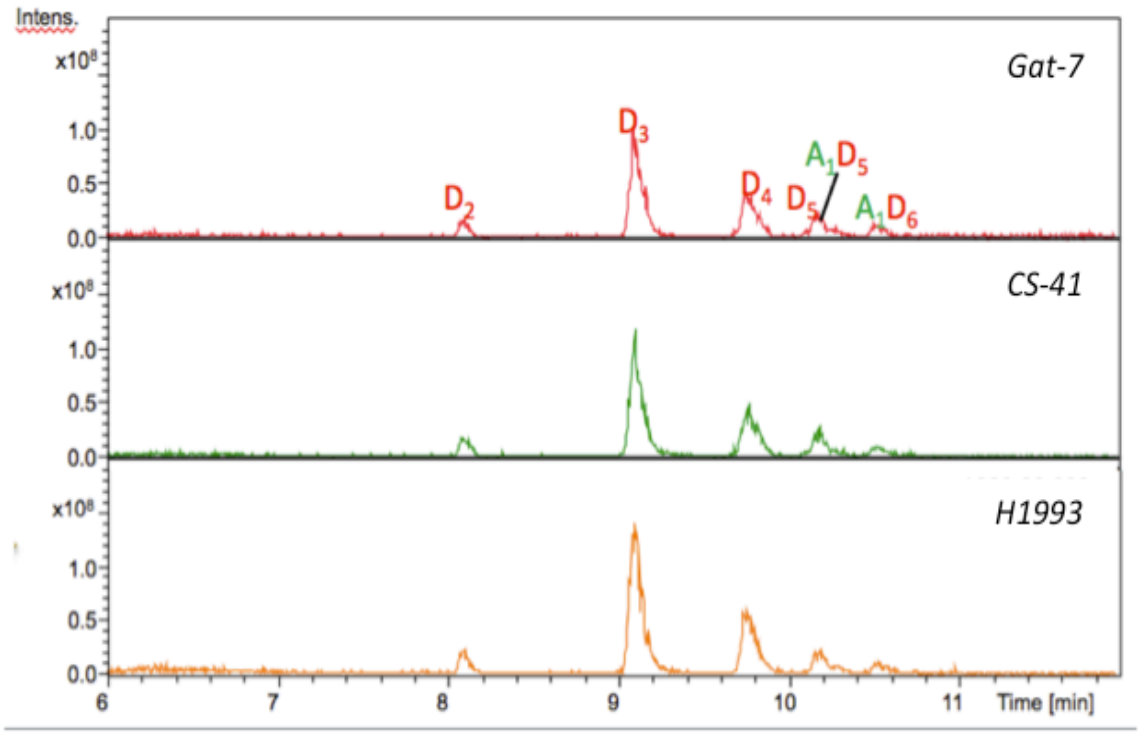

Figure 11 - LC-MS spectra of three microalgal chitosans hydrolysed with chitosanases.

"D" stands for deacetylated monomer and "A" for acetylated monomer. The number is an indication of the length of the oligomer. The spectra were obtained in collaboration with Dr. El Gueddari from the University of Münster.

The same chitosans were also digested with a chitinase (E.C.3.2.1.14), which is a hydrolytic enzyme that binds to N-acetylglucosamine monomers to break down glycosidic bonds in chitin polymers. The chitinase used was from

Serratia marcescens (GenBank ac. No. ABI331431.1). Nevertheless, in this case no hydrolytic activity was detected. The explanation to these results is that the microalgal chitosans assayed are so highly deacetylated that chitinases do not bind to them.

2.2.2.2.3 Determination of the molecular weight of the microalgal chitosans

A screening process successfully identified the presence of chitosans in *Chlorella*. These results were validated by ^{1}H-NMR and by chitosanase digestion of microalgal cell wall fractions solubilized under acidic conditions. ^{1}H-NMR also allowed determining the DA% of the polymers. However, it was still missing to determine the other important characteristic to take into account when describing chitosans to understand its properties, which is the weight-average molecular weight (Mw). This characteristic goes hand in hand with the polydispersity index (Ip), which is essential to understand the variability in the sizes of the different chitosan polymers in one sample. This parameter gives an idea of the quality of the chitosans; the less variability the higher quality. Viscosimetry and light scattering are the most used methods to determine the Mw of chitosans[81,82]. However, only light scattering can be used to obtain information about the polydispersity of the samples[9].

Again, the three strains with the largest chitosan-containing fraction (*GAT-7*, *H1993* and *CS41)* and the strain that had been cultured in open-ponds (*OP*) were selected to determine their Mw and Ip. SEC-MALLS-RI was the method of choice as it is probably the most reliable method for this purpose, it is suitable for the determination of the Ip and it does not require standards[9]. SEC-MALLS-RI briefly consists on a separation step by size exclusion chromatography (SEC), more specifically with gel permeation chromatography, and then the polymers are measured with a multi-angle-laser-light-scattering detector (MALLS) and a refractive index detector (RI). The Mw and Ip determinations were performed in the laboratory of Dr. Moerschbacher from the University of Münster.

As Table 1 shows, the chitosans derived from *Chlorella* resulted to be of rather low molecular weight. The four chitosan samples ranged between 20.5 and 34.6 kDa. This seems to indicate that there could be some considerable variations between *Chlorella* species although more determinations are

required to confirm this observation. When it comes to analyzing the Ip values obtained, it is important to bear in mind that in order for the chitosan sample to be of good quality this value should be lower than 2[9]. Fortunately, the values were between 1.6 and 1.9, so the four of them could be qualified as good quality chitosans as they are in the same range of other medical grade chitosans used[83]. This is a remarkable result taking into account that these chitosans are the product of the first chitosan extraction from microalgae, a process with plenty of room for optimization. These results are in accordance with the hypothesis that chitosans produced enzymatically should be better preserved and thus should be more homogeneous when compared to chitosans obtained using harsh chemical reactions.

The determination of the Mw and Ip values of four different cell wall acid soluble fractions, together with the determination of their DA% by ^{1}HNMR, is a fairly complete characterization of the chitosans produced by *Chlorella.* In all, with all evidences provided, it can be asserted that some *Chorella* strains are capable of naturally making chitosans.

Species	Strain Code	HLB (%)	Chitosan confirmed	DA (%)	Mw (kDa)	Ip
C. sp.	GAT-7	10.9	^{1}H-NMR: Yes Enzymatic: Yes	18	34.6	1.7
C. vulgaris	H1993	2.52	^{1}H-NMR: Yes Enzymatic: Yes	25	28.8	1.6
C. vulgaris	CS-41	2.51	^{1}H-NMR: Yes Enzymatic: Yes	12	20.5	1.9
I. galbana	LB2307	2.09	^{1}H-NMR: No			
C. sacharophila	211/9A	2.03	^{1}H-NMR: Yes	15		
C. sp.	OP	1.35	^{1}H-NMR: Yes	5	24.3	1.8
C. sp	GAT-3	1.13	^{1}H-NMR: Yes	25		
C. sp	GAT-4	0.95	^{1}H-NMR: Yes	-		
C. sp.	GAT-10	0.85	^{1}H-NMR: Yes	16		
T. weissflogii	CCMP1336	0.84	^{1}H-NMR: No			
S. sp.	GAT - 8	0.81	^{1}H-NMR: No			
C. variabilis	NC64A	0.75	^{1}H-NMR: Yes	15		
S. sp.	GAT - 9	0.65	^{1}H-NMR: No			
C. sp.	GAT-1	0.63	^{1}H-NMR: No			
C. zofingiensis	B32	0.49	^{1}H-NMR: No			
C. sp	GAT-2	0.36	^{1}H-NMR: No			
C. reinhardtii	CC124	0.07	Insufficient			
H. pluvialis	K0084	0.02	Insufficient			
S. subspicatus	AC139	0.01	Insufficient			
C. sorokoniana	2805	0.01	Insufficient			

Table 1 - Identification and characterization of chitosans in microalgae.

A fraction possibly containing chitosans was isolated and quantified as the percentage of the weight of the homogenized and lyophilized fraction (HLB). In case there was enough material, the presence of chitosans was confirmed first by ^{1}H-NMR. This technique enables the determination of the degree of acetylation (DA). A reconfirmation of the three best cases was done by digestion with chitosanases. Finally, molecular weights (Mw) and polidispersity indexes (Ip) of the polymers obtained from four strains was determined.

2.2.3 Confirmation of the presence of chitins in microalgae

The screening method developed in subsection 2.2.1 had the aim of determining the presence of chitins and chitosans in microalgae. The screening for chitosans with CAPs has been validated with plentiful evidence in subsection 2.3. *Chlorella,* the genus with a higher signal in the CAPs screening,

has been the only one that, according to the methods utilized, has been confirmed to contain chitosans. In the screening with CBPs, the genera that were pointed as possible new producers of chitins were, in decreasing order: *Scenedesmus, Chlorella, Bracteacoccus* and *Haematococcus.* The purpose of this subsection is to validate the screening method to detect chitins in microalgal cell walls to confirm that indeed these genera are producers of chitins.

2.2.3.1 Monosaccharide linkage analysis of the microalgal cell wall

The results obtained with the screening with CBPs indicated that if there were any chitins in green microalgae, they would be embedded in the cell wall. Moreover, these results were backed by previous studies in which important glucosamine contents were found in the cell wall of *Chlorella* and *Scenedesmus,* the two genera with higher CAPs and CBPs signals in the screening (Figure 7 and 8) [23,53,60–63]. Therefore, an analysis of the polysaccharides present in their cell wall would allow validating the screening method and would provide a definitive proof of the presence of chitins in green microalgae. Moreover, the outcome of such an assay would also be interesting from the point of view that it would provide information about the proportions of chitins and chitosans with respect to the rest of carbohydrates in the cell wall.

A reliable and precise method to determine the carbohydrate composition of the cell walls of microalgae is essential to developing adequate biomass processing strategies, especially when using enzymes targeting specific polysaccharides. As a result, different approaches have been described in the literature for determining the diversity of polysaccharides in the microalgal cell wall[53]. After discussing which approach to follow with experts from the laboratory glycoscience from KTH University in Stockholm led by Dr. Bulone, a method was designed in which a carboxyl reduction, followed by a methylation step and finally a GC-MS analysis were the key steps to perform a monosaccharide linkage analysis of the extracted cell walls of microalgae (personal communications). The presence of chitins and chitosans would be determined by detecting the GlcNAc and GLcN monomers that, linked β-1-4, form the long chains that conforms the structure of the polymers. The process, which is explained in more detail in subsection 2.4.8, was carried out at the laboratory of Dr. Bulone.

Concomitantly, a methodology to extract the microalgal cell wall was developed. Briefly, first the microalgal cells were disrupted using high pressures with a homogenizer. Then, the cell walls were separated from the rest water soluble compounds by centrifugation and solvents were used to eliminate pigments and oils. Finally, enzymes were used to eliminate starch. At the end, the samples were washed with solvents and dried for analysis. The cell wall extraction process is more detailed in subsection 2.4.7.

Two *Chlorella* strains were selected for the glycosidic linkage analysis: *Chlorella sp. GAT-7* and *Chlorella sp. OP.* Although, these particular strains were not in the initial screening with CAPs and CBPs, they were chosen because it had been demonstrated that they were capable of producing chitosans by ^{1}H-NMR and, in the case of GAT-7, also by chitosanse digestion (Figure 11, Table 1 and Appendix 3). It was expected that chitins would be present in the cell walls of these two strains as it is the precursor of chitosans. Studying *GAT-7* was particularly interesting because it was the strain with the largest content of chitosans according to previous analysis. *OP* was relevant because plenty of biomass from the same batch of this strain was readily available at Greenaltech to perform further tests with chitins and chitosans without the need of culturing a large batch of microalgae. Although it was desirable, no more *Chlorella* strains could be included in the assay because it was a long and complicated assay. Instead, it was given priority to having other microlgal species to compare with, such as *I. galbana LB2307* and *H. pluvialis K0084.* Both strains were selected because of their commercial interest. Moreover, *Haematococcus* was amongst the genera with a higher signal in the screening with the CBPs. Instead, *Isochrysis*, because of it low signal in the CBP screening, could be used as a negative control for the presence of chitins.

The results from the glycosidic linkage analysis of the four strains commented above can be seen in Table 2, which shows the 6 most common linkages in each case. The entire list of glycosidic bonds detected for each strain can be found in Appendix 5. As expected, the presence of chitins (4-GlcNAc and t-GlcNAc linkages) is much higher in the two *Chlorella* strains. For both strains, the characteristic linkages of chitins are amongst the five most common ones, representing 8.2 and 6.8 % of all the linkages found for *GAT-7* and *OP*, respectively. The 4-GlcNAc and t-GlcNAc linkages are also present in *I. galbana*

LB2307 but in much lower percentages (only 1.6%). Finally, bonds including GlcNAc were practically absent in the analysis of the cell wall of *H. pluvialis K0084*. With these results, the glycosidic linkage analysis performed in accordance with the CBPs screening because it reaffirms that *Chlorella* is the genus with a higher content of chitins. However, the analysis contradicts the screening because it indicates that there are no chitins in the cell wall of *Haematococcus* and it says that there could be some in the cell wall of *Isochrysis.* In the case of *Isochrysis,* it could be possible that the linkages detected formed part of another polymer that was not chitin, such as a chitin-glucan polymer[24].

	GAT-7		OP		LB2307		K0084	
	Link.	mol%	Link.	mol%	Link.	mol%	Link.	mol%
1	3-Glc*p*	14.5	4-Glc*p*	24	4-Glc*p*	30.7	4-Man*p*	62.8
2	4-Glc*p*	13.9	4-Xyl*p*	16.3	4-Man*p*	12.3	4-Glc*p*	16.1
3	6-Glc*p*	11.7	4-GlcA*p*	11.7	4-Xyl*p*	6.6	6-Man*p*	7.5
4	2-Rhm*p*	9.2	4-Man*p*	7.3	6-Glc*p*	6.3	2-Man*p*	3
5	4-GlcNAc	7.6	4-GlcNAc	5.5	3-Man*p*	4.5	4-Xyl*p*	1.6
6	4-Man*p*	5.4	t-Man*p*	2.9	t-Glc*p*	4.1	t-Xyl*p*	1.1
Other	t-GlcNAc	0.6	t-GlcNAc	1.3	4-GlcNAc	1.3	4-GlcNAc	0.1
Other					t-GlcNAc	0.3	t-GlcNAc	tr
Total GlcNAc		8.2		6.8		1.6		0.1

Table 2 - Presence of the glycosidic linkage 4-GlcNAc in four microalgal cell walls.

Indicated as mol%, this linkage is a clear proof of the presence of chitins. The 6 more common linkages of each cell wall are presented compared to the GlcNAc linkages. "Tr" means that less than 0.1% was detected. The rest of linkages can be found in Appendix 5.

Apart from the validation of the screening with CBPs to confirm the presence of chitins in microalgae, the secondary goal of the glycosidic linkage analysis of the cell walls was to decipher the percentages of chitins and chitosans. This information is very valuable when designing an extraction method to determine the maximum yield. In terms of chitins, the percentage was 8.2% and 6.8% depending for GAT-7 and OP, respectively. However, in the case of chitosans this value could not be obtained. As it can be observed in Appendix 5, none of the glycosidic linkages detected involved the monomer GLcN. The explanation for this is that a glycosidic linkage next to a GLcN is more difficult to be hydrolyzed by TFA than a glycosidic linkage next to a GlcNAc or other

common sugars like glucose or mannose. In other words the less acetylated the polymer is, the more difficult it is to hydrolyze it by TFA and detect its monomers[84]. Thus, in the case of *Chlorella* chitosans, which were determined by ^{1}H-NMR to have a low degree of acetylation, the process yielded oligomers that were not detectable by MS, instead of monomers.. Moreover, as it can be observed if Figure 12, while in the case of GlcNAc the methylation step of the amide gives a N-methyl acetamide, in the case of GLcN this step gives a quaternary ammonium salt. The resulting ammonium salt would not be detected in the GC-MS analysis because it is not volatile[85]. Therefore, because of the reasons mentioned above, only GlcNAc belonging mostly to chitins and maybe in very small proportions to the acetylated parts of chitosans, could be detected. A new glycosidic linkage analysis methodology should be developed in order to be able to detect chitosans with it.

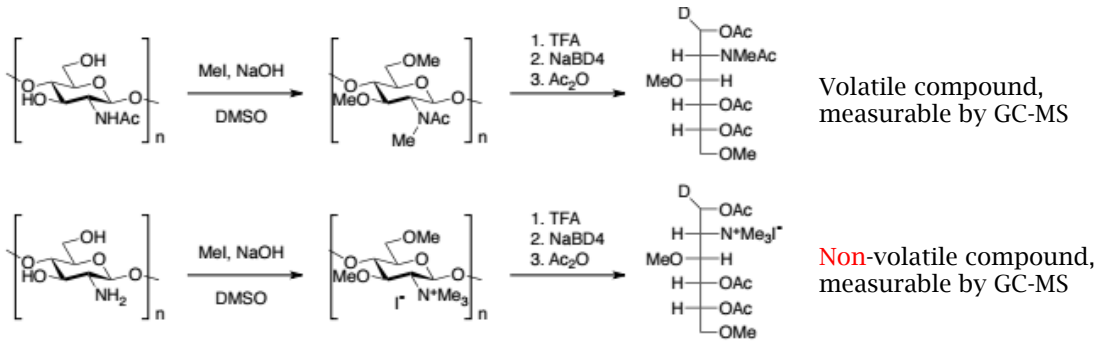

Figure 12 - Generation of a quaternary ammonium salt that is not measurable by GC-MS in the methylation step of the glycosidic linkage analysis of GLcN.

2.2.3.2 Characterization of the acid insoluble cell wall residue

As it is indicated in Figure 9, two fractions were obtained after the chitosan extraction process. The first fraction was soluble in acetic acid and it has been confirmed to contain chitosans. The second fraction, which can be considered as the waste fraction from the first chitosan extraction process, should contain all the other polymers that are not soluble in acidic solutions, water, ethanol and/or acetone, such as chitins. Therefore, the study of this fraction could be useful to validate the CBPs screening method. Moreover, characterizing this fraction would be useful to know how much of the chitins produced by

Chlorella were actually enzymatically deacetylated to chitosans. Furthermore, this fraction could also contain the chitosans that were not solubilized during the extraction process, so it could be the key to understand how efficient the extraction process was.

Table 4 shows the amounts of waste fractions obtained in the extraction of chitosans. As it can be seen, the waste fraction may constitute up to 40-50% of the HLB in *Chlorella* (*GAT-1*) and *Scenedesmus* (*GAT-9*). However, the values in general are between 5% and 25% of HLB for most *Chlorella* strains (*GAT-2, GAT-3, GAT-4, GAT-7, OP, GAT-10, B-32* and *2805*) together with the other two *Scenedesmus* (*GAT-8* and *AC139*) and the *Haematococcus pluvialis* strain assayed (*K0084*). There is a group of strains that contained a waste fraction that was less than 5% of the HLB in which three other *Chlorella* fall into (*H1993, CS-41* and *211-9A*), together with *Isochrysis galbana* LB2307 and *Chlamydomonas reinhardtii CC124*. It seems apparent from this data that the more chitosan found in the acetic acid soluble fraction, the smaller the waste fraction containing other polysaccharides is. The exception is the negative control *C. reinhardtii,* with low values in both cases.

2.2.3.2.1 Characterization of the cell wall residue by ^{1}H-NMR

In order to characterize the waste fraction, ^{1}H-NMR was the method of choice because it had been used successfully to easily and precisely characterize the chitosan extracted from microalgae and, on top of it, it had been proven to be a good method to determine the degree of deacetylation of the sample[78]. However, in order to perform a ^{1}H-NMR the sample must be completely solubilized and none of the waste fractions was soluble when agitating it in 1% HCl at 70ºC overnight, which were the conditions that had functioned with the chitosan fractions.

For this reason a series of tests to find the optimal solubilisation conditions of the waste fractions were carried out. The samples were tried to be dissolved in strong polar solvents with Lithium Chloride (LiCl), a combination that was found in the literature to be used to dissolve chitins by forming a complex with chitins to solubilize it[8]. The conditions tested were DMF, DMF with 5% LiCL DMA and DMA 5% LiCl. Unfortunately, none of them resulted to be good to solubilize the waste fraction. A trial with DMA with 5% LiCl in which 3

freeze/thaw cycles were performed improved the solubility but still it was not enough for ^{1}H-NMR. The difficulty to dissolve the waste fraction was most probably caused by the complexity of the sample and the different dissolution behaviors of its components. Moreover it has to be taken into account that chitin itself is already a difficult to dissolve polymer[8].

2.2.3.2.2 Characterization of the waste fraction by FTIR

Because ^{1}H-NMR could not be performed on the waste fraction and it still was interesting to figure out if there were chitins or chitosans in this fraction, FTIR spectroscopy was attempted. Several techniques can be used to determine the presence of chitins and chitosans in solid state such as solid state NMR[86], FTIR[17,87–89] and for samples in very pure form, elemental analysis[16]. FTIR was chosen because it is a broadly used technique for the characterization of chitins and chitosans and it may even be used to estimate the DA although it is not as easy to interpret as the ^{1}H-NMR and it is not as reproducible. The advantage is that samples do not need to be solubilized for characterization so FTIR can be used with a broad range of DAs[17]. A list of relevant peaks that should be found to identify chitins was obtained from Cárdenas *et al.* [89]. This list is reflected in Table 3, which also shows the difference between the peaks that are expected for α-chitin and β-chitin. Figure 13 A shows the FTIR spectrum of a commercially available chitin (Sigma ref. C7170), which confirms the presence of the 23 peaks for α-chitin indicated in Table 3.

Assigned number	α-Chitin abstorption (cm^{-1})	β-Chitin abstorption (cm^{-1})	Meaning
1, 2	3479, 3448	3479, 3426	nOH
3	3268	3290	$\nu^{as}NH$
4	3106	3102	$\nu^{s}NH$
5	2965	2962	$\nu^{as}CH_3$
6	2927	2929	$\nu^{s}CH_2$
7	2883	2880	$\nu^{as}CH_3$
8 , 9	1660-1627	1656	νC≡O (Amide I)
10	1558	1556	νC-N (C-N-H) + δNH (Amide II)
11	1422	1424	δCH_2
12	1376	1376	δCH + $\delta C\text{-}CH_3$ (Amide II)
13	1312	1314	νC-N + δNH (Amide III)
14	1255	1262	dNH
15	1157	1155	$\nu^{as}C{=}O{=}C$ (ring)
16	1072	1069	νC-O
17	1021	1032	νC-O
18	957	948	γCH_3
19	896	902	γCH (C1 axial) (β bond)
20	746	--	ρCH_2
21	698	692	γNH (Amide V)
22	610	616	γC-O
23	566	--	γC-C

Table 3 –Characteristic peaks found in the FTIR spectrum of chitin.

This list was obtained from Cárdenas et al.[89]. Apart from using them to identify chitins, they are useful to differentiate between α and β-chitin conformations

For this screening several *Chlorella* and *Scenededesmus* species were chosen because they were the ones to have a higher signal ratio in the screening with CBPs presented in subsection 2.2.1. The *Chlorella* species studied were *C. vulgaris H1993, C. vulgaris CS-41, C. saccharophila 211/9A, C. zofingiensis B32, C. sorokoniana 2805, C. sp. GAT-1, C. sp. GAT-2, C. sp GAT-3, C. sp. GAT-4* and

C. sp GAT-7. The *Scenedesmus* species analysed were *S. subspicatus AC139, S. sp. GAT-8* and *S. sp. GAT-9.* Finally, *Chlamydomonas reinhardtii CC124* was selected as the negative control. The waste fractions studied were obtained from the same process in which the chitosans were extracted and analyzed in section 2.2.2.2 (Figure 9).

The quantity of each of the waste fractions obtained and analyzed can be found in Table 4. In this table the number of peaks that are present in the spectrum of each of the strains studied can also be observed. An error margin of +/- 10 cm^{-1} was allowed for each of the peaks to be considered as positive. The right column of Table 3 indicates what each peak in the spectrum is in terms of the atom, the type of vibration ($\nu, \delta, \rho, \gamma$) and the valence of the vibration (symmetric or asymmetric). At first glance it is clear that for all *Chlorella* and *Scenedesmus* species studied that most of the typical peaks from α-chitin are present. In general between 22 and 14 out of the 23 peaks are conserved (Table 4). That is not the case for *Chlamydomonas* as only 9 out of the 23 expected peaks are present, confirming that it does not contain chitins (Table 4). Figures 11B and 11C are examples of the spectra obtained for *Chlorella sp. GAT-7* and *Scenedesmus sp. GAT-8*, showing that most of the peaks expected in α-chitin can be observed in the waste fractions of the two species studied. Appendix 4 contains the FTIR spectra of the rest of waste fractions analyzed.

When it comes to analyzing the individual peaks, peaks 3 and 4, which are indicators of the presence of NH stretching, an important characteristic of glucosamine containing polymers, are not present in any of the *Scenedesmus* species studied and only a subtle curve can be detected in some of the *Chlorella* waste fractions studied. It is important to bear in mind that slight changes in the amount of sample analyzed may result in significant differences with these two peaks. This was observed with the commercial chitin from Sigma Aldrich, as in some cases peaks 3 and 4 were not present. Unfortunately, no more tests were performed with the cell wall residue fractions of the chitosan extraction process from microalgae to try to better identify these peaks through the variation in the quantity of sample analyzed.

Two other peaks that should be commented are peaks 8 and 9. The presence of these two is a typical characteristic that distinguishes α-chitin from β-chitin,

which only has peak 8. In all the *Chlorella* and *Scenedesmus* strains analyzed these two peaks are present, which indicates that the chitin found in the cell wall of these species is α-chitin. The presence of extra peaks, apart from the ones in the list is an indicator of other contaminant substances, something that was already expected in this waste fraction.

Besides providing information about the presence of chitins in the sample, FTIR can also be used to determine its DA. The DA% value was obtained by applying equations II and III from the materials and methods section to the transmittance obtained in the spectrum. As it can be seen in Table 4, all the DA values that could be determined are between 17 and 20%. This result is a major surprise because such a DA is indicating that in the waste fractions, basically only chitosans are found. In fact, this DA% is very similar to the one determined for the chitosans in the chitosan containing fractions. The initial hypothesis was that there could be some chitins that still had not been enzymatically deacetylated to chitosans and therefore they could not be solubilized under acidic conditions. However, the FTIR spectra seem to correspond with the chitosans that were not solubilized after the incubation in acidic conditions. This could be caused by the fact that chitosans are embedded in an intricate mixture of polymers from which it is not easy to separate them. This finding suggests that there is still plenty of room for improvements of the extraction process as an important part of the chitosans are lost in the waste fraction. Last but not least, this also indicates that apparently *Scenedesmus* might be capable of producing chitosans but these cannot be solubilized with the current extraction method. More tests are necessary to confirm the presence of chitosans in *Scenedesmus*.

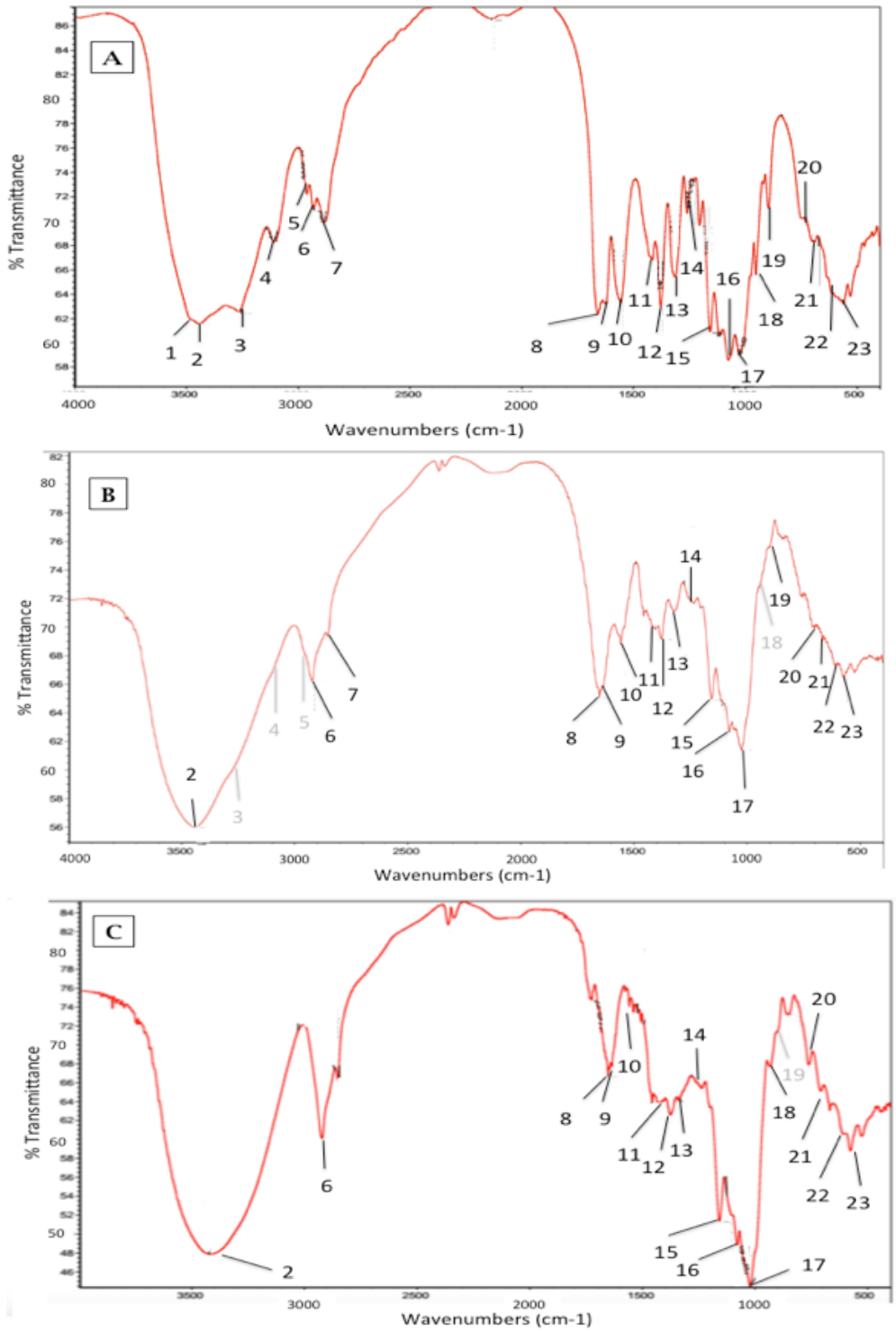

Figure 13 – FTIR spectra of the residual fractions from the Chitosan extaction process.

Comparison between the FTIR spectra of chitin of crustacean origin from Sigma Aldrich (Sigma ref. C7170) (A), and the waste fractions of the chitosan extraction process from *Chlorella sp. GAT-7* (B) and *Scenedesmus sp. (GAT-8)* (C). The peaks marked are the ones specified in Table 3. Some peaks are in faint grey color because they are not clearly visible.

2.2.3.2.3 Characterization of the cell wall residue by monosaccharide linkage analysis

In the FTIR spectra obtained from the waste fractions from the chitosan extraction process, apart from the 23 peaks that are characteristic from α-chitins, other peaks could be observed in the different spectra. This is an indication of the presence of other compounds in the cell wall residue. This was already expected as in this fraction every substance that is not soluble in water, acidic conditions, acetone and/or ethanol should be found. Monosaccharide linkage analysis could be a very useful technique to gain a better knowledge about the composition of these fractions. Moreover, it would corroborate the results from the FTIR analysis that indicate that chitosans accumulate in the waste fractions of the chitosan extraction process of some microalgae.

Because monosaccharide linkage analysis is a highly complex technique it cannot be performed for screening, so only a few samples can be analyzed at a time. The analysis was performed in parallel with the monosaccharide linkage analysis of the entire cell wall already explained in subsection 2.4.1. Hence, the same microalgal strains were tested: two *Chlorella* strains (*C. sp GAT-7* and *C. sp OP*), *H. pluvialis K0084* and *I. galbana LB2307.* The difference was that in this case the sample was the residue from the process to obtain a chitosan rich fraction and not the total cell wall.

The molar percentages of the sum of linkages 4-GlcNAc and t-GlcNAc of each of the strains analyzed can be found in Table 4. As already stated, this linkage is an indication of the presence of chitins and chitosans in a minor degree. The results from the analysis are very similar to the analysis of the cell wall performed in subsection 2.2.3.1; the only difference is that this time no GlcNAc bonds were found in *Isochrysis*. GlcNAc linkages are only present as 12.5 and 13.1% of the linkages found in *Chlorella OP* and *GAT-7* respectively. According to these results, taking into account the percentage of the residual fraction over the entire HLB, 0.94 % and 1.46% of the HLB weight of these *Chlorella* are a polymer formed by GlcNAc linkages. These values are too high to match the results from the FTIR analysis, which indicates that only chitosans are found in this fraction. It is impossible that this high percentage of GlcNAc bonds came only from N-acetylglucosamines found in the highly deacetylated *Chlorella*

chitosans. Therefore, in contradiction with the FTIR, such a high presence of GlcNAc linkages is more likely to be an indication that there are also chitins in the residue of the chitosans extraction process. Hence, the residual fraction is probably a mixture of chitins and chitosans that are not solubilized during the step of incubation in acidic conditions. It would be interesting to have a look at some of the other strains using this technique, specially the ones with a larger cell wall residue fraction like the *Chlorella sp GAT-1* or *Scenedesmus sp. GAT-9*. In case they contained 4-GlcNAc linkages in the same proportions, they could be outstanding new sources of chitins and chitosans if the extraction process was optimized. Another aspect to point out is the fact that the amount of 4-GlcNAc linkages in the waste fraction is considerably higher in comparison to the amount of 4-GlcNAc linkages in the cell wall. This indicates that during the process the residue fraction has been enriched in chitins. The complete monosaccharide linkage analysis of the waste fractions of the strains tested can be found in Appendix 5.

Species	Strain code	Waste fraction (% HLB)	Chitin FTIR peaks	DA%	GlcNAc linkage (mol %)
C. sp	GAT-1	50.97	18/23	17.85	--
S. sp	GAT - 9	44.57	15/23	18.16	--
C. sorokiniana	2805	23.35	17/23	18.05	--
S. subspicatus	AC139	23.07	18/23	18.23	--
C. sp	GAT-2	19.76	16/23	np	--
C. sp	GAT-3	18.54	15/23	19.02	--
C. zofingiensis	B32	18.47	15/23	19.96	--
S. sp	GAT - 8	15.48	18/23	18.63	<0.1
H. pluvialis	K0084	15.21	--	--	12.5
C. sp	GAT-7	11.65	21/23	18.47	--
C. sp	GAT-4	9.85	14/23	18.06	--
C. sp	GAT-10	9.51	--	--	13.2
C. sp	OP	7.2	--	--	--
C. vulgaris	H1993	4.83	21/23	19.35	0.3
I. galbana	LB2307	3.31	--	--	--
C. reinhardtii	CC124	3.06	set-23	np	--
C. vulgaris	CS-41	1.66	19/23	19.06	--
C. Saccharophila	211/9A	0.8	22/23	19.24	--

Table 4 – Analysis of the residual fraction of the chitosan extraction process.

"--" means that the analysis was not performed.. "np" means no peaks, as the peaks required to determine the DA could not be identified in the spectra.

2.3 Discussion

The presence of chitins and chitosans in the cell wall of microalgae was investigated for the first time more than 50 years ago[69]. First indications of the presence of GlcN were enough to generate the hypothesis that chitins are present in the microalgal cell wall. This hypothesis was lately reinforced by the discovery of genes involved in the chitosan production process in some microalgal genomes. Nevertheless, the presence of β-chitin in certain diatoms and α-chitin in *Phaeocystis* were the only cases that had been confirmed[27]. Therefore, the aim of the research explained within this chapter was to finally confirm the previous studies that indicate that, on one hand, there might be

chitins in other microalgal genera apart from *Thalassiosira* and, on the other hand, some species of microalgae might even be capable of naturally producing chitosans. Positive and solid results in this sense have been achieved thanks to the development of a screening method to detect chitins and chitosans in the cell wall of different microalgae that was afterwards validated using standard techniques to characterize chitins and chitosans.

A high-throughput screening method was designed to study a wide number of microalgal species covering a large microalgal phylogenetic variability taking into account that the species selected should be easy to grow. CAPs and CBPs proteins were used to selectively detect chitins and chitosans. Because the technique was limited by the capacity of the enzymes to reach their substrates, it was performed on in intact and homogenized microalgal cell walls. In this way, chitins and chitosans in the exterior or in the interior of cell wall could be detected. The method was semi-quantitative because it only allowed comparing signals between species as no calibration curve was calculated. Figures 7 and 8 are a representation of the fluorescence signals of CAPs and CBPs indicating the presence of chitins and chitosans in different microalgae genera.

On the one hand, microalgae of the genera *Scenedesmus* and *Chlorella* were identified as the most probable chitin producers as they had even larger signals than the positive controls; *Mucor* and *Thalasiossira*. Other genera with considerable high CBP signals, especially in comparison to *C. reinhardtii,* the negative control, were the Chlorophytes *Bracteacoccus* and *Haematococcus*. On the other hand, the CAPs proteins determined that *Chlorella* could be a possible producer of chitosans as it had a much higher signal ratio than the positive control, *Mucor,* and the rest of samples analyzed. The other genera that stood out over the rest where *Scenedesmus*, *Thalassiosira* and *Isochrysis,* to a lesser extent. According to these results, *Thalasiossira* apparently does not only make chitins but it also makes chitosans. It is also relevant to highlight that this diatom is known to produce long chitin fibers that extrude from its cell wall[27]; something that has been accordingly detected, as it is the only one of the genera analyzed that showed clearly a higher signal if it was not disrupted. Instead, the rest of samples that were positive according to the screening required a disruption of their cell wall. This indicates that the chitins and chitosans found in all the genera that were analyzed, apart from

Thalassiosira, are embedded in the cell wall forming the protective barrier. This is especially relevant in the case of *Chlorella,* which is known to secrete chitin fibers if infected by chloroviruses. The fact that in this case the chitins and chitosans have been found in the inner parts of its cell wall is an indication that these polymers are different from the ones this genus produces when it is infected[65].

The standard deviation showed that the values between replicates could vary significantly, especially in the case of *C. vulgaris* and some of the other species, particularly within the CBPs assay. Nonetheless, the variations between replicates did not lead to contradictory results; *Chlamydomonas,* the negative control, which was assayed six different times, always had a very low fluorescence ratio in comparison to the strains with higher signals. It is important to highlight that the variability was influenced by the fact that the fluorescence of the proteins diminished in the course of time as all the values of the second biological replicates, which were obtained two weeks after, were always lower than the first. Another focus of variation could be the differences between batches, as the composition of the cell wall can vary considerably depending on the growth stage and growing conditions[13]. This could be the case for *Mucor*, which had fluorescence ratios that were very low in comparison to some microalgae, especially in the case of the CBPs. More biological replicates are required to determine if these are the normal values for *Mucor* or if, as expected, higher fluorescence values should be measured.

Although the results of the screening indicating that *Scenedesmus, Chlorella, Bracteacoccus and Haemaotococcus* could be new producers of chitins and that *Chlorella* and maybe *Thalassiosira* and *Scenedesmus* could be natural producers of chitosan were quite promising, the methodology used had never been validated. In order to do so, a group of twenty different microalgae strains, picked based on the results from the previous screening, were cultured with the aim of extracting and characterizing the chitosans they should contain. Among the selected microalgae, there were thirteen *Chlorella*, three *Scenedesmus*, one *Thalassiosira*, one *Haematococcus*, one *Isochrysis* and one *Chlamydomonas.* Although not in the list of microalgae probably containing chitosans, *Haematococcus* was included due to its economic importance. Also, *Chlamydomonas* was included as a negative control. A process to separate

chitosans from the cell walls of the selected microalgae was developed (Figure 9).

The acid soluble material, where chitosans should be found, was firstly analyzed by ^{1}H-NMR, a robust and well-established technique for the study of chitosans that can also be used to determine their DA%. The ^{1}H-NMR spectra revealed that the polymers present in the acetic acid soluble fraction of nine different *Chlorella* strains (*GAT7, H1993, CS-41, 211/9A, OP, GAT-3, GAT4, GAT-10* and *NC64A)* were indeed mostly chitosans. Unfortunately, the other 11 strains that went through the chitosan extraction process, including four *Chlorella* and all the *Scenedesmus, Thalassiosira*, *Isochrysis*, *Haematococcus* and *Chlamydomonas* strains, resulted to be negative for the natural production of chitosans. Thus, chitosans could only be detected in 9 out of the 20 strains tested, all of them *Chlorella*. It is also important to highlight that not all the *Chlorella* strains tested contained chitosans. The identity of the polymers was further reconfirmed by digesting some of them with highly specific chitosanases and chitinases. Therefore, by ^{1}H-NMR analysis and enzymatic digestion, the screening methodology developed to detect the presence of chitosans in microalgae using CAPs had been validated. Moreover, the presence of chitosans in some *Chlorella* strains finally was confirmed.

Apart from confirming the presence of chitosans in *Chlorella,* a characterization was performed using standard chitosan characterization techniques. On the one hand, ^{1}H-NMR revealed that the DA% is different in each *Chlorella* strain with values ranging from 5 to 25%, although mostly concentrated between 12 and 18%. On the other hand, SEC-MALLS-RI was used for the determination of the Mw of the polymers produced by *GAT-7*, *H1993*, *CS41* and *OP,* with values of 34.6, 28.8, 20.5 and 24.3 kDA, respectively (Table 1). Hence, *Chlorella* can be defined as producers of natural chitosans of low DA% and low Mw. It is important to highlight that even though the extraction process was not at all optimized the Ip value of all the samples analyzed was below 2, meaning that the quality of the polymers obtained in terms of polydispersity of weights is already comparable to the best chitosans that can be found in the market today after many years of optimizations in their extraction process[83]. This is a positive outcome of the enzymatic deacetylation step performed naturally by *Chlorella* that yields a polymer of very good

quality when compared to conventional chitosans which are a product of a harsh chemical deacetylation step that impairs the structure of the molecule by breaking the glycosidic bonds randomly. The same benefits in terms of quality are to be expected with the pattern of acetylation (PA). While the chemical deacetylation step generates a completely random PA, the enzymatic process is expected to generate a non-random PA. Indeed, an enzymatic fingerprinting analysis to determine the PA of chitosans is currently being developed and preliminary results seem to indicate that microalgal chitosans have a non-random PA (communications with Dr. Stephan Cord-Landwehr, from the University of Münster). Having a non-random PA is synonym of reproducibility in bioactivity, specially when obtaining chitosan oligomers by enzymatic means[13,18].

Amongst the chitosan producers identified, it is important to emphasize strain *GAT-7*, which by far contains the largest amount of chitosans per gram of biomass. In contrast it is a slow grower, and because of that its productivity becomes similar to the rest of *Chlorella* strains. For instance, when looking at the amount of chitosan-containing fraction produced per liter, *211/9A* produced 17.29 mg of chitosan fraction per liter while *GAT-7* made 16.57 mg/L. Consequently, *GAT-7* is the best candidate for growth optimization. Concerning the best conditions for the production of chitosans, no conclusions could be reached in this first analysis. There are no clear differences caused by the growth conditions in *Chlorella* because the results from the most repeated culturing conditions (BBM media, CO_2 supplementation, closed bioreactor) can either be better or worse than growing the cells mixotrophically or in open ponds depending on the strain. A deeper study needs to be carried out with only one strain to determine the best growth conditions for chitosan production. Finally, it is also important to highlight that a wide variety in the amount chitosans produced can be seen between species. Instead, the two *Chlorella vulgaris* tested (*H1993* and *CS41)*, the only species that was repeated, had exactly the same amount of chitosan containing fractions. Nevertheless, the characteristics of these two chitosans varied considerably in DA% (25% versus 12%) and Mw (28.8 kDa versus 20.5 kDa). More extractions and characterizations are required to determine whether these differences depend on the strain or on the growth conditions.

While the CAPs and CBPs screening process had been validated for its capacity to detect chitosans, its ability to detect chitins still had not been corroborated. This was a difficult task because a specific process to separate chitins from the rest of cell wall components had not been carried out. Moreover, chitins were not soluble, and thus could not be analyzed by ^{1}H-NMR like chitosans. Nevertheless, the validation of the screening could still be performed with techniques to study the polysaccharides in their solid state. One of them was to study the entire polysaccharide composition of the cell walls by glycosidic linkage analysis. The other one was to study the fraction of the chitosan enrichment process that was not soluble in acid, i.e. the cell wall residue, were chitins were expected to be. This fraction was analyzed by FTIR and also by glycosidic linkage analysis.

Glycosidic linkage analysis is highly complex and cannot be used to screen several species at the same time. It is for this reason that the CAPs/CBPs assay was chosen for the screening. However, after the CAPs/CBPs assay indicated that *Chlorella* was the genus with higher signal ratio, then it made sense to perform the analysis of the total polysaccharide composition of the cell walls of this genus to confirm the screening. Cell wall fractions were extracted and analyzed by glycosidic linkage analysis. Results revealed that the GlcNAc linkages were present in the cell walls of *C. sp GAT-7* and *C. sp OP* at molar percentages of 8.2 and 6.8, respectively. These were much higher percentages compared to the results obtained for the other species tested: *I. Galbana LB2397* and *H. pluvialis K0084,* with values of 1.6 and 0.1, respectively. When it comes to the monosaccharide linkage analysis of the acid insoluble fractions of the same four strains, it was even clearer that chitins were only present in *Chlorella*, as nothing was found in *Isochrysis* or *Haematococcus.* It is important to highlight that the process to separate chitosans had also increased the amount of chitins in the residual fraction. The molar percentages of the GlcNAc linkages in the acid insoluble residues of *C. sp GAT-7* and *C. sp OP* increased to 12.5% and 13.1%, respectively. In all, the glycosidic linkage analysis successfully confirmed the results obtained with the CBPs screening, indicating that chitins were present in the cell wall of *Chlorella.*

The cell wall residue was also analyzed by FTIR. This technique allowed screening more samples than glycosidic linkage analysis. This was important

because there were interesting samples from which the presence of chitins still had to be confirmed. Amongst these there was the *Scenedesmus* genus, which had had the highest signal in the CBPs screening. There were also *Chlorella* strains with a very large residual fraction such as *Chlorella sp GAT-1,* in which this fraction accounted for more than half of the weight of the homogenized biomass (HLB). Moreover, the FTIR could also be useful to determine the DA% of the samples by measuring the difference in transmittance % between the 1320 and 1420 cm^{-1} peaks. Therefore, 10 *Chlorella*, 3 *Scenedesmus* and 1 *Chlamydomonas reinhardtii* strains were studied. None of the FTIR spectra of the strains tested contained all the typical peaks of chitins. Therefore, it could only be said that some samples had more probabilities of containing chitins. For instance, the spectrum containing most of the expected peaks (22 out of 23) was that of *Chlorella saccharophila 211/9A*. In the case of *Scenedesmus*, two strains (*S. subspicatus AC139* and *S. sp. GAT-8*) contained 18 out of the 23 expected peaks for chitins. The worst spectrum was that of *Chlamydomonas,* in which only 9 peaks coincided (Table 4). Concerning the DA% of all the samples that allowed from which it could be determined, it ranged between 17 to 20%. Surprisingly, no polymers with a higher DA% (chitins) were identified in the FTIR spectra, only chitosans. Finally, the FTIR also clearly indicated that in both the cases of *Chlorella* and *Scenedesmus,* the two typical peaks for α-chitin were observed. To sum up, the FTIR analyses indicated that it was highly probable that α-chitosans of a DA ranging between 17 and 20% accumulate in the waste fraction of the chitosan extraction processes from *Chlorella* and *Scenedesmus*.

Therefore, what the FTIR analyses indicate is that an important percentage of chitosans were not solubilized during the process to obtain a fraction rich in chitosans. This would indicate that the chitosan extraction process developed requires important improvements. Nevertheless, this hypothesis completely disagrees with the results from the glycosidic linkage analysis because the molar percentages obtained for the GlcNAc linkages are too high to support that only highly deacetylated chitosans can be found in the waste fraction. Thus, the results from the monosaccharide linkage analysis disagree with the FTIR spectra and indicate that also chitins are found in the waste fractions. As a compromise between the results of the two techniques utilized to study the

acid insoluble fraction, it could be said that a mixture of chitins and chitosans that failed to be extracted during the process are found in the residual fraction from *Chlorella* and probably from *Scenedesmus.*

In order to better understand the proportions of chitins and chitosans in the residual fraction and also in the whole cell wall, a monosaccharide linkage analysis capable of differentiating between chitins and chitosans would be highly desirable. An approach could be to completely acetylate the sample to convert all GLcN to GlcNAc that could be detected in the MS. In parallel, a non-acetylated sample would also need to be evaluated. The extra amount of GlcNAc linkages detected in the acetylated sample would be attributed to the chitosans in the sample. A method like that would also be very interesting to determinine the amount of chitosans in the samples in comparison to other polysaccharides.

In all, the most important outcome of the research presented within this chapter is that, to our knowledge, this is the first time that chitosans produced naturally by autotrophic microorganisms have been discovered, extracted and characterized. The discovery has been contrasted with different techniques such as CAPs/CBPs, ^{1}H-NMR, chitinase and chitosanase digestion and FTIR. The research performed proofs that certain *Chlorella* strains are capable of producing low molecular weight α–chitosans naturally with a high degree of deacetylation. This is an important statement because before only a selected group of Zygomycetes, Ascomycetes and Basidiomycetes were known to produce this valuable polymer enzymatically and none of them are grown industrially at the scale of Chlorella[13]. In fact, at the moment it is practically impossible to find naturally produced chitosans in the market. Microalgal chitosans might be a solution to some of the problems that chitosans have historically faced, such as the low reproducibility of their bioactivities due to the impairment of the polymer caused by the harsh chemical deacetylation conditions it suffers.

Another important achievement of the research presented in this chapter is the possible identification of chitins and chitosans in *Scenedesmus*. This statement has not been completely proven, as the only techniques supporting it are CAPs/CBPs and FTIR. A confirmation with monosaccharide linkage analysis is required. Moreover, the presence of chitosans in *Scenedesmus* would be proven

much more easily if the modification of the monosaccharide linkage analysis commented above was carried out. In case it was reconfirmed that the polymers were found in the residual fraction of the chitosan extraction process, modifications in the extraction process would be required to achieve the correct separation of the polymers. A way of extracting the chitins and chitosans from the residual fraction is to completely deacetylate it. This action would facilitate the separation of the chemically converted chitosans from the rest of polymers by solubilizing them in acidic conditions.

Other results from the CAPs assay that have not been validated are the production of chitosans by *Thalassiosira.* In the same way as in *Scenedesmus*, a definitive proof could be obtained by analyzing the cell wall or the cell wall residue of *Thalassiosira* following the above-proposed modified version of the glycosidic linkage analysis. Similarly, the production of chitins by *Bracteacoccus* is also a possibility according to the CBPs assay. This can also be confirmed by monosaccharide linkage analysis. Indeed, confirming the presence of chitosans in *Scenedesmus* and *Thalassiosira* is necessary to better understanding the CAPs screening method and its limits. In other words, *Chlorella* was the genus with the highest signal ratio versus auto-fluorescence in the CAPs assay (34 and 16.2 for the two strains), and it has been proven that it produces chitosans. The question now is if the signal ratio of *Thalassiosira* (7.9) or *Scenedesmus (6.2),* which were the second and third highest signal ratios obtained with the CAPs assay, are high enough to indicate the presence of chitosans. The same can be commented about the screening process with CBPs. In this case, *Chlorella* with a signal ratio of 15.2 has been confirmed to contain chitins and the same is expected from *Scenedesmus (*22.6). In the contrary, *Haematococcus*, with a signal ratio of 9 does not contain chitins but *Thalassiosira* with a signal ratio of 9.51 is a chitin producer. Therefore, Bracteacoccus with a signal ratio of 10.6 would be expected to have chitins. Better understanding the capacities of the screening process will allow using it for future applications such as expanding the screening to other species. Moreover, if the method is precisely optimized for a specific strain and its autofluorescence, a calibration curve could be generated to accurately and quickly analyze the conditions that lead to a higher chitin or chitosan production without having to perform all the posterior validations.

The fact that *Chlorella* is one of the microalgae most cultured in the world makes the discovery of these polymers more relevant. Therefore, the chitosans found in *Chlorella* are the basis for the subsequent chapters of this PhD thesis project. In Chapter 3, the molecular biology mechanisms underlying the production of chitosans in *Chlorella* are studied. It is of special interest to understand the enzymatic mechanism underlying the deacetylation step in a species with 25 putative chitin deacetylases. In Chapter 4, the chitosan extraction process is optimized and up-scaled to obtain enough *Chlorella* chitosans to analyze its bioactivities and identify possible applications.

2.4 Materials and Methods

2.4.1 Microalgae culture

The culture conditions vary depending on the strains used and depending on the experiment larger or smaller volumes were required. The recipes of the growth media can be found at Appendix 2. For the CAPs/CBPs assay the cells were cultured as follows: the diatoms *Thalassiosira pseudonana LBFD2* (UTEX), *Chaetoceros gracilis LB2658* (UTEX) and *Phaeodactylum tricornutum 1055/1* (CCAP) were cultured in 300 ml of F/2 x2 culture medium in Erlenmeyer flasks aerated with 5% CO_2 for 8 days when the cultures were at stationary phase. Temperature and light were kept at 25 ˚C and 50 μmol $m^{-2}s^{-1}$, respectively. The zygomycete *Mucor circinelloides* was cultured in 120ml of YPG medium in an Erlenmeyer flask for 5 days when it was harvested in stationary phase. Growth conditions were 28˚C and stirring at 100 rpm. *Chlamydomonas reinhardtii CC124* (Chlamy Collection) was cultured in 470 ml of TAP medium for 9 days, the culture was at stationary phase when harvested. Growth conditions were 50 μmol $m^{-2}s^{-1}$ of light at 25 ˚C. *Dunaliella salina 19/20* (CCAP) was cultured in 130 ml for 10 days and harvested at stationary phase. Growth conditions were 50 μmol $m^{-2}s^{-1}$ of light, 1% CO_2 and 25 ˚C. The culture medium was Dunaliella medium. *Haematococcus pluvialis K0084* (SCCAP) was cultured in 300 ml of Kuhl medium for 14 days at 40 μmol $m^{-2}s^{-1}$, 25 ˚C and 5% CO_2 until reaching the stationary phase. *Chlorococcum sp. GAT-11* was taken to the stationary phase in BBM culture medium aerated with 5% CO_2, at 50 μmol $m^{-2}s^{-1}$ of light and a temperature of 25 ˚C. *Bracteacoccus minor H3801* (CAUP) was cultured in 800 ml of BBM medium for 15 days and harvested at stationary phase. Culturing

conditions were 50 μmol $m^{-2}s^{-1}$, 25 ˚C and aeration with 1% CO_2. *Isochrysis galbana LB2307* (UTEX) was cultured to the stationary phase in 900 ml of F/2x2 medium for 9 days. Growth conditions were 50 μmol $m^{-2}s^{-1}$, 1% CO_2 and 25 ˚C. *Nannochloropsis gaditana AC223* (Algobank Caen) was cultured in a 450 ml Erlenmeyer flask for 18 days and harvested at the stationary phase. Growth conditions were 50 μmol $m^{-2}s^{-1}$ of light, 1% CO_2, 30 ˚C, and F/2 medium. *Chlorella vulgaris CS-41* (CSIRO) was cultured in 325 ml of BBM medium for 22 days and harvested at stationary phase. Growth conditions were 50 μmol $m^{-2}s^{-1}$, 5% CO_2 and 25 ˚C. *Chlorella saccharophila 211/9A* (CAUP) was cultured in 450 ml of BBM culture medium for 16 days and harvested at stationary phase. Growth conditions were 50 μmol $m^{-2}s^{-1}$, 5% CO_2 and 25 ˚C. *Scenedesmus subspicatus AC139* (Algobank Caen) was cultured in 300 ml of F/2x2 medium at 50 μmol $m^{-2}s^{-1}$ for 8 days and harvested at stationary phase. The culture was aerated with 5% CO_2 and the temperature was kept at 25 ˚C.

The strains utilized for the chitosan extraction process were: Chlorella sp. GAT-1, Chlorella sp. GAT-2, Chlorella sp. GAT-3, Chlorella sp. GAT-4, Chlorella sp. GAT-7, Chlorella vulgaris H1993, Chlorella sacharophila 211/9A, Chlorella zofingiensis B32, Chlorella sorokoniana 2805, Chlorella sp. GAT-10, Chlorella vulgaris CS-41, Chorella variabilis NC64A, Chlorella sp. OP, Scenedesmus sp. GAT-8, Scenedesmus sp. GAT-9, Scenedesmus subspicatus AC139, Thalassiosira weissflogii CCMP1336, Isochrysis galbana LB2307, H. pluvialis K0084 and Chlamydomonas reinhardtii CC124.

For the first chitosan extraction process all *Chlorella* and *Scenedesmus* strains (except from *Chlorella vulgaris NC64A*) were cultured under the same conditions. 100 mL pre-cultures were used to inoculate 800 mL cultures to stationary phase. They were cultured autotrophically in an Algaetron in Bold's Basal Media (BBM) at 25ºC with a regular illumination of 100 μmol $m^{-2}s^{-1}$ and a continuous air in-flow containing 2% of CO_2. *Chlorella vulgaris NC64A* was cultured mixotrofically in Modified Bolds Bassal Medium (MBBM) with 100 μmol $m^{-2}s^{-1}$ of illumination, at 25ºC, with continuous shaking at 100 rpm and without aeration. The microalgal powder grown in open ponds was obtained from a commercial producer of *Chlorella (Chlorella sp. OP)*. CCMP1336 was obtained from a 10L culture from the laboratory of Dr. Chris Bowler at the CNRS in Paris. The strain was grown to stationary phase in L1 growth media in

a temperature that ranged between 11 and 16ºC. *Isochrysis galbana* and *Haematococcus pluvialis* were cultured in 100L bioreactors in F/2x2 and Kuhl medium, respectively. The Chlamydomonas strain was cultured mixotrophically to stationary phase in 800 mL of TAP media containing glacial acetic acid as a carbon source. Finally, the *Thalassiosira* was obtained as a pellet from a 10L culture grown to stationary phase at the laboratory of Dr. Chris Bowler at the CNRS in Paris.

The strains utilized for the cell wall monosaccharide linkage analysis were *Chlorella sp. (Gat-7), Chlorella sp. (OP), I. galbana LB2307* and *H. pluvialis K0084.* The strain *GAT-7* was cultured autotrophically in an Algaetron in 100 mL of Bold's Basal Media (BBM) at 25ºC with a regular illumination of 100 μmol $m^{-2}s^{-1}$ and a continuous air in-flow containing 1% of CO_2. 0.120g of dry biomass was used for cell wall extraction. The cell walls of strains *K0084* and *LB2307* were obtained from left over of biomass that had been cultured in 100L bioreactors at Greenaltech's pilot plant under autotrophic conditions in Kuhl and F/2x2 media, respectively. 1.1 g and 2.34 g of dry biomass of *K0084* and *LB2307* respectively were the starting material for the cell wall extraction process.

The recipes for the preparation of the culture media utilized can be found in Appendix 2. Cultures were monitored daily by measuring the optical density (O.D) at 680nm. The biomass of each of the microorganisms was recovered from the liquid culture by centrifuging at 5,000g for 10 minutes and was then immediately frozen in liquid nitrogen and stored at -80 ˚C. When necessary the biomass was solubilized in culture media to a concentration of 40g/L and homogenized by pressure using a cell disruptor, exposing the sample to 2,5 kbars of pressure 5 times. Proper homogenization of the samples was confirmed by optical microscopy.

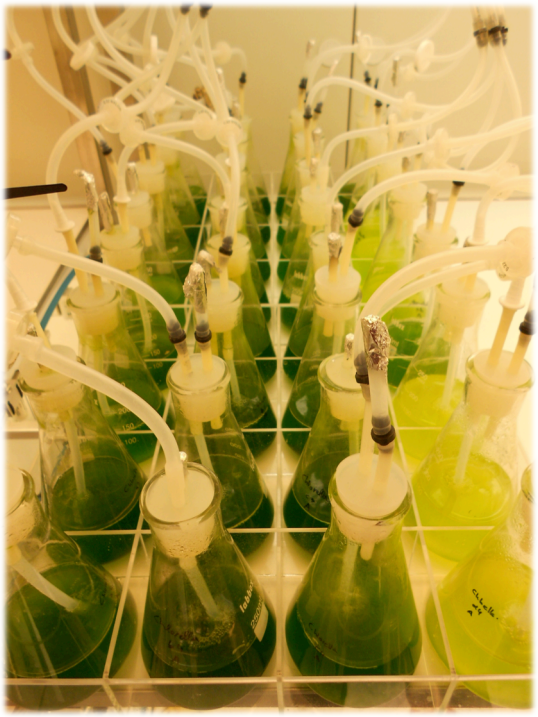

Figure 14 - *Chorella* cultured in 250 ml erlenmeyer flasks

2.4.2 Chitin and Chitosan identification in microalgal biomass using CAPs and CBPs

CBP-eGFP and CAP-eGFP fusion proteins and advise for the design of the screening method were obtained from Tobias Weikert fom the laboratory of Prof. Bruno M. Moerschbacher from the University of Münster, Germany, as part of a collaboration within the Nano3Bio FP7 EU project. The protocol was developed based on previous assays with CAPs and CBPs carried out by the same laboratory group[72,73]. The experiment was performed in Nunclon Delta Surface 96-well plates at room temperature. First the samples were incubated in a 2% BSA PBS solution (mass-volume) for 2 hours under stirring at 100 rpm. Then, 3 washes were performed with Tween 20 0.05% PBS stirring at 100 rpm for 10 minutes each. Next, the samples were incubated in a solution of 1.6 μM CBP or 1.6 μM CAP in 5% BSA TBS for 1 hour stirring at 100 rpm. The negative controls of each sample indicating the autofluorescence of the biomass were incubated in the same way but without CAPs or CBPs. Two more washes were performed before the final reading to remove all those proteins that had not specifically bound to chitin or chitosan. The reading of the fluorescence signal of each of the samples was done with a Biotek FLX800 plate fluorometer

equipped with a 485/20 nm excitation filter and a 528/20 nm emission filter. All centrifugations between incubations and washes were done in a plate centrifuge at 2700 rpm for 10 minutes. Three technical replicates were performed per biomass tested. Moreover, for most strains two or more biological replicates were performed (except from *Mucor, Isochrysis, Chlorococcum* and *Dunaliella* and the non-disrupted biomass of *Thalassiosira, Chaetoceros and Phaeodactylum*). Between four and five strains were tested per plate, among those, *Chlamydomonas* was always included as a negative control in order to detect if the non-attached fluorescent proteins had been washed correctly each time. For this reason, the *Chlamydomonas* biomass was analyzed more than 6 times. All reactives where acquired from Sigma Aldrich.

2.4.3 First microalgal chitosan extraction method

In general, microalgae are grown to stationary phase, harvested at 5,000 g for 10 min and stored at -20ºC in case it is not used immediately. In case commercial biomass is used the biomass is dried at this point. Drying was also performed in some cases at this point to calculate how much biomass was loss with the homogenization step. In the next step microalgae are diluted in growth media in an approximate proportion of 40g biomass/L. Once the biomass is completely dissolved it is homogenized in a cell disruptor (TS Series Benchtop, Constants system) five times at a pressure of 2.5 Kbar. Samples are collected after homogenization and watched under the microscope to confirm proper cell disruption. The homogenized samples are centrifuged at 15,000gs for 20 minutes, the supernatant is discarded and the cell debris is kept to continue with the polymer extraction. Afterwards the homogenized biomass is lyophilized and its weight is determined using a scale. The weight of the homogenized and lyophilized biomass (HLB) contains mostly cell wall debris and is used as a reference to determine the percentage of chitin and chitosan fraction.

The process continues with the deproteination step consisting of the incubation of the homogenized and lyophilized biomass (HLB) in 1:30 w/v of a 2% Sodium Hydroxide solution for 2 hours at 90ºC. After this treatment the proteins are solubilized and separated by centrifugation. Then the pellet is washed with water to reach a neutral pH and right after it is incubated 1:40

w/v with thorough mixing in a solution containing acetic acid 5% during 6 hours at 60ºC. During this incubation the chitosan is solubilized and afterwards separated from the rest of components that precipitate when centrifuged at 10,000gs for 15 minutes.

Chitosans, which are now soluble, are brought to alkaline pH by the addition of a 1,2M sodium hydroxide solution until a final pH of 8-9 is reached. After two hours at this pH chitosans are precipitated at 15,000gs for 20 minutes. Once the chitosans are precipitated they are washed three times with water, two times with ethanol and two more times with acetone. Finally, the chitosans are lyophilised to eliminate any trace solvents. Once this final step has been reached, chitosans are ready for its characterization. The non-soluble fraction in 10% acetic acid is also washed with water and solvents to purify it as much as possible for the analysis of its composition. The value used to compare the quantities of chitin and chitosan fractions between samples is the percentage in weight of these fractions in front of the previously determined HLB weight.

All reactives were acquired from Sigma Aldrich.

2.4.4 ^{1}H-NMR to characterize chitosans

In order to perform the ^{1}H-NMR, 5mg of chitosan are dissolved in Deuterium Oxide with 1% Deuterium Chloride. Samples are left overnight in a magnetic stirrer to ensure complete dissolution of the polymer. After solubilization ^{1}H-NMR is performed following the protocol specified in the article from Lavertu *et. al.*[78]. The only difference is that TMSP-d4 was used as internal reference. All 1H-NMR experiments were performed at the facilities of the NMR Unit of the Scientific and Technological Centers of the University of Barcelona by Dr. M. Antònia Molins Montserrat. The determination of the DA was calculated using the areas of the peaks of protons H1 of both deacetylated and acetylated monomers (H1-D and H1-A):

$$DA\% = \left[1 - \left(\frac{H1D}{H1D+H1A}\right)\right] \times 100 \qquad (I)$$

2.4.5 Digestion of *Chlorella* chitosans with chitinases and chitosanases

Chitosans from *C. sp. GAT-7, C. vulgaris CS-41 and C. vulgaris H1993* were extracted as indicated previously (subsection 2.4.3). To perform the chitosanase digestion, 1 mg/mL of the three chitosans was incubated with chitosanase GenBank ac. No. AFD19011.1 from *Bacillus sp. MN* at a concentration of 1μg/mL overnight at 37ºC in a 50mM sodium acetate buffer at pH 5.2. To perform the chitinase digestion, the same chitosans at a concentration of 1mg/mL were incubated with chitinase GenBank ac. No. ABI31431.1 from *Serratia marcescens* in 50mM sodium acetate buffer at pH 5.8 overnight at 37ºC. The enzymes were removed with 3K PES filters from VWR and analyzed with the LC-MS method described in Harner et. al. 2015[90]. The experiment was performed in collaboration with Dr. Steffan Cord-Landwehr, Dr. Nour Eddine el Gueddari and Jasper Wattjes at the laboratory of Professor Dr. Bruno Moerschbacher at the University of Münster as part of a collaboration within the Nano3Bio FP7 EU project.

2.4.6 Molecular weight and polidispersity index determination of chlorella chitosans

Chitosans from *C. sp. GAT-7, C. vulgaris CS-41 and C. vulgaris H1993* were extracted as indicated previously. The average molecular weight (Mn), the weight-average molecular weight (Mw) and the polydispersity index (Ip) were measured by gel permeation chromatography (Novema® columns from PSS 30Å, 3000Å, 3000Å and guard column; I.D.: 8 mm) coupled on line with a refractive index detector (Agilent Serie 1200 RID®), a viscometer detector (PSS ETA-2010 differential viscometer ®) and multi-angle- laser-light- scattering (PSS SLD 7000 MALLS ®) equipped with a 5 mW He/Ne laser operating at λ = 632.8 nm. Light intensity measurements were derived following the classical Rayleigh-Debye equation allowing to deduce the Mw. The dn/dc was determined from a polynomial based on previous studies that relates the dn/dc with the degree of acetylation. A degassed 0.2 M acetic acid / 0.15 M ammonium acetate buffer (pH = 4.5) was used as eluent. The flow rate was maintained at 0.6 mL/min. The Ip value was obtained by dividing Mw by Mn. The determinations of molecular weights and polidispersity indexes were performed at the laboratory of professor Dr. Bruno Moerschbacher by Dr. Nour

Eddine el Gueddari at the University of Münster as part of collaboration within the Nano3Bio FP7 EU project.

2.4.7 Chlorella cell wall extraction for linkage analysis

Microalgal cells were grown to stationary phase, collected by centrifugation and homogenized as indicated above. The freeze-dried pellet were solubilized in tubes containing 95% ethanol and incubated for 30 min at 65ºC. This process was repeated until the supernatant was completely clear. The pellet was poured in a falcon tube to the 10 mL line and then a mixture of 2:1 Chloroform Methanol was added. This solution was mixed for 1hour at room temperature. The solvent was removed and 70% ethanol was added to the pellet for incubation during 1.5h two times. Subsequently the pellet was incubated for 1h with 80% and then 95% ethanol for 2 hours. All incubations were performed under shaking. Finally the solvent was changed for acetone, and after removing it, the pellet was transferred to a petri dish covered with aluminium foil and let dry overnight. The next day the powder was incubated with 30 ml of 5 U/ml α-amylase (type VI-B from porcine pancreas, Sigma Aldrich) in 0.01M phosphate buffer pH7 during 24h at 37ºC. This process was performed twice removing supernatant by centrifugation. The remaining paste was washed three times for 1 minute with ethanol and then 3 more times with acetone. The final pellet was transferred again to a petri dish recovered with aluminium foil and let dry overnight. The final powder was considered to be the cell wall fraction, which was used for monosaccharide linkage analysis. All reactives were acquired from Sigma Aldrich.

2.4.8 Carboxyl reduction-Methylation-GC/MS analysis

In order to determine the presence of chitins in the *Chlorella* cell wall and the waste fraction of the chitosan extraction process, the linkages between monomers was analysed. This work was performed in collaboration with Dr. Xiaohui Xing from the group of Glycoscience of the KTH Royal Institute of Technology led by Professor Vincent Bulone within the frameworl of the Nano3Bio FP7 EU project. The protocol followed is described bellow.

Uronic acids in a polysaccharide sample (5 mg) were converted to their 6,6-dideuterio neutral sugar counterparts using carbodimide activation at pH 4.75

followed by sodium borodeuteride ($NaBD_4$) reduction at pH 7.0[91]. After dialysis against deionized water for 48 h, the carboxyl-reduced sample was collected by freeze-drying. One milligram of the freeze-dried sample (three technical replicates) was then methylated[92], followed by trifluoroacetic acid (TFA) hydrolysis, reducing end reduction using $NaBD_4$, and acetylation to produce partially methylated alditol acetates (PMAAs), according to the protocol[93]. PMAAs from neutral sugars and amino sugars were then analysed respectively using an SP-2380 capillary column (30 m × 0.25 mm i.d., Sigma-Aldrich) and an Agilent CP-Sil 5 CB capillary column (30 m × 0.25 mm i.d.; Agilent Technologies) on an Agilent 6890/5973 GC-MS System, following the literature[94]. The mass spectra of PMAAs were interpreted by comparing with those of reference derivatives and by referring to literature[91]. The experiment was conducted in duplicates.

2.4.9 Cell wall residue solubility tests for ^{1}H-NMR characterizations

The chitosan waste fraction was obtained as indicated in the description of the chitosan extraction method. The solubility of the samples was studied by dissolving 5 mg of the waste fractions in 1mL of DMF, DMF with 5% LiCL DMA and DMA 5% LiCl. All samples were agitated in a magnetic stirrer overnight. In order to increase the solubility, a trial with DMA 5% LiCl consisting of the same overnight solubilisation followed by three freeze/thaw cycles at -80ºC was carried out. The solubility was compared between the samples by macroscopic observation.

2.4.10 FTIR analysis of the waste fractions of the chitosan extraction process

Chitin and chitosan samples were ground to a very fine powder with KBr and dried thoroughly. The dried mixture was pressed under vacuum in a mould to form a KBr disc containing the sample. Samples of 1 mg were prepared in KBr disk at a concentration of 2% (w/w). The infrared spectra were registered in an FTIR (FT-IR Spectrometer Michelson interferometer) connected to a PC with Omnic software (Thermo Electron Corp., Woburn, MA) to process the data. FTIR was used in the wavenumber region between 4000 and 400 cm^{-1}.

In order to determine the DA using FTIR, the transmittance values were first converted to absorbance using the equation:

$$A = 2 - \log_{10} \%T \quad \text{(II)}$$

Afterwards, the following relation obtained from Brugnerotto *et al.* was used to determine the DA%:

$$A1320/A1420 = 0.3822 + 0.3133 \times DA \quad \text{(III)}$$

3 THE ENZYMATIC PROCESS BEHIND THE PRODUCTION OF CHITOSANS IN CHLORELLA

3.1 Background and aims

When the genome of *Chlorella variabilis NC64A* was sequenced, one of the major observations was that this photosynthetic microorganism had enzymes of the carbohydrate metabolism in much larger proportions when compared to the genomes of other sequenced microalgae[25]. Hence, *C. variabilis NC64A* and probably the rest of other species of the *Chlorella* genus appear to be particularly well equipped with tools for synthesizing and modifying carbohydrates. A proof showing that these tools have been exploited very well by *Chlorella* is its ability to build a particularly robust and difficult to penetrate cell wall, a key attribute of this genus that has allowed it to thrive around the globe. Amongst the many genes encoding for carbohydrate active enzymes, instead of finding homologs of proteins involved in the synthesis of cellulose or hemicellulose, which are major components of the cell walls of land plants, several putative genes related to the formation and remodeling of chitins and chitosans were found[25].

On the side of chitosan production, two putative chitin synthases were identified in the genome of *Chlorella variabilis NC64A*[25]. These proteins are glucosyltransferases in charge of the last step in the elaboration of chitin

polysaccharides, which is to transfer the α-linked GLCNAC sugar from UDP-GLcNAc to the non-reducing end of the growing chitin oligosaccharide (Figure 15)[95]. Moreover, as many as 25 different putative paralogs to chitin deacetylases were identified[25]. These enzymes are capable of hydrolyzing the acetamido group in GlcNAc residues, generating GlcN and acetic acid; thus deacetylating chitins and chitosans[96]. Studies performed to understand the way in which chitosans are synthesized in fungi suggest that chitin synthases operate in tandem with chitin deacetylases, the first enzyme synthesizing chitin, and the second enzyme hydrolyzing the N-acetamido groups of the nascent chitin chains[97]. Additionally, putative paralogs to enzymes involved in the degradation of chitin and chitosan were found in the genome of *C. variabilis NC64A*; more exactly two chitinases and two chitosanases[25]. Chitinases and chitosanases hydrolyze the β-1,4-glycosidic bonds of chitin and chitosan, respectively (Figure 15)[80,98]. The putative existence of the four gene classes involved in synthesizing and modifying chitins and chitosans make *Chlorella* unique, as, except for a plant-type chitinase gene, which is found in land plants (but not in Chlorophytes apart from *Chlorella*), these genes are absent in all the other fully sequenced Viridiplantae species[25]. Such complete machinery had to be useful for the generation and modification of chitosans and finally these polysaccharides have been physically proven to exist in the cell wall of several *Chlorella* species. Chapter 2 of this thesis explains how these chitosans were identified, extracted and characterized.

When focusing on the chitosan biosynthetic pathway, the fact that *C. variabilis NC64A* has chitin synthases is peculiar in microalgae but not so much when taking into account other organisms; chitin is synthesized by an enormous number of living organisms and it is considered to be the most abundant biopolymer in the world after cellulose[52]. Instead, the ability to naturally produce chitosans is very unique, as so far only a small group of fungi of the Zygomycota, Basidiomycota and Ascomycota phyla have been found to be capable of doing so[13]. Thus, the study of *Chlorella's* CDAs (cCDAs) is interesting to understand the biological processes underlying the unique way of naturally producing chitosan by these photosynthetic organisms.

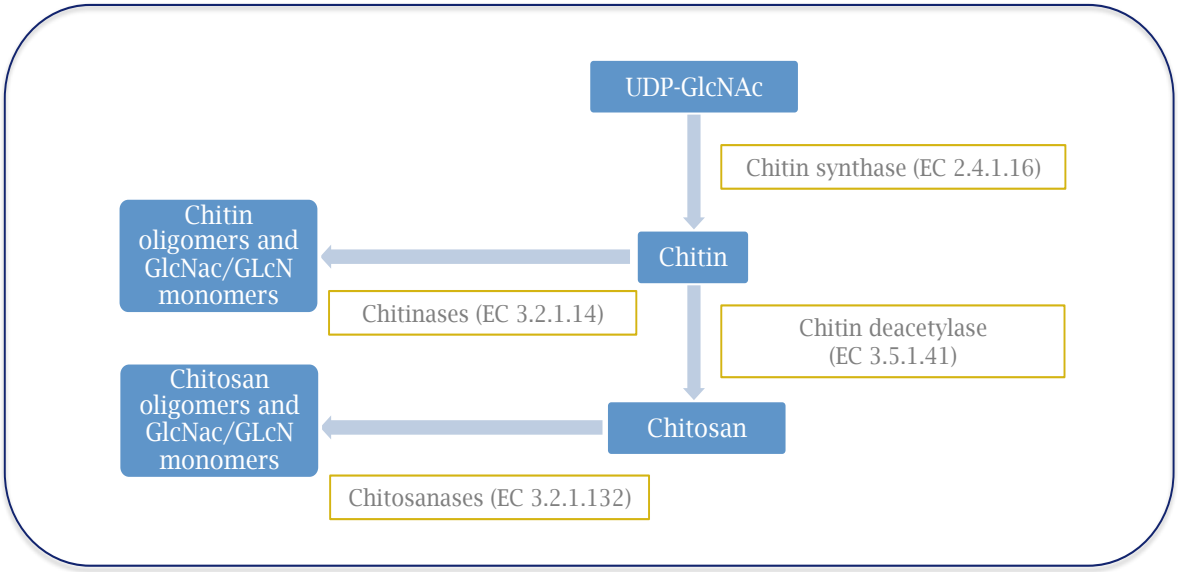

Figure 15 – Putative Enzymes found in *Chlorella variabilis NC64A* that are involved in the synthesis and degradation of chitins and chitosans.

The chitosans that can be found commercially in the market today are produced by chemically deacetylating chitins, mainly from crab and shrimp shells. The chemical deacetylation step involves incubating chitins at high temperatures and concentrated sodium or potassium hydroxide (40-50%) solutions[10]. This process presents several disadvantages; it leads to low quality products with a wide range of molecular weights and random patterns of acetylation, it produces a waste stream with a highly concentrated alkali solution and it consumes considerable amounts of energy[13]. Instead, the enzymatic process of converting chitins to chitosans yields more homogeneous product in terms of chain length, degree and patterns of acetylation, no harmful waste streams are generated and high temperatures are not required. Homogeneous polymers in reproducible batches like the ones that can be produced enzymatically, with specific physical and chemical properties, are required for many high-value applications[90]. Thus, the study of the 25 putative chitin deacetylases in *Chlorella* (cCDA) is also relevant from a commercial point of view.

Along these lines, the enzymatic deacetylation of chitins to make chitosans in *Chlorella* is the most interesting step in the production of these polymers from both perspectives, the study of the biological mechanisms and the development of new or enhanced commercial applications of chitosans. Thus,

discovering the chitin deacetylases responsible for the natural conversion of chitin to chitosan in *Chlorella* is the main goal of the research presented within this chapter. By studying the enzymes from *Chlorella variabilis NC64A,* new CDAs with different and maybe desirable characteristics can be found. 25 putative CDAs is a large number and, to our knowledge, none of them have been studied before. Moreover, *Chlorella* is the only known photosynthetic microorganism capable of fabricating chitosans, hence, the evolutionary distance with other known active CDAs may be translated to new and different properties.

3.2 Results

3.2.1 Identification of active CDAs

CDAs (EC 3.5.1.41) are enzymes that hydrolyze the acetamido group of the GlcNAc units in chitins to generate GlcN and acetic acid (Figure 16). Inside the Carbohydrate Active Enzymes (CAZY) database they have been classified in the Carbohydrate Esterase Family 4 (CE-4) with other members who also share the "NodB homology domain" or "chitin deacetylase domanin"[97,99]. The first active CDA was identified and partially purified from extracts of the fungus *Mucor rouxii*[100]. Later on, some active CDAs were identified and purified from very diverse organisms, such as archaea, marine bacteria, fungi and insects, which in many cases are not even capable of producing chitosans[101]. These enzymes have been characterized and, as their sources, they are very diverse in characteristics and optimal working conditions. Their sizes vary from 12.7 to 150 kDa, their isoelectric point (pI) varies from 2.65 to 4.8, the optimum pH can also vary from 4.5 to 12 and they show a remarkable thermal stability as their optimum temperatures for activity range from 30ºC to up to as high as 60ºC. What is common for most of them is the preference for soluble chitins like glycol chitin or chitin oligomers. The narrow specificity for GlcNAc homopolymers is also a common trait for all CDAs that have been characterized so far. In terms of their cellular localization in fungi, CDAs have been reported either in the periplasm or extracellularly. Periplasmic CDAs are generally tightly coupled to a chitin synthase to rapidly deacetylate newly synthesized chitins before their maturation and crystallization. On the other

side, extracellular CDAs are secreted to alter the physicochemical properties of the cell wall to either protect them from chitinases or to start autolysis. [97,102–104].

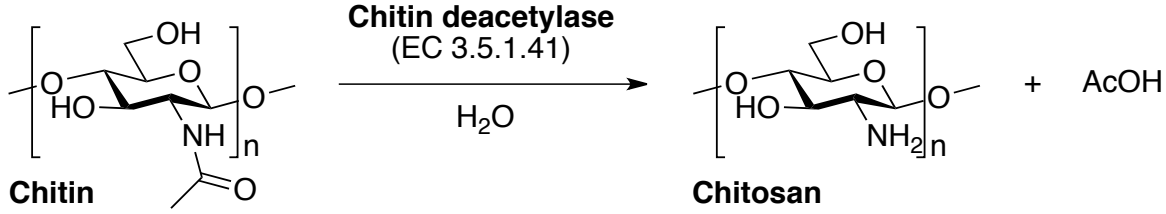

Figure 16 - The catalytic action of chitin deacetylases.

Because CDAs vary so much, their identification, purification and characterization is not an easy and standardized task. In general two approaches to obtain pure chitin deacetylases have been carried out. The first approach consists in the purification of the CDA from intracellular or extracellular protein extracts by applying a combination of different techniques such as precipitation with saturation concentrations of ammonium sulfate and protein purification with size-exclusion chromatography (SEC), immunoaffinity chromatography (IAC), hydrophobic interaction chromatography (HIC) or ion exchange chromatography. After each separation step the protein is monitored by measuring activity of the fractions obtained by either incubating the fractions with soluble chitins and measuring the release of acetic acid or by measuring radioactivity if glycol chitins have radiolabelled *N*-acetyl groups[96,100,105–111].

The second approach is the amplification of the CDA of interest from its natural source by PCR and afterwards clone and recombinantly express it in heterologous hosts such as *E. coli or P. pastoris.* In this case only affinity chromatography is required to purify the recombinant protein using an affinity tag. Afterwards, the presence of the protein is detected by gel electrophoresis[103,112,113]. If possible this second approach is better as higher yields can be obtained and the process is simpler because fewer purification steps are required. However, the heterologous expression of CDAs is not straightforward as CDAs are generally membrane proteins.

In order to increase the possibilities of finding active CDAs in *Chlorella*, both approaches were been pursued; i.e. *Chlorella* CDA expression in *E. coli* and CDA searching in natural protein extracts from *Chlorella*. Because 25 putative CDAs have been identified in *C. variabilis NC64A*[25] and it is a high number especially when trying the cloning and recombinant expression route, a preliminary attempt to identify the most probably real CDAs using bioinformatics tools was carried out.

3.2.2 Identification of probably active *Chlorella* CDAs with bioinformatics tools

The process of chitin deacetylation in *Chlorella variabilis NC64A* is likely to be carried out by several enzymes, as 25 different putative chitin deacetylases were found when its genome was sequenced[25]. However, existing so many of them, it is highly probable that not all of the putative CDAs are active or expressed. Therefore, in this subsection the research performed to bioinformatically identify those putative cCDAs that are most likely to be cCDAs is explained. This work aimed to reduce the number of putative CDAs to be cloned in *E. coli* to study their activity.

When searching for the 25 cCDAs described by Blanc *et al.*[25],only 22 sequences were found in the JGI *Chlorella* genome portal (http://genome.jgi.doe.gov/ChlNC64A_1/ChlNC64A_1.home.html). A new name was given to each putative CDA to ease the interpretation of results (Table 5). These 22 putative CDAs are predicted to be highly heterogeneous as their expected sizes range from 29.5 to 139.2 kDa, although most of them are concentrated between two groups, one averaging 36.2±7 kDa and the other 69.4±7 kDa. Also, most cCDAs are expected to have an isoelectric point (pI) moderately acidic but less acidic than fungal CDAs[102]. Only cCDAS 15 and 17 have a basic pI, something that has not been found amongst the fungal CDAs in the literature[102].

In order to decipher which are the cCDAs with more probabilities of being active, it was attempted to identify cCDAs with more similarities with already identified active CDAs in order to try to infer homology. First, individual BLASTP searches of each cCDA against the whole NCBI database were performed. The goal was to see if amongst the similar proteins or homologous

protein regions found, CDAs already known to be active could be identified. However, results showed significant similarities to putative CDAs from chlorovirus, insects and fungi CDA like proteins that quickly collapsed the results list. Thus, in order to obtain more meaningful results, the BLASTP search was reduced to only Zygomycota, Ascomycota and Basidiomycota, the three fungi phyla that mainly harbor the microorganisms capable of naturally producing chitosans. Still, no previously characterized CDA proteins appeared in the results table.

JGI code	CDA name	ORF (aas)	Mw (kDa)	PI
52845	cCDA1	387	41.9	4.57
56687	cCDA2	311	34.3	5.54
55609	cCDA3	677	73.5	4.52
59447	cCDA4	667	70,5	5.14
58845	cCDA5	684	71.5	5.83
140524	cCDA6	384	38.6	4.49
139978	cCDA7	584	62.1	4.58
142985	cCDA8	624	64.6	5.89
143035	cCDA9	272	29.5	4.26
142762	cCDA10	431	47.1	4.45
142095	cCDA11	299	33	4.27
145232	cCDA12	331	36.7	4.51
144776	cCDA13	648	67.4	4.82
136266	cCDA14	324	35.9	4.78
136204	cCDA15	658	71.4	8.41
136749	cCDA16	490	50.3	6.23
138342	cCDA17	307	34	8.74
138801	cCDA18	650	70.4	4.73
138802	cCDA19	698	73	5.49
138797	cCDA20	392	42	4.36
136163	cCDA21	737	83.1	5.04
54401	cCDA22	1334	139	4.64

Table 5 – Putative *Chlorella* CDAs and expected characteristics

Because the previous results did not help in the search for active cCDAs, sequence similarity searching was performed only between the cCDAs and some fungal CDAs with experimental evidence of deacetylase activity. The fungal CDAs utilized for the analysis were the sequences from the Basidiomycetes *Flammulina velutipes* (Uniprot ID. Q1XGD4)[103] and *Cryptococcus neoformans* (Uniprot IDs. Q5KFG8 and Q96W71)[114], the Zygomycetes *Mucor rouxii* (Uniprot ID. P50325)[115] and *Rhizopus nigricans* (Uniprot ID. Q32XH4)[105], and the Ascomycetes *Saccharomyces cerevisae* (Uniprot IDs. Q06702 and Q06703)[116,117] and *Colletotrichum lindemuthianum* (Uniprot ID. Q6DWK3)[112].

Putative functional similarity can be assessed after a Blast search by analyzing the alignment percentage of identity, the bit-score or the expect value (E)[118]. In this case, the E-value, which is a parameter that describes the number of hits one can expect to see by chance when searching a database, was used to determine the probability of functional similarity[118]. As it can be seen in Table 6, 8 cCDAs (cCDAs 1, 3, 4, 7, 9, 14, 20, 21) showed homology to at least one fungal cCDA. Amongst these, cCDA 9 is clearly the cCDA more similar to the fungal CDAs. Nevertheless, the E-values of the cCDAS are quite discrete when compared to those of the alignments between fungal CDAs. The exception was *C. neoformans* CDA Q5KFG8, which was not homologous to any of the CDAs; in fact it showed less homology than the cCDAs. In this sense the cCDAs with least homology with fungal CDAs were cCDA10 and cCDA19.

	Q1XGD4	P50325	Q06703	Q06702	Q6DWK3	Q32XH4	Q96W71	Q5KGF8
cCDA 9	1.0E-07	4.0E-04	1.0E-04	1.0E-05	--	--	--	--
cCDA 7	6.0E-05	--	--	--	--	--	--	--
cCDA 20	5.0E-04	--	--	--	--	--	--	--
cCDA 4	--	--	2.0E-05	3.0E-06	--	--	--	--
cCDA 1	--	--	--	4.0E-04	--	--	--	--
cCDA 3	--	--	--	5.0E-04	--	--	--	--
cCDA 21	--	--	--	--	8.0E-06	--	--	--
cCDA 14	--	--	--	--	8.0E-04	--	--	--
Q1XGD4	0.0E+00	6.0E-21	8.0E-14	6.0E-14	1.0E-36	6.0E-18	5.0E-20	--
P50325	3.0E-21	0.0E+00	7.0E-29	2.0E-27	9.0E-23	6.0E-84	2.0E-92	--
Q06703	6.0E-14	9.0E-29	0.0E+00	5.0E-127	1.0E-18	2.0E-30	3.0E-28	--
Q06702	5.0E-14	3.0E-27	5.0E-127	0.0E+00	2.0E-20	7.0E-28	1.0E-26	--
Q6DWK3	8.0E-35	2.0E-22	1.0E-18	2.0E-20	0.0E+00	3.0E-18	5.0E-11	--
Q32XH4	2.0E-17	2.0E-78	1.0E-27	2.0E-25	9.0E-16	0.0E+00	1.0E-62	--
Q96W71	4.0E-20	6.0E-92	2.0E-28	2.0E-26	1.0E-11	2.0E-66	0.0E+00	--
Q5KFG8	--	--	--	--	--	--	--	0.0E+00

Table 6 - Similarity between fungal and *Chlorella* CDAs

CDAs in green are *Chlorella* CDAs and CDAs in white are fungal CDAs. The numbers given are the E-values obtained for each alignment. Only E values lower than 0.001 were considered reliable to infer homology[118]. The "--" symbol is given to E-vaues higher than 0.001. All other cCDAs that are not present in this table did not show E-values smaller than 0.001. The UniProt code was used for the fungal CDAs.

Inferring functional similarity based solely on significant local similarity is not completely reliable[118]. An example of it is CDA Q5KFG8 from *C. neoformans,* which is not similar to any of the other fungal cCDAs but instead, it has a similar function. Instead, multiple sequence alignment programs provide more structural, functional, and phylogenetic information than pairwise alignments[118]. For instance, COBALT, simultaneously aligns multiple protein sequences while at the same time uses information about the protein domains[119]. By using this tool, the phylogenetic tree shown in Appendix 6 was obtained. In brief, what it indicates is that, as expected, cCDAs cluster separately from fungal CDAs. In this case, cCDAs 9, 11 and 14 were the ones that shown to be more homologous to fungal CDAs.

Another way of inferring functional homology is to look at the similarity focusing on conserved active site residues[118]. This could be a particularly good strategy for the case of cCDAs that do not have a significant sequence similarity with experimentally characterized fungal CDAs. Thus, 5 motifs that make up the active sites of the deacetylase domain were searched in the sequences of the 22 putative cCDAs. Motif 1 ((TS)(FY)DD) contains two aspartic acid residues, one of them coordinates with zinc or cobalt and the adjacent second one interacts with the acetate released from the substrate[97,120]. As it can be seen in Table 7, all of the putative cCDAs contain this motif except from cCDA19. Motif 2 (H[S/T]xxHP) contains two histidines that bind to a metal and a serine or threonine that forms a hydrogen bond with the second histidine, stabilizing the loop[97,120]. Motif 2 is present in all cCDAs (Table 7). Motif 3 (RxPY) and Motif 4 (D[S/T]xDW) are situated on opposite sites of the active site groove. On one side, Motif 3 has multiple roles including binding to the acetate and coordinating the catalytic aspartate residue. The mutation of the tyrosine residues inactivated completely the peptidoglycan deacetylase of *S. pneumoniae*[97,120]. Indeed, as it can be observed in Table 7, in the case of the putative cCDAs, there are mutations in the tyrosine residue to phenylalanine (cCDA1, cCDA7, cCDA8, cCDA21 and cCDA22), to leucine (cCDA10) or to asparagine (cCDA11). As this specific mutation has been previously proven to be important for the activity of the enzyme, it is probable that the putative cCDAs with this mutation might not be active. Motif 4 forms the other side of the active site groove and the tryptophan should be the most critical residue[97,120]. Motif 4 is equally preserved in all cCDAs, which only have the initial aspartic acid and the following serine. Just in the case of cCDA17 there is a threonine instead of the serine. The tryptophan, however, is not conserved in any of the putative cCDAs (Table 7). Finally, there is also Motif 5 (LxH), which should contain a leucine and a histidine forming a hydrophobic pocket to which the acetate methyl group should bind to and also form a hydrogen bond with the generated acetate[97,120]. In this case the motif could only be identified in putative cCDAs 2, 3, 9, 11 and 21[97,120] (Table 7).

After analyzing 5 motifs of the catalytic site of the cCDAs that according to the literature are important for the deacetylation reaction in active fungal CDAs, it can be concluded that cCDAs 2, 3 and 9 are the most probably active cCDAs. These three cCDAs have all 5 motifs without mutations on the relevant

residues. Only the tryptophan from Motif 4 is missing in these three cCDAs, and in all the rest of cCDAs, something that could compromise the deacetylation activity, as it has been found in the literature[97,120].

CDA number	Motif 1 ((TS)(c)DD)	Motif 2 (H[S/T]xxHP)	Motif 3 (RxPY)	Motif 4 (D[S/T]xDW)	Motif 5 (LxH)
cCDA1	TwDD	HTmtHv	RaPf	DStlp	-
cCDA2	phDD	HTvtHa	RtPY	DStig	fdyaLgH
cCDA3	TnDD	HTlhHv	RaPY	Dssip	eiyaLgH
cCDA4	TnDD	HTvhHv	RaPY	DSsis	
cCDA5	ThDD	HTknHe	RaPY	DStli	
cCDA6	TvDD	HTldHk	RaPY	DSsim	-
cCDA7	ThDD	HTqtHe	RaPf	DSsli	-
cCDA8	TnDD	HTldHi	RgPf	DStit	
cCDA9	SvDD	HTvtHP	RaPY	DSska	pvsiLvH
cCDA10	ihDD	HSktHl	RaPL	DSsii	-
cCDA11	hgDD	HTmtHs	RaPN	DSslt	etwaLtH
cCDA12	ThDD	HTtlHe	RsPY	DSsvi	-
cCDA13	ThDD	HTadHt	RnPY	DStll	-
cCDA14	ThDD	HTvnHs	RaPY	DSsim	-
cCDA15	SqDD	HTmtHs	RaPY	DSslk	
cCDA16	qhDD	HTltHa	RqPY	DSqhd	
cCDA17	ThDD	HSlnHP	RtPY	DTrql	-
cCDA18	ThDD	HTinHi	RnPY	DStlm	
cCDA19	-	HTatHks	RaPY	DStmi	
cCDA20	fhDD	HTktHk	RaPY	DSsli	-
cCDA21	ThDD	HTvsHv	RdPf	DStin	iregLfH
cCDA22	ahDD	HTedHi	RaPf	DSsiv	

Table 7 - Identification of motifs from active fungal CDAs in C. Variabilis NC64A putative Chitin deacetylases.

In case the amino acid is conserved it is shown in capital letters.

3.2.3 Identification of putative CDAs that are transcribed to RNA

In an effort to continue identifying those putative cCDAs with more probabilities of being active, the transcription of the 22 genes to RNA was studied by RT-PCR. In order to do so, a process to extract the RNA from *Chlorella* had to be developed because no previous references could be found in the literature. This was a particularly complicated task due to the difficult to penetrate *Chlorella* cell wall. Briefly, *C. variabilis NC64A* was cultured to late exponential and to late stationary phases in duplicates to see if a difference in the translation of the cCDAs to RNA could be observed according to the growth stage. Then the RNA was extracted and purified as explained in subsection 3.4.3. The amount of RNA extracted was considerably higher in the case of the samples collected at exponential phase, probably as a result of a harder cell wall composition in the stationary phase. Because of the higher RNA concentration and the slightly better 260/280 and 260/230 nm ratios, the RNA obtained from the cells at exponential stage was chosen to perform RT-PCR. Once the RNA was extracted it was converted to cDNA using a reverse transcriptase as explained in subsection 3.4.4. After the cDNA was available, the transcription of each of the 22 cCDA genes to RNA was analyzed by amplifying fragments from cDNA, with an intercalated intron to differentiate cDNA from contaminant DNA.

As Figure 17 shows, sequences of the expected sizes of 19 out of the 22 putative CDA's were amplified from their cDNA (cCDAs 1, 2, 3 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 17, 18, 20, 21). The positive controls, which were made for the cDNA of the 18s gene were also well amplified. The 19 amplified DNA fragments were afterwards reconfirmed to belong to the expected cCDA by sequencing. These results are clear arguments in favor of the hypothesis that the chitin deacetylation reaction is carried out by several different enzymes at the same time.

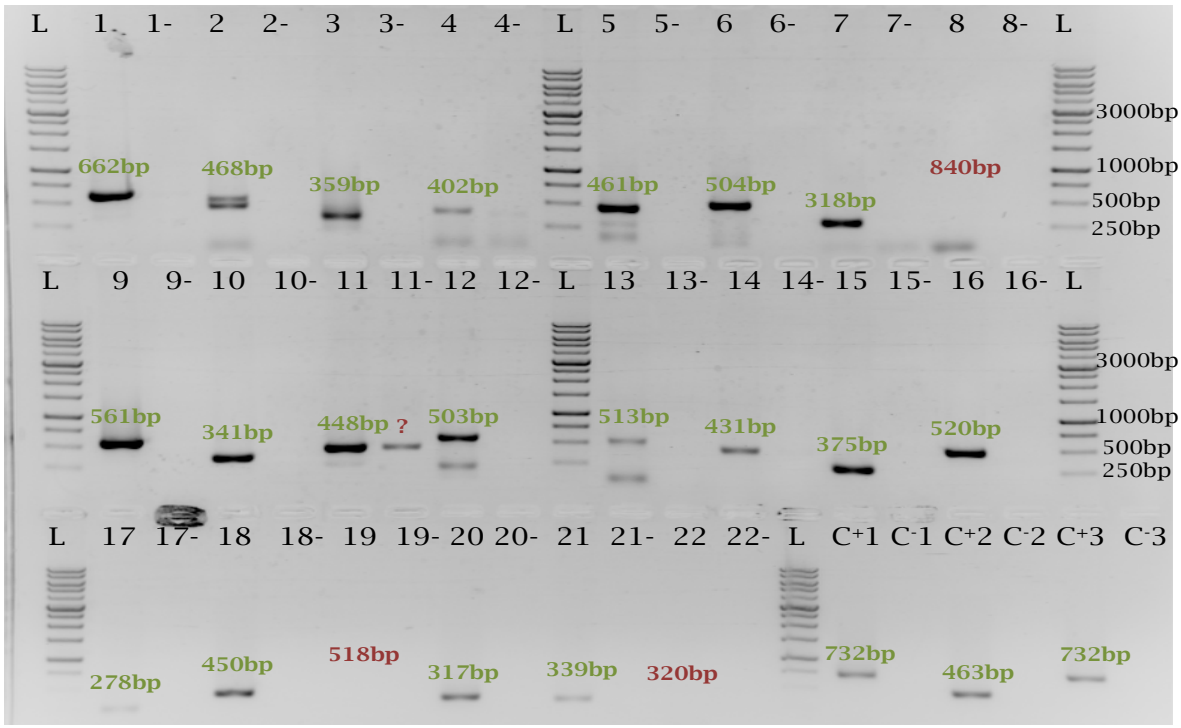

Figure 17 - Putative cCDAs that are transcribed to RNA by RT-PCR.

19 out of the 22 putative cCDAs are transcribed to RNA. The expected size of the putative cCDAs is indicated and colored in green if a band of the correct size was amplified and in red if no band was amplified. Three different PCR reactions were required to amplify all the fragments; the positive controls and negative controls of each of these PCRs are found at the end of the gel.

3.2.4 Identification of active CDAs in *Chlorella* protein extracts

In the quest for finding the CDAs that give *Chlorella* the rare capacity of enzymatically converting chitins to chitosans, bioinformatics and molecular biology tools have been employed. According to the analysis of the protein sequences using bioinformatics tools, cCDA 9 was identified as the most probably active CDA in the *C. variabilis NC64A* genome. However, thanks to molecular biology tools to analyze the DNA that is transcribed to RNA, it seems apparent that several enzymes govern the deacetylation mechanism in this microalgae, as 19 different cCDAs have been amplified from the cDNA of *C. variabilis NC64A*. In this subsection, the results from using proteomics tools to identify the CDAs present in the protein extracts of *C. variabilis NC64A* are explained.

3.2.4.1 Preparation of *C. variabilis NC64A* protein extracts

As indicated in the introduction of this chapter, several natural CDAs have been previously identified, purified and characterized from fungal protein

extracts, obtained in some cases from the growth media as secreted extracellular proteins[96,107,108] and in other cases from mycelial extracts.[105,109–111]. Similarly, the same *C. variabilis NC64A* cultures utilized to identify the cCDAs transcribed to RNA were used to obtain protein extracts from the growth media and from the cells. This included 4 cultures, two of them grown to stationary phase and two of them grown to exponential phase. On one hand, the protein extracts from the media were prepared by collecting the growth media after harvesting, then precipitating the proteins with ammonium sulfate and finally dialyzing them to eliminate all the salts. To be sure that the proteins were concentrated enough, a part of them were concentrated by membrane ultrafiltration. On the other hand, the cellular protein extracts were obtained by disrupting the harvested cells by homogenization. All the samples prepared for testing the deacetylase activity are found in the diagram of Figure 18. In total 8 different samples were obtained: samples EEC (Extracellular proteins, Exponential growth phase and Concentrated), EEN (Extracellular proteins, Exponential growth phase and Non-concentrated), ESC (Extracellular proteins, Stationary growth phase and Concentrated), ESN (Extracellular proteins, Stationary growth phase and Non-concentrated), CES (Cellular proteins, Exponential growth phase and Soluble), CEN (Cellular proteins, Exponential growth phase and Non-soluble), CSS (Cellular proteins, Stationary growth phase and Soluble) and CSN (Cellular proteins, Stationary growth phase and Non-soluble).

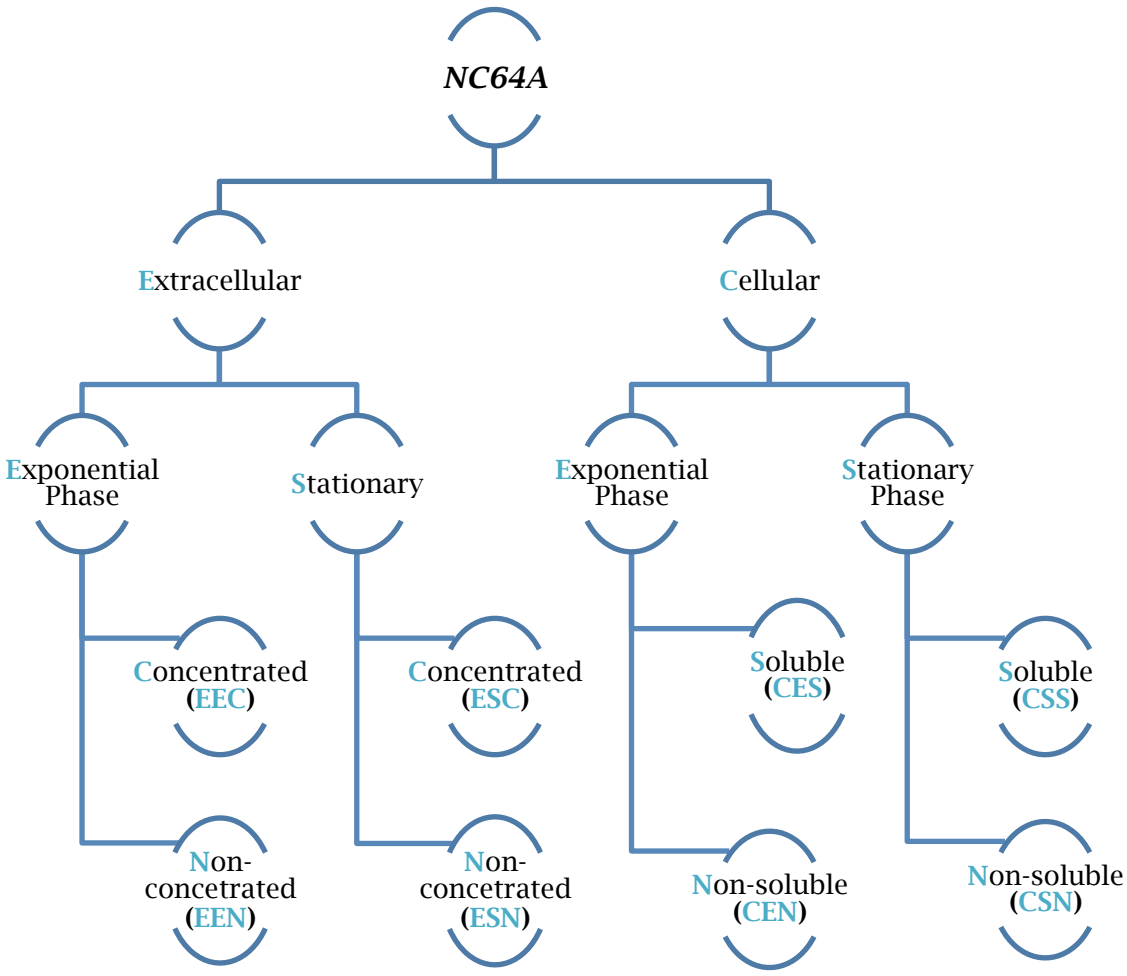

Figure 18 - *C. variabilis NC64A* extracts prepared to identify active CDAs

3.2.4.2 Chitin deacetylase activity of *C. variabilis NC64A* protein extracts on chitin oligomers (A4)

Before identifying any specific CDA in the protein extracts, it was first necessary to check if there was any chitin deacetylase activity at all. The first experiment to do so consisted in the incubation of the samples with chitin tetramers and afterwards the detection of the deacetylation of these oligomers by mass spectrometry (MS). This methodology can also be applied for the detection of chitinase activity in the extracts. The experiment was performed in collaboration with Lea Hembach, from the laboratory of Dr. Moerschbacher in the University of Münster. The samples were incubated at pH 4.5 and pH 7

and 37ºC during four days; plenty of time to ensure that in case there was any active chitin deacetylase in the sample it would be detected. To avoid having misleading results, the protein extracts had previously been checked with the mass spectrometer for the presence of chitin oligomers but none were found. The results obtained after the incubation can be found in Table 8. As it shows, there was chitin deacetylase activity in all samples tested but only at pH 7. Also, in all cases only one monomer out of four had been deacetylated. Surprisingly, the extracellular extracts were the ones to have a higher deacetylase activity; in fact, the most active sample was the extracellular extract of the culture grown to stationary phase. Moreover, chitinase activity was also found in all protein extracts at pH 4.5.

Protein extract	pH	CDA-activity (A4)				Chitinase activity
		A3D1	A2D2	A1D3	D4	
EEN	4.5	-	-	-	-	-
	7	+++	-	-	-	+
ESN	4.5	-	-	-	-	-
	7	++++	-	-	-	+
CES	4.5	-	-	-	-	-
	7	+	-	-	-	+/++
CEN	4.5	-	-	-	-	-/+
	7	+/++	-	-	-	+/++
CSS	4.5	-	-	-	-	+
	7	++	-	-	-	++
CSN	4.5	-	-	-	-	-
	7	++	-	-	-	++

Table 8 - Chitinase and Chitin Deacetylase activity of *C.sp NC64A* protein extracts on chitin tetramers (A4)

The name of the samples tested corresponds to the explanation of Figure 18. In the description of the oligomers the letter "A" is for Acetylated and "D" is for Deacetylated monomers; initially the oligomer was A4. In all cases where deacetylation was detected, only one monomer was deacetylated. The symbol "+" is an indication of activity and the symbol "-" means that no activity was detected. "/" indicates that different activity was observed between duplicates.

3.2.4.3 Chitin deacetylase activity of *C. variabilis NC64A* protein extracts on chitin polymers (Glycol chitin)

After proving that the extracts were active on chitin oligomers, the next step was to measure the activity of the protein extracts on chitin polymers (glycol chitin). This experiment was also performed in collaboration with Lea Hembach, from the University of Münster. In this case, polyacrylamide gels containing glycol chitin were prepared at pH 4.5 and pH 7 and were used to perform a Dot Blot test by adding on top of them a drop of the extracts[121]. The gels were incubated for 20h at 37ºC, a much shorter period of time in comparison with the test on chitin oligomers to reduce chitinase activity. Afterwards, the gels were stained with calcofluor-white, a fluorescent stain that binds selectively to chitin. The calcofluor-white stain reveals the presence of chitin under UV light, a trait that is useful to detect any change occurring in the chitin structure[122]. In case black dots appear under U.V. light after the first incubation, i.e. no chitin is detected in the place where the protein extracts were dropped, it is an indication that the chitin that was present in the gel has been depolymerized by the action of a chitinase from the extract. Then, in order to detect deacetylase activity, the gels are semi-depolymerized using a mixture of sodium nitrate and concentrated sulfuric acid, a process that washes away the chitosan (but not the chitin) in the gels. The appearance of new black spots under U.V. light in the calcofluor-white stained gels is a sign of chitin deacetylase in the extract. Ideally, to easily determine CDA activity, no chitinase activity should have been found previously.

Six different cellular and extracellular *C. variabilis NC64A* protein extracts were selected for the Dot blot assay (MEC, MEN, MSC, MSN, CES, CEN). Also, as a positive control for the detection of chitinase activity, the chitinase from *Serratia marcescens* (ChiB) was used[90]. A fungal CDA of known activity that had not been published yet was used as a positive control for chitin deacetylase activity. In the previous experiment with the chitin oligomers, chitin deacetylase activity was observed only at pH 7. However, in this case, due to technical problems in the handling of the gels, results could only be obtained from the gel at pH 4.5. The good news was that chitin deacetylase activity on glycol chitin was observed at pH 4.5 in all samples tested. Figure 19 shows how after incubating the chitin glycol loaded gel with the different protein extracts, chitinase activity was only observed on the positive control (ChiB). Instead,

after eliminating the chitosan from the gel, new black spots appeared in all samples as an indication of chitin deacetylase activity. CES sample (cellular proteins, exponential growth phase and soluble fraction) appeared to be the most active of the samples tested per volume, as the same volumes were loaded in all cases but not the same quantity of proteins. To sum up, results show that CDAs are capable of deacetylating chitin oligomers at pH 7 and glycol chitin at pH 4.5 and most probably also at pH 7.

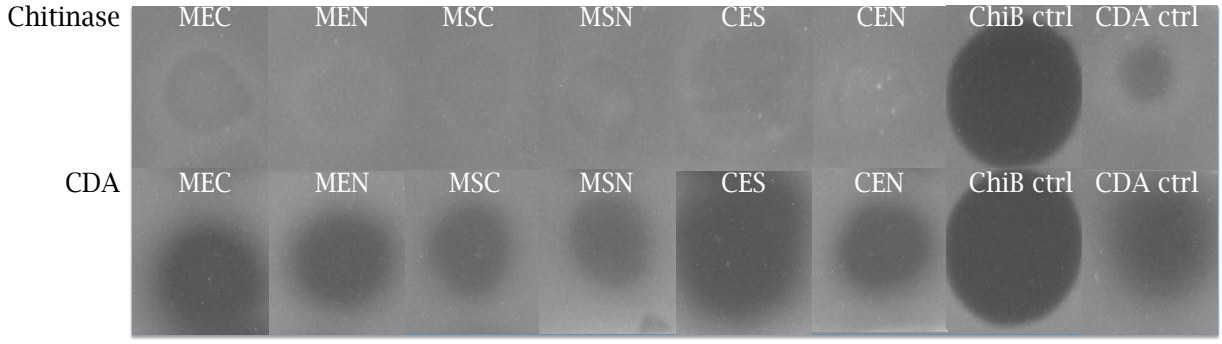

Figure 19 – Dot Blot activity assay to find chitinase and chitin deacetylase activity of *C. variabilis NC64A* extracts on glycol chitin.

On top, the black spots (symbol of chitinase activity) only appear in the case of the positive control (ChiB), in all other cases only the shape left by the drops can be observed. The bottom row shows how, after eliminating the chitosan from the gel, black spots appear in all samples, indicating deacetylase activity. These dots were only observed where the extracts had been dropped.

3.2.4.4 Identification of CDAs by gel zymography

The confirmation of the capacity of the *C. variabilis NC64A* extracts to deacetylate chitin glycol opened the door to perform gel zymography. This technique is useful to detect enzyme activity by allowing the enzymatic reaction to happen in situ after the proteins from the extract have been separated by gel electrophoresis[121,123,124]. In this case, the proteins of the *NC64A* extracts were separated in a semi-native gel electrophoresis to attempt to see bands with chitin deacetylase activity on glycol chitin. These bands could be afterwards cut out and sequenced using mass spectrometry to identify the enzymes causing the deacetylation. These experiments were carried out in collaboration with Lea Hembach from the University of Münster.

In the first zymography, protein extracts from *C. variabilis NC64A* (MEC, MSC, CES) were separated in a semi-native SDS-PAGE. Then, the gel was incubated tightly coupled with an overlay gel containing 1% glycol chitin to allow for the deacetylation reaction to occur. Subsequently, the overlay gel containing chitin was incubated with calcofluor-white and the gel was developed under a UV transiluminator. No black spots appeared at this stage, meaning that no chitinase activity was detected (Figure 20, zymography 1). Then, in order to determine the deacetylase activity, the gel was depolymerized using a mixture of nitrous and sulfuric acid and observed again under a UV lamp. The results showed clear signals of deacetylase activity in protein extracts from both the media and the disrupted cells (Figure 20, zymography 1). As stated above, a very good aspect of this technique is the fact that the positive bands can be cut out from the original gel and sequenced to identify the proteins in them. Two different bands of the protein extract ESC, one around 60kDa (Band 1) and the other one around 35kDA (Band 2) were sequenced using a mass spectrometer. The obtained polypeptide sequences were compared to the whole NC64A proteome and, as Table 9 shows, 7 different putative *Chlorella* chitin deacetylases (cCDAs 1, 3, 4, 6, 7, 10, 21) were identified.

After analyzing the results from the first zymography, it was decided to repeat the experiment for several reasons. First, as it can be observed in Figure 20, the proteins under 35 kDA could not be detected. So, in the second zymography, the electrophoresis separation was better controlled to prevent small proteins from escaping from the gel. Secondly, several different chitin deacetylases were identified in each of the bands sequenced, thus, it had not been possible to determine which of them were really active, as the activity could be attributed to only one of the detected cCDAs. To attempt to obtain individual cCDAs per band, the separation was improved by incrementing the amount of polyacrylamide in the separation gel from 7% to 15%. Finally, only three out of the 8 *NC64A* extracts were tested in the first gel zymography, so in the new experiment more samples were included (EEC, ESC, CES, CEN, CSS twice and CSN).

As it can be seen in Figure 20 (zymography 2), chitin deacetylase activity is observed approximately at the same sizes in the extracts EEC, ESC, CES and CSS. This repeated pattern corresponds with Table 5, which indicates that most

cCDAs are either of 36.2±7 kDa or 69.4±7 kDa, so it might not necessarily mean that the same cCDAs are present in EEC, ESC, CES and CSS extracts; it could be different proteins with the same sizes. Hence, a statement indicating that the same cCDAs are found in all the types of samples with deacetylase activity tested, independently of the growth stage or cellular and extracellular localization, cannot be made without sequencing. Another observation is the fact that the extracellular protein extracts show more activity per amount of protein loaded in the gel, something that can be explained by the fact that it is a purer extract. It is also important to highlight that no activity is observed in the insoluble protein extracts (CEN and CSN). Six bands (Bands 3 to 8) were cut from the ESC extract and were sequenced by MS. The obtained polypeptides were compared to the whole *C. variabilis NC64A* putative proteome and Table 9 shows the putative cCDAs identified this time (cCDAs 3, 4, 7, 10, 14, 16 and 21). A very good result is that cCDA 7 appeared with a high spectral count in bands 7 and 8 as the only cCDA present. As a result, to our knowledge, cCDA 7 (JGI code: 139978) is the first CDA from *Chlorella* to have been empirically demonstrated to deacetylate chitin. In this second zymography it can be appreciated that there is still plenty of space for the gel to continue running to improve protein separation and increase the probabilities of obtaining individual bands.

When comparing the zymography assays 1 and 2, cCDAs 3, 4, 7, 10 and 21 appear in both experiments showing a high number of spectral counts. Putative cCDAs 6 and 1 are only present in the first gel and putative cCDAs 14 and 16 are only present in gel 2. However, it must be taken into account that cCDAs number 6 and 16 are the ones with a lower spectral count in both cases (Table 9). Thus, it is fair to say that the technique is reproducible and the results are robust because the proteins that are more present are the same in the two experiments. The most commonly present cCDAs in the extract studied (cCDAs 3, 4, 7, 10 and 21) have been identified in different bands of different sizes. However, the sizes shown are unreliable because the proteins are not fully denatured. Furthermore, isoforms of each enzyme could be present, something that also happens in fungal deacetylases [97,101,110,125].

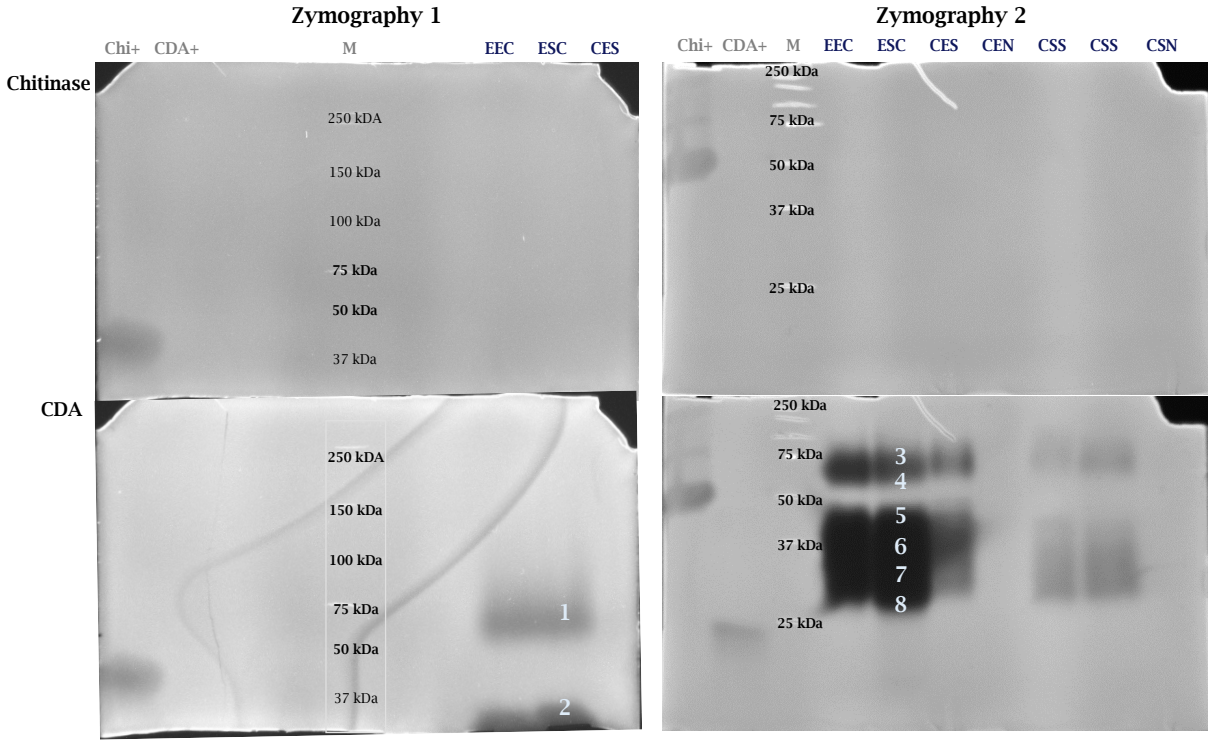

Figure 20 - Gel zymography to identify active CDAs in *C. variabilis NC64A*.

Gels after staining with Calcofluor-white to check for chitinase activity (top) and after depolymerizing the gel to check for chitin deacetylase activity (bottom). The gels on the left belongs to zymography 1 and the gels on the right to zymography 2. The numbers indicate the bands that were cut out from the gels. All bands cut came from extract ESC.

Band	Size (kDa)	C. Variabilis NC64A chitin deacetylases (Protein Spectral Count)								
		1	3	4	6	7	10	14	16	21
3	70		5	8			13	5	1	15
1	65	3		2	1	6				3
4	60			18			4			
5	45					8				3
6	40					11				12
2	35		12	3			5			1
7	35					6				
8	30					4				

Table 9- Chitin deacetylases from *C.variabilis NC64A* identified in 8 different bands showing chitin deacetylase activity.

Bands 1 and 2 belong to the first zymography and the rest to the second zymography.

In parallel with the second zymography, a third zymography was carried out. The idea behind this experiment was to improve the separation of chitin deacetylases by increasing the interactions with the gel through the incorporation of 1% chitin glycol in the same gel in which the proteins were ran, instead of using an overlay gel like in the previous zymographies[123]. The same protein extracts as in the second zymography were loaded. The experiment was successful because sharper bands showing chitin deacetylase activity were obtained. Moreover, it was much easier to cut the bands for sequencing as it could be done directly from the gel showing activity. Two bands from extract ESC were cut and sequenced using MS but, unfortunately, the presence of 1% chitin glycol in the bands appeared to be an impediment for sequencing. Therefore, a fourth zymography experiment was performed reducing five times the amount of glycol chitin incorporated in the gel (0.2%). Two bands were cut again from extract ESC but the low concentrations of chitin glycol still impeded the sequencing. It can be concluded then that the incorporation of the substrate in the gel increases the resolution and the probabilities of identifying individually active cCDAs. However, so far it has been impossible to sequence the bands obtained from the gels incorporating glycol chitin.

3.2.5 Identification of active *Chlorella* CDAs by recombinant expression in *E. coli*

3.2.5.1 Cloning and transformation of *C. variabilis NC64A* CDAs in *E. coli*

In the first zymography experiment 7 different cCDAs were identified in two bands showing chitin deacetylase activity. The fact that these 7 cCDAs had also been found in the cDNA reaffirmed these results. Because none of these cCDAs appeared alone in the bands it was not possible to figure out if all or only some of these cCDAs were active. To answer this question, genes of cCDAs 1, 3, 4, 7, 10 and 21 were selected to be amplified by PCR from the cDNA of *C. variabilis NC64A* to then be cloned in a construct for recombinant expression in *E. coli* (cCDA 6 was left out because it had only one protein spectral count).

Primers for the amplification of the 6 different putative cCDAs were designed and several PCRs were carried out in which different temperatures in the annealing cycle, the addition of DMSO or the use of different DNA polymerases

were tested. At the end only cCDAs 1, 3, 4 and 10 could be amplified. These four bands were cloned into a pET-22b(+) vector by digesting them with restriction enzymes and then ligating the complementary extremes using a T4 DNA ligase. The new ligated constructs were transformed into chemically competent *E.coli* cells and the bacteria containing the plasmid were selected by ampicillin resistance. Because the selection with ampicillin only ensures that the clone carries the B-lactamase gene and not the gene of interest (GOI), the clones were screened for the presence of the cCDAs by colony-PCR. After the screening, clones with the expected band size of cCDAs 1, 3 and 10 were identified. Plasmidic DNA was extracted from the selected clones and sequenced to confirm that apart from the correct sizes, the sequences were also correct and no mutations had occurred during the cloning process. The analysis of the sequences revealed one clone with the expected sequence of cCDA 3. In the case of cCDA 10, the three clones sequenced had an insertion of 9 bps at position 1267. This insertion could be simply that the predicted cDNA is not correct. Because this insertion did not cause a change in the reading frame, the sequence was considered to be good. In cCDA1, several errors were found; in all clones sequenced, a deletion of 18 bps was detected (from bp 883 to 900). Moreover, in one of the clones a deletion of 306 bp was detected in bp 137 (clone 1.4) and a deletion of 36 bps was seen in the other two clones in bp 490 (clones 1.5 ad 1.6).

3.2.5.2 Recombinant expression of *C. variabilis NC64A* CDAs in *E. coli*

Clones cCDA 1.4, 1.5, 3.4, 10.3 and 10.6, a WT Rosetta strain as a negative control and a positive control containing *Colletotrichum lindemuthianum* CDA (ClCDA) were cultured and induced with IPTG for recombinant chitin deacetylase production. Protein extracts from these clones were obtained by disrupting the cells with one freeze/defreeze cycle followed by sonication. Soluble proteins were purified by affinity chromatography taking advantage of the His-tag of the pET-22b(+) vector and separated by SDS-PAGE electrophoresis. cCDA 1 approximate expected size is 42kDA, the expected size for cCDA 3 is 75kDA and 47 kDA for cCDA 10. However, results not presented here showed that affinity-purified protein extracts were still very impure and no traces of overexpressed proteins of the desired sizes could be

observed. The cause was determined to be that too much protein had been loaded per column and that the washing step had not been stringent enough.

A new SDS-PAGE electrophoretic separation was performed with three different types of samples. The first sample was the soluble proteins extracts of the 7 induced cultures (cCDAs 1.4I, 1.5I, 3.4I, 10.3I, 10.6I, Rosetta control I and ClCDAI). The second type of samples was the insoluble protein extracts from the same 7 induced cultures together with two non-induced cultures (cCDA 3.4 and 10.3) that was solubilized by denaturating the proteins in 8M urea. The third type of samples was two soluble induced extracts purified by affinity chromatography (cCDA 3.4IHis and 10.3IHis), but this time using more stringent conditions compared to the first purification commented above. Figure 21 shows how a band of the expected size was found in all induced cultures but, unfortunately, only in the insoluble fractions. For this reason, from the purification of the soluble fraction (cCDA 3.4IHIS and 10.3IHIS) nothing could be obtained. Amongst all soluble fractions the only recombinant protein present was that of the control ClCDA. The insoluble fractions, although they clearly contained the cCDAs, could not be tested for chitin deacetylase activity because the proteins had been denaturated after the incubation in 8M urea; a renaturation treatment was necessary.

Results so far indicated that more efforts were needed on the protein expression and extraction process in order to obtain soluble and active cCDAs. Therefore, it made sense to focus on a single cCDA, so cCDA 3.4 was selected because it seemed to be the one showing a higher expression (Figure 21). This time, different induction times and temperatures were tested to see if in any of the cases some of the expressed proteins appeared in the soluble fraction. Inductions were done at 30ºC during 1h, 3h and 5h and also at 20ºC during 1h, 5h and 16h all with 1mM IPTG. Moreover, in order to obtain more soluble proteins, 0.1% Triton reagent was added to the lysis buffer. Additionally, in order to solubilize the insoluble proteins deposited in the pellet after sonication, the pellet was re-sonicated in RIPA buffer. This buffer, because it contained 1% Triton and 0.1% SDS was expected to allow the solubilization of membrane proteins but, because it was not as harsh as 8M urea, it was expected that it would not inactivate them. Finally, the resulting pellet obtained after sonication in the presence of Triton reagent and sonication in

RIPA buffer, was solubilized by incubation in 8M urea to see if the cCDA still stayed in this most insoluble fraction. From this new extraction scheme, three different fractions were obtained: one containing the soluble proteins in a buffer with Triton, another one containing insoluble but presumably still active proteins in RIPA buffer, and finally another fraction containing inactive insoluble proteins in a buffer containing urea.

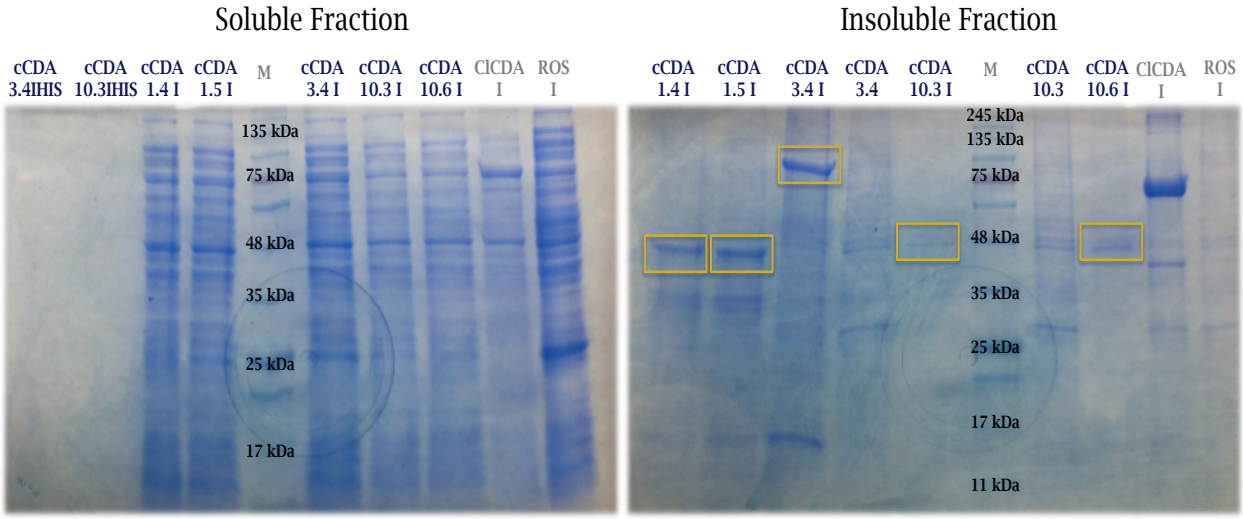

Figure 21 – Protein extracts of *E. coli* clones expressing putative CDAs 1, 3 and 10 from *C. variabilis NC64A.*

The gel on the left shows the soluble proteins and the gel on the right shows the insoluble protein extracts solubilized in 8M urea. The expressed proteins are located in the insoluble fractions in all cases. "I" indicates that the culture was induced with IPTG. "HIS" indicates that the extract was purified using nickel affinity columns.

The protein profiles of the three protein fractions commented above can be observed in Figure 22 (gels 1 to 4). At the soluble protein extracts containing Triton again no overexpressed band is observed at the expected size (75kDa). In the case of the insoluble proteins solubilized in RIPA buffer, the band is visible in samples induced at 30ºC during 1 and 5 hours and in samples induced at 20ºC during 5 and 16 hours. However, a faint band is also observed in the negative control (Rosetta strain) induced at 20ºC during 16 hours. Finally, the proteins solubilized in 8M urea are again the ones with clearer bands at the expected size, especially in the extracts from samples induced for 1, 3 and 5 hours at 30ºC and 5 and 16 hours at 20ºC. Contrary to what was

expected, few proteins got dissolved in the RIPA buffer as it can be seen when comparing the extracts with the soluble and 8M urea extracts.

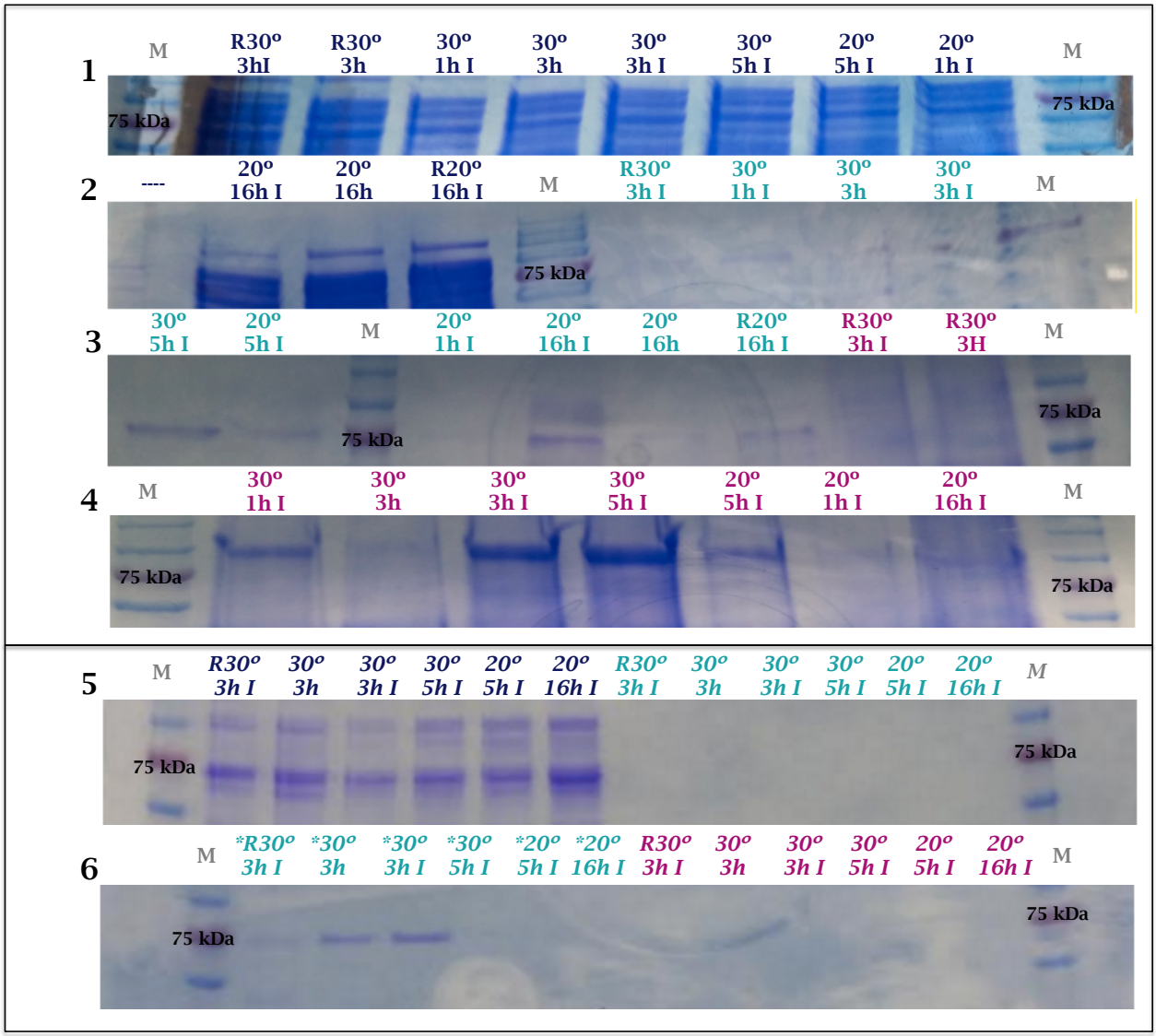

Figure 22 – Results from different strategies assayed to express cCDA 3.4 in *E.coli* clone cCDA3.4

The six gels are divided in two groups; the four gels on top contain soluble protein extracts (Blue), insoluble protein extracts solubilized in RIPA buffer (Cyan) and insoluble protein extracts solubilized in 8M urea (Fuchsia). Gels 5 and 6 contain samples following the same color code as the samples in gels 1-4, but the difference is that their buffer was changed by membrane ultrafiltration (samples with a * symbol in gel 6) or were purified using histidine affinity columns (the rest of samples from gel 5 and 6). The name of each sample indicates if it is the Rosetta clone cCDA3.4 or if it is the Rosetta with no extra genetic modifications ("R"). The name also indicates the temperature and the time of induction, and in case IPTG was added the name of the sample contains an "I". The "M" letter indicates that the marker was loaded in that well. The expected band size of cCDA 3.4 is around 75kDa.

Some of the samples from the three buffers were purified using histidine affinity columns (30º3h I, 30º5h I, 20º5h I, 20º16h I, control of induction 30º 3h and untransformed control R 30º3h I). However, the purification of the soluble fractions in Triton buffer did not yield a completely pure extract and a 75kDa band is observed with approximately the same intensity in the clones tested and in the non-transformed control (Figure 22-5). Also, the purification of the RIPA and urea extracts with the affinity columns did not yield any protein visible by Coomassie staining (Figure 22-5 and 22-6). In parallel, the samples in RIPA buffer were concentrated by membrane ultrafiltration. This was done because this technique, apart from concentrating the sample, also allows changing the buffer and it was hypothesized that a buffer exchange could be necessary when testing the deacetylase activity of the samples. These samples were loaded in wells 2-7 of gel number 6 and the band is clearly visible in the clone induced at 3h and 30ºC. Nonetheless, less intense bands can also be appreciated in the non-induced control and the non-transformed control (Figure 23-6).

3.2.5.3 Chitin deacetylase activity of *E. coli cCDA 3.4* protein extracts on chitin oligomers (A4) and chitin polymers (chitin glycol)

Results presented in Figure 22 showed that it was possible that cCDA 3.4 could be in an active form in some of the extracts solubilized in RIPA buffer. Although it had not been appreciated in the Coomassie stained gels, it was also a possibility that little amounts of active protein were being expressed in the soluble extracts, especially after the addition of 0.1% triton reagent. In order to test these hypotheses, the soluble extracts containing Triton, the non-soluble extracts solubilized in RIPA buffer and the purifications by affinity chromatography of the two types of extracts, were tested for their capacity to deacetylate chitin oligomers and chitin polymers (chitin glycol).

For each type of extract, 6 samples were assayed (30º3h I, 30º5h I, 20º5h I, 20º16h I, control of induction 30º 3h and untransformed control R 30º3h I); hence 24 different extracts were tested. The test on oligomers was done by incubating the protein extracts with A4 chitin oligomers in activity buffer at pH 7 overnight and then analyzing the results by MS; exactly the same protocol applied to the *Chlorella* protein extracts explained above (subsection 3.2.4.2),

only that this time the incubation was performed for a shorter period of time. The analysis of activity on chitin polymers was done with a Dot blot activity assay in the same way it was done for the protein extracts of *Chlorella* (subsection 3.2.4.3). The difference was that the activity was only tested at pH 7.

Unfortunately, no activity on chitin oligomers or on chitin polymers was detected in any of the extracts tested. Only the positive controls showed activity in both cases so no errors affecting the enzymatic deacetylation reaction and its revelation occurred in the process. The results from the Dot Blot activity gel can be observed in Figure 23. In all, it can be concluded that so far no chitin deacetylase activity has been observed on cCDAs expressed in *E.coli.*

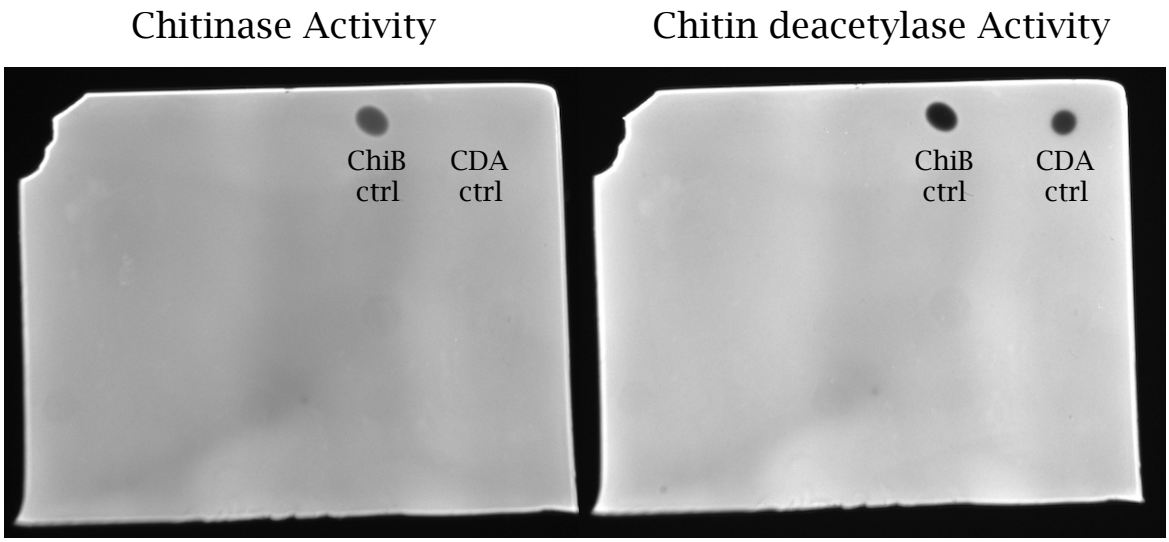

Figure 23 – Dot Blot activity assay to find chitinase and chitin deacetylase activity on glycol chitin of protein extracts of *E. coli cCDA3.4* clone.

Chitinase and chitin deacetylase activity is only observed in the positive controls (ChiB ctrl and CDA ctrl). No activity is detected in any of the 24 protein extracts assayed. The samples tested were: 30º3h I, 30º5h I, 20º5h I, 20º16h I, control of induction 30º 3h and untransformed control R 30º3h I, all of them obtained as soluble extracts, histidine-affinity purified soluble extracts, insoluble extracts in RIPA buffer and histidine-affinity purified insoluble extracts in RIPA buffer.

3.3 Discussion

Chitosans discovered in the cell wall of *Chlorella* might have interesting commercial applications. *Chlorella* chitosan differ from the chitosans in the market by its enzymatic production; a process that yields more reproducible polymers in terms of molecular weight, degree of acetylation and pattern of acetylation in comparison to the chemical processes currently undertaken. The enzymes in charge of the deacetylation reaction are known as chitin deacetylases and, unexpectedly, up to 25 of them may be working actively in *Chlorella*. The aim of this chapter was to explain the research performed to identify active chitin deacetylases in *C. variabilis NC64A.*

After reviewing the techniques used for the isolation of the CDAs studied so far, we acknowledged that basically two approaches had been followed. On the one hand, especially on the first chitin deacetylases identified, researchers attempted to purify the protein from cellular and extracellular protein extracts of the organism of interest. On the other hand, the characteristics of the hydrolytic enzyme were revealed after cloning and recombinantly expressing the putative CDA in a heterologous host such as *E. coli* or *P. pastoris*. The second approach offered some advantages such as the ease of purification and the possibility of obtaining larger quantities of expressed proteins. However, recombinantly expressing 25 different proteins is an arduous task and it is even more so in the case of CDAs, which often are membrane proteins[97].

Bioinformatic tools were used to try to reduce the list of putative cCDAs to study by selecting those that were more similar to known active CDAs and then trying to infer functional homology. A local protein sequence alignment software (BLASTp), a multiple sequence alignment program (COBALT) and the identification of conserved motifs in protein sequences were used to conclude that cCDAs are not similar to known active fungal CDAs. Thus, the information gathered with the tools utilized was not enough to reduce the list of cCDAs to be heterologously expressed. Results indicated that if one cCDA should be the most similar to active fungal CDAs, it would be cCDA 9.

Molecular biology tools were also used to study the 22 cCDAs. A process to extract the *C. variabilis NC64A* RNA was developed because no standard protocol was found in the literature. Once the RNA had been retro-transcribed to cDNA, it was used to identify the cCDAs that are transcribed to RNA and

thus obtain more conclusive results about which could be the most probably active cCDAs. However, 19 out of the 22 proteins analyzed were amplified from the cDNA, so the list of proteins to express had been barely reduced. Only putative cCDAs 8, 19 and 22 were not detected in the RNA and, thus, were discarded from the list of proteins to express in *E. coli*. Utilizing qPCR could be the next approach to analyze the transcription and better predict which of the putative cCDAs is indeed active by detecting and quantifying the most highly translated ones. Such a tool could also be very useful to study the production of chitosans in *Chlorella,* the variations according to the growth conditions and the biological reasons for producing these polymers.

The cDNA obtained from the RNA extraction process was also necessary to amplify by PCR the genes to be cloned and heterologously expressed in *E. coli.* It was not until results from proteomics tools to identify active CDAs in *NC64A* were obtained that it was possible to select a smaller group of cCDAs for recombinant expression in *E. coli.* In the first zymography experiment, several protein spectral counts of putative cCDAs 1, 3, 4, 7, 10 and 21 appeared in two sequenced bands showing chitin deacetylation activity (Table 9). Because none of these cCDAs appeared alone in the bands showing chitin deacetylase activity, it was not possible to know if all or only some of these cCDAs were indeed active. They all coincided with the cCDAs found in the cDNA of *Chlorella* and, apart from cCDA 10, they also corresponded with the cCDAs determined to be more similar to fungal CDAs according to the p-BLAST analyses (Table 6). Although, cCDA 9, which was the most probably active cCDA according to the bioinformatics analyses, was not amongst the cCDAs identified in the bands showing deacetylase activity and most of the positive cCDAs (except from cCDAs 3 and 4) could be inactive due to modifications in the catalytically important tyrosine residue from Motif 3 (Table 7), the results from the zymography were determinant to choose the cCDAs to be recombinantly expressed in *E. coli*. Therefore, cCDAs 1, 3, 4, 7, 10 and 21 were selected for amplification by PCR for recombinant expression in *E. coli.*

Because cCDAs 4, 7 and 21 failed to be correctly amplified by PCR, only cCDAs 1, 3 and 10 were included in the first experiment to express cCDAs in *E. coli.* These proteins could only be expressed in the insoluble fraction and could only be detected after solubilization in a buffer that did not allow the analysis

of the deacetylation activity because it contained 8M urea. For this reason, the number of clones analyzed in the subsequent experiment was narrowed to only cCDA 3.4 to focus on finding an expression strategy that yielded soluble proteins. cCDA 3.4 was chosen because it was the only one of the three cCDA sequences that had no mutations and also because it was the one with a higher recombinant yield.

Despite different times and temperatures of induction were tested, no signs of cCDA 3.4 in the soluble fraction were observed. Only when solubilizing the insoluble pellet in RIPA buffer and in 8M urea the protein could be detected. Hoping that the RIPA buffer would not impair the activity of the enzyme, its capacity to deacetylate chitin oligomers and polymers was analyzed. Soluble proteins were also included in the assay just in case small amounts of enzyme were present in this fraction and could be detected by the high resolution of the activity assay. Nevertheless, no activity was found in any of the extracts tested. Although one of the extracts that could have been active, the one containing the samples concentrated by membrane ultrafiltration, could not be tested because there were already many samples to be analyzed, it is highly probable that a better expression strategy still needs to be designed to obtain more soluble proteins to confirm the deacetylation activity of cCDA 3.4. New approaches should include using the *E. coli* strain Lemo 21, which allows for the tunable expression of difficult clones by varying the levels of Lysozyme, the natural inhibitor of T7-RNA polymerase[126]. This strategy has previously been successful with other CDAs that had been difficult to express (personal communications with Shoa Naqvi and Lea Hembach from the laboratory led by Dr. Moerschbacher, at the University of Münster). If this approach does not succeed, a last resource would be the renaturation of the proteins in 8M urea by slowly changing the buffer.

Fortunately, zymography studies of protein extracts from *C. variabilis NC64A* yielded positive results concerning the identification of active cCDAs. Apart from cCDAs 1, 3, 4, 7, 10 and 21, which have already been commented above that were identified in the first zymography, cCDAs 6, 14 and 16 were also discovered in the sequencing of several protein bands showing chitin deacetylase activity in subsequent zymography experiments (Table 9). In total four different zymography assays were performed with the goal of separating

the proteins as much as possible to obtain bands with chitin deacetylase activity in which only one cCDA was present. This was a possible way of demonstrating that the cCDA was capable of deacetylating chitin on its own, hence, an indication that it was active. Amongst the cCDAs identified, cCDA7 was found alone in two of the bands sequenced. With this evidence, it can be concluded that cCDA7 is, to our knowledge, the first active CDA to ever have been identified in Viridiplantae.

The information gathered so far about cCDA7 (JGI code: 139978) is that this chitin deacetylase enzyme has 584 aminoacids, its predicted molecular weight is 62.1 kDa and its predicted isoelectric point is 4.58. cCDA7 contains the NodB homology domain between aminoacids 32 and 157. Inside this domain is where the catalytically relevant residues for the deacetylation reaction are located (Table 7). Motif 1 is situated between aminoacids 39 and 42, Motif 2 is located between aminoacids 96 and 101, Motif 3 is composed by aminoacids 136-140 and finally the not so clearly present Motif 4 seems to be at position 158-162. Moreover, the protein contains a transmembrane region spanning from aminoacids 423 to 445. This indicates that the aminoacids preceding this transmembrane region, including the catalytic domain, are located outside the membrane, and the succeeding aminoacids are situated inside. Notwithstanding, we have empirically demonstrated that cCDA7 is secreted to the growth media. Furthermore, the zymography assays are also an indication that this new cCDA is capable of deacetylating chitin polymers in the form of chitin glycol. Still, there are plenty of characteristics about this new enzyme to be discovered; an upcoming task is to elucidate in more detail the catalytic mechanism, the substrate specificity and the optimal working conditions, in the same way as it has been done with other active CDAs identified so far.

Zymography assays still could be useful to find other active cCDAs by simply improving the separation of the proteins by increasing the time of the electrophoresis, playing with concentrations of acrylamide and certainly by augmenting the size of the gels. The strategy of incorporating chitin glycol in the gel in which the proteins are separated needs to be redesigned. It is probable that a compromise concentration of chitin glycol that still show chitin deacetylase activity and does not cause problems with the mass spectrometer can be found. Moreover, it is important to bear in mind that so far only the

bands of the extracellular protein extracts have been analyzed. So, other cCDAs, as it could be the case for cCDA9, are going to be found in the cellular extracts that have already shown activity and maybe some of them will also be found individually.

Results presented here corroborate the hypothesis that the conversion of chitin to chitosan in *Chlorella* is performed by several chitin deacetylases at the same time. The question that rises is, why is this step so relevant to *Chlorella* to devote so many resources to it? cCDAs are probably very important in the cell wall formation and, as an extension of this, they could play a key role in the *Chlorella*-pathogen interactions. Besides the production of chitosan, another very particular trait of the *Chlorella* species is the large amount of viruses that have been discovered to infect them. These *Chlorella* viruses attach to the surface of the host cell, and then release polysaccharide-digesting enzymes to degrade it and penetrate to deliver the viral core to the host cytoplasm. Chitinases, chitosanases, glucanases, amongst others, are the sort of enzymes released[127]. Thus, after discovering that *Chlorella* secretes many different cCDAs to the media, it is plausible that these enzymes work modulating the cell wall, probably with other polysaccharide modifying enzymes, to regulate the interaction with pathogens like chloroviruses.

Understanding the mechanisms underlying the capacity of *Chlorella* to produce chitosans using so many enzymes is also very relevant to understand how to modulate the culture parameters in order to produce the most chitosan of the desired quality. Moreover, it could be possible to mimic these mechanisms and use the enzymes *in vitro* to produce tailored chitin oligomers with potentially valuable applications in fields like medicine, cosmetics or agriculture. With so many putative CDAs, it is highly expectable that there might be some of them showing new characteristics that could complement the CDAs that have already been characterized. One of the most requested traits that would boost the usage of these enzymes in the industry is the capacity to deacetylate natural crystalline chitin, without having to degrade it to oligomers or convert it to chitin glycol beforehand. Being so many, one of the 19 putative cCDAs that have not been discarded to be active yet may be capable of doing so more efficiently than the CDAs characterized so far.

3.4 Materials and Methods

3.4.1 Bioinformatic analysis of the putative cCDAs

22 sequences of putative CDAs from *Chlorella NC64A* were obtained from the JGI Genome Portal. The information about the expected molecular weight and isoelectric point of each protein sequence was obtained from the ProtParam software (ExPASy). A file containing all the sequences was uploaded and the Mw and pI were computed on average resolution.

Pairwise sequence alignments were done with the tool Blastp from NCBI. Alignments with E-values smaller than 0.001 were considered to be homologous[118]. In relation to the algorithm parameters, the Expect thresholds and the Word size were set to 10 and 6 respectively. The matrix chosen for the scoring was BLOSUM 62. The Gap costs were 11 for existence and 1 for extension. Finally, the conditional compositional score matrix adjustment option was selected and no filtering and masking options were selected.

Multiple sequence alignments were performed with the tool COBALT from NCBI. The parameters set were the standard: In the alignment parameters, the gap penalties were -11 and -1 for the opening and the extension respectively. The End-gap penalties were -5 and -1 for the opening and the extension respectively. In the Constraint Parameters the Constraint E-value was set to 0.003, RPS BLAST was chosen to guide the alignment and the option of finding conserved columns and recomputed alignment were activated. Finally, the use of query clustering parameters was activated, and was set to a word size of 4, a maximum clustering distance of 0.8 and a the regular alphabet was selected.

The motif search to identify active residues of the active site was performed with the motif search tool of the CLC Main Workbench 6.2 program.

The identification of transmembrane helices was done thanks to the software TMHMM (DTU).

3.4.2 *Chlorella* culture for RNA and protein extraction

The same cultures were grown to extract RNA and proteins to study the presence of CDAs. Cells from *C. Variabilis NC64A* were cultured to both exponential and stationary phase. *NC64A* was cultured mixotrophically in 150 mL of MBBM medium in the presence of 10ug/mL of tetracycline (Sigma

Aldrich) to avoid contamination, at 25ºC, shaking at 100 rpm and a light intensity of 50 μE. Cells were grown in two replicates during 96h to late exponential phase and during 296 hours to late stationary phase. The growth of the cultures was monitored by measuring the O.D at 680nm and also by counting the number of cells using a Neubauer chamber.

3.4.3 Extraction of RNA from *C. varibialis NC64A*

The protocol was designed based on the protocol "RNA extraction of Chlamydomonas, 20110323" from the JGI website with some modifications[128]. After several iterations the final protocol was as follows: First, cells were cultured as indicated above. Then samples of $1x10^8$ and $5x10^8$ cells were harvested separately for RNA extraction by centrifugation at 2000 RCF for 10 minutes in 15mL ultra resistant Falcon tubes. The supernatant was decanted immediately and the pellet was solubilized in 1mL of fresh lysis buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20 mM EDTA, 2%SDS, 1 mg/ml Proteinase K, all solubilized in nuclease-free water). Then, 10mL of TRIzol® (Thermo Fischer Scientific) was added and the cells were incubated for 5 minutes at ambient temperature and then frozen in liquid nitrogen and moved to a -80ºC freezer for later use.

In order to homogenize the cells, the frozen cells were thawed in ambient temperature and pipetted up and down ten times. Then, to separate the lipids, the sample was centrifuged at 12,000 RCF for 10 minutes at 4˚C. The resulting pellet contained the extracellular matrix material (ECM), polysaccharides, and high molecular weight DNA, while the supernatant contained the RNA. In high fat content samples, a layer of fat collects above the supernatant. The cleared supernatant was transferred to a new tube and let stay at room temperature for 5 minutes to permit complete dissociation of the nucleoprotein complex. Then, 0.2 mL of chloroform was added per 1 mL of TRIzol® reagent used for homogenization and the mixture was shaken vigorously for 15 seconds. The sample was left for 2-3 minutes at room temperature (RT) and centrifuged at 12,000 RCF for 15 minutes at 4˚C. The colorless upper aqueous phase containing the RNA representing approximately 50% of the total volume was pipetted out and placed in a new tube.

Using RNAse free material, 5mL of isopropanol was added to the aqueous phase and the mixture was incubated at room temperature for 10 minutes. Then, the sample was centrifuged at 12,000 RCF for 10 minutes at 4ºC and the supernatant was eliminated leaving only the RNA pellet. The RNA was washed by the addition of 10 mL of 75% ethanol, vortexed briefly and centrifuged at 7500 RCF for 5 minutes at 4˚C. The wash was discarded and the pellet was let dry for 10 minutes. Finally, the pellet was solubilized in 20 μL of RNase-free water by incubating the sample in a heat block set at 60˚C for 15 minutes and pipetting up and down several times.

In order to fully eliminate the genomic DNA from the sample, the RNA was brought to 87.5 μL with RNAse-free water and was incubated for 10 min at RT with 10 μL of buffer RDD and 2.5 μL of DNAse I stock solution (Qiagen RNa-se free DNase set). Afterwards, the RNA was further cleaned following the instructions of the miRNeasy Mini Kit from Qiagen. Finally, the quality of the RNA was checked with a Nanodrop 1000 (Thermo Fisher Scientific). The 260/280 and 260/230 nm signal ratios were used to measure the quality of the RNA. For the 260/280 a value similar to 2.1 is expected and for the 260/230 ratio a value higher than 2 is expected. The quality of the RNA was re-confirmed by gel electrophoresis by loading 400 ng in an agarose gel.

3.4.4 RT-PCR

Total *C.variabilis NC64A* RNA was prepared as previously described. The corresponding cDNA was prepared from 1 μg total RNA following the protocol of Thermoscript (Invitrogen) using a 10 : 1 mixture of oligo dT and random hexamer for priming. Before adding the RNA and the primers to the general mix, they were mixed together and denaturalized by incubation at 65ºC for 5 min. followed by incubation on ice. The following are the cDNA synthesis reaction temperatures and times: 25˚C 10′, 42˚C 10′, 50˚C 30′, 55˚C 30′, 60˚C 20′, 85˚C 5′. Afterwards, the reactions were treated with 1uL RNaseH for 20 min at 37ºC.

3.4.5 Detection of putative cCDAs in the cDNA of *C. variabilis NC64A*

Once the cDNA was obtained, it was attempted to amplify representative fragments of each individual cCDNA by PCR using the primers and under the conditions indicated in Table 10.

3 PCRs with different conditions were carried out to meet the requirements of amplification of all the fragments. In all cases, a mix containing 14.8μL of water, 2uL of PCR buffer 10X, 0.4μL of 10mM DNTPS (Bioron), 0.4μL of 10mM forward and reverse primers, 1μL of cDNA and 1 μL of RedTaq® DNA Polymerase was prepared. The PCR program was changed in each of the 3 cases. In PCR one it consisted of an initial denaturation step of 2 min. at 95ºC. Then, 35 repeating cycles of 55s at 95ºC for denaturation, 55s at 49ºC for annealing and 40s at 72ºC for elongation were carried out. The final step was an elongation step of 8 min. at 72ºC. PCR number two consisted of the same steps; the only difference was that the annealing temperature was 55ºC and each elongation step was performed during 50s. Finally, PCR 3 consisted of the same conditions of PCR 1 despite the annealing temperature, which was 57ºC. All PCR reactions were performed with the Thermocycler Eppendorf Mastercycler Gradient. The PCR fragments obtained for each of the cCDAs were sequenced using the same primers from Table 10 in Stab Vida. The primers were synthesized by Sigma Aldrich. All reagents, unless otherwise indicated, were purchased from Sigma Aldrich.

CDA	Fwd. Primer	Rev. Primer	Length (bps)	Pcr
1	F357 CCCTCCACCTACTTCATCTC	R358 CCCATGGCATACTCAATAAACT	662	2
2	F401 GGATTGAGGAGGAGATTGG	R402 TTCTGAAGGTAGGGCTTGT	468	1
3	F359 CGACTCCTCCTCCAACAAC	R360 TCTCCTCCTCCTCATCGCT	359	2
4	F361 TGAGCGTGGAGATGGATGT	R362 TGGAGGTAGAGGAGTCGGT	402	2
5	F363 GAAGGATGTGAGGGAGGAG	R364 GGGAGTGGATGAAGATGGG	461	2
6	F365 GGACATTGTGGGGTTCAGGG	R366 GTGTGTGGCTTGGTGAGGG	504	3
7	F367 TGGGAGGCTTGTTTGTGGG	R368 ACGGAGAGCAAGAGGGAGA	316	3
8	F403 AGCCCGAGTATGTGCAGAA	R404 ATGTGTTTGGGAGCGAGAG	840	2
9	F373 CAGGCATCAGCAACCCAAA	R374 CCCAAGACAGGAAAGCCACAA	561	3
10	F375 TGCTGGTTTGGTGCTGTGT	R376 GTGCGTCTTGCTGTGGATG	341	3
11	F377 TGCTTCCACTCCATGTACT	R378 CCAACTTCCAAATCAACAACC	448	2
12	F379 GGTGGTGGCGATGTATGAGG	R380 GGATGAACACGGGGAAGGG	503	3
13	F381 TGTGGTGTGGATGTAGATGGG	R382 TGTGGAAGGACGGGTACGA	516	2
14	F383 AGGGAGTATTTGGTGGAGGAG	R384 AGGAAGATGGGGAAGGGGG	431	3
15	F385 ACTTCATCCTCTTTTCCCA	R386 CCCATTCCTCGTAGTCATA	375	1
16	F387 CCATCACCCCTACCACCTAC	R388 CACACTGGCACCTCAAACA	520	2
17	F407 CACTCAACCACCCAAACCC	R408 CATAGTCCACAAACCGCGTC	278	3
18	F391 GGGATGAAACGGAGGAGGA	R392 GGATGTAGATGGGGAAGGG	450	2
19	F393 CCCCTCTACATCCACACAC	R394 GGCCATTTCTTGCACAAAC	518	2
20	F395 CCATCTTCATCCACACACCC	R396 CCCCTCCACTTCCTCCTCA	317	2
21	F397 TAAAGGAGACGGTGGGGGA	R398 TGATGGTGGAGTCGTAGAG	339	2
22	F399 CTCTTCATCCACACGCCCT	R400 TCCTCCTCCACCACATCCC	320	3
C1	F41 GTCAGAGGTGAAATTCTTGGATTTA	R42 AGGGCAGGGACGTAATCAACG	732	1 , 3
C3	F413 GTTCTTAGTTGGTGGGTTG	R414 TAGGTGGGAGGGTTTAATG	463	2

Table 10 - Primers and conditions used for the amplification of the 22 CDA sequences from *C.variabilis NC64A* cDNA.

A control for each PCR condition was included, C1 for PCRs 1 and 3 and C3 for PCR 2.

3.4.6 Protein extraction from *C. variabilis NC64A*

After culturing the microalgal strains as indicated in subsection 3.4.2, the part of the pellet that was not selected for RNA extraction was recovered from the liquid culture by centrifuging at 5,000g for 10 minutes and afterwards immediately frozen in liquid nitrogen and stored at -80 ˚C. The supernatant, containing the growth media, approximately 150 mL was kept and the recommended concentration of SIGMAFAST protease inhibitor cocktail was dissolved in it immediately (Sigma Aldrich). Subsequently the media was filter-sterilized using a Stericup® filter unit (Millipore) and kept at 4ºC.

The proteins from the growth media were precipitated at 4ºC by adding ammonium sulfate to 80% saturation at 4ºC and letting precipitation occur overnight. The next day, the samples were centrifuged at 20.000 RCF for 30 min. at 4ºC. The precipitated proteins were slowly solubilized in 5mL of MilliQ water and dialyzed against PBS in three cycles of 2 hours each, which included the addition of a new PBS buffer each time. Then, the dialyzed proteins were concentrated 20X by membrane ultrafiltration using Vivaspin 500 columns (Sartorius Stedim Biotech). The presence of proteins in these fractions was confirmed by gel electrophoresis and staining the proteins with Coomassie Blue (Sigma Aldrich).

The cellular proteins were obtained from the frozen pellets, which were thawed and solubilized in 10 mL of a previously chilled to 4ºC and filter-sterilized extraction buffer composed of PBS, the recommended concentration of SIGMAFAST protease inhibitor cocktail and 7mM Phenanthroline (Sigma Aldrich). The cells were disrupted by homogenization in a cell disruptor (TS Series Benchtop, Constants system) five times at a pressure of 2.2 Kbar. Then, soluble proteins were separated from the insoluble ones by centrifugation at 12.000 RCF for 10 min. at 4ºC. Finally, 33% of previously sterilized glycerol was added to all the protein fractions obtained, cellular and extracellular, which were thoroughly mixed and stored at -80ºC. The cellular protein fraction was quantified using a Bradford reagent (Sigma Aldrich) and the extracellular protein fraction was quantified using the Qubit Protein Assay Kit protocol (Thermofisher). A scheme of the protein extracts obtained can be seen in Figure 18.

3.4.7 Chitin deacetylase and chitinase activity on chitin oligomers

The detection of chitin deacetylase and chitinase activities in *Chlorella* protein extracts was achieved in a buffer containing a final concentration of 50mM sodium bicarbonate, 0.4 mg/mL of chitin tetramers (Megazyme) and 25% v/v of the protein extracts. In one case the buffer was adjusted to pH 4.5 and in the other to pH 7. All samples were incubated at 37ºC during 96 hours. Analyses of chitin and chitosan oligomers were done using a Dionex Ultimate 3000RS UHPLC system (Thermo Scientific) coupled to an evaporative light scattering detector (Model Sedex 90LT, Sedere) and an ESI-MSn-detector (amaZon speed,

Bruker). The specific conditions for the separation and detection of the oligomers can be found in Hamer et al. 2015[90]. The experiments were performed in duplicates. i.e. with two different protein extracts for each condition.

The chitinase and chitin deacetylase activity on *E. coli* protein extracts was performed in the same way as explained above. The differences were that only pH 7 was tested and the activity buffer, instead of 50mM sodium bicarbonate, contained TEA buffer; according to previous experiments with other CDAs, no difference should be observed between the two buffers. Moreover, the incubation was done during 16 hours instead of 96 hours and each sample was tested only once, no duplicates were done..

These experiments were performed in collaboration with Lea Hembach, at the laboratory of Dr. Bruno Moerschbacher at the University of Münster within the framework of the Nano3,,Bio FP7 EU project.

3.4.8 Dot blot to determine chitin deacetylase activity in chitin polymers (glycol chitin)

First a polyacrylamide gel containing 10% polyacrylamide and 1% glycol chitin was prepared. Glycol chitin was prepared from glycol chitosan (Sigma Aldrich) at the laboratory of Dr. Bruno Moerschbacher at the University of Münster. Once the gel had polymerized, 5μL of each Chlorella protein extract, including the controls, were dropped on top of the gel. Then, the gel was incubated in a wet chamber during 20 hours at 37ºC. Instead, 3μL were dropped on top of the gel when testing *E. coli* protein extracts and the incubation was carried out for 16 hours. The positive control for chitinase activity was the purified chitinase ChiB (ABI331431.1) form *Serratia marcescens.* The positive control for chitin deacetylase activity was a purified CDA of fungal origin that at the time this thesis was written it had not been published, so it was kept confidential. After the incubation, the gel was washed and then stained with a Calcofluor-white solution for approximately 10 minutes at room temperature. The Calcofluor-white solution consisted of 0.5M Tris HCl pH 8.9 and 0.01% Calcofluor-white. After rinsing out the excess of staining solution with water, chitinase activity was determined by the appearance of black spots in the specific positions where the samples had been dropped when exposed to U.V. light in a

transiluminator. In order to detect the chitin deacetylase activity, the same gels were depolymerized. The depolymerization step was carried out by incubating the gels for 10 minutes at room temperature under the hood in 20 mL of depolymerization solution. The depolymeriszation solution was prepared by adding 550μL of sulfuric acid to 20mL of 5.5mM sodium nitrite and mixing under the hood until no more green/yellow steam was produced[124]. After depolymerization, the gel was washed twice in water. Finally, the gel was exposed to U.V. light in a transiluminator to detect new black spots in the glycol chitin gel, caused by the deacetylation activity of the extracts tested. These experiments were performed in collaboration with Lea Hembach, at the laboratory of Dr. Bruno Moerschbacher in the University of Münster within the framework of the Nano3Bio FP7 EU project.

3.4.9 Identification of chitin deacetylases by gel zymography

Chitin deacetylase activity is detected in a semi-native SDS-PAGE in which the proteins are not denatured with Β-mercaptoethanol and are not boiled. The protocol followed is based on a modification of the method used by Jean Trudel et al.[124] made in the laboratory of Dr. Moerschbacher at the University of Münster[121,123]. The loading buffer is made of 8% (w/v) SDS in 0.25 M Tris HCl at pH 6.8, 20% (v/v) glycerol, and 0.4% (w/v) bromophenol blue. The stacking gel was prepared containing 4.8% polyacrylamide and the separation gel contained 7% polyacrylamide in the first zymography and 15% in the other assays. In the cases in which chitin glycol was embedded in the gels, the gels were prepared as in zymography 2 with the addition of 1% (v/v) chitin glycol in zymography 3 and 0.2% (v/v) chitin glycol in zymography 4. The overlay gel was prepared like the Dot blot gel (10% polyacrylamide and 1% chitin glycol). 5 μg of protein extracts were loaded per well in the first two zymografies. In zymography 3 the amount of protein loaded per well was adjusted between 3 to 12 μg based on previous results to reduce saturation and increase the possibility of finding individually active proteins per band. In zymography 4, a serial dilution of the sample was loaded in the gel (0.5μg/mL of protein per well and then 0.1, 0.05, 0.025, 0.0125, 0.00625 and 0.00156 μg/mL of protein per well). The electrophoresis was run in all cases between 3 and 6 hours at 4ºC between 10 and 30 mA. After the gel was run, the proteins regained their correct conformation by washing away the SDS with three sequential washing steps of

1 hour; the first one in Activity buffer with 1% Triton X-100, the second one in Activity Buffer with 0.5% Triton X-100 and the last one in Activity Buffer only. The activity buffer is composed of 50 mM sodium bicarbonate at pH 7. In the case of zymographies 1 and 2 at this point the overlay gel was carefully placed on top of the gel used for separating the proteins with electrophoresis. Then all gels from all zymographies, with or without overlay gel were wet with activity buffer and incubated in a wet chamber for 20h at 37ºC. Then, the development of the gels to detect chitinase and chitin deacetylase activities under UV light in a transilluminator was performed like in the Dot blot activity assay explained above.

3.4.10 Cloning of cCDAs into the vector for recombinant expression in *E.coli*

cCDAs 1, 3, 4, 7, 10 and 21 were tried to be amplified from the cCDA of *C. variabilis NC64A* by PCR using primers in Table 11. cCDA 1 and cCDA 10 were amplified in PCR 1. PCR 1 was performed in 20μL containing 9.6μL of milli-Q water, 4 μL of 5X Superfi™ Green Buffer 1 (Thermo Fisher Scientific), 0.4 μL of 10mM of DNTP mix (Bioron), 4 uL of GC enhancer (Thermo Fisher Scientific), 0.2 uL of Platinum™ Superfi™ DNA Polymerase (Thermo Fisher Scientific), 0.4 uL of 10mM fwd. and rev. primers and 1uL of cDNA from *C.variabilis NC64A* obtained as explained above. The PCR program started by a denaturation step of 33s at 96ºC. Then, 35 repeating cycles of 10s at 96ºC for denaturation, 10s at 56.4ºC for annealing and 40s at 72ºC for elongation were carried out. The final step was an elongation step of 5 min. at 72ºC. cCDAs 3, 4, 7 and 21 were tried to be amplified in PCR 2. PCR 2 was made with the same components as PCR 1 with slight changes in the PCR program: the annealing temperature was 59.2ºC and the elongation time was 80s. Faint cCDA 3 and 4 bands were obtained and cut out and purified from the agarose gel using GenElute™ Gel Extraction Kit from Sigma Aldrich. PCR2 was repeated using the purified bands as template to obtain more DNA for cloning. In this case the annealing temperature was lowered to 55.4ºC.

cCDA	Fwd. Primer	Rev. Primer	Expected size (bp)	Cloning strategy
1	F435 ttttGGATCCtATGCAGCTGATTCAGGGAC	R436 ttttCTCGAGGCAGGTCTTGGTGCACGA	1182	3':BamHI 5': XhoI
3	F437 aaaaGAGCTCaATGCAGCGCTGCCGGCTC	R438 AAAACTCGAGCAGCTGCTTACGACTGTTCTT	2052	3':SacI 5': XhoI
4	F439 AAAAGGATCCAATGCAGGCGTCTAAGATCA	R440 ATATAAGCTTCTCCTGCTGCCGCGGCTG	2022	3':BamHI 5': HindIII
7	F441 aaaGGATCCaATGCAAGCCCTGCTGCTG	R442 AAAACTCGAGCGAGGCGGGCAGCAGCTC	1772	3':BamHI 5': XhoI
10	F443 AttAGGATCCtATGGAGCGCCTACCACTT	R444 aaaaCTCGAGGGCAGTCGGGGGGCTGGT	1314	3':BamHI 5': XhoI
21	F445 aaaaGGATCCaATGAATCTCGCCGCGGCA	R446 ttttCTCGAGCGCCGCCATCTTCTCCTCC	2232	3':BamHI 5': XhoI

Table 11 – Primers and restriction enzymes used in the PCR to clone cCDAs to pET-22b(+) vector

From PCRs 1 and 2, the sequences of cCDA 1, 3, 4 and 10 were obtained; cCDA 7 and 21 were not amplified. It was decided to continue with these sequences as four different proteins to express in *E. coli* was already a high number. The sequences, together with the plasmid pET-22b(+) (Novagen) were digested using the restriction endonucleases indicated in Table 11. XhoI restriction enzyme was obtained from Sigma Aldrich, SACI from New England Biolabs and BamHI and HindIII from Bioron. Afterwards, inserts and vector were separated by agarose gel electrophoresis to check the correct digestion. pET-22b(+) vector was digested as a control with each enzyme individually. After finding that all

digestions had worked well, vector and insert sequences were cut out from the gel and purified as indicated above. Then, each sequence was quantified using the Qubit DS DNA BR assay kit (Thermo Fisher Scientific). Afterwards, each cCDA was ligated with the correspondingly digested vector using T4 DNA ligase from Takara. The ligation reaction was performed in 10 μL containing a 1:3 molar ratio of vector:insert, 0.7 μL of ligase and 1 μL of ligation buffer at 20ºC for four hours.

2 μL of the ligated DNA were transformed into Rosetta™(DE3) competent cells, which is an *E. coli* strain designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. Competents cells were obtained chemically by dissolving a exponential growth culture in TSS buffer (LB with 10% PEG, 5% DMSO and 35mM magnesium chloride, all at pH 6.5)[129]. The transformation protocol consisted in thawing the chemically competent cells, and after 5 min. in ice, incubating them 30 min with the ligated DNA in ice for 30 min. Afterwards, a heat shock was induced by incubating the cells in a water bath for exactly 45 seconds. The cells were incubated for 1 hour in LB media at 37 ºC. Finally, the transformed cells were inoculated in agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The plates were incubated over night at 37ºC and the next morning the grown colonies had been selected by their ability to grow in chloramphenicol and ampicillin.

To ensure that the resistant colonies carried the Gene of Interest (GOI), colony PCR was performed. Colony PCR simply consists on doing a PCR using directly the transformed colonies without the necessity of extracting the DNA in advance. The PCR was designed so that one primer annealed to the vector (R91) and the other one annealed to the inserted cCDA (Table 12). A colony was touched with a pipette tip, and the attached material was then dissolved in 20 uL of PCR mix (15.8 μL of water, 2 uL of PCR buffer 10X, 0.4 μL of 10mM DNTPS, 0.4 μL of 10mM of the fwd. and rev. primers and 1 μL of RedTaq® DNA Polymerase). The PCR program initiated with a denaturation step of 2 min. at 95ºC. Then, 35 repeating cycles of 55s at 95ºC for denaturation, 55s at 52.5ºC for annealing and 90s at 72ºC for elongation were carried out. The final step was an elongation step of 8 min. at 72ºC. 8 colonies of each cCDA were necessary to identify several correctly transformed clones of cCDA 1 and 3. Instead, more than 30 colonies of clones of cCDA 4 and cCDA 10 were

screened and positive colonies were only found in the case of cCDA 10. Positive clones were stored at -80ºC after adding 0.15 mL of glycerol to 0.85 ml of the logarithmic-phase *E. coli* culture.

cCDA	Fwd. Primer	Rev. Primer	Expected size (bp)
1	F357 CCCTCCACCTA CTTCATCTC	R91 CCTCCTTTCAG CAAAAAAC	1076
3	F359 CGACTCCTCCT CCAACAAC	R91 CCTCCTTTCAG CAAAAAAC	669
4	F361 TGAGCGTGGA GATGGATGT	R91 CCTCCTTTCAG CAAAAAAC	880
10	F375 TGCTGGTTTGG TGCTGTGT	R91 CCTCCTTTCAG CAAAAAAC	1411

Table 12 - Primers used and expected band size in the colony PCR to screen the presence of the cCDA sequences in the transformed *E. coli* colonies.

3.4.11 cCDA expression in *E.coli*

E. coli Rosetta clones cCDA 1.4, 1.5, 3.4, 10.3 and 10.6, a Rosetta WT as a negative control and a positive control containing *Colletotrichum lindemuthianum* CDA AY633657 gene (ClCDA) were cultured overnight in 5mL of LB culture medium. Different antibiotics were added depending on the strain: 34μg/mL of chloramphenicol and 100 μg/mL of ampicillin in the case of the clones, only chloramphenicol in the case of the untransformed Rosetta strain and 100 μg/mL of ampicillin together with 25 μg/mL in the case of the ClCDA. The laboratory of Dr. Bruno Moerschbacher from the University of Münster kindly shared the strain containing the plasmid with ClCDA gene. The following morning, 50mL of LB with the same antibiotics commented above was inoculated with 1% (v/v) overnight culture. Each culture was inoculated twice, one for induction with IPTG and the other one as a control of induction. Cultures grew at 37ºC with vigorous shaking at 220 rpm. After 4h, $O.D._{600nm}$ was between 0.6 and 0.85 and cultures were induced with 1mM IPTG. Then, all cultures, the induced and non-induced ones, were grown for 3 more hours in

the conditions indicated above. Afterwards, cultures were harvested by centrifugation at 12,000 RCF for 3 min, and the pellets were washed 3 times in PBS buffer and frozen in liquid nitrogen and then at -80ºC.

Protein extracts were obtained by disrupting the cells by thawing the frozen pellets on ice and then sonicating them. Prior to sonication each pellet was solubilized in 5mL of ice-cold lysis buffer (50mM Tris HCl pH 6.8, 100mM NaCL, 1mM DTT, SigmaFast protease inhibitor cocktail (Sigma Aldrich), all at pH 7.5). Sonication was done in 8 cycles of 15s of sonication at 20% intensity with pauses of 25s in between and then 4 cycles of 15s of sonication at 30% intensity and pauses of 25 seconds. Soluble proteins were separated from the insoluble material by centrifugation at 10,000 RCF at 4ºC for 20 minutes. 2.4 mL of the soluble fraction was stored at 4ºC for purification by affinity chromatography. The rest of soluble proteins left were stored at -80ºC after adding 20% (v/v) sterile glycerol and freezing them in liquid nitrogen. The insoluble fraction was stored directly at -80ºC.

The pET-22(b+) expression vector carries a His-Tag, so 2.4 mL of the supernatant (soluble proteins) were purified by affinity chromatography following the protocol for native protein purification of His-Select® Spin Columns (Sigma Aldrich). All clones, induced and non-induced and the WT Rosetta strain went through the purification process. The equilibration buffer contained 1mM imidazole, the washing buffer contained 5mM imidazole and the elution buffer contained 250 mM Imidazole. The purified proteins were eluted in 200uL of elution buffer and stored at 4ºC. Then, 32 μL of the purified proteins were mixed with 5x loading buffer (0.25% v/v Bromophenol Blue, 50% v/v glycerol, 10% v/v SDS, 0.25M Tris-HCl pH 6.8 and DTT 0.5M, all reagents from Sigma Aldrich), and separated by SDS-PAGE gel electrophoresis in Any kD™ TGX™ Precast Protein Gels (Biorad) for 30 minutes at 200 V in a Mini-PROTEAN® electrophoresis cell (Biorad). The running buffer was composed of 25mM Tris, 192mM Glycine and 0.1% SDS (w/v). Once, the SDS-PAGE electrophoresis process had finished, the gel containing the separated proteins was coomassie stained for 4 hours using Brilliant Blue G solution (Sigma Aldrich), and distained with several incubations with Distaining solution containing 10% (v/v) acetic acid and 10% (v/v) isopropanol.

Because no cCDAs were expressed in the previous experiment, two cCDAs (cCDAs 3.4 and 10.3) were purified from the soluble extracts but this time under more stringent conditions. The affinity purification protocol followed using His-Select® Spin Columns (Sigma Aldrich) was the same as before with slight changes: to avoid saturating the column, 0.6 mL of protein extracts were purified this time instead of 2.4 mL. Moreover, the washes were more stringent as three washes were performed instead of two and the concentration of imidazole was 10mM instead of 5 mM. Also, to check for the possibility that the proteins were all in the insoluble fraction, the pellet after disruption of all the induced cultures and the non-induced cCDA 3.4 and 10.3 cultures was dissolved in 3ml of a denaturation solution (50mM Tris HCl pH 7.5 and 8M urea) for 1h at 30ºC and under vigorous shaking (300rpm). Then the denatured protein extracts, together with the two purified extracts and also the soluble fraction of all the induced cultures were separated by SDS-PAGE electrophoresis as indicated in the previous paragraph. Then the gels were stained and distained as stated above.

Because all cCDAs expressed were located in the insoluble fraction, a new culture and induction experiment was designed to try to obtain some soluble cCDAs. The clone cCDA 3.4 was cultured in the same way as in the previous experiment. Cultures were induced again with 1mM IPTG but at 30ºC for 1h, 3h and 5h and at 20ºC for 1h, 5h and 16h. Controls of induction were collected at 3h when grown at 30ºC, and at 16h when grown at 20ºC. In addition, an untransformed Rosetta was cultured as a negative control and induced 3h at 30º and 16h at 20ºC. The cells were harvested at the indicated times and lysed as stated above. This time, however, the lysis buffer was prepared differently to increase the solubility of the proteins (50mM sodium phosphate, 0.3M sodium chloride, 5 mM imidazole, 0.1% Triton and SigmaFast protease inhibitor cocktail, pH 8; all reagents from Sigma Aldrich). The soluble proteins were separated from the insoluble pellet by centrifugation for 20 min, at 10,000gs at 4ºC. The pellet was resuspended in 1.5mL of RIPA buffer and sonicated as indicated above to obtain more soluble but still active proteins (50mM sodium phosphate, 0.3M sodium chloride, 5 mM imidazole, 1% Triton, 0.1%SDS and SigmaFast protease inhibitor cocktail, pH 8; all reagents from Sigma Aldrich). Again, the soluble proteins were separated from the pellet by centrifugation. Finally, the pellet was solubilized by incubating it for 1h in the

same RIPA buffer containing 8 M urea. The samples were then separated by SDS-PAGE electrophoresis, stained and distained as indicated above. All soluble protein fractions were stored at -80ºC after the addition of 25% (v/v) sterile glycerol. The insoluble proteins in urea were stored at the 4ºC.

To try to obtain clearer results the protein extracts obtained in the paragraph above were tried to be purified using His-Select® Spin Columns (Sigma Aldrich). 6 soluble extracts and insoluble extracts solubilized in RIPA buffer and in 8M urea (30º3h I, 30º5h I, 20º5h I, 20º16h I, control of induction 30º3h and untransformed control R 30º3h I) were purified following the protocols and buffers of the His-Select® Spin Columns. For the soluble proteins and the insoluble proteins in RIPA buffer the protocol for native proteins was followed and in the case of the proteins in urea, the protocol for denatured proteins was followed. 5mM imidazole was added to the equilibration buffer (none in the case of the urea extract), 10mM imidazole to the washing buffer (5mM in the case of the urea extract) and 250mM imidazole to the elution buffer. In order to modify as little as possible the composition of the initial buffers in which the proteins were dissolved, 20% glycerol was added to all the buffers (equilibration, washing and elution buffers). Moreover, to all buffers used in the purification of the soluble extracts, 0.1% Triton was added and in the case of the purification of the proteins in RIPA buffer, 1% Triton and 0.1% SDS were added. All buffers were prepared at pH 8.

With the goal of augmenting the concentration of the sample, while at the same time exchanging the buffer to one that could be used to test deacetylation activity in the future, the same 6 RIPA samples were concentrated by membrane microfiltration using Vivaspin 500 columns (Sartorius Stedim Biotech). In this case, 500 μL of sample were loaded into the column, and centrifuged at 15,000gs at room temperature until the volume of the sample was reduced to 250 μL. Then, 250 μL of a buffer containing 50mM sodium phosphate, 300mM sodium chloride and 20% glycerol was added. This procedure was performed three times. The last centrifugation step was performed until the final volume was approximately 75 μL.

4 CHITOSAN PRODUCTION SCALE-UP AND THE STUDY OF ITS BIOACTIVITIES

4.1 Background and aims

In Chapter 2 of this thesis project it was discovered that microalgae from the *Chlorella* genus can produce chitosans. Then, in Chapter 3 it was reconfirmed that *Chlorella* has the ability to produce chitosans naturally by preparing protein fractions containing putative chitin deacetylases that are capable of deacetylating chitins *in vitro*. In this new chapter, the results from an initial functional characterization of *Chlorella* chitosans are explained. The aims were, on the one hand, to see if these newly discovered polymers truly behaved like chitosans and then, on the other hand, to start deciphering what *Chlorella*-derived chitosans might be good for to pinpoint possible applications. Before explaining the results, the process to choose relevant bioactivities to be studied is explained, starting by a review of the possible applications of chitosans that was made trying to highlight the cases in which there are already commercial products. Then, some of the applications in which *Chlorella* chitosans have more probabilities of succeeding according to its characteristics were selected. Before studying the chosen applications, the process to extract chitosans from *Chlorella* was optimized and up-scaled.

4.1.1 Possible applications of chitosans

Depending on their characteristics, chitosans can be used in a very wide spectrum of applications, such as medicine, pharmacy, human and animal nutrition, cosmetics, agriculture, biochemistry and biotechnology, water treatment, textile and paper industries, amongst others[7,8,11,12,130–134].

4.1.1.1 Chitosans for biomedical applications

Chitosans are very useful for biomedical applications because they are biodegradable, biocompatible and non-toxic. One of the most studied medical applications of chitosans is the treatment of wounds, burns and scars. Apart from the properties commented before, their mucoadhesive properties, together with antimicrobial bioactivities, promotion of angiogenesis, haemostatic capacity, analgesic effect, improvement of tissue regeneration and anti-inflammatory properties, are all recognized attributes necessary for the treatment of wounds, burns and scars[135–137]. Due to all of these interesting characteristics, several derivatives from chitins and chitosans have been commercialized for this purpose in the form of membranes, fibres, hydrogels, chitosan-coated medical devices, etc. Some companies with such kinds of wound healing products in the market are HemCon (Patch PRO, Bandage PRO, ChitoFlex® PRO, GuardaCare® XR PRO, ChitoGauze® PRO, SynaeroTM, Dental Dressing PRO etc), Medoderm (Quractiv®), Genbiotech (Proderm®), Celox Medical (Celox™ Rapid Gauze, Celox™ Gauze, Celox-A™ Applicator, Celox-A™ Granules), Marine Polymer Technologies (Syvek NT™, SyvekExcel™) Medtronic (Novashield®), SAM Medical (Chito-SAM™), RevMedX (Xtat™), CoreLeader Biotech (Hemo-Fiber and Hemo-Pad), Scion Biomedical (Clo-Sur PLUS P.A.D™), Lunainc (Chitoseal™), IMS (Chitodine®), Hemostasisllc (ExcelArrest® XT), amongst others. Especially interesting is the case of Marine Polymer Technologies, as they use very high molecular weight chitin fibbers, which they call poly-n-acetyl glucosamine, obtained from a diatom of the *Thalassiosira* genus. They claim that this polymer is different from chitosans because the fibbers are completely acetylated and have a unique tertiary structure[138–144]. The interesting wound healing properties of chitosans, peculiarly the antimicrobial and biodegradable capacities have also been exploited to make surgical sutures like the ones sold by Medovent.

Tissue engineering is the other biomedical field in which chitosans have been thoroughly investigated due to their minimal foreign body reactions, antibacterial nature, biodegradability, biocompatibility and ability to be molded into various geometries. 3-D supports made of chitosans or mixtures of chitosans with other polymers that improve the physical, chemical and biological properties (collagen, alginate, gelatin, carboxymethyl cellulose, PHBV, polylactic acid, polyglycolic acid, etc.) and/or other substances (Hydroxyapatite, Nano silicon dioxide, Keratin nanoparticles, calcium phosphate, bioactive glass, chondroitin, etc.), have been used with excellent results in bone and cartilage tissue ingeneering[145–151]. Smith and Nephew (BST-CarGel®), Ceramed (k-IBS®) and Oligomedic (JointRep™) are examples of companies with products for cartilage and bone tissue regeneration containing chitosans. In the field of orthopedic tissue engineering the already commented characteristics, especially the fact that chitosans can be degraded in a controlled rate by human enzymes such as lisozymes, have been found to be valuable to make chitosan a good bioresorbable material for implants[152,153]. Moreover, chitosans used as coating of other materials like titanium, improves the integration and cellular attachment of implants because of its minimal foreign body reaction, antimicrobial and antioxidant properties[154,155]. Furthermore, 3-D scaffolds made of chitosans alone have been used with promising results for the fabrication of bioengineered open ventricles[156]. The repair and regeneration of nerve injuries has also been demonstrated to be facilitated with the use of chitosans[157]. Medovent manufactures nerve guides to support the repair of peripheral nerve injuries (Reaxon®). Other cells that have demonstrated to be successfully grown in scaffolds made of chitosan are hepatocytes[158] and respiratory epithelial cells[159]. Chitosans have also been studied as a skin replacement for individuals who have suffered extensive losses of skin generally because of burns[160,161]. Finally, chitosans can also be used for *in-vitro* spermatogenesis. Indeed, chitosans have been key to be able to produce human spermatozoa *in-vitro.* This technology from Kallistem (Artistem®) is currently under preclinical development. In aesthetic medicine, hydrogels made of chitosans alone or mixtures of collagen and chitosans have been proven to be successful as dermal fillers, especially because of its anti-inflammatory properties [162,163]. Merz Pharma has recently been granted a patent for the use of chitosan beads cross-linked with citrate ions as a dermal filler[164].

4.1.1.2 Chitosans for pharmaceutical applications

Moving towards the pharmaceutical arena, the uses of chitosans in drug delivery systems have been thoroughly investigated. Chitosans due to its cationic character, have properties such as controlled drug release, mucoadhesion, pH-dependent in situ gelation and degradation, transfection enhancement, permeation enhancement and efflux pump inhibitory effects, which can be further improved by chemical modifications. Such properties, together with its non-toxic characteristics, are very appreciated for a wide range of drug delivery systems, including oral, ocular, nasal, vaginal, buccal, parenteral, intravesical or skin drug delivery systems. Of special interest is the fact that the pH sensitivity of chitosan can be used to prepare nanocarriers that specifically deliver drugs targeting the tumor acidic microenvironments. Chitosans and chitosan-derivatives in the form of capsules, powders, gels, solutions, emulsions, microspheres and nanocapsules have been investigated to deliver drugs to cure different conditions[165–170]. In this area companies like Viscogel AB commercialise formulations for time-controlled drug release based on chitosans (Viscogel™ and Viscoemulsion™). An example of a vaccine adjuvant containing chitosan is VaccigGrade™ commercialized by Invivogen. A nail lacquer, containing Ciclopirox and a chitosan derivative (hydroxypropyl-chitosan) for optimal topical delivery, was developed by Polichem (Almirall) to solve onychomycosis and is commercially available worldwide under different brand names (Onytec®, Polinail®, Niogermox®, Niogermos®, Kitonail®, Privex®, Fulcare®, Rejuvenail®and Myconail®). Archimedes Pharma has developed a nasal absorption enhancer (Chisys®) which Javelin Pharmaceuticals uses for his alternative product for parenteral morphine (Rylomine ®) that reached phase 3 clinical trials but no information has been found on whether or not it has been marketed. To finish commenting other medical/pharmaceutical applications of chitosans it is important to highlight the evidence showing the anticancer and anti-inflammatory properties of chitosan derivatives and chitosan oligosaccharides[171–173].

4.1.1.3 Chitosans for human and animal nutrition applications

In the field of human nutrition, chitosans have mainly been marketed as a dietary supplement due to its hypocholesterolomic activity and its capacity to entrap lipids[174,175], although some studies indicate that these benefits are not

statistically significant[176]. Many different products such as Liposan Ultra® from Primex or Chitosan Forte from Arkopharma are commercialized with these claims. A recent review highlights the potential of chitosans and chitosan oligosaccharides as nutraceuticals for the prevention, delay or treatment of age-related diseases thanks to their antioxidative, anti-inflamatory, antidiabetic, hypocholesterolemic and anticancer properties[177]. Nutragenesis commercializes a nutraceutical exploiting the just mentioned benefits of chitosans (Orisett®). Mastix Medica recently introduced a chitosan based sugar-free chewing gum (RenaGum™) that helps patients with chronic kidney disease maintain normal serum phosphate levels. The use of chitosans in animals and fish nutrition industry has also been studied and the main benefits they offer are improved growth performance and feed conversion ratio thanks to the biological effects caused by the immunomodulatory, anti-oxidative, antimicrobial and hypocholesterolemic properties of chitosans[178,179]. Epakitin® from Vetoquinol is an example of a food supplement for improvement of renal functioning in dogs and cats and Biovita-P from Kun Poong Bio is commercialized for farm animals and fish.

4.1.1.4 Chitosans for cosmetic applications

The cosmetic industry has largely benefited from chitosans. The antimicrobial effects of chitosans have been shown to reduce and prevent bad breath, dental plaque and caries[180,181]. Elmex and Chitodent commercialize toothpastes and Synedent markets oral rinses, all containing chitosans. In skin care products the moisturizing, film forming and thickening capacities of high molecular weight chitosan polymers together with water resistance and the previously mentioned antimicrobial and anti-infammatory capacities of all chitosans are highly valued[11]. Different companies commercialize chitosans for skin care applications such as Primex (ChitoClear®) or Clariant (Velsan®Soft and Vitipure®). Other companies like Almirall (Zeloglin®) uses chitosans as active ingredients in their dermatological formulations. The capacity to absorb humidity and the antibacterial properties are exploited in deodorants[11]. Louis Widmer commercializes a chitosan based aluminum salt free deodorant. When added to hair-care formulations, chitosans increase stability to high humidity and reduce electrostatic charges[11]. Icelandic Secret Volumizing Shampoo

(Swanson Ultra) and Ciclosan® (Almirall) are examples of shampoos commercialized claiming the benefits of chitosans indicated above.

4.1.1.5 Chitosans for agricultural applications

Chitosans are also very useful in agriculture as plant elicitors to improve plant health and crop yields and in the reduction of pesticide use. Chitosans have shown to have, antibacterial, antifungal, antiviral and insecticide effects[182]. An interesting example is the use of chitosans for the biological control of parasitic nematodes by attracting chitinolytic bacteria that destroy the eggs and cuticles of young nematodes[11]. At the same time, chitosans induce host defense responses in plants by activating many different mechanisms (lignification, ion flux variations, chitinase and glucanase activation, generation of active oxygen species, phytoalexin biosynthesis, etc.)[182]. Moreover, chitosans have been used to encapsulate biocides and thus reduce the amount of pesticides required by increasing their availability and controlling their release[183]. Furthermore, chitosans induce favorable changes in the metabolism of plants that lead to increase germination when applied as coating agent in seeds[182]. Additionally, chitosans capacity to form films and reduce stomatal opening results in antitranspirant activities that are effective to palliate the water stress effects of climate change[184]. Finally, the film forming capacity is also exploited in the post-harvest and preservation of fruits and vegetables[185]. AltosanCu, Altosan B/Zn (Altinco), Kaitosol® (Kaitosol) and ChitoPlant® (ChiPro) are examples of products containing chitosans that are sold as plant elicitors claiming benefits like higher yields and reduced crop diseases.

4.1.1.6 Other applications of chitosans

Chitosans can be applied in a wide range of other industries. The coating of foods with chitosans to prevent spoilage and the use of chitosan-based active packaging materials can be useful for preserving and extending the shelf life of foods. Moreover, chitosan is recognized as non-toxic and thus it can be consumed together with the preserved food. Combining chitosans with other film-forming biopolymers, such as plant and animal protein or polysaccharides, can lower the high costs of this application to the food industry[185–187].

In biochemistry, chitosans are useful for the immobilization of enzymes to make them more stable and to be able to reutilize them. Several enzymes from many applications such as biosensors, laundry detergents, chromatography or aromatization of beverages, amongst others, have been immobilized with chitosans[188–192]. The use of chitosans for the separation of proteins and other substances such as steroids by chromatography has also been reported[193,194].

Another application in which chitosans are commercially present is waste water treatment because of their ability to decrease energy consumption in reverse osmosis membranes by making them more permeable and their capacity to prevent microorganisms from attaching to the membranes[195,196]. Moreover, chitosans have the outstanding properties of binding and separating heavy metals, dyes and proteins and inducing flocculation to remove substances from solutions[197–199]. Indeed, due to the flocculating capacities, chitosans have also been shown to be useful for the flocculation of microalgal cultures to ease and reduce energy consumption in the harvesting process[200–204]. Chitosans have also been used to culture microalgae, which have been reported to be highly viable in chitosan based immobilized systems and achieve better efficiencies in the removal of nitrate and phosphates from water[205,206]. BioLog Heppe or Dungeness Environmental Solutions (Chitovan) are examples of companies offering chitosans for the wastewater treatment industry.

Chitosans are also used to improve the properties of some of the most used materials in our society. On one hand, papers are coated with chitosans to improve their printability, strength and water barrier properties[207,208]. On the other hand, chitosans in textiles are known to have antimicrobial properties, to reduce shrinkage, to improve dying by requiring less water and chemicals in the dying process and making dyes last longer[209]. Swicofil (Crabyon®) and Canepa and ItalDenim (Kitotex®) are examples of companies including chitosans in their fashion and home textile products.

Finally, chitosans blended with less expensive biodegradable materials can also be used as bioplastics for the manufacturing of 3D objects with high toughness and water resistance at competitive prices[210]. Chitosans have also been proposed as biodegradable plastics that could provide functionality (antimicrobial and antioxidant) to food packaging[211,212].The European Union FP7 n-CHITOPACK project is an example of initiatives in this direction.

4.1.2 Where could *Chlorella* chitosans be applied?

4.1.2.1 *Chlorella* chitosans are expensive to produce

As it is commented above, chitosans are used or could be used in a huge variety of applications making it complicated to decipher which could be the most appropriate ones for *Chlorella* chitosans. The first limitation of microalgal chitosans to reach the market is its high production costs. Although the process to extract chitosans from *Chlorella* is apparently simpler than the chitin extraction process from crustaceans, which afterwards needs to be converted to chitosans, the costs of the starting material are higher when using microalgae. Unlike in the case of crustaceans, the cell wall of *Chlorella* from which chitosans are extracted is currently not available as a waste, because the biomass is sold in the nutraceutical industry without separating the fiber rich cell wall. On top of it, the percentage of natural chitosans in *Chlorella* is much lower compared to the amount of chitins in the crustaceans used in the chitin extraction process nowadays. Nonetheless, the prices of chitosans as a raw material are very diverse and they may go from as little as 10$/Kg for applications in water treatment to over 20,000 $/kg for more quality demanding applications like biomedicine[20]. Microalgal chitosans are expected to be placed amongst the chitosans of best quality because of the advantages they offer (Table 18, Chapter 5). An important one is the fact that they are enzymatically produced, and thus should have a non-random pattern of acetylation, making these biopolymers more reproducible in terms of activity from batch to batch. Moreover, its vegetable origin makes it safer and reduces the possibility of inducing irritations derived from immunogenic proteins of crustacean origin. Hence, although microalgae are an expensive raw material for chitosans, the new characteristics it offers may open doors for niche high value applications.

4.1.2.2 Regulations and marketing favor *Chlorella* chitosans in some markets

The regulations on the use of chitosans play in favor of microalgal chitosans in front of commercially available chitosans mainly because of their non-animal origin and because of their enzymatic and natural origin. On one hand, from a regulatory perspective, the non-animal origin is especially valued in biomedical applications as it may considerably reduce the time to market of new medical

devices. According to the 17th rule of the European Medical Device Directive 93/42/EEC – OJ 169/ 12.7.93, all medical devices containing chitin or its derivatives are incorporated in class he III. Instead, medical devices incorporating microalgal chitosans entail lesser risks because of their vegetable origin and thus could also be classified as I, II or IIb. In practice, this means that microalgal chitosans are a good option for newly developed medical devices, especially the ones that have no equivalent existent product. These devices would require simpler and shorter clinical trials if they were not in class III because its safety can be demonstrated much more easily. On the other hand, the natural origin is clearly valued in the organic agriculture field. In this case, Commission Implementing Regulation (EU) No 354/2014 correcting Council Regulation (EC) No 889/2008 indicates that chitosans, unlike naturally available chitin, are not in the list of raw materials approved for organic agriculture due to the chemical process required for their production. Naturally available chitosans in microalgae should not have a problem to be approved for organic agriculture. It would be possible that in such a situation agriculture would be willing to pay higher prices for microalgal chitosans.

The vegetable and natural origins are also an advantage for *Chlorella*-derived chitosans over other chitosans from a marketing perspective. The industry in which this advantage could be more largely exploited is the cosmetics industry. Consumers prefer cosmetics containing ingredients of vegetable origin instead of animal origin, especially if these last ones entail more risk of allergic reactions. Moreover, certifications like Ecocert® or the Cosmos-standard recognizing the highest standards of organic, sustainable and natural cosmetics that are safe to be used, should be more easily obtained with microalgal chitosans. Due to the expected high prices of microalgal chitosans, niche applications such as high-end cosmetics could offer possibilities within this market.

4.1.2.3 Physicochemical characteristics of *Chlorella* chitosans and possible applications

By taking into account the high production costs and the advantages of *Chlorella* chitosans in front of conventional chitosans from regulatory and marketing perspectives, the markets that seem more suitable for *Chlorella*-derived chitosans seem to be:

- as a raw material in the biomedical and pharmaceutical markets,
- as an ingredient for the high-end cosmetics industry,
- and maybe as a final product in the organic agriculture business.

After this first segmentation, it is easier to choose relevant bioactivities to be studied that match with the physicochemical properties of microalgal chitosans characterized so far. Research has revealed that not all chitosans are good for every application, indeed, factors such as the structure, the Mw, the DA, the Ip, the PA, among others, are cardinal for the bioactivities of these polymers. Hence, the information available in the literature about the performance of chitosans that are physico-chemically similar to microalgal chitosans can be used to extrapolate properties to the newly discovered polymers. Chitosans obtained from *Chlorella* so far are characterized by an α conformation, a low Mw of approximately 20 to 40 kDa and a low DA ranging from 5 to 25% (Table 2).

According to the literature, chitosans with similar characteristics to *Chlorella* derived chitosans are especially good for antimicrobial activities, because low DAs are good for the electrostatic binding to membranes and the creation of a permeabilization effect that prevents the transport of solutes. Moreover, the low Mw is essential to reach and inhibit important mechanisms from the nucleus such as RNA and protein synthesis[10]. Nevertheless, it is important to highlight that the link between the characteristics of chitosans with its antimicrobial bioactivity might also be microbe specific[213].

Chlorella-derived chitosans also seem to be especially appropriate to have an antioxidant effect as it has been demonstrated that low DA and Mw improve the scavenging effects of free radicals and the metal ion chelating potency of chitosans[10].

Gene delivery is apparently another application in which the natural Mw and DA of *Chlorella* chitosans could be perfectly well suited to offer a good balance between DNA protection and intracellular unpackaging efficiency[10,214].

There are other interesting applications in which *Chlorella* chitosans do not completely fit like the three cases commented above but still should offer a good performance:

Low DA and low Mw chitosans, especially oligomers, which can also be obtained *in situ* and *in vivo* thanks to the capacity of lysozymes to degrade chitosans to these smaller molecules, are also desired characteristics for wound, scar, burn and ulcer healing applications[10,215].

In the preparation of drug delivery systems such as microspheres and nanocapsules, low Mw and low DA chitosans are more easily dissolved, making the process easier. Moreover low Mw chitosans accelerate the drug release rate and are better for the preparation of small size particles, the distribution of which is more easily controlled if the Ip of the polymer is also small. Nonetheless, in the case of drug delivery systems, higher Mw and lower DA chitosans are also requested to reduce hydrophobicity, increase loading capacity, increase drug release rate and obtain a more homogeneous morphology[10,83].

Chitosans from *Chlorella* should also be well suited to applications in tissue engineering as low Das and Mw correlate with lack of toxicity and good cell proliferation[216]. However, research indicates that a higher Mw is desired to obtain a better structure that is degraded less easily and to have a polymer with optimal mucoadhesive properties. Fortunately, the structure is an aspect that can be improved by mixing microalgal chitosans with other polymers[10]. The analgesic and haemostatic properties of chitosans would also by ideal if the chitosans from *Chlorella* had the same DA and a higher Mw[10].

When analysing the antitumor activity, the bioactivity is found in low Mw chitosans, especially oligomers, but no information was found about the effects of the DA[10,173].

Finally, the low molecular weight and degree of acetylation of the naturally produced chitosans in *Chlorella* also seem very appropriate to have high plant

elicitor activities, although optimal results have been obtained with even smaller chitosans[217].

Other possible applications were not commented either because they did not fit the physico-chemical properties found in *Chlorella* chitosans or because they require low priced chitosans or simply because no information linking physico-chemical properties and performance was found. Nonetheless, the information gathered thus far was enough to start testing some of the hypothetical activities that microalgal chitosans should have.

4.2 Results

4.2.1 Production of 1 gram of *Chlorella*-derived chitosans

Before starting the functional characterization of microalgal chitosans it was necessary to produce these chitosans in enough quantities for the tests. In chapter 2 of this thesis project (subsection 2.2.2.1), the first method to extract *Chlorella* derived chitosans was developed. The simple protocol, depicted in Figure 9, describes how to obtain chitosans from *Chlorella* biomass by following three main sequential steps: disrupting the cells, eliminating the proteins and solubilizing chitosans in an acidic solution. This methodology resulted to be very good to isolate a fraction in which chitosans were identified. However, the physical aspect of the samples denoted that something else, apart from chitosans was present. The color of the samples was dark green or even black, while the color of chitosans should be white or slightly beige or brown in the worst cases. Thus it was decided that, in order to obtain meaningful results from the analysis of the bioactivities of microalgal chitosans, it was necessary to work with purer samples. Hence, there was a need to develop an optimized version of the method to extract chitosans from *Chlorella*.

4.2.1.1 Optimization of the method to extract chitosans from *Chlorella*

The only one of the techniques utilized to study the characteristics of microalgal chitosans in Chapter 2 that could provide some information about the degree of purity of the extracted microalgal chitosans was ^{1}H-NMR. While the physical aspect of the polymers clearly reflected the presence of

contaminant substances, the ^{1}H-NMR spectra looked surprisingly clean; only small contaminant peaks were seen in some cases (Appendix 3). The explanation found for this phenomenon was that maybe the main contaminants were the abundant pigments present in *Chlorella*. Very low quantities of these pigments are required to completely stain a material, so probably the pigments present were under the threshold of detection of the technique. Hence, it was decided that it was necessary to use more solvents to eliminate those pigments. Knowing beforehand that chitosans have an outstanding capacity to adsorb dyes and pigments, it was decided that the decoloration step could be more useful if performed at the beginning, before free chitosans have the opportunity to interact and adsorb the pigments[199]. This added step would also have a positive impact on cell wall lipid removal at this point, maybe helping in the destruction of the cell wall structure and in the liberation of chitosans in the subsequent steps.

At the same time, other modifications in the extraction process were proposed to try to further improve the quality of *Chlorella* chitosans. Firstly, it was decided to avoid working with dried biomass as doing so probably obstructed the extraction of chitosans making them more tightly trapped together with other fibers from the cell wall. Another reason to work with wet biomass was that drying the biomass probably made it much harder to eliminate the pigments. All initial extractions had been performed with dried biomass in order to calculate the mass losses between steps. Thus it was decided that only a small aliquot would be dried to know the initial biomass content but the extraction should be performed using wet biomass. Secondly, the temperature of the incubations in the extraction process was changed to room temperature, as it was possible that high temperatures could harm the polymers and could make it more difficult to obtain a white product. In order to counteract the lower efficacy at low temperature, each extraction step had to be performed for a longer period of time. Hence, in the new extraction process the duration was extended, the temperature of all the steps was maintained at room temperature and the starting material was kept wet.

In Chapter 2, apart from characterizing the fraction containing chitosans, the left over fraction, which contained all the material that was not soluble in acetic acid, was also characterized (see subsection 2.2.3). A glycosidic linkage

analysis of the fraction indicated that chitins were highly present in this fraction. Moreover, FT-IR spectroscopy revealed that chitosans that had not yet been solubilized still remained in this fraction that was insoluble in acidic conditions. Therefore, from the characterization of the waste fraction two observations were made that could improve the process. On one hand, more attention had to be paid to the chitosan solubilization step. Probably better mixing and longer periods of time were required for a better solubilization. On the other hand, developing a process to chemically deacetylate the left over fraction and recover the insoluble chitin converted to chitosans could be useful to obtain more chitosans from the same biomass. This second chitosan would not have the advantage of being naturally produced but would still be of non-crustacean origin.

To sum up, several changes were proposed to the first version of the chitosan extraction process from microalgae in order to obtain chitosans of higher quality and a higher yield per unit of biomass (Figure 24). The scheme of the previous process can be found in Figure 9 (Chapter 2). Briefly, the main differences between the two processes are four:

1. the elimination of the drying steps before and after cell disruption,
2. the addition of a depigmentation and delipidation step after cell disruption,
3. the temperature reduction and increase in incubation time and
4. the addition of a chemical deacetylation step to recover more chitosans from the fraction that is insoluble in acetic acid.

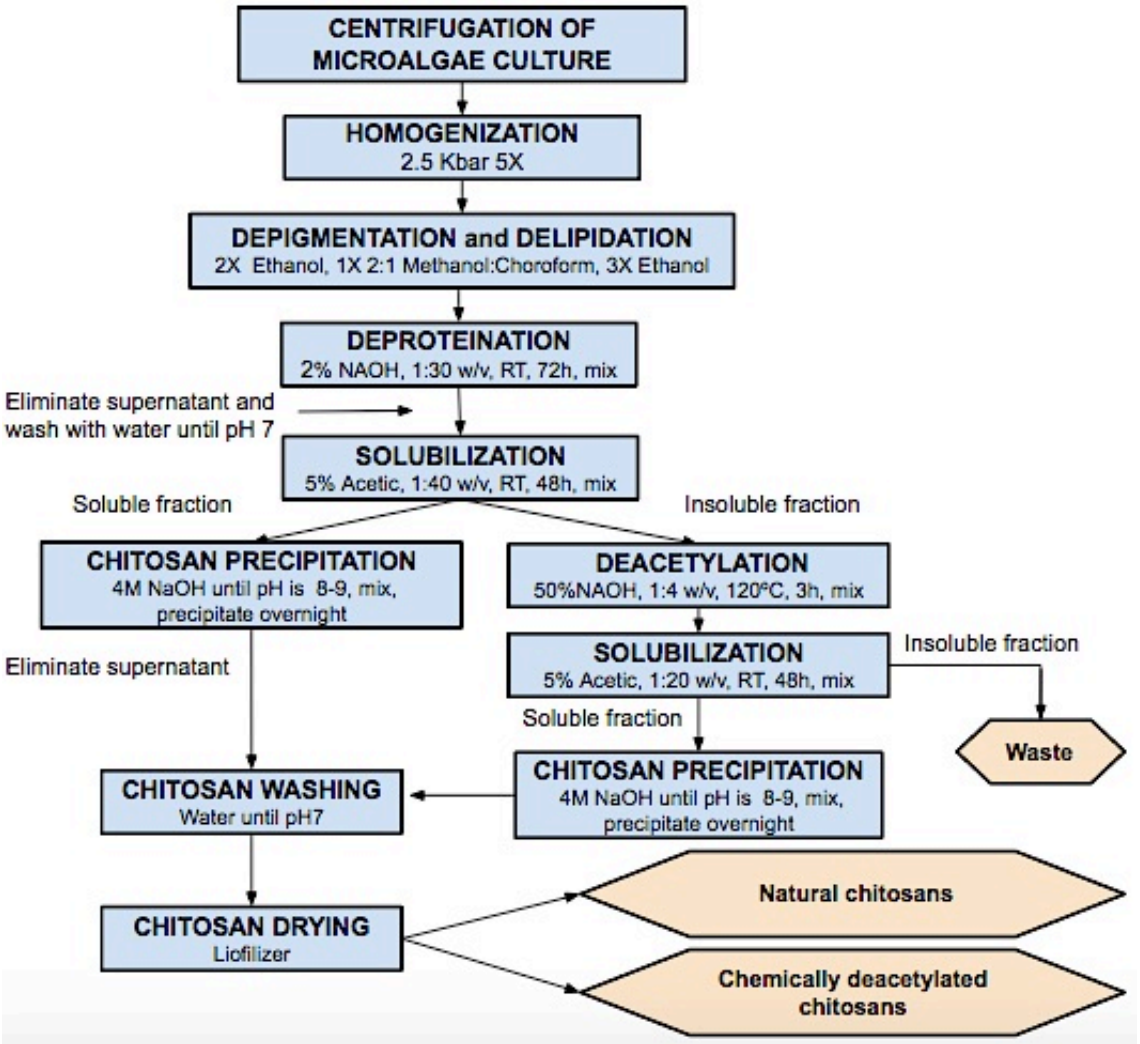

Figure 24 – Schematic representation of the microalgal chitosans extraction process optimized.

The results from this new process could be immediately observed in the physical appearance of the chitosans obtained by comparing them with chitosans previously obtained with the old extraction method. Figure 25 shows the positive effects on the final color of the new process in two dimensions. On the one hand, both the old and new extraction methods were performed on dry biomass of *C. sp. OP* and the new method clearly improved the coloration from black (Figure 25-A) to light brown (Figure 25-C). On the other hand, simply changing the state of the initial biomass from dry to liquid was enough to observe a change in the color of the chitosans from black (Figure 25-A) to dark beige (Figure 25-B). Nevertheless, where it is undeniable that the new process

has an outstanding positive effect is when it is applied to wet biomass. In this case the color of the resulting chitosans was completely white and sometimes slightly beige (Figure 25-D).

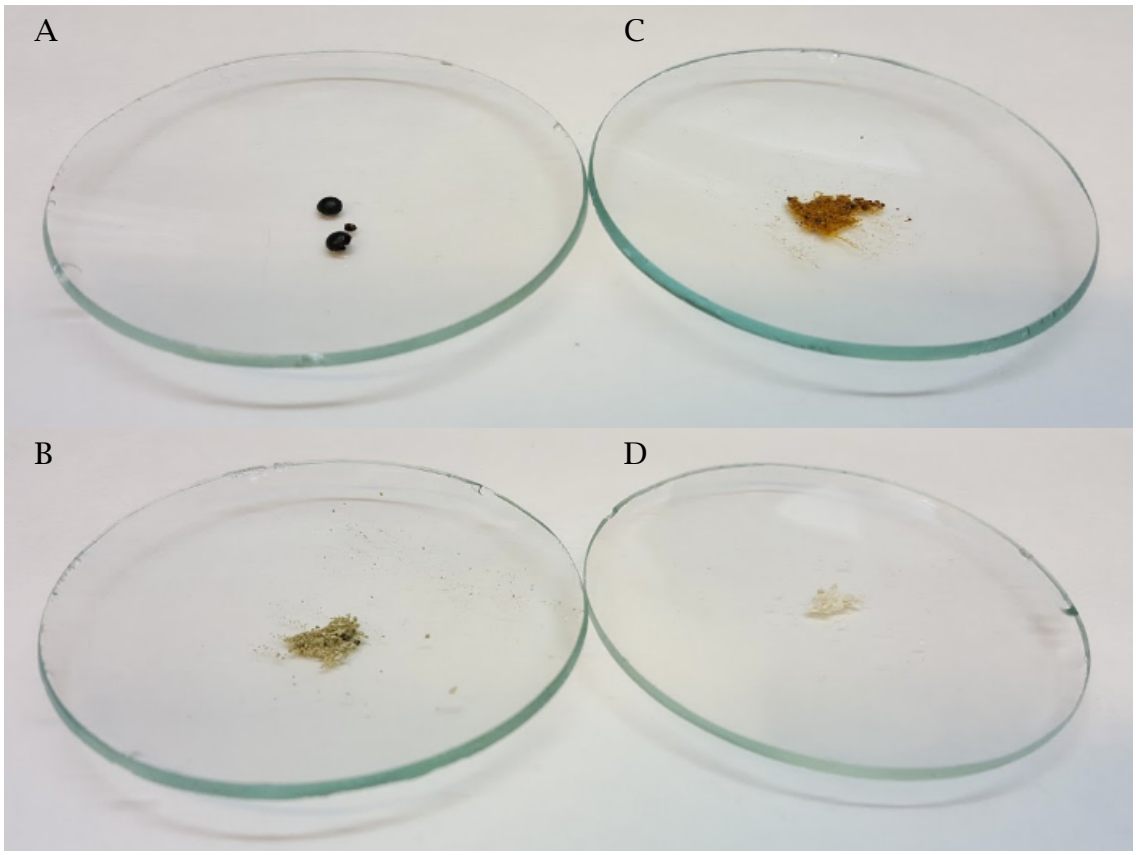

Figure 25 – Changes in the physical appearance of microalgae chitosans after the optimization of the extraction process.

C. and D. show the improved depigmentation capacity of the optimized extraction process over the initial procedure (A and B). The new method is efficient to obtain white *Chlorella* derived chitosans if wet biomass is used as a raw material (D). Results are less optimal if the starting biomass is dry.

The goal of the new extraction process was not only to improve the color of microalgal chitosans but also to obtain polymers of enhanced structural quality. It was expected that the new procedure, because it is smoother, would affect the polymers structure much less and thus would yield polymers of better quality. A good way of measuring the improvement in quality is by comparing the polidispersity indexes (Ip) of the old and new processes performed on the same biomass. However, because the determination of the Mw and Ip values requires considerable efforts, few of the extracted microalgal chitosans had been characterized, and, until that point, none of them had been

extracted following the new procedure. Therefore, no comparisons could be made to reach conclusions concerning the improved physical characteristics of the polymers.

The next subsection explains the application of the new extraction process to obtain 1 gram of *Chlorella* derived chitosans. As already commented, these chitosans will be used to perform the first functional characterization of microalgal chitosans. Hence, the goal is to characterize these chitosans before testing their bioactivities. Therefore, in the subsection below, a comparison of the characteristics of chitosans obtained with the two processes, the old and the new one, is made. 1 gram of material was considered to be enough to perform some bioactivity tests that require little quantities of chitosans. Two extractions were performed in order to have chitosans from two different species in stock to test bioactivities. The two species chosen were *C. sp. OP* and *C. sp. GAT-10*.

4.2.1.2 Production of 1 gram of chitosans from *C. sp. OP*

With the aim of simplifying the process, the first extraction at 1-gram scale was performed using biomass grown in open-ponds. *C. sp. OP*. was readily available and chitosans had already been found in its cell walls so it was ideal for testing the new process at this new scale in a quick and straightforward way. Prior to attempting the extraction, ten independent extractions of chitosans were performed using this biomass to determine the expected yield, which was found to average 0.43% of the dry weight. The 1-gram scale extraction was performed with 500g of raw material so slightly over 2g of naturally produced chitosans were expected. However, at the end of the extraction process, only 0.547g of natural chitosans (0.11% of the dry weight) and 1.178g of chemically deacetylated chitosans (0.24% of the dry weight) could be collected from *C. sp. OP*. The names that were given to the natural chitosans and the chemically deacetylated chitosans obtained in this batch were A10.3 and A10.D, respectively.

The low yield can be mostly attributed to miscalculations regarding the percentage of dry biomass that the centrifuge could retain in its pellet. Based on other species, the percentage of dry biomass in the wet pellet recovered was expected to be around 30%. However, in the case of *C. sp. OP* it was only 12% dry biomass. Hence, approximately 2/3 of the raw material had not been

retained as pellet and had been wrongly discarded after homogenization. Moreover, another explanation for the low yield is that the process of solubilization of natural chitosans was not very efficient as it can be corroborated by the fact that the proportions of chemically deacetylated chitosans versus natural chitosans were too high in favor of the first ones. The reason for the low solubilization was that the cells had not been as well disrupted as they normally do when the experiments are performed in lower volumes, something that was corroborated under the microscope.

The physical appearance of chitosans A10.3 and A10.D can be seen in Figure 26. As it shows, the color of both polymers is rather brown. The main reason for this is the fact that dry biomass was utilized and, according to previous results, this clearly has an effect on the ability of the process to reduce pigmentation.

As table 13 reflects, the most relevant structural characteristics of chitosans A10.3 and A10.D were determined (DA, Mw, and Ip). The DA values of both samples were 5% for A10.3 and 1.7% for A10.D; the chemical deacetylation reaction practically deacetylated all the monomers. The Mw of the natural chitosans (18.8 kDA) was the lowest of all molecular weights determined by then, as all *Chlorella*-derived chitosans up to that moment had been in the 20 – 35 kDA range. What is striking is the difference in Mw between this sample and the chemically deacetylated one, which almost doubles it (32.4 kDA). Apparently, only chitosans of the smallest molecular weight were solubilized, while the larger ones stayed in the pellet.

The best news from the characterization of these chitosans came from their Ip value, which only was 1.4 (Table 13). This is a considerable improvement with respect to the extraction of chitosans from the same biomass following the first process, as it had an Ip of 1.8 (Table 1). In fact, 1.6 had been the best Ip obtained when using the first chitosan extraction process and it was obtained from a laboratory culture under fully controlled conditions, unlike *C. sp. OP,* which was bought dry elsewhere. Even the chemically deacetylated chitosans, although they had gone through a harsh process, still had a better Ip (1.5) in comparison to all the samples extracted with the anterior protocol. With these results, it can be asserted that the new extraction protocol yields chitosans with improved physicochemical characteristics compared to the initial process.

4.2.1.3 Production of 1 gram of chitosans from *C. sp. GAT-10*

The second attempt to obtain chitosans from *Chlorella* at the 1-gram scale was performed using fresh biomass. In this case, species *C. sp. GAT-10* was used as raw material. This strain, as it can be observed in Table 2, had also been proven to contain chitosans. Table 2 also shows that there are several other strains that in theory contain a lot more chitosans, so at first glance, choosing strain GAT-10 might not seem like the best possible option. However the growth of strain GAT-10 in a parallel project was carefully optimized to make it the best *Chlorella* of the whole library in terms of growth. The key of this particular strain and its ability to grow so fast is that it underwent a growth optimization process to habituate to a mixotrophic regime. Thus, even though the percentage of chitosans it contained was low in comparison to other strains like *C. sp GAT-7* and *C. sacharophila 211/9A,* it was of accumulating so much more biomass that its productivity was superior. To be more concrete, *GAT-7* and *211/9A,* the two strains with a higher concentration of chitosans per liter according to the first screening, accumulated 16.5 and 17.2 mg/L respectively. Instead, thanks to the growth optimization, strain *GAT-10* was now capable of producing up to 33.9 mg/L. This was determined based on eight independent extraction tests performed from laboratory scale mixotrophic cultures of strain *1230.* From these tests, the average content of natural chitosans was determined to be 0.38% of dry weight.

In order to perform the second chitosan extraction at a scale of 1 gram, 279g of dry biomass of *C. sp. GAT-10* were accumulated after culturing it mixotrophically in 15L photobioreactors in a semi-continuous fashion. In this case, the biomass was kept wet, only some aliquots were dried to determine the dry weight. With the amount of biomass accumulated and knowing that approximately 0.38% of it accounted for chitosans, it was expected that exactly 1 gram of chitosans should be collected. Fortunately, the expected yield was reached and surpassed. After following the updated version of the chitosans extraction protocol, 1.275 grams of natural chitosans were extracted (0.455% of the dry weight biomass). Moreover, from the chemical deacetylation of the cell wall residues that had not dissolved in acetic acid, 1.640 grams of chitosans were collected (0.586% of dry weight biomass). Therefore, in total 1.041% of the biomass weight was collected in the form of chitosans. The large amount of

chitosans found after the chemical deacetylation step is an indication that an important fraction of the natural chitosans available had not been efficiently captured. As in the case of batch A10.3, it was caused by the difficulties encountered to completely disrupt *Chlorella* at these larger volumes. The name that was given to this batch of chitosans from strain *GAT-10* was A11. Thus, the natural chitosans sample from this batch was named A11 and the chemically deacetylated chitosans A11.D.

As figure 27 shows, the color of the two chitosans obtained from A11 is considerably lighter in comparison to chitosans A10.3 and A10.D. While the same process was applied, the only difference was the state of the biomass, as in the case of batch A11, it was not dried at any point. Hence, these results reconfirmed that drying the biomass has a clear negative effect on the elimination of pigments. It is also interesting to highlight that while the chemically deacetyated sample (A11.D) is light beige or almost white, the natural sample (A11) is considerably darker (light brown). This reveals that the pigments were not completely eliminated before the precipitation of the natural chitosans and thus got firmly attached to them. Then, the harsh chemical deacetylation step finished depigmenting the sample.

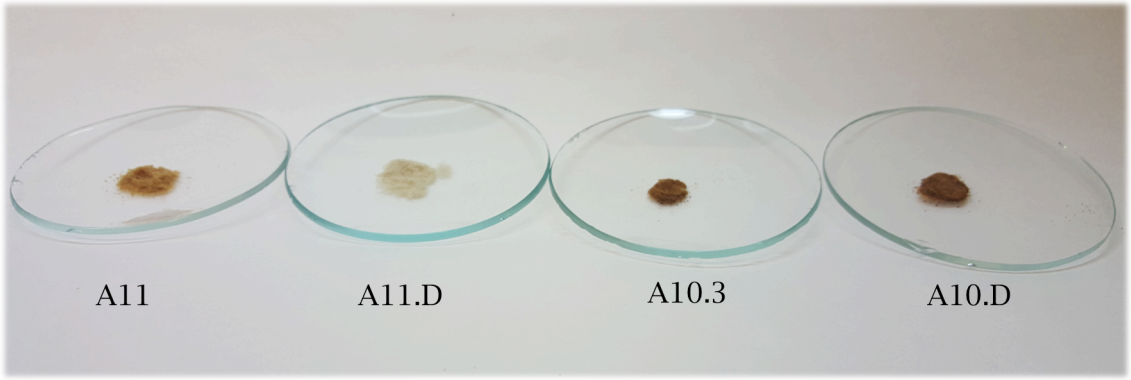

Figure 26 – Physical appearance of the four microalgal chitosans obtained from the extraction process at 1 g. scale.

Namely: A11: Natural chitosans from *C. sp, GAT-10.* A11.D: Chemically deacetylated chitosans from *C. sp, GAT-10.* A10.3: Natural chitosans from *C. sp, OP* A10.D*:* Chemically deacetylated chitosans from *C. sp, OP*

The DA% of the natural chitosans (10.3%) were in the expected values compared to the previous milligram scale extractions; all showing DA% in the ranges of 7 to 14 %. Outstandingly, the Mws were the highest determined so far, especially in the case of the chemically deacetylated fraction, which almost tripled the highest size obtained until that moment (Tables 2 and 13). As it happened with fractions A10.3 and A10.D, it was observed that the size of the chemically deacetylated chitosans was much higher. This larger size could be the reason why these chitosans do not dissolve as well.

With respect to the Ip parameter, the values of A11 and A11.D were larger than those of A10.3 and A10.D, something that initially was not expected due to the higher quality of the biomass. The larger difference in sizes between chitosans A11 and A11.D compared to the difference in sizes between A10.3 and A10.D probably contributed to the increased Ip values obtained (Table 13). Hence the quality of the starting material is not everything that counts to obtain chitosans with low Ip values, it seems that there are also variations between strains and growth conditions.

Chitosans	Strain	Deacetylation	Yield	Color	DA (%)	Mw (kDa)	Ip
A10.3	*OP*	Natural	0.11%	Light Brown	5	18.81(±8.5%)	1.4
A10.D	*OP*	Chemical	0.24%	Light Beige	1.50%	32.4 (±8.3%)	1.5
A11	*GAT-10*	Natural	0.46%	Brown	10.30%	39.0 (±7%)	1.7
A11.D	*GAT-10*	Chemical	0.59%	Brown	2.60%	90.6 (±11%)	1.8

Table 13 – Structural characteristics of the four microalgal chitosans obtained from the extraction process at 1-gram scale

4.2.2 Properties of *Chlorella*-derived Chitosans

Thanks to the optimization and scale-up of the extraction process, hundreds of milligrams of different microalgal chitosans were produced, representing enough material to perform an initial functional characterization of these newly discovered polymers. Moreover, the new chitosans were purer, had a lighter color and characteristics that are comparable to commercially available chitosans of the highest quality. Thus, it made sense to start deciphering their capabilities.

As reviewed at the beginning of this chapter, plenty of different bioactivities are attributed to chitosans. Based upon the characteristics of microalgal chitosans, some of these bioactivities seem to be more probably found in them. Taking into account the quantities of material available for tests and in accordance to the conclusions drawn from the analysis of the possible applications of *Chlorella*-derived chitosans, broad-spectrum bioactivities such as antimicrobial, wound-healing and nanocapsules-forming properties were studied.

4.2.2.1 Antimicrobial properties

One of the most studied, useful and unique properties of chitosans is their antimicrobial activity[12,218]. Many investigators have shown that chitosans have a very broad antimicrobial spectrum, affecting bacteria, fungi and viruses[218,219]. The study of this bioactivity is of great interest because it is a trait that is desirable in many of the possible applications of chitosans, including biomedicine, cosmetics and agriculture. Moreover, the growing demand for a more rational use of chemicals and more natural sources of preservatives in the food and cosmetics industry has also fostered the research on this property of chitosans[12]. The antimicrobial activity is species and strain specific, and is affected by the type of growth media utilized *in vitro*[15,213,218]

Although the antimicrobial activity of chitosans is well documented, its mode of action is complex, involving diverse and random events that might ultimately lead to cell growth inhibition or death[15]. Three different mechanisms have been proposed. First, the interaction of positively charged chitosans with negatively charged cell membrane components (like phospholipids or proteins) is the most accepted inhibitory mechanism. This electrostatic interaction results in extensive cell surface alterations affecting the permeability and leading to internal osmotic imbalances. Since this mechanism is based on electrostatic interactions, the more freely available cationic amines, i.e. the lower the DA, the higher the antimicrobial activity is expected. In the same way, the more negatively charged the surface of the microbial cell wall is, the larger inhibitory effect should be noted, although studies demonstrating the contrary have also been found[218]. These contradictions lead to the research of other modus operandi such as the second proposed mechanism, which involves the binding of chitosans to DNA, leading to inhibition of mRNA and

protein synthesis[218]. Nevertheless, many authors suggest that the possibility that chitosans would be able to pass through the cell wall of microorganisms is rather unlikely[15]. In any case it is clear that if this mechanism is to be effective at causing growth inhibition, low or very low Mw chitosans are required[10]. A third mechanism of action has been described, involving the chelating activity of chitosans; the polymers selectively bind to trace metals inhibiting their use and hence inhibiting growth and the production of toxins[12].

Thus, according to the literature, the characteristics of microalgal chitosans (low DA and low Mw) should be especially well suited to affect the growth of microorganisms at least in two different ways; the intake of solutes and disruption of osmotic balances on one side and the inhibition of important mechanisms occurring in the nucleus such as RNA and protein synthesis on the other side[10]. Moreover a third mechanism, which involves the chelation of metal ions, could also benefit from the microalgal chitosan properties, as apparently low DAs are better for the removal of metal ions[10]. Moreover, many researchers have highlighted that in order to have antimicrobial properties, chitosans should have a low molecular weight that is not lower than 10kD[15], making microalgal chitosans even more suitable to have this property.

With the aim of confirming the antimicrobial properties of microalgal chitosans, a test taking into account the specific solubility problems of chitosans was developed with *E. coli*. After the test had showed to work correctly in a reproducible fashion, it was extended to the common pathogenic bacteria *S. aureus* and *P. acnes*. These two species allow widening the screening on the efficacy of microalgal chitosans to gram-positive and anaerobically grown bacteria. At the same time, *S. aureus* and *P. acnes* are interesting targets for biomedical, pharmaceutical and cosmetics applications.

4.2.2.1.1 Inhibitory properties of microalgal chitosans against Escherichia coli

E. coli is a rod shaped gram-negative bacteria commonly found in the lower intestine of warm-blooded organisms. While most strains are harmless, some serotypes can be pathogenic mainly by causing food poisoning to their hosts. However, the main reason why this bacteria was chosen was because it is an easy and fast growing model microorganism, and thus it is the

ideal organism to set up a test to reproducibly explore the antimicrobial properties of microalgal chitosans.

Several reports can be found in the literature showing the antibacterial efficacy of chitosans against *E. coli*. However, when comparing these reports it is absolutely impossible to have a concrete idea of the concentration of chitosans required to inhibit its growth as minimum inhibitory concentration (MIC) values range from as little as 20 ppm to up to 1300 ppm[218,220]. This is because, as already commented, the antimicrobial activity depends on many factors such as the strain and the physiological phase, the growth media and its ionic strength and pH or, of course, the characteristics of the chitosans utilized. Therefore, it is not very useful to provide an absolute MIC value per strain as it is completely dependent on the experimental settings and it cannot be compared to previous results obtained elsewhere[12]. For this reason, it was decided that it would be more interesting to make a benchmark comparison with a commercially available chitosan of similar characteristics.

One of the most commonly used methods to test the antimicrobial activity of molecules is the broth microdilution assay. It was the method of choice to test the chitosans derived from *Chlorella* mainly because it allows the quantification of the inhibition and the generation of MIC values and minimum bactericidal or fungicidal concentration values (MBC or MFC). This is an advantage over the disk diffusion assay, which is the other commonly used method and it is rather qualitative. Moreover, the broth microdilution assay, because it is a miniaturized assay, requires a low amount of reagents and little space[221].

The broth microdilution method to test antimicrobial activity briefly consists on the preparation of a previously determined dilution of the antimicrobial substance in a suitable growth medium dispensed in 96 well plates. Then, the wells are inoculated with a standardized microorganism suspension and incubated for a specific period of time at which the growth of the culture (turbidity) is measured. The lowest concentration of the molecule being tested that prevents growth represents the MIC[221]. If more concrete results are required, viable counts can be obtained from growing aliquots of the cultures from each well on agar plates. This action allows the determination of the MBC

and MFC values, defined as the lowest concentration reducing the inoculum by 99.9% within 24h[15].

As it can be observed in the Materials and Methods section 4.4.3 of this chapter, a modification had to be introduced in the standard broth microdilution assay. The dilution of the chitosans and the posterior growth of the bacteria had to be performed in media diluted ten-fold because otherwise each time the chitosans were added to the culture, substances precipitated from the media. This phenomenon occurred not only in the case of *Chlorella* chitosans but also in the case of conventional chitosans of crustacean origin. By diluting the nutrient rich media, the precipitation was avoided.

The *Chlorella*-derived chitosans used in the antimicrobial assay against *E. coli* were *C.sp OP* A10.3. The extraction and characterization of these chitosans is explained in subsection 4.2.1.2. The Mw and DA of these chitosans were 18.81 kDA and 5%, with an Ip of only 1.4. As a control, the commercially available chitosans from Sigma Aldrich with more similar characteristics to A10.3 were used (low molecular weight and 15-25% DA). A control with a known antibiotic assay was also included (Ampicillin). The bacterial growth after incubation with the chitosans was not monitored by turbidity; instead at each time point an aliquot was inoculated on agar plates to obtain the colony forming units (CFU).

Results are presented in Figure 27, where the inhibitory power of the three substances tested (conventional crustacean chitosans, *Chlorella*-derived chitosans and ampicillin) is compared. The charts are the result of a duplicate assay in which the concentrations were adjusted to obtain clear MBC values. These fine adjustments could be done thanks to previous assays (results not shown). Hence, although only duplicates of the experiment were performed, it can be said that the results are reproducible by looking at the previous experiments. It should be noted that the maximum number of colony counts that could be detected was $1x10^8$, over that number the growth was confluent and uncountable, so all confluent plates were recorded as if they had $1x10^8$ CFU/mL, although they probably had more.

The first thing to be noted is that the ten-fold diluted media still contained enough nutrients to allow *E. coli* to grow, thus confirming that the method works correctly. The MIC values, i.e. the minimum chitosan concentration preventing growth of *E. coli,* are clearly lower for microalgal chitosans

compared to crustacean chitosans, although this better performance fluctuated in the course of time. At time 4 hours, all chitosan solutions clearly had an effect on the growth of *E. coli* as it was almost completely inhibited in all cases. It could be a possibility that the sudden change of culture media to a new one diluted ten-fold affected the bacteria at the beginning, however, Figure 27 shows that the control without chitosans or ampicillin was able to thrive in this new media from the beginning. After 8 hours the MIC raised and a difference could be already observed between the two chitosans. While the crustacean chitosans needed to be between 50 and 100 ppm to keep the CFU counts lower than at time 0, the chitosans from *Chlorella* required between 20 and 30 ppm. Finally, at 24 hours, the MIC again raised so that in the case of the chitosans from Sigma Aldrich between 200 and 400 ppm were needed to maintain the inoculum at equal or lower bacterial levels compared to the start. Instead, at 24 hours, the MIC of the microalgal chitosans was only between 20 and 30 ppm. The same behavior was observed in the case of the ampicillin, as the MIC was between 0 and 6.25 ppm at 4 hours and then it raised to somewhere between 6.25 and 12.25 ppm at 8 and 24 hours.

The MBC values, indicate the minimal concentration of chitosans required to kill 99.9% of the initial inoculum in 24h. However, at the time this experiment was performed, this definition was misinterpreted. It was understood that the MBC indicated a reduction of 99.9% with respect to the normal growth of the microorganisms, represented by the positive control containing no antimicrobial substances. As a result of this misinterpretation, the design of the experiment did not allow for enough resolution to detect the MBC values; the maximum reduction of the inoculum that could be detected was between 99.73% and 99.82% depending on the initial cell count on each case. Hence, for the sake of comparing the results, an adjusted MBC value (aMBC) was defined: the minimal concentration of chitosans required to kill 99.7% of the inoculum in 24 hours. Thanks to this slight modification, very clear conclusions can be reached, as the aMBC values of microalgal chitosans for *E. coli* were one order of magnitude lower than the aMBC values of chitosans from crustaceans. While the aMBC of the natural chitosans was only between 40 and 30 ppm, halfway 400 and 200 ppm of the chemically deacetylated chitosans from crustaceans were needed to see the same effects. In other words, 400ppm of chitosans of animal origin were required to have no CFUs in the agar plate, representing an

inhibition of at least 99.81% of the initial inoculum. Instead, with the chitosans of vegetable origin, no CFUs appeared on the agar plate at 24h using only 40ppm, representing an inhibition of at least 99.76% of the initial inoculum. Surprisingly, the amount of *Chlorella*-derived chitosans needed to completely reduce the growth of *E. coli* at 24h is not very far away from the amount of ampicillin required for the same purpose (between 12.5 and 25 ppm). In this case, with 25 ppm of ampicillin, no colonies appeared on the agar plate, thus inhibiting the culture at least 99.78%. If the initial interpretation of the MBC is applied, i.e. the inhibited cultures are compared to the control containing no antimicrobials at 24 hours, 400ppm of S.A. 448869, 40ppm of A10.3 and 25ppm of ampicillin were capable of reducing the growth of *E. coli* at least 99.95%. The MIC and aMBC values for each antimicrobial substance are shown in Table 14.

In the overall, the results obtained, when compared to the literature, situate the chitosans from *Chlorella* amongst the most inhibitory against *E. coli* ever tested[218]. A small change should be included in the test in the future, which is to increase its resolution to $1x10^9$ CFU/mL, so that it can be used to determine the MBC values.

	MIC (ppm)			aMBC (ppm)
	4h	8h	24h	24h
S.A. 448869	<50	<100	<400	<400
C.sp OP A10.3	<20	<20	<30	<40
Ampicillin	<6.25	<12.5	<12.5	<25

Table 14 - Susceptibility of *E. coli* to *Chlorella* chitosans compared to crustacean chitosans and ampicillin.

The MBC values were adjusted so that instead of indicating a reduction of growth of at least 99.9%, they indicate a reduction of 99.7%. (aMBC).

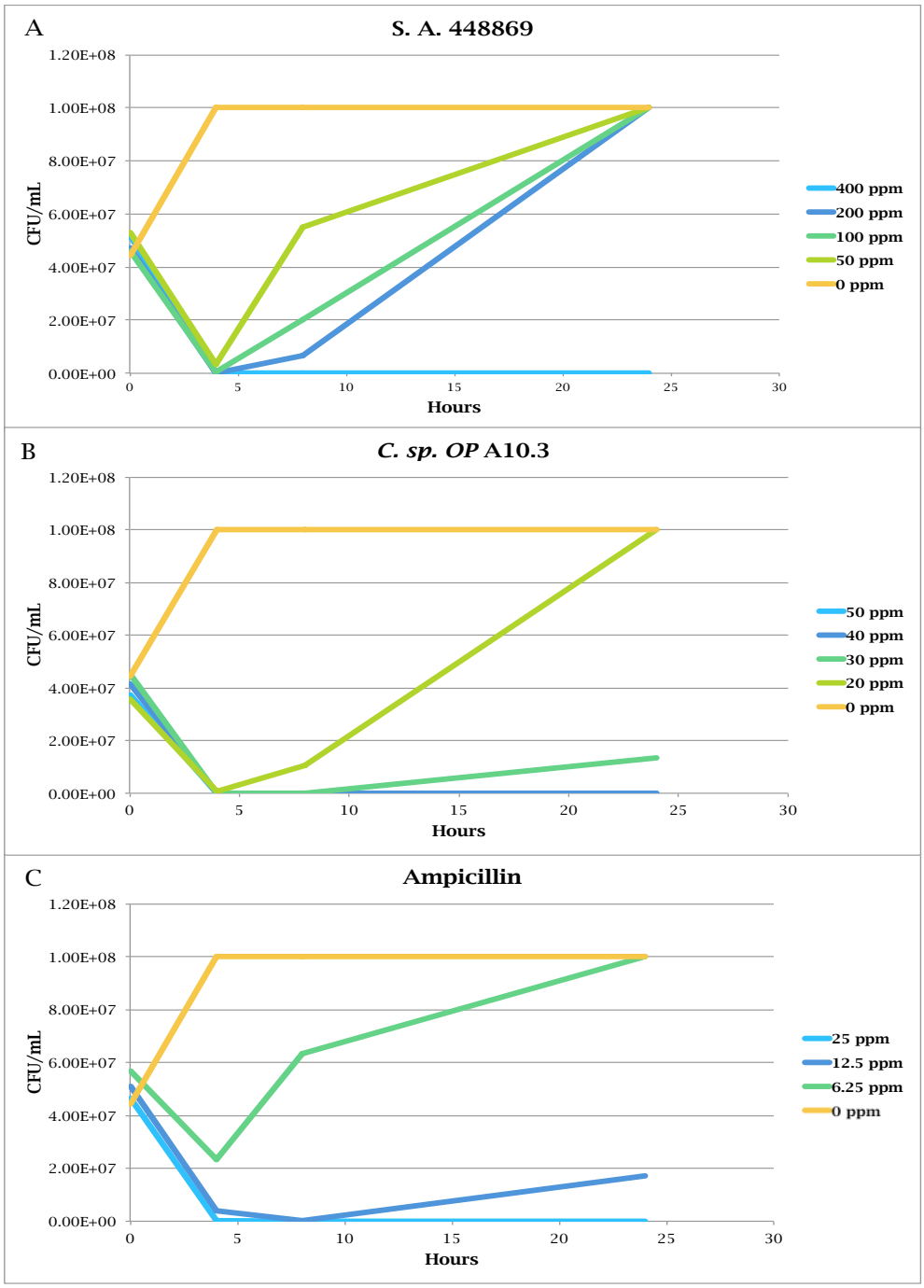

Figure 27 - Inhibitory effects of microalgal chitosans A10.3 over *E. coli* growth compared to crustacean chitosans and ampicillin.

The inhibitory effect of three different compounds is compared, namely: A. Conventional chitosans of crustacean origin (Sigma Aldrich ref.448869) B. Chitosans obtained from *Chlorella sp. OP* (Batch A10.3) C. Ampicillin, a known effective antibiotic against *E. coli*. The test was limited to counting $1x10^8$ CFU/mL, so any further growth could not be monitored and was assigned the value $1x10^8$ CFU/mL. Also, it is important to highlight that the colors of the lines represent different concentrations of antimicrobial substances in each graph. The charts are the results of two technical replicates.

4.2.2.1.2 Inhibitory properties of microalgal chitosans against Staphylococcus aureus

In the previous section it is proven that microalgal chitosans are effective in the inhibition of the gram-negative bacterium *E. coli.* In order to have a better idea of the antimicrobial spectrum of action of these newly discovered polymers, *S. aureus*, a gram-positive bacterium that is easy to grow in the laboratory, was chosen.

Although *S. aureus* is commonly found in the skin microbiome, it is regarded as a pathogenic skin colonizing bacteria. The skin of more than 90% of patients affected by atopic dermatitis (eczema) is colonized by *S. aureus,* in comparison with less than 5% of healthy individuals[222]. Atopic dermatitis, characterized by skin inflammation and itchiness is a chronic skin disorder that affects more than 15% of US children and 2% of adults that has doubled or tripled in industrialized countries over the past three decades[222]. Moreover, atopic dermatitis cause patients to scratch themselves, a behavior that worsens the symptoms and increases the risk of developing additional skin infections. Other skin disorders in which *S. aureus* is involved include pimples, impetigo, boils and carbuncles, cellulitis, folliculitis, scalded skin syndrome and abscesses[220]. Reducing the bacterial load have been demonstrated as an effective strategy to reduce clinical severity of these skin infections and the most common treatments include topical applications[222]. Thus, discovering new antimicrobial molecules that can be used topically to inhibit the growth of *S. aureus* is of great interest. This is especially relevant in the case of *S. aureus,* which is notorious for its ability to become resistant to antibiotics. Indeed, the troubles caused by multi-drug resistant genotypes of *S. aureus*, like the methicillin resistant strains (MRSA), have been increasing to reach global epidemic proportions[223,224]. Chitosans are of particular interest as they can also be used to impregnate or coat materials to prevent *S. aureus* colonization and thus prevent epidemia[209,225].

Several reports presenting the use of chitosans to inhibit the growth of *S. aureus* can be found[15,209,213,218,220]. As in the case of *E. coli,* it is impossible to have a more or less precise idea of the amount of chitosans needed to inhibit its growth because MIC values range from as little as 20 ppm to as much as 1200 ppm[218,220]. This is a consequence of the many factors that have an incidence in

the inhibitory power of chitosans that have not been standardized to perform the antimicrobial assays, such as the strain utilized, the growth conditions and the characteristics of the chitosans. Therefore, as with *E. coli,* the experiment to check the antimicrobial activity of *Chlorella* chitosans against *S. aureus* was designed to compare the inhibition power of microalgal chitosans against commercially available chitosans produced by conventional means that had previously been proven efficient in the literature[15]. Hence, the ability of three different chitosans to inhibit the growth of *S. aureus* was assayed. Two of these chitosans came from *Chlorella sp. OP*: on one hand the naturally available chitosans extracted by solubilization in acidic conditions (A10.3; DA: 5%, Mw: 18.8 kDA and Ip: 1.4) and, on the other hand, the chitosans obtained from the chemical deacetylation of the residual fraction (A10.D; DA: 1.7%, Mw: 32.4 kDA and Ip: 1.5). The third chitosans used came from crustaceans, (Sigma Aldrich ref. 448869) and were selected for being the ones with more similarities to the two *Chlorella* chitosans (Low Mw and DA 15-25%). The broth microdilution method that was successfully set up for *E. coli* was also applied in this case.

In Figure 28 it can be observed how different concentrations of the three chitosans tested inhibit the growth of *S. aureus* at different time points in a period of 24h. It should be noted that the maximum number of colony counts that could be detected was $5x10^9$, over that number the growth was confluent and uncountable, so all confluent plates were recorded as $5x10^9$ CFU/mL. All cultures at time zero hours were confluent and it can be observed that after the addition of chitosans this confluence was eliminated in only four hours. The MIC value determined with this experiment at 4, 8 and 28 hours for the crustacean chitosans was 100 ppm, and for the naturally produced chitosans *C. sp. OP* A10.3 and the chemically deacetylated chitosans *C. sp. OP* A10.D it was 25 ppm. However, it must be said that all the concentrations of all the chitosans tested were inhibitory to *S. aureus,* so less concentrated solutions of chitosans should have been tested in order to detect the exact MIC values for each time point because it is likely that the values are lower than the ones obtained. At 24 hours, the growth of the *S. aureus* cultures containing 100 ppm of crustacean chitosans and the growth of the *S. aureus* cultures containing 25 ppm of chemically deacetylated microalgal chitosans began to point upwards. So, it is likely that the MIC values at 24 hours for these two

chitosans are not much lower than the values given. This is not the case for the natural *Chlorella* chitosans.

Precise MBC values cannot be given with this experiment because the definition of the MBC requires knowing the initial cell count, something that could not be determined because all cultures were confluent. Nevertheless, if the maximum number of colonies that could be counted ($5x10^9$ CFU/mL) was considered as the cell count at time zero for all the cultures, an approximate MBC value can be obtained. Along these lines, the concentrations of chitosans capable of reducing the bacterial load 99.9% for the crustacean chitosans was exactly 200 ppm. Contradictorily, although it cannot be observed in the graph, 400ppm of these same chitosans inhibited only 99.4% of the culture. So, 400 ppm could not be considered inside the MBC definition. This incongruence is easily explained by human error as in such high dilutions tiny deviations can result in important errors. In the case of the *Chlorella* chitosans the MBC values are much clear; at 100 ppm no colonies were capable of surviving at 24 hours, so at least 99.9% mortality was induced. In fact, the growth at 50 ppm of natural and chemically deacetylated microalgal chitosans was inhibited 98.9% and 99.3% respectively, thus indicating that the MBC is probably very close to 50 ppm. The values of inhibition of *S. aureus* growth by chitosans 448869, A10.3 and A10.D are captured in Table 15.

In all, it can be said that the experiment performed has allowed determining in a semi-quantitative fashion the antimicrobial properties of one crustacean and two microalgal chitosans against *S. aureus.* Results indicate that the two chitosans of vegetable origin are at least four times more effective than the chitosans of animal origin. According to the literature, in the same way as in *E. coli,* the values presented here place *Chlorella* derived chitosans amongst the most effective chitosans against *S. aureus* ever tested[218]. In order to improve the precision of this experiment, the initial cell count should be better determined. Moreover, a higher resolution is also necessary, as, with the current experimental design, the maximum inhibition that can be detected is 99.9%. So at least a further 1/10 dilution would be necessary in order to have clearer values and better determine the MBC.

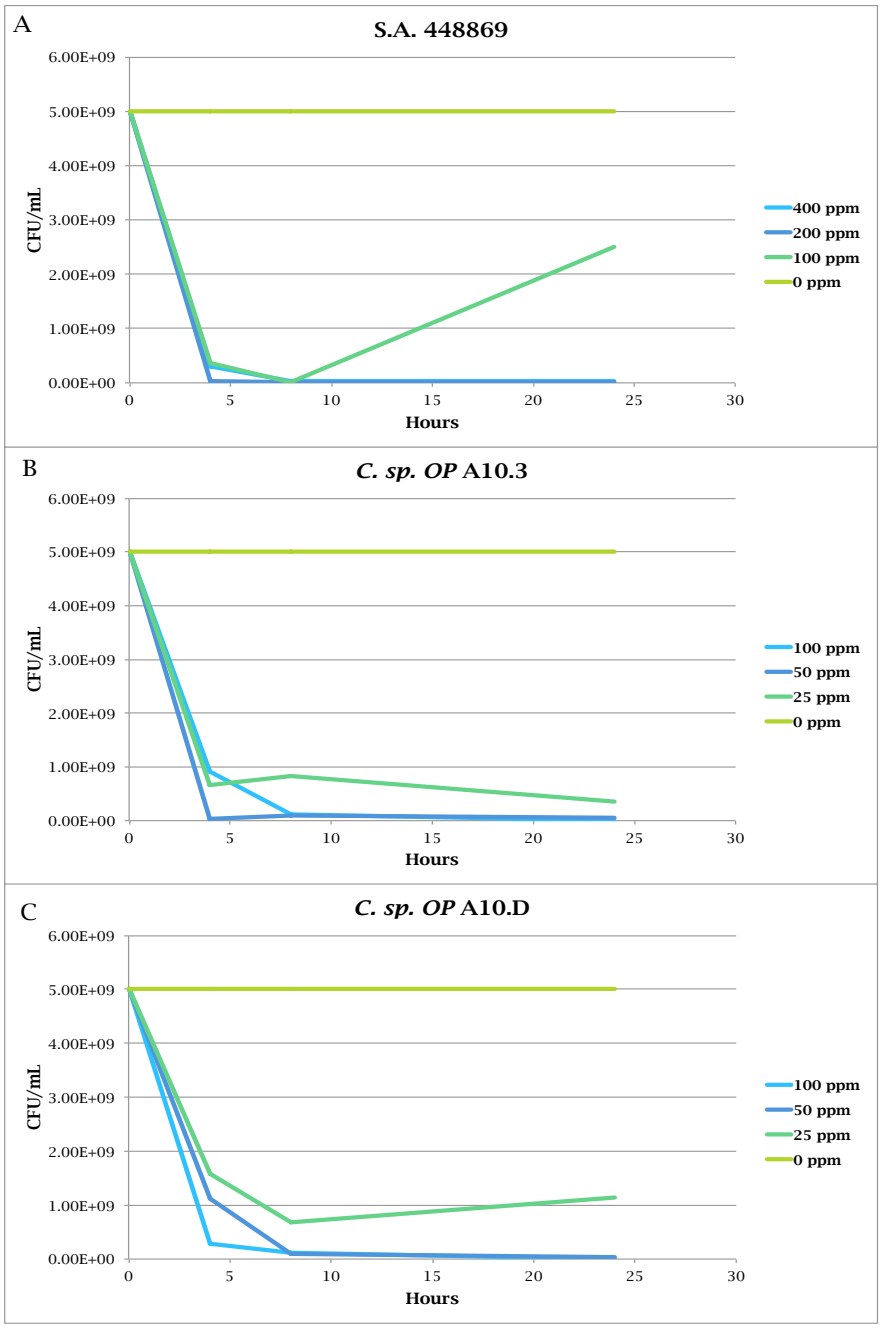

Figure 28 - Inhibitory effects of microalgal chitosans A10.3. and A10.D on *S.aureus* growth compared to crustacean chitosans.

The inhibitory effect of three different compounds is compared, namely: A. Conventional chitosans of crustacean origin Sigma Aldrich ref.448869. B. Natural chitosans produced by *Chlorella sp. OP.* C. Chemically deacetylated chitosans from *Chlorella sp. OP.* The test was limited to counting $5x10^9$ CFU/mL, so any further growth could not be monitored and was assigned the value $5x10^9$ CFU/mL. Also, it is important to highlight that the colors assigned do not correspond to the same concentrations of chitosans, as crustacean chitosans were more concentrated. The charts are the results of two technical replicates.

	MIC (ppm)			*MBC (ppm)
	4h	8h	24h	24h
S.A. 448869	<100	<100	<100	200**
C. sp. OP A10.3	<25	<25	<25	<100
C. sp OP A10.D	<25	<25	<25	<100

Table 15 – Susceptibility of *S. aureus* to *Chlorella sp. OP* A10.3 and A.10D chitosans compared to crustacean chitosans.

*Because the initial cell count could not be determined exactly, the values presented here are based on the supposition that the initial cell count was $5x10^9$ CFU/mL, the highest number of colonies that could be counted. In fact this value is probably quite higher, and hence, the MBC values are probably lower. ** The MBC value obtained was incongruent with the fact that 400 ppm were not capable of reducing the inoculum 99.9% (only 99.4%).

4.2.2.1.3 Inhibitory properties of microalgal chitosans against P. acnes

Microalgal chitosans have already been demonstrated to be effective against one gram-negative bacterium (*E. coli)* and one gram-positive bacterium *(S. aureus*), both of them grown aerobically. With the intention of further expanding the information about the antibacterial properties of microalgal chitosans, this time an anaerobic microorganism was selected: *Propionibacterium acnes.*

P. acnes is a facultative anaerobe that forms part of the natural human skin microbiota, inhabiting mainly in the face, retroauricular crease, chest and back, supported by sebaceous glands[222]. This commensal skin bacterium is well known for its relation to acne, a skin condition affecting 80% of the population at some point in their lives[226]. An increase in sebum in the skin, in periods like puberty in which the pilosebaceous unit matures, leads to the perfect environment to allow *P. acnes* to prosper. This bacterium secretes lipases, proteases and hyaluronidases that injure the skin and activate pro-inflammatory cascades[222]. Moreover, *P. acnes*, is also associated with surgical site infections originated when, after an incision, exposed tissues are contaminated with endogenous flora. *P. acnes* is capable of forming biofilms,

especially in inserted implants, and this may cause serious postoperative complications because biofilms make bacteria more resistant to host defenses and antimicrobial agents[227].

As in the case of *S. aureus, P. acnes* have increasingly developed resistance to common antibiotics[226]. In the case of acne, other common treatments include the use of benzoyl peroxide, salicylic acid or retinoic acid, which are highly effective but at the same time have several adverse effects including toxicity, irritation or discoloration of the skin. In fact, in the case of retinoic acid, restrictions on its use are implemented by the FDA[226,228]. Therefore, alternative treatments are required to reduce *P. acnes* related complications. Chitosans, because of its inherent antimicrobial activity coupled to its ability to coat materials used in implants to prevent *P. acnes* biofilm formation, could be a potential solution. Indeed, a report showing the antimicrobial capacity of chitosans of different molecular weights against *P. acnes* was recently published[228]. It indicates that the MIC of chitosans with similar characteristics to microalgal chitosans (Mw 25-35 kDa and 8-9% DA) was around 32 ppm[228]. Other reports commenting the antimicrobial properties of chitosans against *P. acnes* tested the polymer in the form of nanoparticles[228,229].

An experiment to test the antimicrobial properties of *Chlorella*-derived chitosans against *P. acnes* was carried out based on the broth microdilution method developed for *E. coli*. In this case two microalgal chitosans, extracted from two different *Chlorella* strains as indicated in subsection 4.2, were utilized (*C. sp. OP* batch A10.3; DA: 5%, Mw: 18.8 kDA and Ip: 1.4 and *C. sp. GAT-10* batch A11; DA: 10.3% Mw: 39 kDA and Ip: 1.7). As a control, chitosans of crustacean origin with similar characteristics were also tested (S.A. 448869).

A difference with respect to the assays performed on *E. coli* and *S. aureus* was that, because *P.* acnes is more susceptible to changes, instead of following the same culture at different time points, different microtiter plates containing the same conditions were prepared for each time point. Thus, a measurement of the inhibition of growth of the same culture in the course of time could not be performed. This means that MBC values could not be obtained with this experimental setting. Moreover, the CFU/mL at time zero was determined to be the same for all conditions and it was the result of an average of 14 different cell-counts. This can be observed in Figure 29, where all conditions start from

the same point ($5.69x10^7$ CFU/mL), although the standard deviation of the mean was considerably high ($3.55x10^7$ CFU/mL).

The next aspect to highlight from Figure 29 is the behavior of the control sample (0 ppm). At time point 4.5 hours, an important reduction of the cell count occurred. This is probably a result of the disturbance caused by changing the microorganism to a ten-fold diluted media in the 96-well plate, that was not in anaerobic conditions at time zero. Then, although the culture seemed to recover at 17h, no growth was observed with respect to the initial inoculum after 27.5 hours. Therefore, it can also be concluded that no MIC values can be obtained from this experiment, as the positive control containing no antimicrobial substances already inhibits the growth of *P. acnes* at the conditions tested.

Notwithstanding, although MIC and MBC values cannot be given, Figure 29 clearly shows that samples containing chitosans dramatically reduce the initial inoculum of *P. acnes*. While the CFU/mL values of the negative control remain more or less steady after 27.5 hours, all the chitosans, at concentrations as low as 20 ppm, almost completely reduced the initial inoculum. Moreover, if the data is analyzed further from what can be seen in the charts, it can be asserted that microalgal chitosans A11 are the best inhibitors. The total elimination of colonies corresponds to a reduction of 99.56%, of the initial average inoculum ($5.69x10^7$ CFU/mL). This reduction is achieved by chitosans A11 concentrated at 80 ppm in only 4.5 hours. Instead, the other two chitosans require 17 hours to achieve the same results. Furthermore, in order to reduce cell counts to zero at 27.5 hours (99.56% reduction with respect to the average initial cell count), 40ppm of chitosans A10.3 and 448869 are required. Instead only 20 ppm of A11 is necessary. Finally, it is also important to point out that all concentrations of the three chitosans tested were more inhibitory to *P. acnes* than 12.5 ppm of ampicillin.

To sum up, this last antimicrobial assay, while being the least precise of all and not allowing the calculation of MBC and MIC values, is still enough to show that chitosans have a strong inhibitory power against *P. acnes.* In this case no major differences between microalgal chitosans and crustacean chitosans were observed, although *Chlorella* derived chitosans A11 performed slightly better than the other two chitosans tested. Future anti-*P. acnes* assays should include

changes in the experimental protocol in order to obtain more accurate results. Improved growth conditions are necessary so that the sample without chitosans is able to grow correctly. Nevertheless, the results obtained with this experiment, indicating that 20 ppm of chitosans A11 and 40 ppm of chitosans A10.3 and 448869 are necessary to completely reduce the initial inoculum of *P. acnes*, are in agreement with the MIC values found in the literature for chitosan powders of similar characteristics (32 ppm)[228].

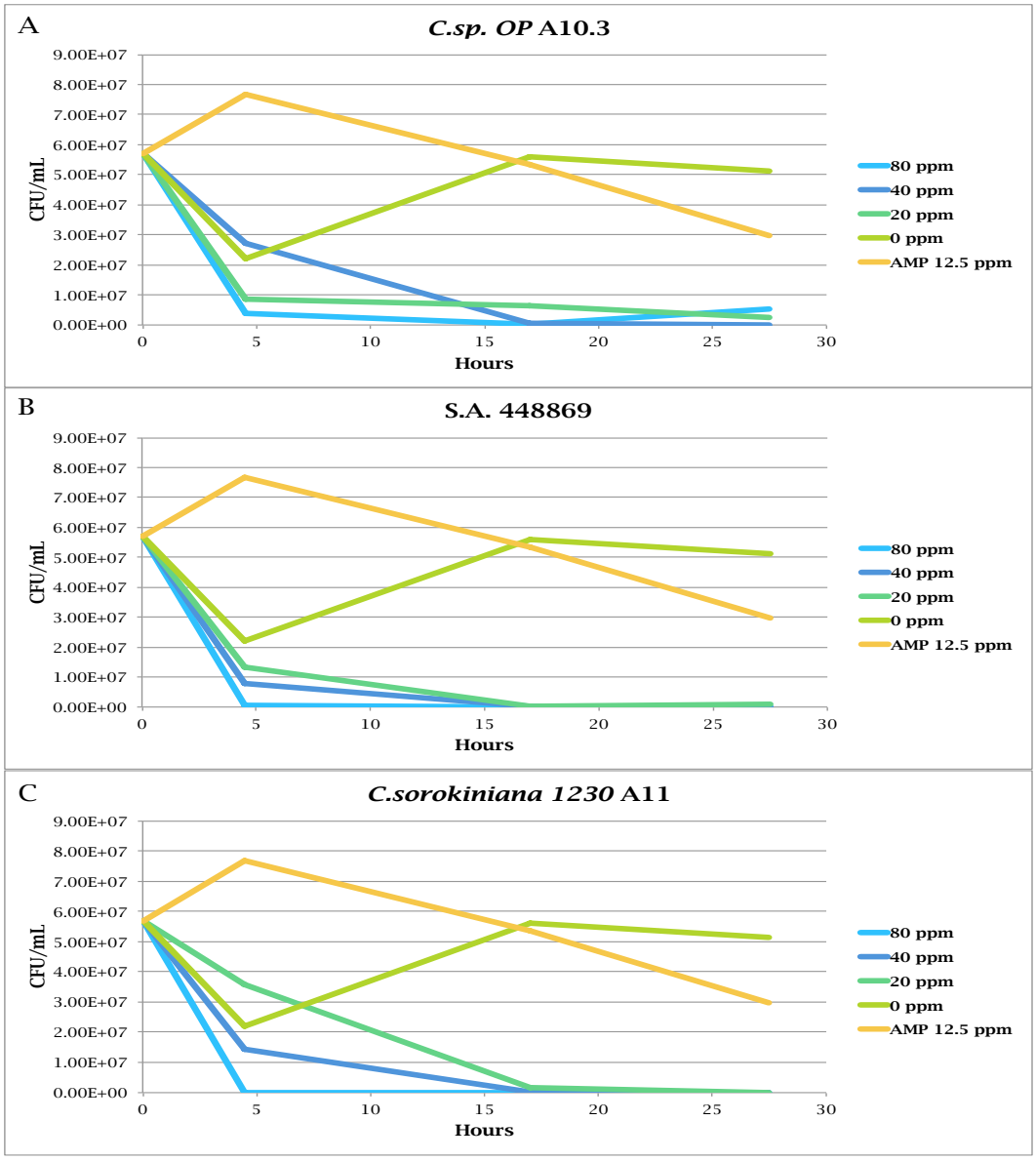

Figure 29 - Inhibitory effects of microalgal chitosans A10.3. and A11 on *P.acnes* growth compared to crustacean chitosans.

Namely: A. Chitosans obtained from *C. sp OP* (batch A10.3). B: Conventional chitosans of crustacean origin (Sigma Aldrich ref. 448869). C: Chitosans obtained from *C. sp. GAT-10* (batch A11).

4.2.2.2 Skin wound healing properties

After analyzing all the possible functions of chitosans in general and linking these to the characteristics of microalgal chitosans, wound healing was hypothesized to be one of the probable applications of these polymers. The chitosans from *Chlorella*, because of their low DA and Mw values should theoretically have good wound healing properties[10]. Studying this bioactivity provides information about how *Chlorella*-derived chitosans have an incidence on cell migration and proliferation. This knowledge is not only important for applications such as wound, scar and burn dressings and devices[230], but also for tissue engineering applications. Moreover, cosmetics related claims such as skin regeneration or anti-aging can also be formulated based on molecules that induce cell proliferation.

There are several different assays that can be performed to test wound healing *in vitro* and one of the most commonly used ones is the scratch assay. It is a simple and economical method to test cell migration and proliferation that is based on the creation of an artificial gap on a confluent cell monolayer that the cells will then try to close until cell-cell contacts are re-established[231]. Generally, the gap is created by simply scratching the cells with a p200 pipet tip. One major advantage of this technique is that it mimics to some extent the migration of cells *in vivo*, especially the epidermal skin cells (mainly keratinocytes) in the event of a wound. Another advantage is that migration can be objectively measured with microscopy or with fluorescent plate readers.

However, as any other laboratory technique, the scratch assay has some drawbacks. The main one in this case is the need for numerous replicates because of a lack of reproducibility when performing the scratch[231]. Because this disadvantage was even more important in our case due to our lack of experience with these assays, it was decided to use the ORIS™ wound healing assay. The difference of this assay with the average scratch test is that the gap created is more reproducible. In this case the cells are seeded in wells containing a physical barrier (stopper) to create a cell-free zone in the center of a 96-well plate. Once a confluent monolayer has been achieved around the "stopper", it is removed causing the same effect of the scrape with the pipette tip in the scratch assay, only that in this case the gap is more reproducible and fewer replicates are required. The substances that are being tested for their

wound healing properties are added to the media at this point. Cell migration can be easily detected with a microscope or fluorescent plate readers. An adapter allows measuring the fluorescence of only the cells that have migrated and populated the initial "cell-free" zone, eliminating in this way the contamination signal emitted by other cells that are in the well but did not migrate.

The conditions of the assay performed can be observed in Table 16. The chitosans utilized were A10.3 from *Chlorella sp. OP* at a concentration that is kept confidential. The negative control to which all other conditions were compared to was grown on DMEM media. The other samples were cultured on the same media supplemented with 10% Fetal Calf Serum (FCS) for optimal growth. Of the samples in supplemented media, a negative control in which only water was added to the growth media was prepared. Also, a positive control was prepared by adding 100 μg/mL of Epidermal Growth Factor (EGF). This Growth Factor is known to stimulate cell migration and is usually used as a positive control for scratch assays[232].

DMEM	**DMEM + 10% FCS**
No product (Basal)	No product (Basal)
	EGF (100ng/mL)
	Chitosans (Yμg/mL)

Table 16 - Experimental conditions of the wound healing assay

As Figure 30 shows, the *Chlorella*-derived chitosans very clearly induced wound closure *in vitro* in human keratinocytes cultured in growth media supplemented with 10% FCS. The bioactivity is even higher than that of the positive control, which contains EGF at concentrations that are in excess[232]. Therefore, the results are clear to show that, as hypothesized based on results of chitosans with similar characteristics available in the literature; *Chlorella* chitosans have wound healing bioactivity in human keratinocytes grown in serum rich media.

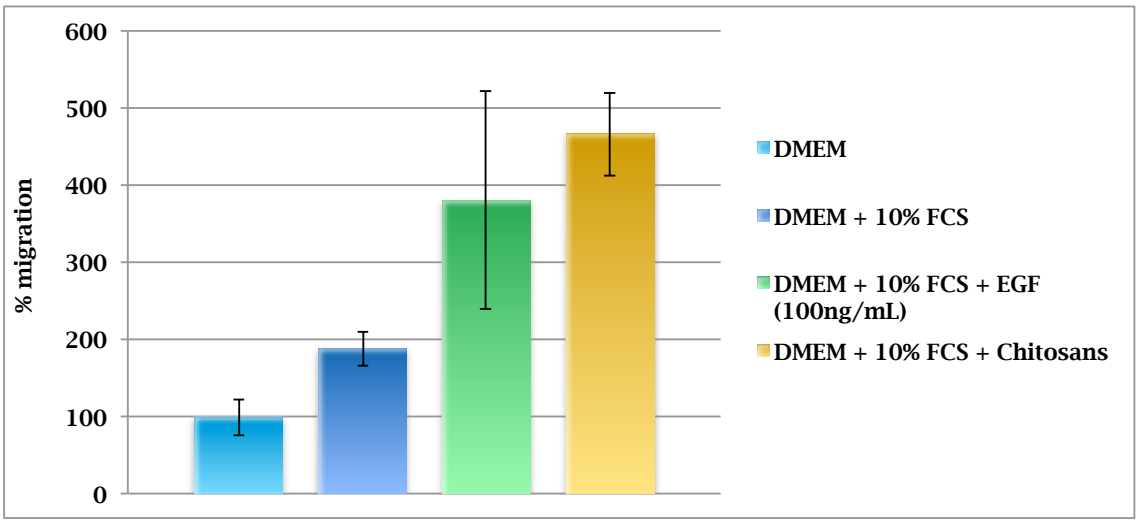

Figure 30 - Wound-healing properties of *Chlorella sp. OP* chitosans in supplemented growth media.

The graph shows the percentage of migration in relation to the negative control (DMEM media with no added product; Basal).

4.2.2.3 Nanocapsule-forming properties

In the introductory subsection of this chapter the utilization of chitosans for the elaboration of drug—delivery vehicles was reviewed. Many different drug delivery systems based on nanoparticles have been investigated such as liposomes, micelles, nanospheres, niosomes, nanocapsules, solid lipid nanoparticles, microemulsions and carbon nanotubes. Letchford *et al.* provide a comprehensive review in which the different structures of theses nanoparticles are described[233]. Among these, colloidal nanocapsules (NCs) comprised by an oily core, lecithin and a hydrophilic coat of chitosan have been at the focus of intense research due to the promising results obtained in the delivery of drugs with undesired characteristics such as facile degradation, low water solubility or poor bioavailability[234,235]. Several active compounds have successfully been encapsulated and delivered using NCs such as the lipophilic low molecular weight drugs diazepam, docetaxel and the alkaloid capsaicin. The hydrophobic hormone progesterone, the lipophilic glucocorticoid clobetasol-17-propionate or the biotechnologically produced peptides Salmon

Calcitonin and Cyclosporine and the oxidation sensitive and scarcely orally bioavailable hormone melatonin have also been encapsulated. Also, the hepatitis B surface antigen was encapsulated for the development of needle free vaccination approaches[83,234,236–240]. The presence of chitosans in these nanoparticles is key to protect and provide effective mucosal and transdermal administration[83,236]. Furthermore, these delivery systems offer several other interesting advantages such as high drug encapsulation efficiency, lack of toxicity/irritation, the possibility of controlling the drug-release rate and the capacity to withstand freeze drying for long term storage and stability[234,241].

It is also important to highlight the specific capacity of this encapsulation method to deliver lipophilic compounds such as many microalgal pigments that can be used as active ingredients in the cosmetics industry. This trait, together with the targeted delivery to the epidermis, which is also a very interesting characteristic for the cosmetics industry, finished tipping the scale to attempt to prepare the first Chlorella derived nanocapsules. In theory, as commented in the introductory section of this chapter, the characteristics of the microalgal chitosans produced so far match well with the required characteristics for nanocapsule formation. Low Mw and high DA chitosans have already been used for the preparation of efficiently packed small size nanocapsules[83].

Calvo *et al.* introduced the protocol to obtain these chitosan-coated nanoemulsions, by the process of spontaneous emulsification driven by solvent displacement[234]. Briefly, the procedure consists on a homogenous liquid–liquid nucleation that occurs when an organic solvent (such as ethanol), previously in the organic phase together with an oil (such as Mygliol 812) and a surfactant (such as lecithin), migrates to an aqueous phase. This process yields an oil and water nanoemulsion stabilized by the surfactant adsorbed at the interface. The chitosans are dissolved in the organic phase before they are in contact with the aqueous phase. At the moment in which the nanoemulsion is formed, the chitosans interact with the negatively charged phospholipids of the surfactant to finally yield the core–shell colloidal nanocapsule structure[83]. It is because of this binding of the chitosans to the negatively charged surface of phospholipids present in the emulsion that their zeta potential is reversed from negative to positive. This positive zeta potential is what has shown to

improve the properties of the colloids regarding their stability in presence of biological cations. Moreover, the positive zeta potential is what improves adherence to mucus and biological membranes[237]. According to Letchford *et al.*, the nanocapsules resulting from the solvent displacement process should measure between 100 and 300 nm[233].

A first assay was designed together with experts in the matter from the laboratory of Dr. Francisco Goycoolea, at the University of Münster. In order to test if *Chlorella*-derived chitosans could be a good raw material for the elaboration of nanocapsules, a benchmark comparison with commercially available chitosans was performed. Two chitosans from *Chlorella sp. OP* were tested; one of these chitosans was naturally available and extracted by solubilization in acidic media (A10.3) and the other one was obtained after a chemical deacetylation process of the waste fraction (A10.D). The extraction process is explained in more detail in subsection 4.2.1.1. Then, two commercially available chitosans were selected from a library of chitosans in order to have positive controls with similar characteristics to the microalagal chitosans; one with similar characteristics to A10.3 (HMC 90/5), and the other one with similar characteristics to A10.D (HMC 95/20). The characteristics of the four chitosans used are described in Table 17.

	HMC 90/5	HMC 95/20	A10.3	A10.D
Mw (kDa)	20.1	52.4	18.81 ± 8.5 %	32.40 ± 8.3 %
Ip	1.8	1.4	1.4 ± 12.4 %	1.5 ± 12.7 %
DA (%)	5	2	5	1.7

Table 17 – Characteristics of the chitosans used in the first nanoencapuslation test.

Three independent batches of nanocapsules were prepared; the characteristics of which are plotted in Figure 31. These characteristics were obtained using Dynamic Light Scattering (DLS). Regarding the size, it can be said that all nanocapsules resulted to be between 120 and 180 nm (Figure 31-A). What is important to highlight is the fact that the differences between batches using the same chitosans were larger than the mean differences between the

chitosans used for the coating. Hence, it can be concluded that no significant differences were observed with respect to size. When looking at another parameter like the polydispersity index (Ip) similar conclusions were reached. Polydispersity of all batches was between 0.10 and 0.24, but no significant differences were observed between the commercial and the microalgal chitosans (Figure 31-B). In the same way, the derived count rate showed higher deviation between batches than between chitosans (Figure 31-D). This last parameter is an indicator of the quality and stability of the sample and is also useful to compare the individual conditions of the measurements. Finally, the only parameter showing slight differences was the mean zeta potential. The mean zeta potential was somewhat lower in samples prepared with microalgal chitosans, although only samples prepared from HMC 95/20 and A10.3 showed statistically significant differences ($p = 0.0264$) (Figure 31-C). It is also important to highlight that, in terms of polydispersity, derived count rate and zeta potential, *Chlorella* derived chitosans A10.3 showed fewer variations between batches.

The first study about the fabrication of nanocapsules with chitosans from *Chlorella* indicate that these recently discovered polymers exhibit very similar results in size, polydispersity and derived count rate, when compared to commercially available chitosans. Only slightly lower zeta potential values were measured in emulsions coated with microalgal chitosans possibly due to their greater water content or slightly lower purity when compared to the traditional, shrimp-sourced chitosans. This would result in a lower concentration of *Chlorella*-derived chitosans versus conventional chitosans covering the surface of the nanocapsules.

In all, these results demonstrate that microalgal chitosans, although not yet produced in an optimal industrial way, have a great potential as a raw material for the preparation of nanocapsules for drug delivery as they perform practically as well as biomedical grade chitosans.

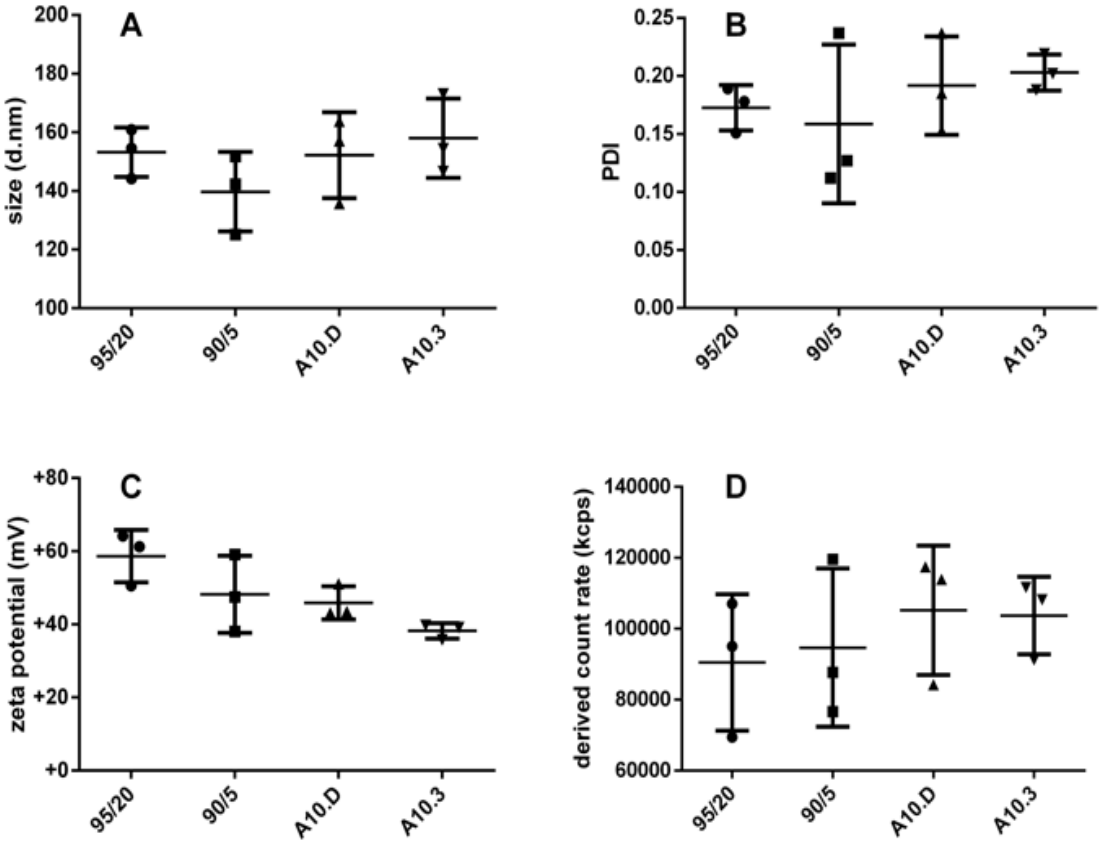

Figure 31 - Physical properties of nanocapsules produced with two commercially available chitosans and two microalgal chitosans.

Namely, A- Size, B- Polidispersity, C- Zeta potential and D- Derived count rate. The results correspond to mean and standard deviation of experiments conducted in triplicate. The plot was borrowed from the report prepared in collaboration with Dr. Francisco Goycoolea and Stefan Hoffmann from the University of Münster.

4.3 Discussion

In this thesis project it has been revealed that the cell wall of certain species of the *Chlorella* genus contain considerable quantities of chitosans. Many different bioactivities are attributed to these polymers with applications in fields as diverse as biomedicine, pharmacy, cosmetics, biochemistry, water treatment or agriculture, amongst many others. The idea behind this chapter was to first analyze the literature to identify possible bioactivities according to the characteristics of *Chlorella*-derived chitosans and then see if these polymers behaved as expected.

However, before any analysis of the properties of chitosans from *Chlorella*, it was necessary to have polymers of acceptable quality, purity and quantity. Up to that moment, only tens of milligrams of polymers had been obtained with a first version of the extraction process. That procedure yielded microalgal chitosans with a correct ^{1}H-NMR spectra but showing a very dark color. Hence, before scaling up the process to the 1-gram scale to have enough material to test bioactivities, the extraction process had to be optimized to eliminate the contaminating substances. In order to reduce the color of the final product, a solvent depigmentation step that was also expected to reduce the lipid content of the sample, was introduced right after the cell disruption step. Moreover, the biomass was not dried at any point before the chitosans were obtained to ease pigment elimination. In order to preserve the natural characteristics of the polymers and again facilitate the elimination of pigments, the use of high temperatures was eliminated, and instead, the incubation steps now performed at room temperature where prolonged. Finally, the other important change that was introduced in the extraction method was the addition of a chemical deacetylation step to recover chitosans from the acid insoluble fraction. The scheme of the new process can be observed in Figure 24.

The new method quickly showed improvements on the coloration of the chitosans from *Chlorella*. With wet biomass from strain *C. sp GAT-7,* extracted at laboratory scale, completely white chitosans were obtained, thus clearly reaffirming the need to work with wet biomass as a raw material (Figure 25). This was a very good result as it showed that there is no need to use bleaching agents for the elimination of microalgal pigments, as these agents are known to damage the chitosan structure even after a brief exposure[10]. However when

the method was applied to the extractions at scale of 1 gram, results were not as satisfying in this sense. The color of chitosans A10.3 and A10.D were dark brown because the raw material used in this extraction batch was dry. The color of the chitosans obtained using wet biomass (A11 and A11.D) was considerably lighter; light beige in the case of the chemically deacetylated polymer, although not white. Nevertheless, the colors obtained were much lighter than those obtained in the initial extraction procedures and were considered to be acceptable for an initial functional characterization of *Chlorella*-derived chitosans.

Thanks to the analysis of samples A10.3 and A10.4, the positive effects of the new extraction method on the structure of chitosans from *Chlorella* could be observed. The Ip value, the most relevant parameter to determine the quality of chitosans, was calculated to be only 1.4 and 1.5 for the naturally extracted and for the chemically deacetylated polysaccharides, respectively. These are the best Ip values seen in microalgal chitosans so far. What is more important is the fact that these Ip values are comparable to chitosans of the highest quality. For instance, the chitosans from Sigma Aldrich used in the antimicrobial tests, characterized in the same way as microalgal chitosans by Dr. Nour Eddine El Gueddari from the University of Münster, have an Ip of 1.7. More significantly, in the same laboratory, 30 different chitosans that are commercialized in the biomedical field were characterized. A comparison between the Ip values of these 30 chitosans with chitosans A10.3, revealed that only 9 out of the 30 chitosans presented better Ip values, being 1.2 the lowest of all Ip values determined. Regarding the effect of the new extraction method on the other relevant characteristics, such as the Mw and the DA, the values were coherent with the previous extractions from the same species. Only the fact that chemically deacetylated chitosans have a much larger Mw in comparison to natural chitosans was surprising and should be confirmed with further extractions. In case it was confirmed, it would be interesting to undersand how and why chitins in the cell wall are digested right after or right before the natural deacetylation process.

A11 was expected to show even better structural properties than those of A10.3 as the biomass and the chitosan extraction process had been controlled from cradle to crave. Therefore the Ip value was expected to be at least as good

as that of A10.3. However, the Ip value was a bit higher (1.7 and 1.8), although still in the range of biomedical chitosans. In fact with these samples some impurities were also observed in the ^{1}H-NMR spectrum. It might be possible that the higher oil contents of this mixotrophically strain could make it more complicated to extract the chitosans. Another aspect to highlight is the much larger Mw values obtained for chitosans A11, especially A11.D. This is the first time that the Mw of chitosans of a mixotrophically grown *Chlorella* strain has been analyzed for the Mw, therefore it could be possible that the presence of organic carbon sources in the media could increase the size of the polymers. What has been documented in the production of fungal chitosans is the fact that a larger supplementation of nitrogen increases the molecular weight of chitosans[13]. The growth media of *C. sp. GAT-10* is optimized and contains high quantities of nitrogen too. Therefore high nitrogen levels could also be an explanation for the high Mw of chitosans A11. To our knowledge, this has never been documented because this is the first time that chitosans are produced with photosynthetic organisms, so further research is necessary to confirm these relationships.

The most important conclusion that can be reached from chitosans A10.3, A10.D, A11 and A11.D is the confirmation that the new process works successfully at the scale of 1 gram because the processes yielded the expected amount of chitosans. Thus, in all, it can be concluded that the chitosans obtained with the new extraction process, although more colored than wished, are of acceptable characteristics to perform the biofunctional characterization.

Despite the good results obtained with the new method to extract microalgal chitosans there are many aspects that can be optimized to increase the quantity and quality of chitosans obtained from *Chlorella*. In terms of improving the quantity, there is an area that has not been explored in this thesis project: strain growth optimization. Many parameters can be adjusted to identify the growth conditions that lead to a higher chitosans production rate either by ameliorating the growth of the strain or by identifying the mechanism that induce each cell to produce more polymers. Some examples are: the composition of the growth media including nutrient deprivation, the addition or elimination of sugars to make the microalgae grow either autotrophically, mixotrophically or heterotrophically, the light intensity, the

aereation and CO_2 concentration, the detection of inhibitory products, the pH or the temperature[242–245]. Strain *C. sp. GAT-10* is an example of the amazing improvements in biomass productivity that can be obtained by optimizing the growth conditions of these strains that are not yet industrially exploited. *GAT-10*, although not among the strains containing more chitosans per gram of dry biomass according to the initial screening (Table 2), has become the most productive strain thanks to the optimization of its growth. Strain *C. sp. GAT-7,* the one containing the largest concentration of chitosans of all the *Chlorella* screened so far, would greatly increase its productivity if its optimal growth conditions were identified.

At the same time, apart from improving growth, identifying the conditions that lead to the highest generation of chitosans per cell would allow increasing the productivity of all *Chlorella* strains. Important variations in the chitosan content have been observed in a same strain grown different days. That is not the case when the same biomass is analyzed several times. Thus, there are conditions that vary between growth batches that affect the generation of chitosans that should be identified. For instance, it has been demonstrated that small variations in the percentage of CO_2 supplied to the culture greatly affects the percentage of amino sugars in the cell wall of different *Chlorella* strains[246]. The only factor that, according to the literature, is known to induce the production of chitin-like polymers in *Chlorella* up to date is the infection with *Chlorella* viruses[65,67]. Therefore, infecting *Chlorella* with their species-specific chlorovirus at the end of the culture is another strategy that could be studied to increment the production of chitins or chitosans[247].

Another aspect that should be improved to obtain a higher yield at the 1-gram scale is the solubilization of chitosans in acid. Results seem to show that an important proportion of the natural microalgal chitosans is not being recovered. Possible solutions could be an improvement in the agitation during the incubation or a better pretreatment of the sample to eliminate substances that could interfere in the process of solubilization in acid. This pretreatment includes a better disruption of the cells, as this was not completely achieved in any of the two 1-gram extractions performed.

In order to improve the quality of the polymers, the depigmentation step needs to be further improved to obtain whiter chitosans. The reason why A11 was

not white at the end, even though the biomass had not been dried, should be studied. It could simply be that there are differences between the compositions of the biomasses that require individualized solutions. For instance, the biomass of *C. sp GAT-10* contains more lipids because of its mixotrophic metabolism[243], so it might need more incubations with organic solvents to completely eliminate lipids, in which pigments are soluble. Finally another aspect that should be optimized is the amount of reagents used in each extraction step. So far, the process is being carried out using reagents largely in excess, but if the process was more carefully studied, stoichiometric amounts of reagent could be calculated and in this way a lot less reagents would be required, less water would be needed to wash them away and the chitosans would suffer even less alterations.

The successful implementation of the new extraction process allowed the production of hundreds of milligrams of four different chitosans from *Chlorella* (A10.3, A10.D, A11 and A10.D). Therefore, it was possible to perform the first functional characterization of microalgal chitosans. Three properties were studied: the antimicrobial, wound-healing and nanocapsule-forming properties. The three of them were selected from all other possible properties of chitosans taking into account different factors. First, it was determined that the most probable applications of microalgal chitosans were biomedicine, pharmacy, luxury cosmetics or organic agriculture. This conclusion is based on one hand on the high costs of production and, on the other, the advantages from marketing and regulatory standpoints that these new polymers offer because of their natural and non-animal origin. Then, broad-spectrum bioactivities that could be useful for these markets were predicted based on the physicochemical properties of the microalgal chitosans and the information available in the literature about similar polymers. Another important aspect that was also taken into account in the selection of properties to test was the still relatively low amounts of *Chlorella* chitosans available. Hence, the antimicrobial, wound-healing and nanocapsule-forming properties matched all these premises.

The fact that chitosans are capable of inhibiting the growth of bacteria, fungi, viruses and insects is a trait that is highly desirable in several of the possible

markets of microalgal chitosans such as biomedicine, cosmetics and organic agriculture. Moreover, according to the literature, the intrinsic characteristics of these microalgal chitosans (low Mw and high Da) seem to be perfectly designed to have powerful antimicrobial capacities. Therefore, in order to confirm the forecasts, a method capable of comparing the antimicrobial capacities of microalgal chitosans with those of crustacean origin having similar characteristics was designed. This was not a straightforward task as chitosans are not soluble in the nutrient rich growth media of bacterial cultures and having soluble chitosans is essential to be able to determine the antimicrobial capacity[226]. The insolubility issues were solved by diluting the media and the method was successfully set up for *E. coli* and then used to test the antimicrobial activity against *S. aureus* and *P. acnes.*

Although each experiment with each microorganism had particular issues, microalgal chitosans showed clear antimicrobial activities in all cases. Moreover, against *E. coli* and *S. aureus*, this inhibitory activity was much higher than the antimicrobial activity of crustacean chitosans of similar characteristics[15]. More specifically, at least 10 times less and 4 times less chitosans from *Chlorella* were necessary to completely inhibit the growth of *E. coli* and *S. aureus*, respectively. According to Zheng *et al.*, *E. coli* is more affected by low Mw chitosans, while high Mw chitosans are better targeted to inhibit *S. aureus*[213]. This could explain why *Chlorella* chitosans, which have a smaller molecular weight, were so much more effective in the case of *E. coli.* When comparing the inhibitory concentrations of *Chlorella* chitosans against *E. coli* and *S. aureus* with those found in the literature, they are situated amongst the best inhibitory concentrations reported in both cases (Tables 14 and 15)[218]. In the case of *P. acnes,* a clear inhibitory action was also observed at low concentrations of *Chlorella* chitosans. However, this time the inhibitory concentrations were similar between the conventional and microalgal polymers.

In all, the antimicrobial test developed accomplished its main function, which was to demonstrate that *Chlorella* chitosans have a strong antibacterial activity as compared with commercially available chitosans that have been reported to have antimicrobial activity in the literature[15]. In the case of *E. coli,* the test resulted to be very accurate and allowed determining very precise MIC values

(Table 14). The only modification required for future tests is to increase the dilution of the cultures inoculated in the agar plates in order to increase the resolution of the test so that it is useful for the determinination of the MBC values. Regarding *S. aureus,* the test performed correctly but the concentrations of chitosans assayed resulted to bee too high, as they were all inhibitory (Table 15). Therefore, another assay in which chitosans are less concentrated is required to determine more exact MIC and MBC values. Furthermore, the cultures need to be further diluted at time zero in order to obtain countable colonies and not a confluent agar plate. The initial cell count is necessary to be able to determine the MBC values. Finally, the test on *P. acnes,* although it was enough to show a clear inhibitory effect of chitosans, should be modified because the conditions assayed without chitosans were already inhibitory for the microorganism. Hence, it was not useful to determine MIC or MBC values. It is probable that a less diluted growth media will be required but this will affect the solubility of chitosans. Maybe the assay can still be performed in richer media by simply calculating the solubility of chitosans and taking it into account in the results.

Important considerations must be taken into account when the inhibitory effects seen *in vitro* are extrapolated to *in vivo* applications. The antimicrobial effects depend on many intrinsic parameters of each experiment such as the media, its ioinic strength, the pH, the temperature, etc. For instance the antimicrobial activity of chitosans is known to increase at lower pH. This means that, microalgal chitosans, if applied topically in a lotion at the pH of the skin (around 4.7), the action should be higher[248]. This also means that the application setting has to be well defined and controlled, in order to avoid unfavorable interactions, or loss of its activity once the chitosans are introduced in a final formulation. Another aspect that must taken into account is the capacity of chitosans to improve the immune response of the host, including human, animals or plants. Thus, this means that it might be possible that lower concentrations are required to see antimicrobial activities *in vivo*[175,183,229].

Now that a test to study the antimicrobial capacity of microalgal chitosans has been set up, it would be worth testing the activity of chitosans against other pathogens. For instance, strains of *S. aureus* and *P. acnes* that have generated

antibiotic resistances are interesting targets, as there is a need to develop new therapeutics to treat and prevent their infections without adverse side effects. As Champer *et al.* show, the physical attack mechanism of chitosans, having several random modes of action, is more difficult to circumvent, unlike conventional antibiotics that affect specific mechanisms of the microbial machinery[226]. Hence multiple changes in the cell are necessary to acquire resistance to chitosans. Moreover, any developed resistance, would most likely be non-specific, such as a reduction in surface charge or increase in surface hydrophobicity. These changes may reduce bacterial virulence and are difficult to be accomplished simultaneously. Along these lines, none of 8 different cell lines of *P. acnes* developed resistance to chitosans when studied[226]. Hence, chitosans might be an excellent tool to fight against these pathogens that are becoming increasingly resistant to antibiotics. At the same time, it would also be interesting to expand the assays to pathogenic fungi. Some possible targets could be *Malassezia furfur* associated with several skin conditions including dandruff or *Candida albicans* and *Aspergillus brasilliensis*, the challenge microorganisms applied in preservative challenge testing in cosmetics.

The second property that was evaluated was the bioactivity of microalgal chitosans as wound-healing inducers. In the method utilized to test this hypothesis, cell migration and proliferation is measured according to the capacity of cells to cover an artificial gap formed in the middle of a confluent layer of cells. Hence, this test was not only chosen for the information it could provide about wound healing applications but also for all possible applications in which a positive effect in cell proliferation and migration is desired, such as tissue engineering or anti-aging cosmetics.

An in vitro assay was performed on keratinocytes, the most abundant cells on the skin epidermis. The most important outcome was the finding that microalgal chitosans have an outstanding capacity to induce cell migration and proliferation. A preliminary assay (data not shown) indicated that microalgal chitosans are only effective in case the cells are being grown in a fully supplemented growth medium. Indeed, this is not the first time in which it has been observed that the presence of serum clearly has an effect on the wound-healing properties of chitosans. Howling *et al.* found that the mitogenic

response induced by chitosans on fibroblasts was dependent on serum concentration[216]. Hence, the results obtained here reaffirm the previously reported wound healing mechanisms of chitosans in which they bind, stabilize and activate serum components such as heparin and growth factors[216].

The assay performed was in general very reproducible and robust, while at the same time provided logical results. Only in the case in which the EGF was added as a positive control, a higher error was observed, mainly due to the chaotic behavior it induced on the cells. Therefore very few aspects need to be ameliorated. The most important one is the addition of a control containing chitosans that are known to have wound healing effects for comparison with *Chlorella* chitosans. In this case, conventional chitosans were not added in order to limit the size of the expensive assay.

The wound healing properties that can now be attributed to chitosans from *Chlorella sp. OP* (A10.3) probably can be extrapolated to the other naturally *Chlorella*-derived chitosans obtained so far as they all have similar characteristics. In the same way, it is expected that the capacity to induce cell proliferation can be extrapolated to other cell types, although that cannot be affirmed until it is proven. In all, the results from the study of the wound healing properties of *Chlorella* chitosans show that these new polymers are inducers of cell proliferation and migration. As a result, they show a great potential for applications like wound healing, tissue engineering or cosmetics.

Finally, microalgal chitosans A10.3 and A10.D, produced as shown in subsection 4.2.1.2 were analyzed for their capacity to make nanocapsules. Nanocapsules are drug delivery systems that are especially successful in the transdermal and mucosal delivery of lipophilic compounds that, if not protected, are easily degraded. Therefore, the results from the study of this property are relevant for future applications of *Chlorella* chitosans in the pharmaceutical and cosmetics industries.

Nanocapsules were successfully formed with conventional and microalgal chitosans following the protocol from the laboratory of Dr. Francisco Goycoolea at the University of Münster. The analysis of the characteristics of the *Chorella* nanocapsules indicated that the size (125 -170 nm) was in the

lower range of the expected sizes according to the description of nanocapsules made by Letchford *et al.* (100-300nm)[233]. This size also corresponded with the size of the particles obtained using the conventional biomedical grade chitosans of similar characteristics (HMC 90/5 and HMC 90/20). Hence, this is a confirmation that low Mw and high DA chitosans produce efficiently packed small size nanocapsules[83]. As with the size, other parameters analyzed, such as the derived count rate and the polydispersity, indicated that there were no statistically significant differences between the crustacean and microalgal chitosans. Instead, when the zeta potential of the nanocapsules was studied, it was found that in the case of microalgal chitosans these values were a bit lower. The explanation for this phenomenon relies on the fact that microalgal chitosans are not yet at the level of purity of biomedical grade chitosans thus resulting in lower quantity of chitosans coating the nanocapsules. It is important to bear in mind that the coating of the colloids with chitosans is what reverses the zeta potential from negative to positive, providing the colloids stability and the property to adhere to mucus and biological membranes. Thus, a reduction in the zeta potential means a reduction in stability and adherence. However zeta potentials of 40-50mV, are already very good when compared to the values of other nanocapsules found in the literature and thus should be more than enough to show good mucoadhesive properties and good stability[83,237–239].

Once the formation of nanocapsules has been found to be successful with microalgal chitosans, the next logical step is to try to encapsulate a bioactive compound. Indeed, initial tests in this regard are being undertaken. Astaxanthin, a fat-soluble pigment produced by microalgae, was selected because the potential application of this molecule in many different industries is being hampered by its instability[249]. Indeed, as already commented before, one of the main advantages of chitosan coated nanoemulsions is the increase in stability. Following the same encapsulation procedure explained in subsection 4.4.5 and dissolving an astaxanthin rich oleoresin extract from *Haematococcus pluvialis* in the organic phase, the bioactive has been encapsulated successfully in nanocapsules made with *Chlorella* chitosans. The size of the nanocapsules ranged between 185 and 218 nm and the zeta potential ranged from 40 to 46 mV depending on the chitosan from microalgae used (A10.3 or A.11). Unfortunately, the experiment is not yet finished because

two main parameters are still being calculated: the optimal amount of astaxanthin to encapsulate and the stability gain of astaxanthin. Nonetheless, the results obtained so far are already a confirmation that microalgal chitosans can be used for the successful encapsulation of lipophilic bioactives.

To sum up, microalgal chitosans, because of their physicochemical properties, were hypothesized to have good antimicrobial, wound healing and nanocapsule-forming properties. The results from the experiments presented in this chapter confirm these hypotheses. Indeed, *Chlorella* chitosans have performed outstandingly in the three cases. In all, this is just an initial functional characterization of chitosans but it is important to keep in mind that there are many other potential bioactivities that *Chlorella* chitosans probably have according to their physicochemical characteristics.

4.4 Materials and Methods

4.4.1 Optimized chitosans extraction from *Chlorella*

First, microalgae are cultured, harvested and frozen according to the specific characteristics of each strain. The extraction process is performed preferably using wet biomass. The only time in which this is not accomplished is when the biomass used has been bought elsewhere, as commercial suppliers provide it in a dry format. In order to homogenize the biomass, it is defrosted and diluted in growth media at a proportion of 40g/L. Once the biomass is completely dissolved, it is homogenized in a cell disruptor (TS Series Benchtop, Constants system) five times at a pressure of 2.5 Kbar. Samples are collected before and after homogenization and watched under the microscope to confirm proper cell disruption. The homogenized samples are centrifuged at 15,000gs for 20 minutes, the supernatant is discarded and the cell debris is kept to continue with the polymer extraction.

The process continues with the depigmentation and delipidation of the homogenized biomass with solvents. In order to do so first the biomass is incubated with ethanol 95% in a proportion of 1:20 w/v. This is performed twice for at least 2 hours each time under thorough agitation. Then the biomass is incubated for 1 hour under strong agitation with a mixture of chloroform and methanol 2:1 at a proportion of 1:8 w/v. Then, the cells are

incubated in three subsequent steps of two hours under strong agitation in which the concentration of ethanol is increased (70%, 80% and 95%). The biomass is collected from each incubation step by centrifugation at 8,000gs during 20 min.

Afterwards, the deproteination step consists on incubating the biomass in 1:30 w/v of a 2% Sodium Hydroxide solution for 72 hours at room temperature under strong agitation. After this treatment the proteins are soluble and separated by centrifugation. Then the pellet is washed with water to reach a neutral pH and right after it is incubated 1:40 w/v under thorough mixing in a solution containing acetic acid 5% during 48 hours at room temperature. During this incubation the chitosans are solubilized and afterwards separated from the rest of components that precipitate when centrifuged at 10,000gs for 15 minutes.

Chitosans, which are now soluble, are brought to alkaline pH by the addition of a 4M sodium hydroxide solution until a final pH of 8-9 is reached. After overnight incubation at this pH chitosans are precipitated at 15,000gs for 20 minutes. Once the chitosans are precipitated they are washed at least 5 times with abundant water until the pH of the water used for washing is the same as the water before washing. Finally, the chitosans are dried by lyophilization to eliminate any trace solvents. Once this final step has been reached, chitosans are ready for its characterization.

The non-soluble fraction in 5% acetic acid is washed with water one time and then incubated in a proportion of 1:8 in 50% sodium hydroxide for 3 hours at 120ºC and under thorough mixing, during this period deacetylation occurs. The solution is diluted by adding the same volume of water and then the pellet is recovered by centrifugation at 10,000gs for 20 minutes. Then, the chemically deacetylated chitosans are separated by solubilization in 5% acetic acid during 48h under thorough mixing. The solubilized chitosans are separated from the insoluble material by centrifugation during 15 min at 20,000gs. Then, the same procedure to precipitate, wash and dry the natural chitosans explained above is applied to the chemically deacetylated chitosans.

All reactives were acquired from Sigma Aldrich.

4.4.2 Production of 1g of chitosans from *Chlorella*

Two different microalgae biomasses were used. On one hand, the biomass (*C. sp. OP)* was obtained from a trusted supplier of *Chlorella* to be able to perform trials efficiently, without having to run large *Chlorella* cultures each time. This strain had been grown in open ponds and was supplied dried.

On the other hand, the biomass from *C. sp. GAT-10* was obtained from the pilot plant of Greenaltech S.L. and kept wet and frozen until the moment of extraction. The microalgae was cultured in a semi-continuous fashion in two 15L photobioreactors, one having a diameter of 0.12m and the other one 0.24m. The growth media utilized was Bolds Bassal Medium (BBM) supplemented with 20 g/L of glucose and 2 g/L of sodium nitrate. The addition of glucose converts the growth regime in mixotrophic as the microorganism uses both photosynthesis and glucose metabolisms for growth. The cultures were aerated from seven aereation points located at the bottom of the photoreactor with 6.7 L/min of air and 0.08 L/min of carbon dioxide. The light was variable depending on the amount of natural light. In general, the light intensity varied between 100 and 160 μE m−2 s−1.

The procedure to obtain the desired biomass was as follows: pre-inoculum cultures were prepared in erlenmeyers flasks and then subsequently scaled-up 1:5. The bioreactor was inoculated so that the total 15L ended up being at a final concentration of biomass of 0.1g/L. Afterwards the culture is grown to the end of the exponential phase. At this point, half of the culture is harvested and the other half is replenished to the same level it was before (15L) with freshly prepared media. The phase of the culture was controlled by optical density at 680 nm (OD680nm). The harvested biomass was collected by centrifugation for 10min at 3000gs in three batches of 2.8L. Then, the biomass was frozen and kept at -20ºC until the moment of extraction.

The extraction of the natural and chemically deacetylated chitosans was performed as indicated in subsection 4.4.1 with small changes due to the increased volumes. The cell disruptor used in the laboratory scale extractions (TS Series Benchtop, Constants system) was too small for the large volumes of the difficult to disrupt *Chlorella* cells that had to be treated this time. Therefore, a larger but less potent homogenizer was used (Homolab 2, FBF Italy). With it, the biomass went through a pressure of 1kBar five times. This

was not enough to disrupt the cells so the liquid had to be passed through the smaller size homogenizer (TS Series Benchtop, Constants system) only one time at a pressure of 2.5 Kbar. The disruption of the cells was controlled by observation under the microscopy. The increased volumes also made it too complicated to work with bench-top centrifuges. Instead, for the recovery of the biomass after homogenization, a separator was used (Westfalia Separator, Mineraloil Systems GmbH Model OTC3-02-137) at a flow rate of 1 L/min. This separator had the capacity to harvest 0.8 Kg of wet biomass. Two and three rounds of centrifugation were done for *C. sp. OP* and *C. sp. GAT-10*, respectively.

Dr. Nour Eddine El Gueddari from the University of Münster and myself characterized the four chitosans obtained as explained in subsections 2.4.4 and 2.4.6.

4.4.3 Determination of the antimicrobial activity of chitosans

The antimicrobial test was developed thanks to the advices of Dr. Nour Eddine El Gueddari from the University of Münster in the framework of the Nano3Bio EU FP7 project. The microalgal chitosans used came from the batches A10.3 and A10.D from *C. sp OP* and the batch A11 from *C. sp. GAT-10,* which were obtained as indicated in subsection 4.3. A10.3 had the following characteristics: DA: 5%, Mw: 18.81(±8.5%) kDA and Ip: 1.4. A10.D had the following characteristics: DA: 1.5%, Mw: 32.4(±8.3%) kDA and Ip: 1.5. A11 DA: 10.3%., Mw: 39 kDA(±7.0%) and Ip: 1.7 .Dr. Nour Eddine El Gueddari and myself characterized them as indicated in subsections 2.4.4 and 2.4.6. The crustacean chitosans used were purchased from Sigma Aldrich (Ref. 448869). Finally, ampicillin was also acquired from Sigma Aldrich.

The *E. coli* strain tested was GC5™ (Sigma Aldrich) and the *S. aureus* strain was ATCC 6538. Both strains were grown aerobically in Mueller Hinton Broth (Sigma Aldrich) at pH 5.9 in an incubator at 37ºC under vigorous shaking (225-250 rpm). The *P. acnes* strain tested was ATCC 6919, which was grown anaerobically inside an anerobiosis jar or a plastic zip bag. The anaerobic atmosphere was generated with anaerobic generator sachets (ref. 230-096124 Scharlab) and the absence of oxygen was monitored with anerobiosis indicating

stripes (ref. 230-096118 Scharlab). The growth media in which it was grown at pH 5.9 was Clostridial Nutrient Medium from Sigma Aldrich (ref. 27546).

The determination of the antimicrobial activity was performed by the broth microdilution method. Stock solutions of 1.1 fold the highest final concentration of chitosans or ampicillin tested were prepared in PBS (Sigma Aldrich) and filter-sterilized. The rest of concentrations were also prepared 1.1 fold by doing a serial dilution of the stock solution to obtain the desired concentrations to be tested. All solutions contained the same amount of acetic acid and were filter sterilized. 180 μL of each solution were added to the wells of a 96-well flat-bottom polystyrene microtiter plate (Thermo Fisher Scientific). Concomitantly, the strains were grown in the respective growth conditions to an optical density at 600 nm (OD600) of 0.5. Subsequently, 20 μL of these cultures were added to the respective wells to end up having 200 μL of a culture containing the desired concentration of chitosans in a broth diluted 1/10. It had to be performed in this way because if the media was not diluted, many things precipitated from the growth when mixed with the chitosans. Each condition was studied in duplicates, except from the negative control (0 ppm), which was the same for all the conditions and thus was tested in triplicates. The cultures where incubated at 37ºC in the growth chamber without shaking and 10 μL aliquots were removed at predetermined intervals. Then, the aliquots were diluted and inoculated into agar plates to record survival counts after overnight incubation at 37ºC inside the growth chamber (72 hours in the case of *P.acnes*). The same well was studied along the different time points for *E. coli* and *S. aureus.* Instead, in the case of *P. acnes,* different 96 well plates were prepared for each time point in order to avoid disturbing the anaerobic conditions each time. The experimental setting needs to be designed so that 99.9% inhibition of growth can be detected in order to determine MBC values.

4.4.4 Wound healing assays

The chitosans used came from the A10.3 batch of *Chlorella sp. OP* which was well characterized by Dr, Nour Eddine El Gueddari as indicated in subsections 2.4.4 and 2.4.6; DA: 5%, Mw: 18.81(±8.5%) and Ip: 1.4. A 100 times concentrated chitosan stock solution was prepared using 5% stoichiometric excess of acetic

acid. The solution was filter sterilized. The Human Keratinocyte cell line used was CRL-2310™ from ATCC. Oris™ Cell Migration Assay Kit (collagen coated) ref. CMACC5. 101 was used to measure cell migration.

Cells were cultured in Keratinocyte-Serum Free Medium (Gibco) supplemented with 0.05 mg/mL of bovine pituitary extract (BPE) and 35ng/mL of recombinant human EGF (Sigma Aldrich). Cells were kept at 37ºC in incubators with 5% CO_2 and 90% humidity. The viability of the cells was controlled with Trypan Blue staining (Merck Millipore). All assays were performed when the cell line was at 80-90% confluence and over 90% viability.

The experiment was performed following the instructions from the Oris™ Cell Migration Assay Kit. 80,000 thousand cells were seeded per well in 100 μL of DMEM medium supplemented with 10% FCS (Thermo Fischer Scientific) in the 96 collagen-coated well plates from the kit. Cells were cultured overnight in the incubator to permit cell attachment. The following morning the stoppers and the media were gently removed and the wells were washed to remove unattached cells. Then, 100 μL of the different media with the substances to be tested were added to each well (Table 16). After 24 hours of incubation (18 in assay number 3), cells were stained with 5μM Calcein AM (Thermo Fischer Scientific) and the fluorescence signal, corresponding to the cells that had migrated and occupied the "cell-free zone", was quantified using a Fluoroskan Ascent™ microplate fluorometer (Thermo Fischer Scientific). The excitation wavelength was set to 485 nm and the emission wavelength to 538nm. The signal from a well with no cell migration was considered to be the background of the technique and this value was subtracted from all the obtained values. Assay number one was performed in duplicates and the other two assays were performed in triplicates.

4.4.5 Nanocapsule formation process

The microalgal chitosans (A 10.3 and A10.D) used in the encapsulation process were obtained as indicated in subsection 4 from *C. sp. OP*. The conventional chitosans (HMC 95/20 and HMC 90/5) were kindly provided by HMC. All chitosans were characterized in the degree of acetylation, molecular weight and polydispersity by Dr. Nour Eddine El Gueddari from the University of

Münster and me as indicated in subsections 2.4.4 and 2.4.6. The characteristics of the chitosans used can be found in Table 17.

The formation of nanocapsules was performed in collaboration with the laboratory of Dr. Francisco Goycoolea at the University of Münster in the framework of the Nano3Bio FP7 EU project, more specifically Dr. Beatriz Santos Carballal and Steffan Hoffmann. The encapsulation process was performed as follows: chitosans were dissolved in a 5% stoichiometric excess of hydrochloric acid. Nanosystems were prepared according to a slightly modified procedure introduced by Calvo *et al.* Briefly, an organic phase consisting of 62.5 μL Miglyol 812® (Sasol) and 500 μL of a 40 mg/mL ethanolic lecithin solution (Epikuron 145 V, Cargill) was filled to 5 mL using ethanol. The organic phase was quickly poured over 10mL of aqueous phase with a chitosans concentration of 0.5 mg/mL. The milky dispersion was concentrated in a rotavapor to 4-5 mL and topped up to 5 mL using water. The characteristics of the resulting nanocapsules were measured using a Malvern Zetasizer NanoZS (Malvern Instruments).

5 GENERAL DISCUSSIONS AND FUTURE PROSPECTS

Chitins and chitosans are described as a family of linear polysaccharides consisting of varying amounts of β(1→4) linked GlcNAc and GLcN. What differentiates both polymers is the ability of chitosan to be dissolved in liquid acidic solutions, as a result of a higher percentage of GLcN units. The deacetylated units yield free amino groups that, at slightly acidic conditions, convey positive charges to the polymers, making them the only known polycationic polysaccharides. Therefore, chitosans interact with polyanionic biomolecules such as proteins and nucleic acids and polyanionic phospholipidic membranes and sulfated polysaccharides like the human glycosaminoglycans at cell surfaces. As a consequence of these interactions, chitosans have been reported to have many bioactivities that make them the most advanced and promising biofunctional polymers.

Chitins are mainly commercially extracted from the exoskeleton of shrimps, prawns, crabs, and other crustaceans and the inner shells of squids. Currently, most commercial production of chitosans is based on the chemical deacetylation of chitins, being chitosan the main derivative of chitin. However, the harsh processing required to convert chitins into chitosans and its crustacean origin are drawbacks hampering the applicability of chitosans in the market. On the one hand, the effects of the harsh chemical reactions required to deacetylate chitins are difficult to control, thus creating important

differences between batches. The structure of some of the polysaccharides gets impaired under such conditions, resulting in an increase in the polydispersity of sizes. Moreover, the pattern of acetylation, which has also been demonstrated to have an incidence on the bioactivities, is not controllable and thus completely random. These issues are crucial because they make the biofunctionalities of these polymers poorly reproducible, a major drawback causing the development of chitosan-based products to lag far behind expectations. Furthermore, the chemical deacetylation process is also problematic from the point of view of environmental sustainability, due to the large amounts of concentrated alkaline solutions generated. On the other hand, the origin of these polymers has also been a major concern for biomedical applications as material sourced from animals are more difficult to be approved by the regulatory agencies because of the adverse reactions they may cause. In a similar fashion, the possible allergic reactions and the difficulty of marketing products of crustacean origin in the cosmetics industry has hampered its development. Additionally, there is an extra limitation caused by the origin, which is the seasonal limitation of seafood shell supply (Table 18).

Some publications indicated that the cell wall of some microalgae contains important concentrations of glucosamines. In fact, the presence of chitins had already been proven in some diatoms like *Thalassiosira* and the haptophyte *Phaeocystis* and some authors had suggested the presence of a chitin-like polymer in *Chlorella*[24,26,27]. Moreover, the presence of chitin deacetylases in the genome of *Chlorella variabilis NC64A* was another argument in favor of the presence of chitosans in microalgae[25]. Therefore, it was decided to further explore these photosynthetic microorganisms to confirm if any of them had the capacity to make chitins and, especially, chitosans. Chitosans directly extracted from microalgae would solve the problems of currently commercially available crustacean chitosans commented above from both sides; the processing and the origin (Table 18). Moreover, extracting chitosans from the cell wall would be an added value to a part of the microalgae that is generally not valorized (Figure 32).

A high-throughput screening method to look for chitins and chitosans in microalgae was developed. It relied on the use of CAPs and CBPs, which are

fusion proteins that have the ability to selectively bind to chitins or chitosans and be detected by fluorescence. With this methodology, the presence of chitins and chitosans in *Chlorella* was finally confirmed. This finding was validated with the use of standard techniques for the characterization of these polymers such as ^{1}HNMR, FTIR, glycosidic linkage analysis, chitinase and chitosanse digestion and HPSEC. Therefore, with no doubt, it can now be asserted that some *Chlorella* strains are capable of producing chitins and deacetylate them to chitosans naturally. This discovery converts *Chlorella* into one of the few photosynthetic organisms to fabricate chitins and the only one described so far to naturally produce chitosans. Moreover, a method has been designed and validated to search for chitins and chitosans in microalgae that can be used for further screenings or to identify the growth conditions that lead to a higher chitosan production.

Apart from *Chorella*, the *Scenedesmus* genus also accumulated evidence from the CAPs and CBPs screening and the FTIR analysis indicating that it is capable of producing chitins and maybe chitosans. Other indications of the screening test that are also waiting to be validated are the presence of chitosans in *Thalassiosira* and fabrication of chitins by *Bracteacoccus.* A modified glycosidic linkage analysis of the cell walls that was capable of differentiating between chitins and chitosans would be the ideal test to confirm the above-mentioned findings. Confirming the presence of chitins and chitosans in these strains would provide more information about the screening process and its detection limits. It is also important to highlight that so far some microalgae of the Chlorophyta, Heterokontophyta and Haptophyta divisions have been screened for the presence of chitins and chitosans. Nevertheless, there are still many other species from these divisions or the so far non-studied divisions to be analyzed (Glaucophyta, Rhodophyta, Cryptophyta, Dinophyta, Euglenophyta and Chlorarachniophyta).

Chlorella is one of the microalgal genera that are cultured in higher quantities worldwide. It is estimated that at least 2,000 tones of it are produced annually mainly for dietary supplements and it is one of the few microalgae utilized for human consumption[250]. All of these attributes made it the ideal microalgae for the production of natural chitosans and hence the characteristics of its polymers and its bioactivities were further studied.

The chitosan containing fractions of 13 different *Chlorella* strains were analyzed by ^{1}H-NMR. This analysis showed that 9 of the 13 strains had produced chitosans, indicating that apparently not all *Chlorella* are capable of producing chitosans. Moreover, ^{1}H-NMR was useful to determine the DA% of 8 of the 9 *Chlorella* strains containing chitosans. The spectra indicated that the DA values of *Chlorella* chitosans ranged from 5 to 25%, although mostly concentrated between 12 and 18%. At the same time, the Mw of the chitosans derived from four different *Chlorella* strains was determined by an aqueous HPSEC-MALLS-RI System. According to all the analyses carried out so far, the Mw of the naturally produced microalgal chitosans ranges between 20.5 and 39 kDA. Moreover this analysis also allowed the determination of the polidispersity of the sizes of the chitosans in the sample (Ip). Outstandingly, the value was below 2 in all cases, indicating that *Chlorella* chitosans were already at the same level of polidispersity of sizes than commercially available chitosans used for biomedical purposes. In all, it was concluded from the characterization of the first chitosans extracted from *Chlorella* that these biopolymers had in general a low DA% and a low and fairly homogeneous Mw.

Once the polymers had been extracted and it had been confirmed that they were chitosans, the next goal was to see if they behaved like chitosans. Therefore, the next step was to scale-up the chitosan extraction process from *Chlorella* to obtain enough polymers for an initial functional characterization. Nevertheless, while chitosans are expected to be of a white-beige color, the microalgal chitosans obtained up to that moment had been dark green, almost black. This was an indication that, although it was not visible in the ^{1}HNMR spectra, the microalgal pigments and the lipids in which they are soluble had not been completely eliminated during the process to extract chitosans. Having the purest possible chitosans was paramount to obtain meaningful results with the biofunctional characterization. Therefore, the extraction process was optimized by adding some extra steps to eliminate pigments and by performing each incubation step for a longer period of time and at room temperature. Moreover, thanks to the characterization of the fraction non-soluble in acid of the first extraction process, it was identified that chitins and chitosans were embedded within this fraction. Hence, in order to obtain a larger amount of polymers per gram of biomass, a step to chemically deacetylate the residual fraction was added. In all, with the optimized

extraction process, two different chitosans would be obtained, one that was naturally produced by *Chlorella* and the other one, which was a result of the chemical deacetylation step.

The new extraction process was successful to obtain white chitosans only if wet biomass was used as a starting material, demonstrating that drying the biomass was detrimental for pigment elimination. As a consequence of the good results obtained, the new process, which so far had been performed at the mg scale, was brought up to the g scale with two different *Chlorella* strains: *Chlorella sp. OP* and *Chlorella sp. GAT-10.* While the Mw and DA% of these chitosans stayed in the range of previously obtained chitosans, what was revealing was the fact that the Ip values of the chitosans obtained had been lower compared to the extractions performed so far from *Chlorella*. Moreover these extractions confirmed that the yield was the same at this scale than at the mg scale. Therefore, it was concluded that the extraction had been successfully scaled up to the production of approximately 1 gram of chitosans. The next step will be to scale-up to tens and hundreds of grams. Centrifuges at these larger scales are very costly, therefore the possibility of changing the centrifugation steps for filtration steps should be carefully studied.

The fact that the color and the quality of chitosans had been improved, together with the fact that hundreds of milligrams of each chitosans had been obtained, indicated that these four chitosans could be used for the first biofunctional characterization. As such, three properties were analyzed: the antimicrobial, wound-healing and nanocapsule-forming properties. The study of these bioactivities was selected from the immense amount of properties generally attributed to chitosans based on the physicochemical characteristics of microalgal chitosans and the literature available about the bioactivities of similar chitosans. Moreover, in order to make this first biofunctional characterization relevant for future market applications, the advantages and disadvantages of the chitosans from *Chlorella* with respect to crustacean chitosans were taken into account.

The most important objective of the biofunctional characterization was to see if *Chlorella* chitosans indeed behaved like chitosans. The assays performed showed that the chitosans from *Chlorella* truly had antimicrobial properties, wound-healing properties and were capable of forming nanocapsules.

Therefore, it was confirmed that *Chlorella* chitosans did not only had the characteristics of chitosans but also had its bioactivities. More specifically, the antimicrobial assays showed that the same amount of chitosans or less, compared to the most similar chitosans from Sigma Aldrich, which were known to have antimicrobial properties, were required to inhibit the growth of the bacteria tested (*E. coli, S. aureus* and *P.acnes).* The best result was obtained with *E. coli*, which was inhibited with approximately ten times less natural *Chlorella* chitosans in comparison to the crustacean chitosans. In the case of *S. aureus, Chlorella* chitosans were four times better than the chitosans from Sigma Aldrich and in the case of P. acnes, chitosans behaved similarly. Even though standard MIC protocols cannot be used when dissolving chitosans and each MIC value is largely dependent on the conditions of each study, it is still relevant to highlight that the values obtained for the chitosans from *Chlorella* against *E. coli* and *S. aureus,* were amongst the best ever published[218].

Regarding the wound healing properties, the assays performed with keratinocytes revealed that natural *Chlorella* chitosans have wound healing properties if the cells are in nutrient rich media, confirming that one of the wound healing mechanisms of chitosans is to bind, stabilize and activate serum components such as growth factors[216]. Finally, both naturally and chemically deacetylated chitosans derived from *Chlorella* were also equivalent to biomedical grade chitosans in their capacity to form nanocapsules for drug delivery. The only difference observed was that *Chlorella* chitosans had a lower zeta potential, something that was considered to be caused by the presence of impurities in the sample.

Altogether, the data presented here serves as an initial physicochemical and biofunctional characterization of the chitosans from *Chlorella.* Nonetheless, on the side of the physicochemical properties much remains to be done, especially on the analysis of the purity. Although the ^{1}HNMR of the microalgal chitosans obtained are very clean, the color of the chitosans and the low zeta potential of the nanocapsules made with these chitosans seem to indicate that the chitosans are not yet completely pure. One way to determine the purity of the sample with respect to other carbohydrates would be to perform a glycosidic linkage analysis that was capable of detecting GLcN linkages, a test that has already been proposed to solve other open questions from this thesis project.

Indeed, Dr. Xing from KTH University is already setting up such method in Stockholm. Another test that will be performed is a complementary test that is being developed by Jasper Wattjes at the University of Münster. It is based on the digestion of the polymers with chitinases and chitosanases to obtain oligomers that can be quantified by MS. Additionally, determining the ash content of the chitosans could provide more information about impurities such as salts and minerals. The metal content must also be determined if *Chlorella* chitosans are to be used for biomedical applications.

Another characteristic that should be confirmed about microalgal chitosans is their non-random pattern of acetylation, a clear advantage in front of chemically deacetylated chitosans in terms of reproducibility of bioactivities. As it has already been commented, temptative analyses indicating that microalgal chitosans have a non-random PA have been carried out with an enzymatic fingerprinting method developed by Dr. Cord-landwehr from the University of Münster. Nonetheless, some troubles were encountered due to low DA of some microalgal chitosans to determne the PA with this methodology. Otherwise, the PA might also be determined by 13C NMR, the standard technique used for the determination of the PA[18].

On the side of the biofunctional properties, there is also a great deal to do. In terms of the antimicrobial properties, the test on *P. acnes* needs to be improved in order to be able to determine the MIC and MBC values. Moreover, the antifungal and anti-film forming properties are planned on being studied to broaden the knowledge of the antimicrobial properties of *Chlorella* chitosans. With respect to the wound healing capacities, it would be interesting to perform a benchmark against other biomedical grade chitosans. Regarding the nanocapsule forming properties of the microalgal chitosans tested, the next test to be performed is to encapsulate bioactive molecules to measure the advantages of encapsulating with microalgal chitosans in terms of delivery, bioavailability and stability. Finally, of course, there are many other bioactivites that would be interesting to test, especially those in which *Chlorella* chitosans are predicted to be very well suited: antioxidant effects and gene delivery.

Many doors have been left open for further research. Notwithstanding, the initial goals of screening microalgae, characterizing the polymers and

determining some of their bioactivities, have been accomplished. *Chlorella* chitosans have many characteristics that are advantageous in front of conventional crustacean chitosans and therefore have the opportunity to reach the market in niche applications (Table 18). Nonetheless, higher scales need to be tested to see if the yields are maintained and to have a better idea of the costs of production. *Chlorella* is currently sold mainly as a dietary supplement, for the benefits of its remarkable richness in proteins, lipids, polysaccharides, pigments and vitamins. The strong cell wall containing the chitosans prevents its native form from being digested by humans; therefore it needs to be disrupted to be bioavailable. Nevertheless, *Chlorella* powders are sold with the broken cell wall to be able to claim that they are fiber rich nutraceuticals and to avoid having to separate the cell wall[251,252]. Therefore, in the current status, biomass would need to be produced exclusively for the production of chitosans, as there is no cell wall left overs available, hence considerably affecting the final cost in the process to extract chitosans from *Chlorella*. In order to reduce the cost of production, a biorefinery approach in which chitosans are extracted from the cell wall and the rest of its components are left for nutritional supplement purposes would definitely make the process more cost effective. Even more interesting would be to bring the biorefinery concept to its maximum by extracting the different valuable components from the biomass of *Chlorella* separately (Figure 32)[252]. Indeed, such valuable compounds can be made more bioavailable and stable by encapsulating them with chitosans.

The discovery of chitosans in *Chlorella* also raised the question of how these photosynthetic microorganisms were capable of fabricating these polymers. Although the metabolic pathway leading to the production of these chitosans necessitated the action of several different enzymes, the most interesting ones were the chitin deacetylases. While chitins occur in many organisms, the natural presence of chitosans is very rare. Before the discovery of chitosans in *Chlorella*, only a few fungi were known to be able to perform the chitin deacetylation step naturally. Moreover, the process to convert chitins to chitosans in *Chlorella* was of particular interest because 25 different putative

chitin deacetylases had been identified in the genome of *Chlorella variabilis NC64A*[25].

Therefore it was intended to identify which of the large list of 25 putative cCDAs of *Chlorella variabilis NC64A* was indeed an active CDA. The best way of doing so was to recombinantly express the sequences encoding these proteins in *E.* coli because this allowed to obtain high amounts of pure proteins to be studied afterwards. Nevertheless, recombinantly expressing 25 different proteins that should tentatively be hard to express because of the nature of membrane proteins was a humongous task. Therefore, the list of proteins to express was attempted to be reduced to a list containing the most probably active cCDAs using bioinformatic, molecular biology and protein biochemistry tools.

Only 22 out of the 25 sequences were found in the JGI database. These sequences were analyzed using BLASTp, COBALT and conserved motif search to try to identify those that were more similar to active fungal cCDAs. However, little similarity was observed. cCDAs were only similar between them or similar to putative CDAs from chlorovirus. Only cCDA 9 was apparently more similar to the active CDAs analyzed. Hence, bioinformatic tools were not effective at reducing the list of proteins to be expressed in *E. coli.* Therefore, the transcription of the cCDAS to RNA was studied in *Chlorella* to try to reduce the list by eliminating those sequences that did not transcribe to RNA. However, 19 out of the 22 cCDAs were found in the cDNA of *C. variabilis NC64A.* Thus, only 3 out of the 22 sequences could be discarded after studying the transcriptome (cCDAs 8, 19 and 22).

The next approach to reduce the list of proteins to be expressed in *E. coli* was to analyze *C. variabilis NC64A* protein extracts. The chitin deacetylase activity of cellular and extracellular protein extracts was first tested on chitin oligomers and polymers and it was confirmed. Hence, several zymography assays were carried out to try to identify cCDAs in protein bands that showed chitin deacetylase activity against chitin glycol. Thanks to these assays, 9 different cCDAs (1, 3, 4, 6, 7, 10, 14, 16 and 21) were identified in different bands showing chitin deacetylase activity. This was a confirmation that many different enzymes are involved in the mechanisms to deacetyate chitins in *Chlorella*. Amongst these putative cCDAs, only cCDA 7 (JGI code: 139978)

could be confirmed to be an active cCDA because it was found without any other putative cCDAs in two bands with chitin deacetylase activity. Although the characteristics of this enzyme need to be validated in purified samples, cCDA7 seems to be the first active cCDA to be identified amongst all viridiplantae organisms. The upcoming task will be to elucidate the catalytic mechanism, the substrate specificity and the optimal working conditions for this newly discovered enzyme, in the same way as it has been done with other active CDAs identified so far from other microorganisms.

The proteomic analyses finally allowed reducing the list of putative cCDAs to be expressed in *E. coli.* Putative cCDAs 1, 3, 4, 7, 10 and 21 were selected for this purpose because they had large protein spectral counts in the first active protein bands that were sequenced. All of these proteins were attempted to be expressed in *E. coli,* but, due to problems with the correct amplification of the genes or issues with the correct expression of the proteins, none of them have been demonstrated to be an active cCDA so far by this method. Only proteins 1, 3 and 10 have been expressed but as insoluble inclusion bodies that so far could not be demonstrated to be active. Further research is required in this regard and in the near future other expression vectors and strains will be tested.

With the information gathered so far it seems apparent that the mechanisms of *Chlorella* to deacetylate chitin are very complex and governed by many enzymes. Therefore, *Chlorella* is likely to play an important role in the future research of chitin deacetylases. It is possible that in the future some of these enzymes might be used for the in vitro production of chitosans with targeted characteristics[90].

Another topic that emerged from the discovery of chitosans in *Chlorella* that remains untapped is the function of these polymers in their lifecycle. This is especially relevant after the evidence gathered in this thesis project indicating that many resources of *Chlorella* are devoted to the production of chitins and chitosans. Only for the deacetylation of chitins it has been demonstrated that at least 19 different enzymes are being transcribed to RNA and at least 9 of them are present in active protein extracts. As Burczyk *et al.* pointed out, the chitinous-like wall may play a protective role and may contribute to their

resistance against factors which may be able to decompose the inner cell wall layer[23]. Blanc *et al.* went further with this thesis indicating that the chitinous material has been paramount in the success of *Chlorella* genus. They state that it has probably reached such a cosmopolitan distribution because most parasites failed to penetrate its chitinous cell wall[25]. At the same time, chitosans might also be involved in the sinking mechanism of this microalga as Durkin *et al.* suggest is the case for the chitin produced in diatoms[27]. This would allow changing the location once there are no nutrients in its surroundings. Finally, after reviewing the literature about the antimicrobial properties of chitosans, the low but higher than 10kDA Mw and low DA of *Chlorella* chitosans seem to be perfectly designed for this property[10]. Releasing chitosans and chitooligosaccharides when chlorovirus tries to digest the cell wall with its chitinases and chitosanasases to trespass could be a defense mechanism of *Chlorella*[127]. This would make sense taking into account that the activity of chitosans against plant viruses has been demonstrated[219]. If it was confirmed, the *Chlorella* cell wall would be something more than a simple tough barrier that is difficult to penetrate; it would be a functionalized barrier that has been carefully tailored to have the best antimicrobial properties along the millions of years of evolution of the *Chorella* genus. In all, whatever the reasons are for *Chlorella* to produce chitins and chitosans, understanding them is the best approach towards increasing chitosan production

Source of chitosans	Advantages	Disadvantages
Crustacean Shells[10]	Well established method for industrial production	Harsh chemical deacetylation affecting polymer quality
	Cheap raw material	Highly alkali effluents
	High concentration of chitins in the raw material	Demineralization treatment to eliminate $CaCO_3$
	High Mw chitosans reducible to any size	High temperatures
		Dependence on seasonal variations
		Random PA
		Not a natural product
		Immunogenic / medical devices (Class III)
		Immunogenic / poor marketing for cosmetics
Chlorella cell wall	Controlled conditions from generation to extraction (heavy metal control)	Not scaled up to industrial levels
	Mild acidic and alkali effluents	High raw material costs if it is not a biorefinary model
	Free of protein contaminations / allergic reactions	Low percentage of natural chitosans
	Vegetable origin	
	Non-Random PA	
	High reproducibility (expected)	
	Low polydispersity index	
	Mild acidic and alkali effluents	
	No seasonal variation	

Table 18 - Advantages and disadvantages of *Chlorella* chitosans in front of crustacean chitosans

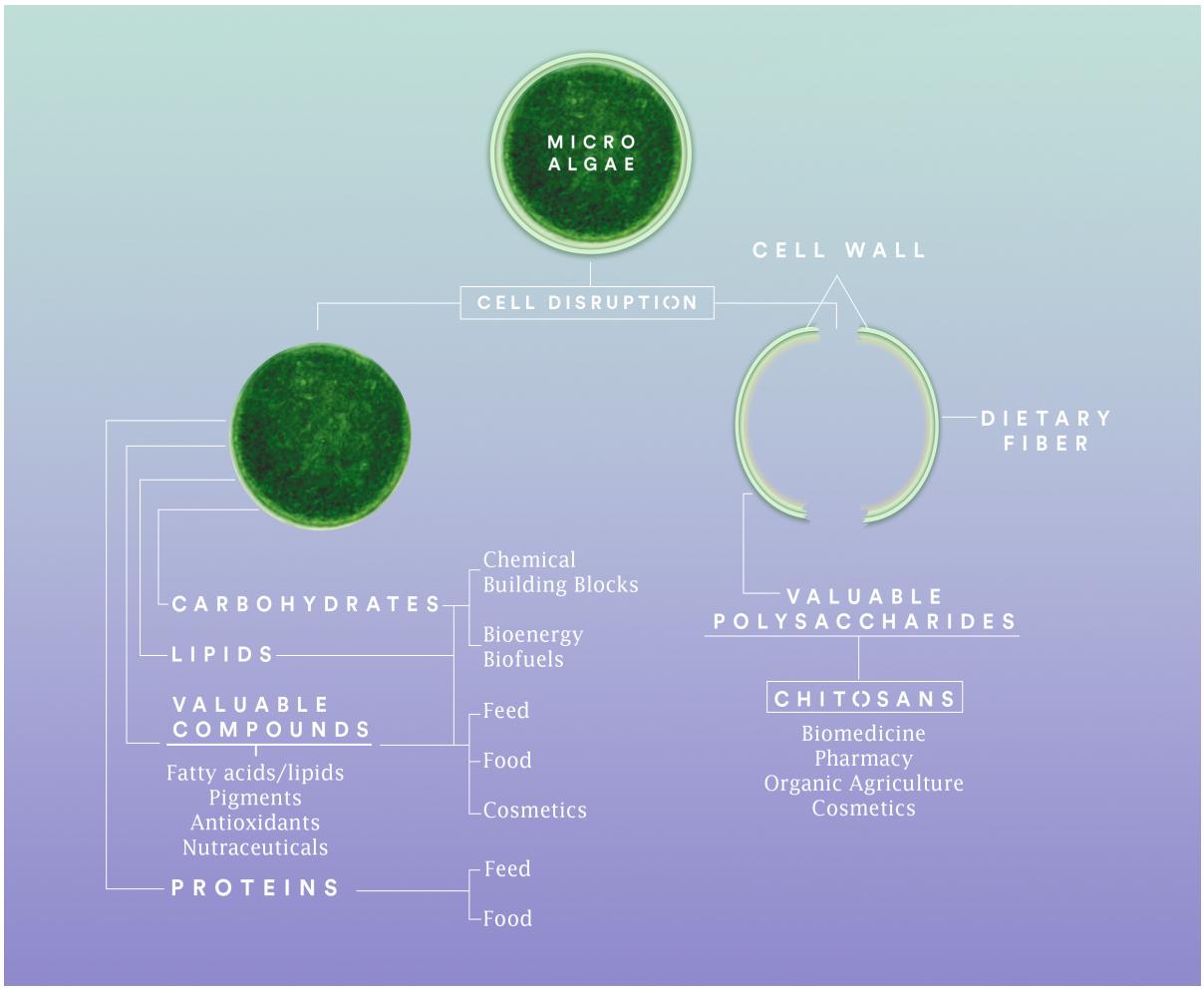

Figure 32 - Chitosan, a new product that can be obtained from the low value microalgal cell wal

6 CONCLUSIONS

i. A methodology was developed to screen microalgal cell walls for the presence of chitins and chitosans. This method, which can be used in a high-throughput fashion, is based on the specific recognition of chitins or chitosans by chitin binding proteins (CBPs) or chitosan affinity proteins (CAPs), Through the use of this technique, chitins and chitosans have been identified in *Chlorella*. Furthermore, according to the screening, it is also highly probable that *Scenedesmus* might produce chitins and/or chitosans and that *Thalassiosira* might produce chitosans.

ii. The screening method was validated using standard chitin and chitosan analysis techniques. The presence of chitosans in *Chlorella* cell wall fractions was corroborated by ^{1}HNMR, chitinase/chitosanase digestion and FTIR. The presence of chitins in other in *Chlorella* cell wall fractions was verified by glycosidic linkage analysis.

iii. A method to extract natural chitosans from *Chlorella* cell walls was developed. It consists of six subsequent steps: cell disruption, depigmentation / delipidation, deproteination, chitosan solubilization, chitosan washing and chitosan drying. This procedure allows extraction of enzymatically produced chitosans of clear color, low DA, low Mw and low Ip values.. The chitins and chitosans left in the cell wall, as a

residual fraction of the process, can also be extracted if a deacetylation step is carried out. The process to extract chitosans from *Chlorella* has been patented (Patent WO 2016/096986 A1).

iv. The chitosans extracted from *Chlorella* have been characterized. According to the ^{1}HNMR analyses performed so far, *Chlorella* chitosans have a low degree of acetylation, ranging from 5 to 25% depending on the strain, but mostly concentrated between 12 and 18%. According to HPSEC-MALLS-RI, the *Chlorella* chitosans analyzed thus far have a low molecular weight, which ranges from 20.5 to 34.6 kDA, depending on the strain. The same technique was useful to determine the Ip value, which was lowered up to 1.4 for natural chitosans extracted according to the method explained in conclusion III.

v. An initial study was performed to identify the enzymes behind the natural production of chitosans in *Chlorella variabilis NC64A*. .At least 19 out of the 22 putative CDA genes analyzed were proven to translate to RNA. From these, 9 putative CDAs were identified in sequenced extracellular protein extracts showing chitin deacetylase activity. This indicates that many enzymes are probably involved in the chitin deacetylation process in *Chlorella*. Out of these, cCDA7 (JGI code: 139978) is the only one that was found alone in a band with chitin deacetylase activity; therefore, it is the first CDA from *Chlorella* that has been proven to be active. The expression of putative CDAs from *Chlorella* in *E. coli* to analyze their properties has yet to be accomplished.

vi. A functional characterization of the chitosans from *Chlorella* was carried out. The ability of natural *Chlorella* chitosans to inhibit the growth of *E. coli, S. aureus* and *P. acnes* was demonstrated. Moreover, natural *Chlorella*-derived chitosans have shown to be successful in inducing keratinocyte proliferation and migration, thus showing potential for skin wound healing applications. Finally, natural and chemically deacetylated chitosans from *Chlorella* were shown to be capable of forming nanocapsules for drug delivery applications.

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8 APPENDICES

APPENDIX 1 – DETERMINATION OF CHITIN OR CHITOSAN PRESENCE USING CAPS AND CBPS

The following tables indicate the exact values obtained using the CAPs and CBPs assay as plotted in Figures 7 and 8.

Species	CAPs (Whole)	Stand. Dev. (whole)	CAPs (Disrupted)	Stand. Dev. (Disrupted)
M. circinelloides	6.76	-	5.94	-
C. reinhardtii	1.84	0.93	1.73	0.38
T. pseudonana	7.89	-	7.22	0.74
P. tricornutum	2.2	-	3.77	1.4
C. gracilis	1.84	-	2.13	0.51
N. gaditana	2.25	0.34	5.21	0.89
I. galbana	5.6	-	3.84	-
Chlorococcum sp.	1.1	-	1.95	-
D. salina	1.8	-	2.19	-
S. subspicatus	1.64	0.54	6.22	2.2
Bracteacoccus sp.	1.74	0.17	2.62	1.16
H. pluvialis	1.24	0.05	2.08	0.71
C. vulgaris	10.66	1.81	34.18	23.88
C. saccharophila	7.8	2.95	16.18	3.15

Table 19 – Fluorescence ratio obtained when using CAPs to detect chitosans in microalgae

Species	CBPs (Whole)	Stand. Dev (Whole)	CBPs (Disrupted)	Stand. Dev (Disrupted)
M. circinelloides	2.22	-	1.6	-
C. reinhardtii	2.53	0.56	2.02	0.31
T. pseudonana	14.2	-	9.51	0.84
P. tricornutum	1.77	-	3.11	1.17
C. gracilis	1.74	-	3.34	0.39
N. gaditana	2.5	0.43	4.28	0.43
I. galbana	2.36	-	1.77	-
Chlorococcum sp.	1.21	-	3.34	-
D. salina	1.96	-	1.44	-
S. subspicatus	2.11	0.39	22.57	3.07
Bracteacoccus sp.	4.28	0.06	10.57	4.75
H. pluvialis	2.48	0.24	9	4.77
C. vulgaris	2.38	1.09	15.24	11.24
C. saccharophila	5.02	3.22	17.29	1.8

Table 20 – Fluorescence ratio obtained when using CBPs to detect chitosans in microalgae

More precisely, values are a ratio between the fluorescence signal after incubation with CAPs/CBPs and the auto-fluorescence of each biomass. The standard deviation value given is for two biological replicates; in case there is no value it means that only one replicate was done. Each biological replicate is the average of three technical replicates

APPENDIX 2 – CULTURE MEDIA RECIPES

1 - FORMULATION OF BBM (BOLD'S BASAL MEDIUM) and MBBM (MODIFIED BOLD'S BASAL MEDIUM) (Bold 1949, Bischoff and Bold 1963)

(BBM for growing Chlorella, Scenedesmus, Chlorococcum and Bracteacoccus)

(MBBM for growing Chlorella variabilis NC64A)

· BBM Stock Solutions

1) 25.0 g $NaNO_3$ / L

2) 2.5 g $CaCl_2.2H_2O$ / L

3) 7.5 g $MgSO_4.7H_2O$ / L

4) 7.5 g K_2HPO_4 / L

5) 17.5 g KH_2PO_4 / L

6) 2.5 g NaCl / L

7) 50.0 g disodium EDTA, 31.0 g KOH / L

8) 4.98 g $FeSO_4.7H_2O$ per liter acidified H_2O (Acidified H_2O is 999.0 mL d-H_2O + 1.0 mL concentrated H_2SO_4)

9) 11.42 g H_3BO_3 / L

10) 8.82 g $ZnSO_4.7H_2O$, 1.44 g $MnCl_2.4H_2O$, 0.71 g MoO_3, 1.57 g $CuSO_4.5H_2O$, and 0.49 g $Co(NO_3)_2.6H_2O$ per liter d-H_2O

Note: Stock solution 10 takes weeks for all of the salts to dissolve. Use as suspension until then.

· BBM Preparation

to 950 mL of d-H_2O add:

10.0 mL of stock solutions 1, 2, 3, 4, 5 and 6

1.0 mL of stock solutions 7, 8 and 9

2.0 mL of stock solution 10

· MBBM Preparation

to 950 mL of d-H_2O add:

- 10.0 mL of stock solutions 1, 2, 3, 4, 5 and 6

- 1.0 mL of stock solutions 7, 8 and 9

- 2.0 mL of stock solution 10

- 1.0 gm of bacto-peptone

- 5.0 gm of sucrose

· Tetracycline (filter sterilized, 10 μg/mL final concentration) is added after the media is autoclaved and cool.

· For MBBM plates, agar is added to 1.5% before autoclaving

· For MBBM soft agar (for tittering), agar is added to 0.75% before autoclaving.

- Level to 1 L with deionized water

2 - FORMULATION OF DUNALIELLA MEDIUM (Zhi-wey et al 2010)

(For growing *Dunaliella*)

· Dunaliella Stock Solutions

1) 25.0 g $NaNO_3$ / L

2) 3.9 g $NaH_2 PO_4.2H_2O$ / L

3) 7.5 g $MgSO_4.7H_2O$ / L

4) 3.67 g KCl /L

5) 10.5 g $NaHCO_3$/L

6) 2.5 g $CaCl_2.2H_2O$ / L

7) Fe salting:

0.15g $Na_2EDTA \cdot 2H_2O$ / L

0.24g $FeCl_3 \cdot 6H_2O$ / L

8) 11.42 g H_3BO_3 / L

9) 79 g $CuSO_4 \cdot 7H_2O$ /L

10) STOCK SOLUTION H5-BG11

	(For 1L)
$ZnSO_4 \cdot 7H_2O$	0.44 g
$MnCl_2 \cdot 4H_2O$	3.62 g
$Na_2MoO_4 \cdot 2H_2O$	0.782 g
$CuSO_4 \cdot 5H_2O$ Stock solution [79 g/l]	2 ml
$Co(NO_3)_2 \cdot 6H_2O$ Stock Solution [49.4 g/l]	2 ml
HBO Solution	500 ml

· Dunaliella medium Preparation

to 500 mL of d-H_2O add the ingredients as indicated in the following table

	1 l
NaCl	87.66 g
$NaNO_3$ [25 g/l]	16.8 ml
$NaH_2PO_4.2H_2O$ [3.9 g/l]	4 ml
$MgSO_4.7H_2O$ [7.5 g/l]	164 ml
KCl [3.67 g/l]	20 ml
$NaHCO_3$ [20 g/l]	80 ml
$CaCl_2.2H_2O$ [2.5 g/l]	17.6 ml
*Fe-Salting liquid	1 ml
H5-BG11	1 ml

*Must be sterilized by filtration and be added to the medium after autoclaving it.

- Adjust to pH 7.5

- Level to 1 L with deionized water

- To prepare this medium solidified in agar 15 g/l of agar should be added prior autoclaving

3 - FORMULATION OF TAP MEDIUM

(For growing *Chlamydomonas*)

· TAP Stock Solutions

1) TAP salts

	(For 1L)
NH_4Cl	15 g
$MgSO_4.7H_2O$	4 g
$CaCl_2.2H_2O$	2 g

2) TAP Phosphate

	100 ml	50 ml	200 ml	500 ml
$K_2HPO_4.3H_2O$	3.77 g	1.885 g	7.54 g	18.85 g
KH_2PO_4	1.44 g	0.72 g	2.88 g	7.2 g

3) Hutners Trace Elements*

	1 L	500 ml	200 ml	100 ml
$ZnSO_4.7H_2O$	22 g	11 g	4.4 g	2.2 g
H_3BO_3	11.4 g	5.7 g	2.28 g	1.14 g
$MnCl_2.4H_2O$	5.06 g	2.53 g	1.012 g	0.506 g
$CoCl_2.6H_2O$	1.61 g	0.805 g	0.322 g	0.161 g
$CuSO_4.5H_2O$	1.57 g	0.785 g	0.314 g	0.157 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.10 g	0.55 g	0.22 g	0.11 g
$FeSO_4.7H_2O$	4.99 g	2.495 g	0.998 g	0.499 g
EDTA disodium Salt dihydrate	50 g	25 g	10 g	5 g

* EDTA is added last at 70ºC. The solution must be left for two weeks agitating it once a day before using it. The final solution is purple and has a yellowish-brown precipitate that must be eliminated by filtration.

·TAP medium Preparation

- to 900 mL of d-H_2O add the ingredients as indicated in the following table

	(For 1 L)
Tris	2.42 g
TAP salts	25 ml
TAP phosphate	3.75 ml
Hutner's trace elements	1ml
Glacial acetic acid	1ml

-Adjust to pH 7.0

- Level to 1 L with deionized water

- To prepare this medium solidified in agar 15 g/l of agar should be added prior autoclaving

4 - FORMULATION OF F/2 MEDIUM

(Guillard and Ryther 1962, Guillard 1975. Handbook of microalgae.)

(for Nannochloropsis)

(F/2x2 for Thalassiosira, Chaetoceros, Phaeodactylum, Isochryisis and Scenedesmus)

· F/2 Stock Solutions

1) 75.0 g $NaNO_3$ / L

2) 5 g $NaH_2PO_4.2H_2O$ / L

3) 30 g/l $Na_2SiO_3 \cdot 9H_2O$*

*Silicates can be omitted if the strain does not require them

4) Trace metals solution

For 1 L of water dissolve:

3.15 g $FeCl_3 \cdot 6H_2O$

4.36 g $Na_2EDTA \cdot 2H_2O$

1 ml $MnCl_2.4H_2O$ [180g/l]

1 ml $ZnSO_4 \cdot 7H_2O$ [22g/l]

1 ml $CoCl_2 \cdot 6H_2O$ [10g/l]

1 ml $CuSO_4 \cdot 5H_2O$ [9.8g/l]

1 ml $Na_2MoO4 \cdot 2H_2O$ [6.3g/l]

5) Vitamins solutions

For 1 L of water dissolve:

200 mg Thiamine· HCl (Vitamin B_1)

20µl Biotin (Vitamin H 50g/l)

40µl Cyanocobalamin (Vitamin B_{12} 25g/l)

·F/2 medium Preparation

In 500 mL of filtered sea water:

	1L	500 ml	2L	5L
$NaNO_3$ [75g/l]	1 ml	1.5 ml	6 ml	15 ml
$NaH_2PO_4.H_2O$ [5g/l]	1 ml	400 ml	1.6 ml	4 ml
*$Na_2SiO_3.9H_2O$ [30g/l]	1 ml	263 ml	1.052 ml	2.63 ml
*Trace metals solution	1 ml	0.5 ml	2 ml	5 ml
*Vitamins solution	0.5 ml	0.25 ml	1 ml	2.5 ml

- Adjust to pH 7.0

- Level to 1 L with deionized water

- In case it brings silicates it cannot be autoclaved it has to be filter sterilized

5 - FORMULATION OF KUHL MEDIUM

(for Haematococcus)

· Kuhl stock solutions

1) Solution A

101.1 g/L KNO_3

1.47 g/L $CaCl_2.2H_2O$

2) Solution B

24.64 g/L $MgSO_4.7H_2O$

3) Solution C

62.1 g/L $NaH_2PO_4.H_2O$

8.9 g $Na_2HPO_4.2H_2O$

4) Solution D: K micronutrients solution

	For 1 L
H_3BO_3	61 mg
$MnSO_4 \cdot H_2O$	169 mg
$ZnSO_4 \cdot 7H_2O$	287 mg
$CuSO_4 \cdot 5H_2O$	2.5 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	12.4 mg

5) Solution E: (FE-EDTA complex)

In 800 mL:

6.9 g /L $FeSO_4.7H_2O$

9.3 g/L Na_2 - EDTA.

Heat up to boiling temperature. Bring to 1L with water

· Kuhl medium Preparation

In 800mL:

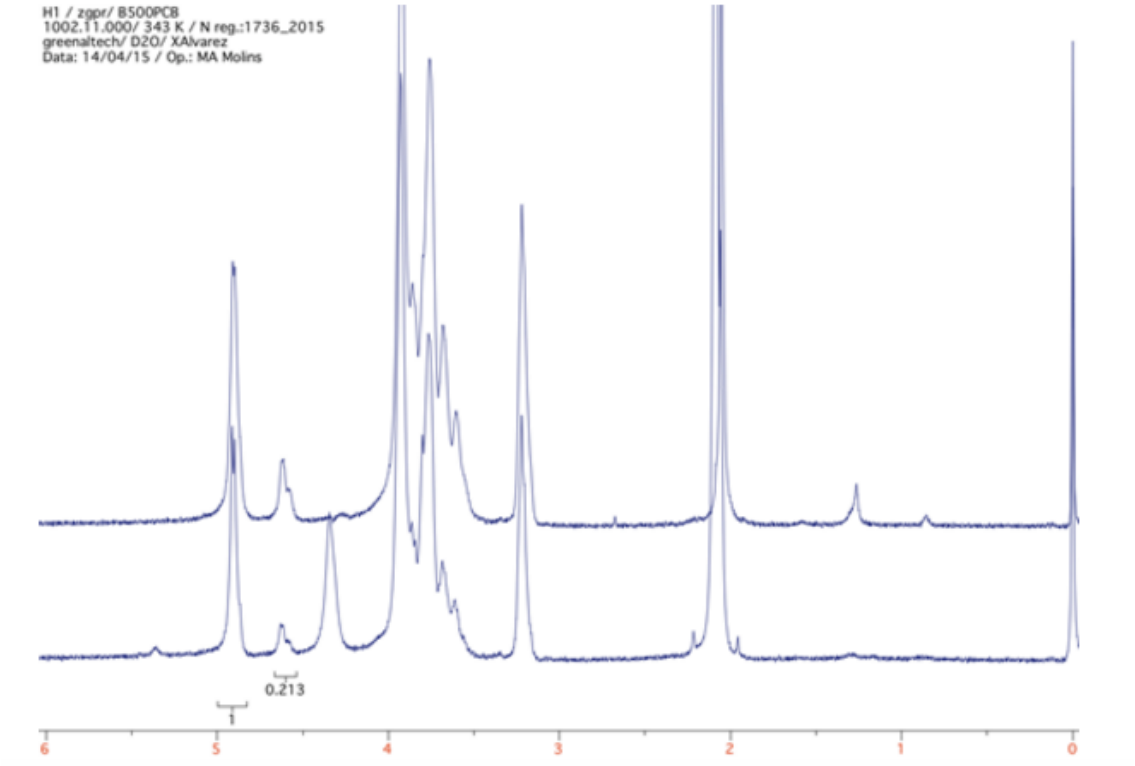
	For 1 L
SOLUCIÓ A	10ml
SOLUCIÓ B	10ml
SOLUCIÓ C	10ml
SOLUCIÓ D (K micronutrients)	1ml
SOLUCIÓ E (Fe-EDTA complex))	1 ml

- Level to 1 L with deionized water and autoclave.

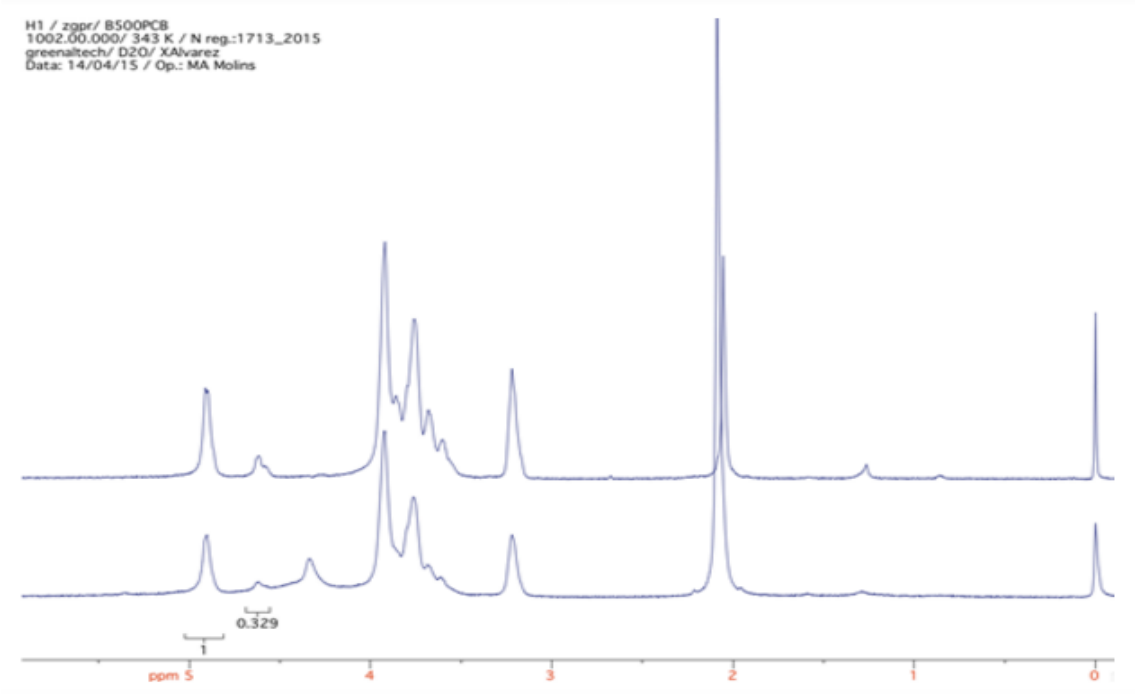
APPENDIX 3 – ^{1}H-NMR SPECTRA OF ALL THE MICROALGAL SPECIES SCREENED LOOKING FOR THE PRESENCE OF CHITOSANS

In case there are two spectra, the spectrum on top if that of Sigmal Aldrich (ref. 448869) and the spectrum below is that of microalgae in different cases.

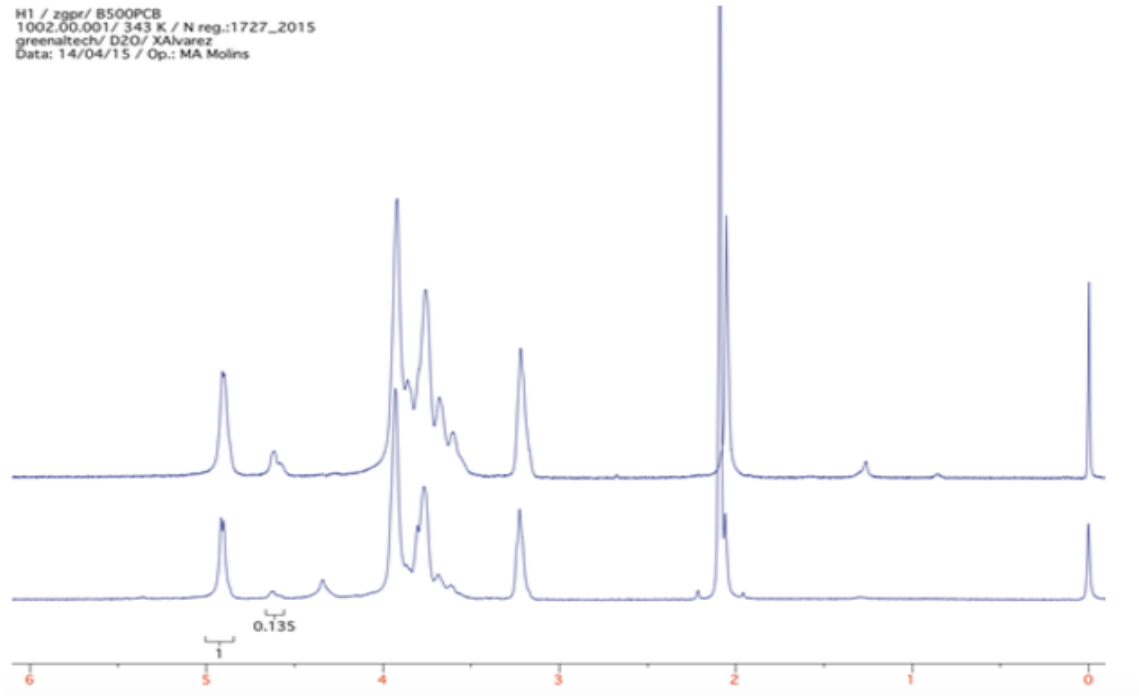
C. sp GAT-7



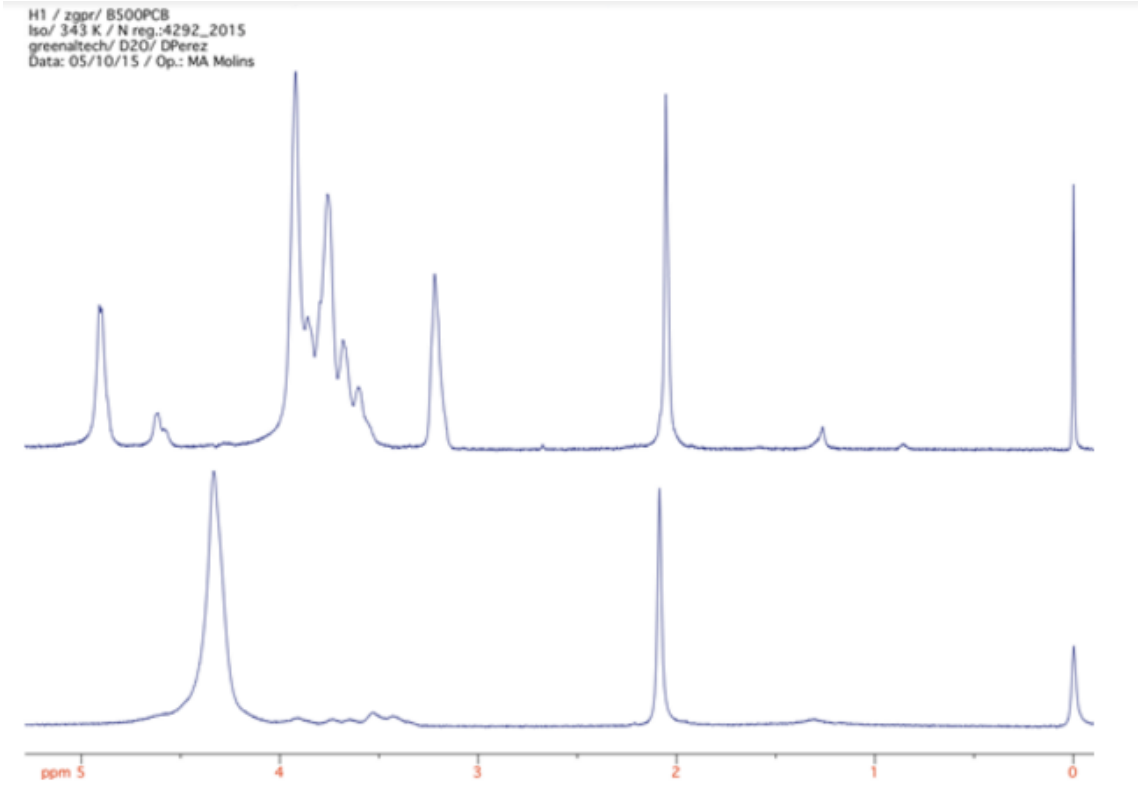
C. vulgaris H1993



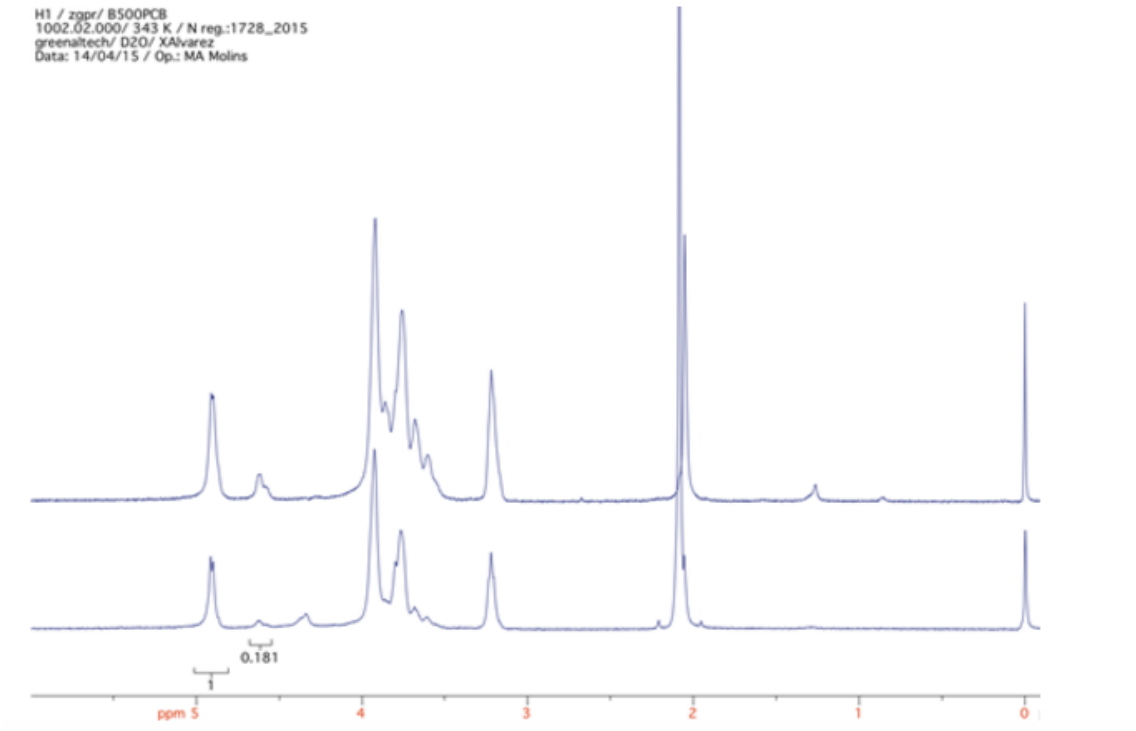
C. vulgaris CS-41



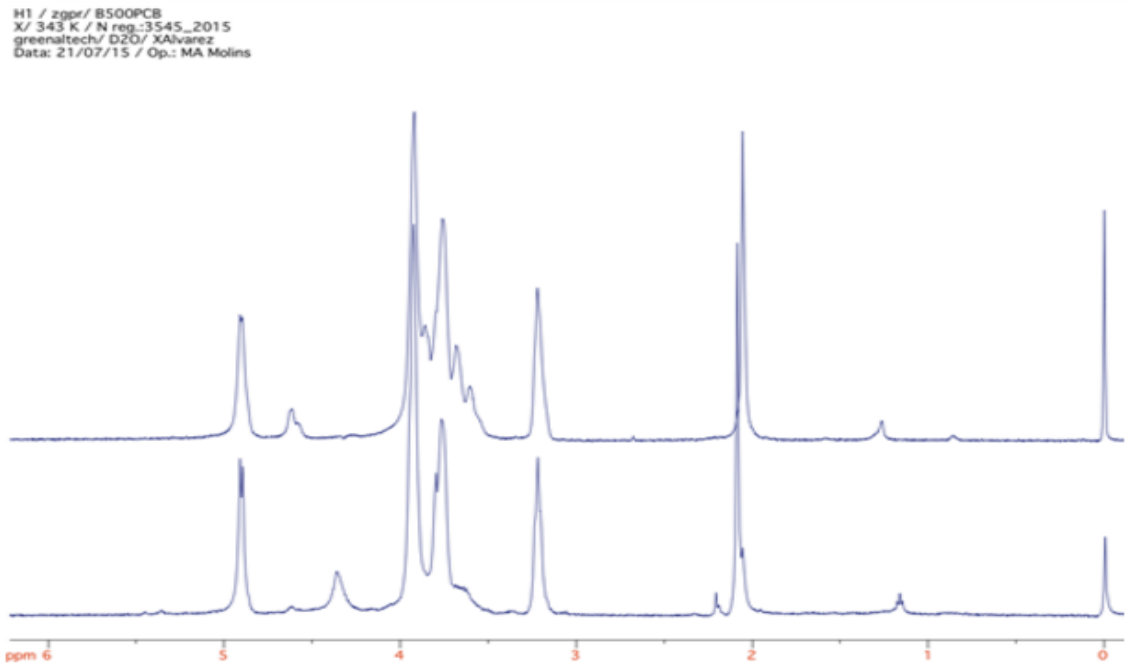
I. galbana L2307



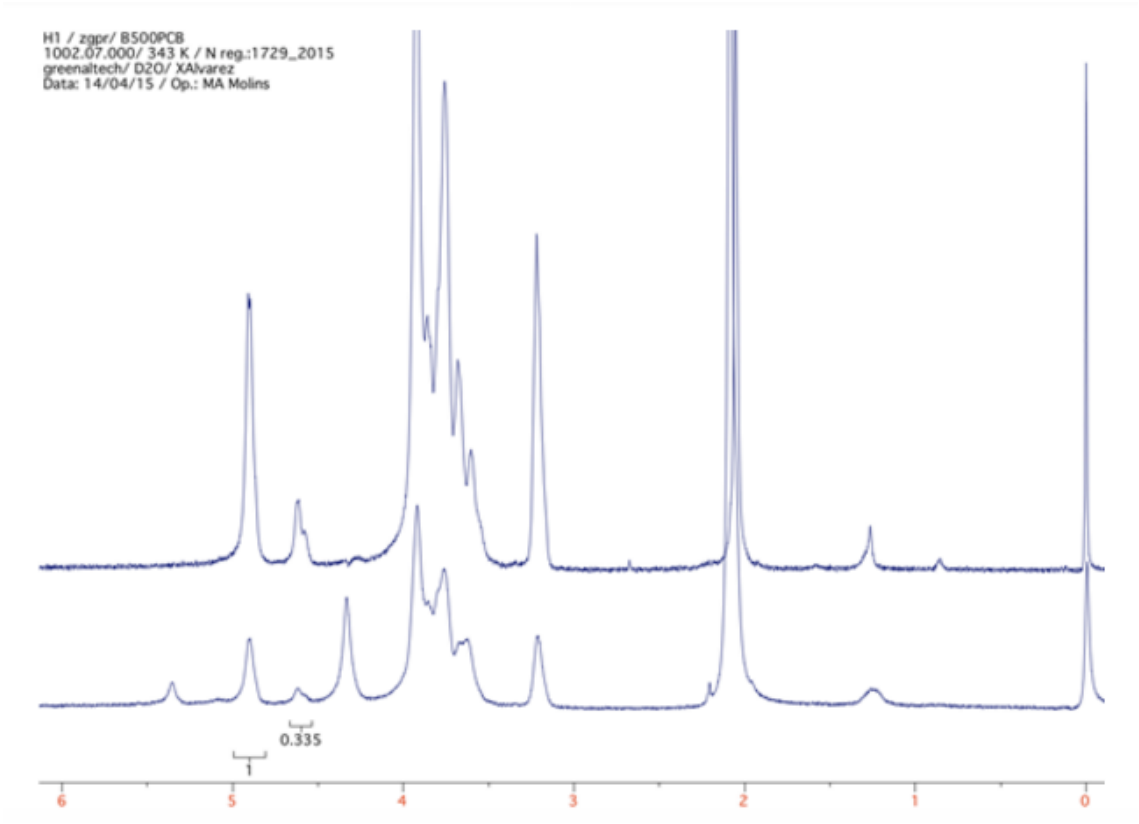
C. saccharophila 211/9A



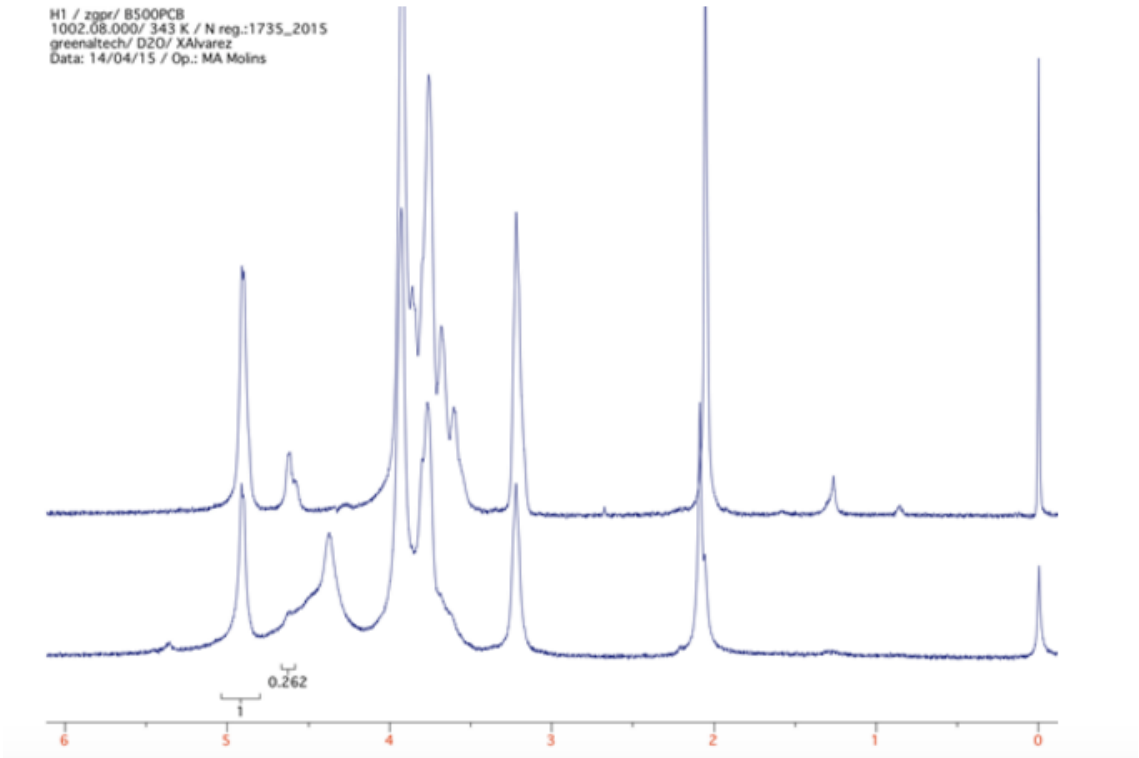
C. sp. OP



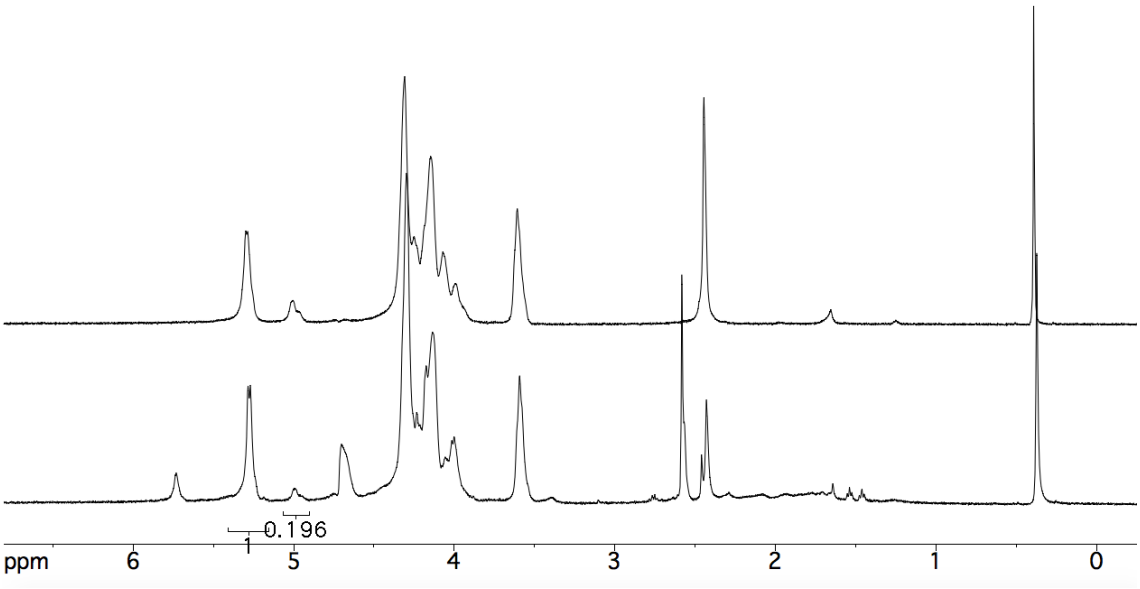
C. sp. GAT- 3



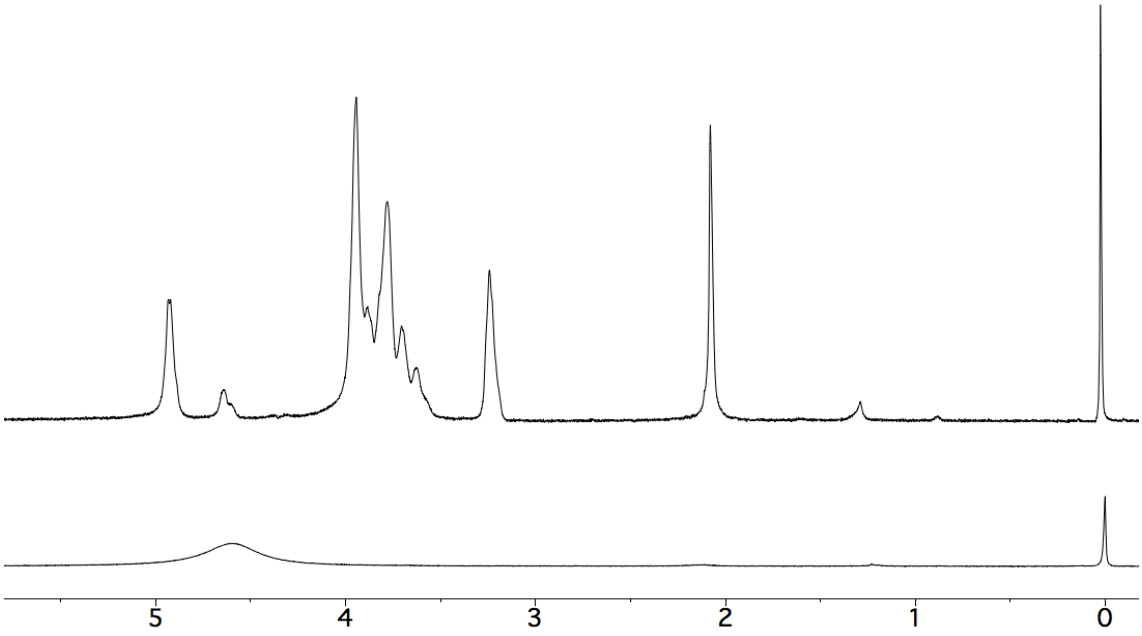
C. sp. GAT- 4



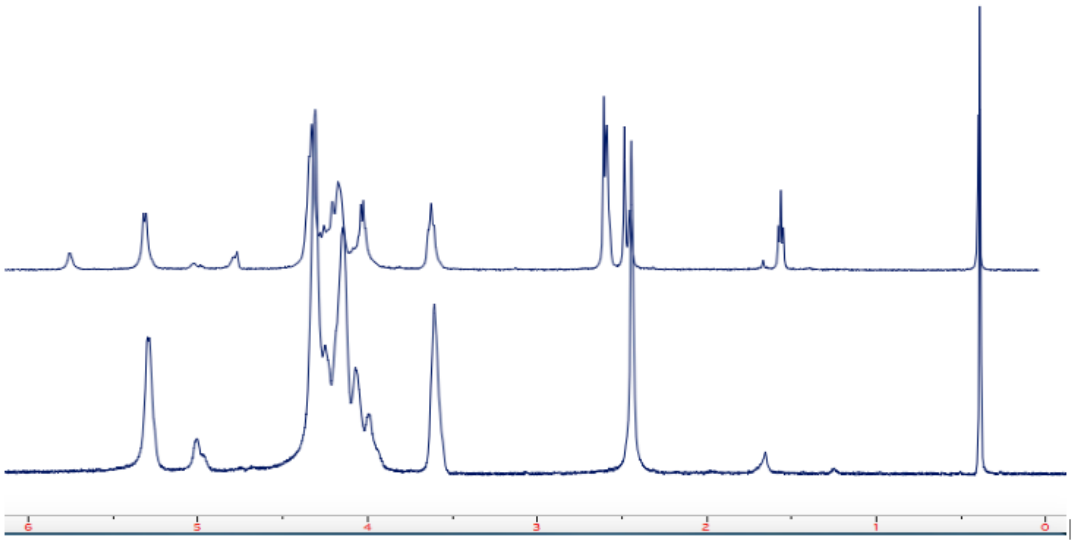
C. zofingiensis B32



T. pseudonana LBFD2

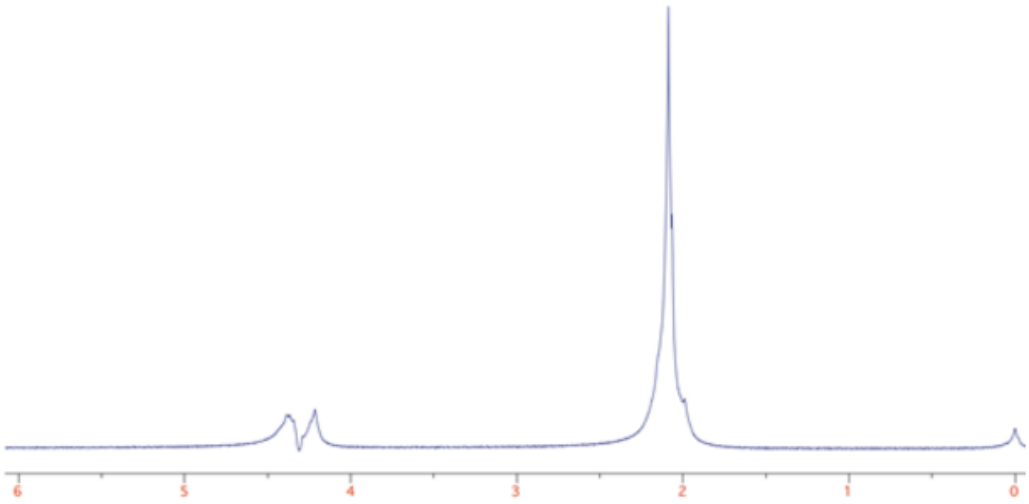


C. variabilis NC64A

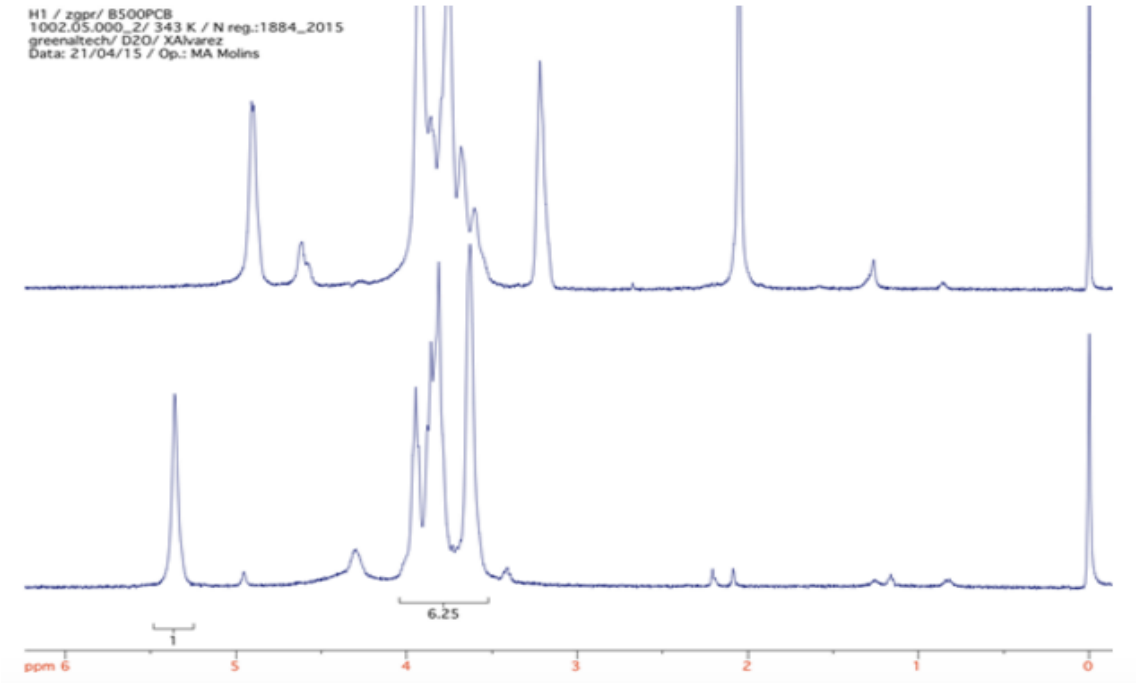


Bottom SIAL / TOP chitosan NC64A

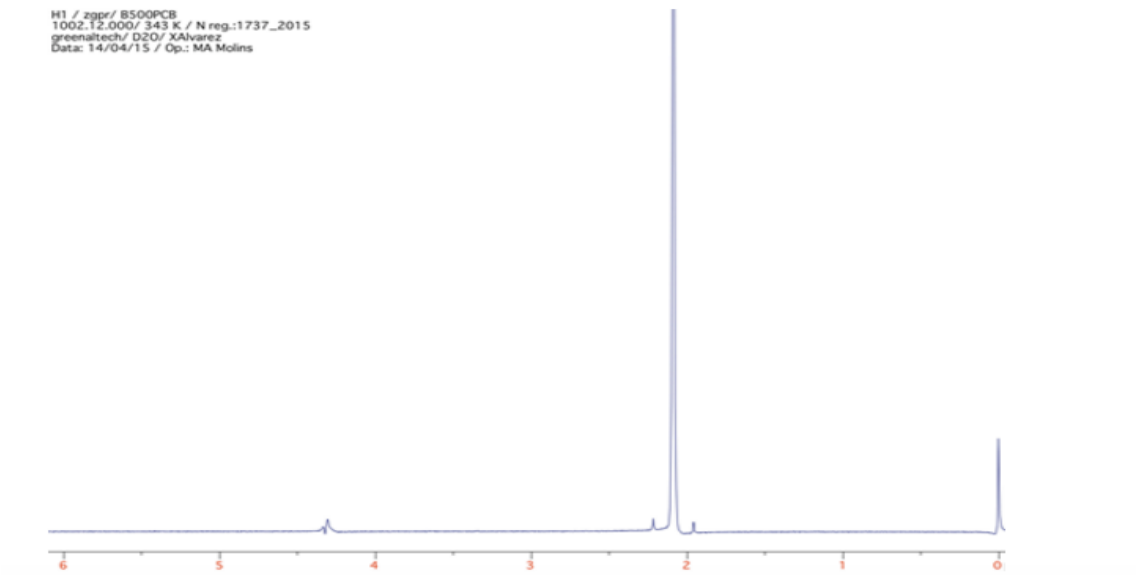
S. sp. GAT - 9



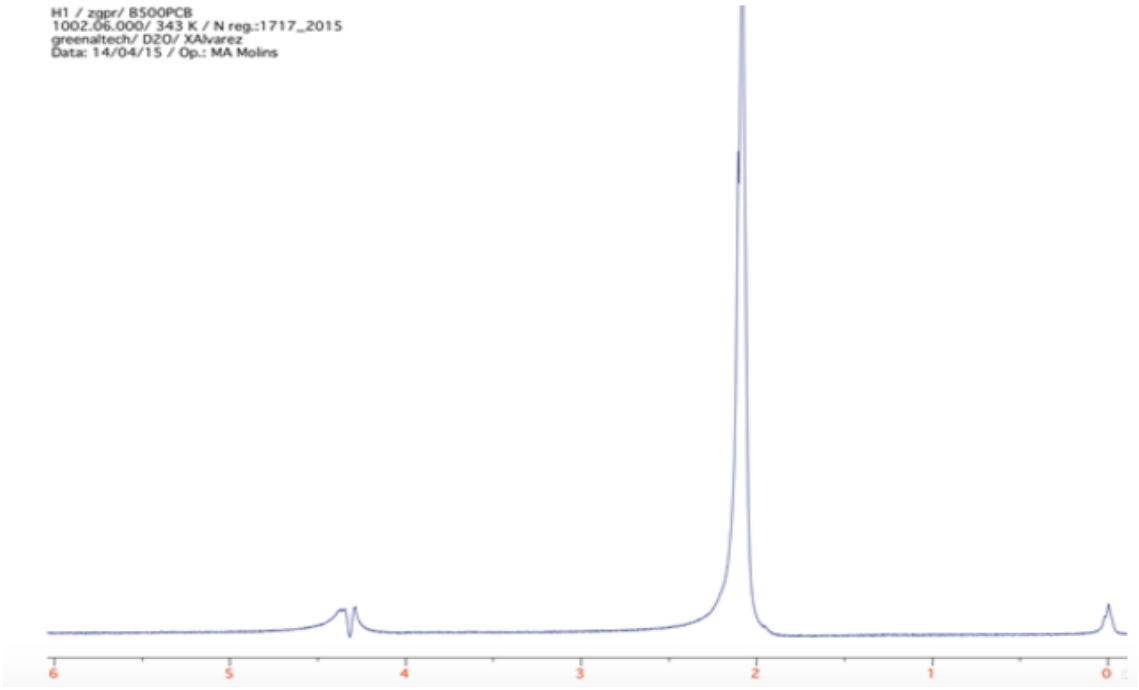
C. sp. GAT - 1



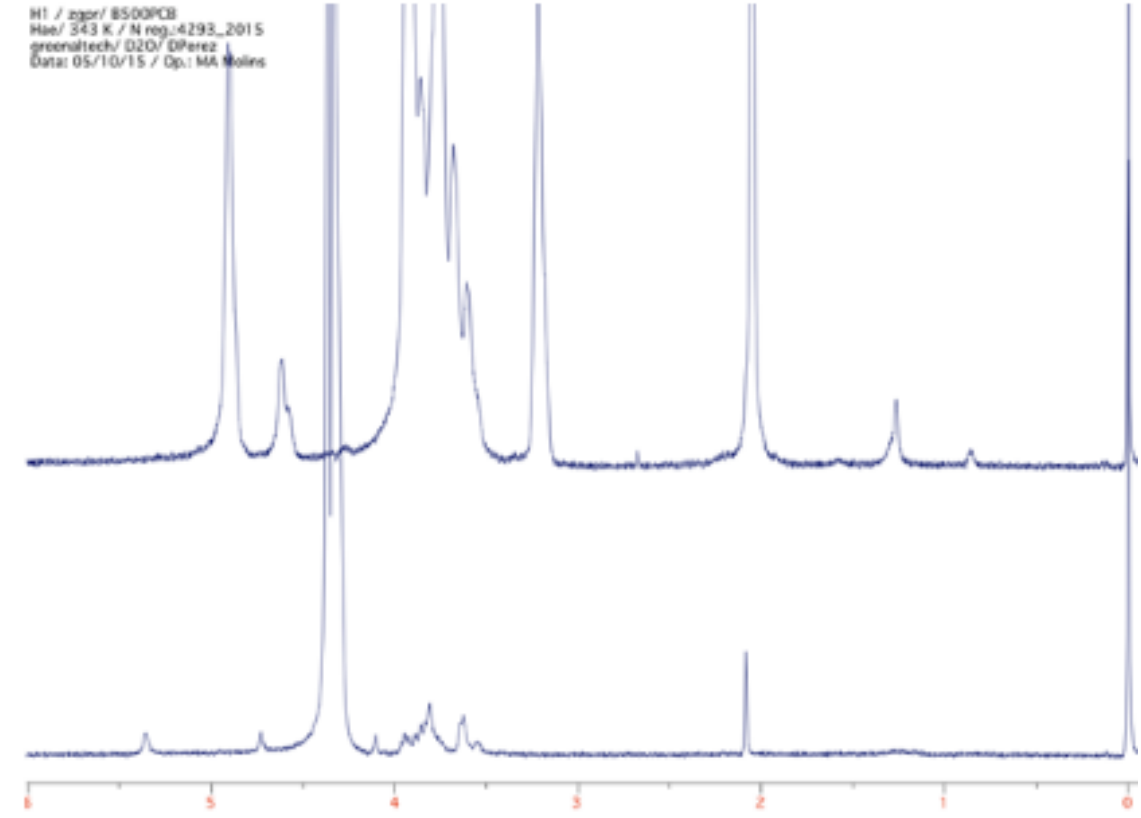
C. zofingiensis B32



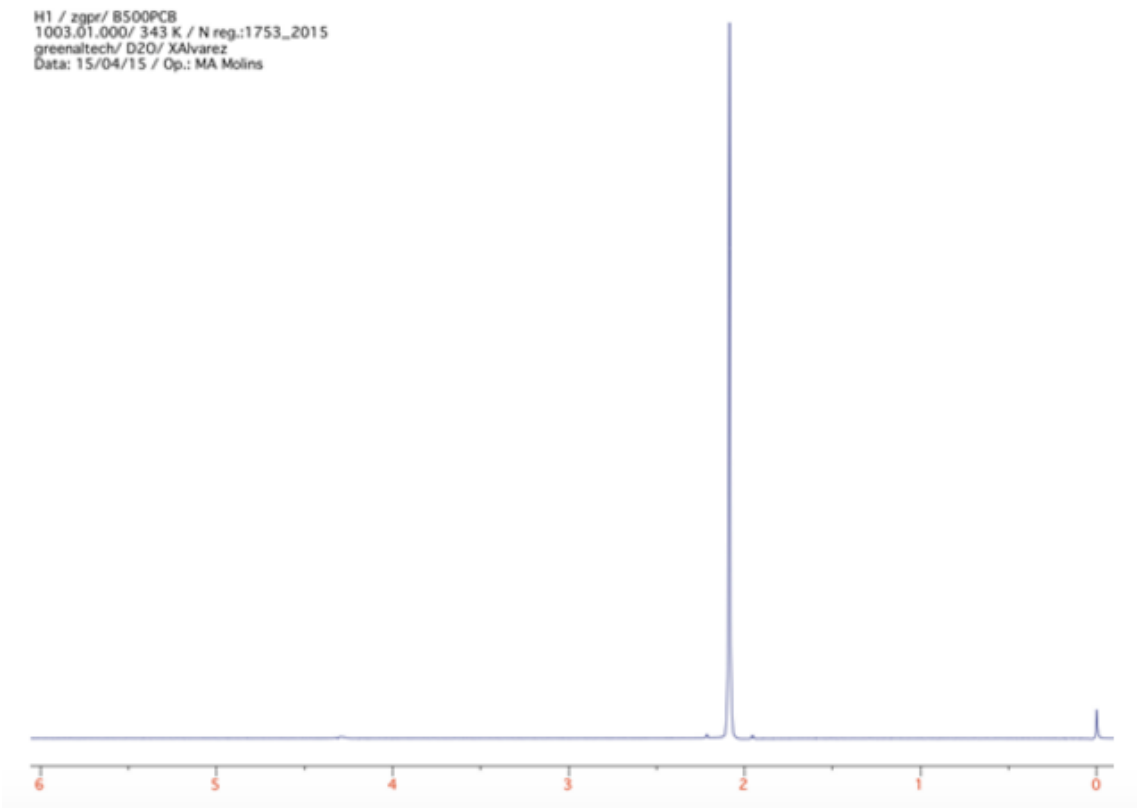
C. sp. GAT - 2



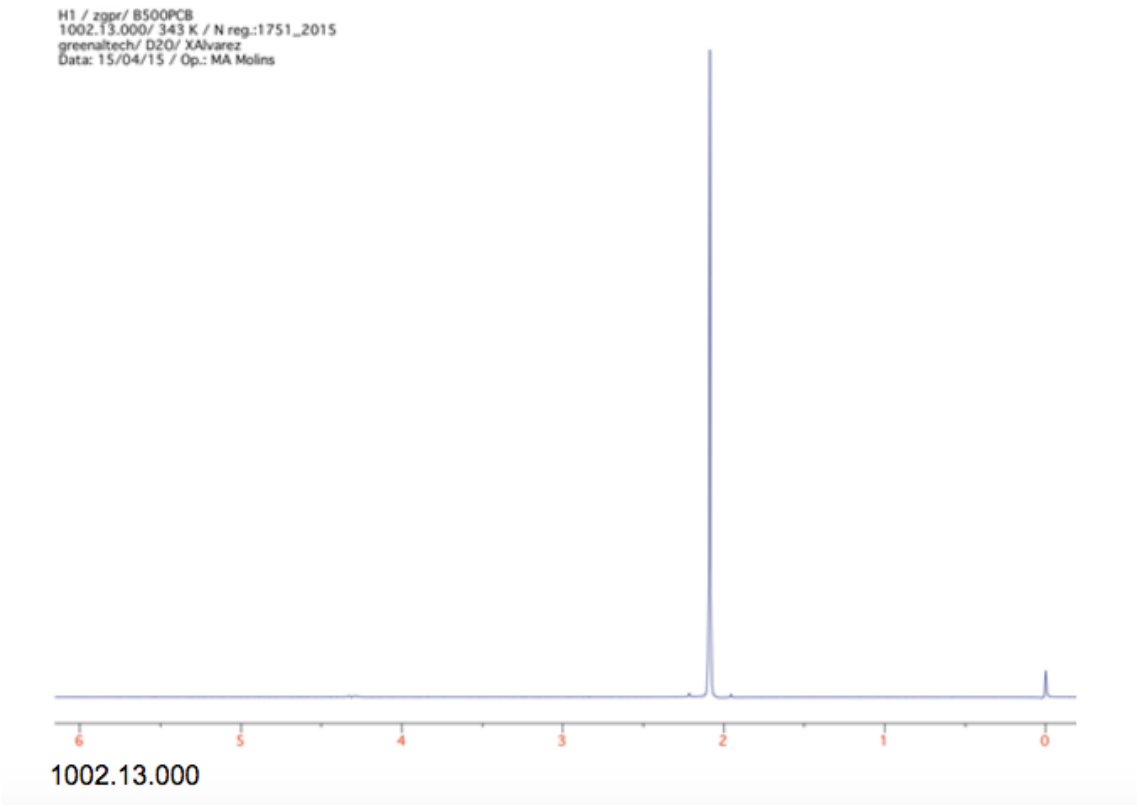
H. pluvialis K0084



S. subspicatus AC.139

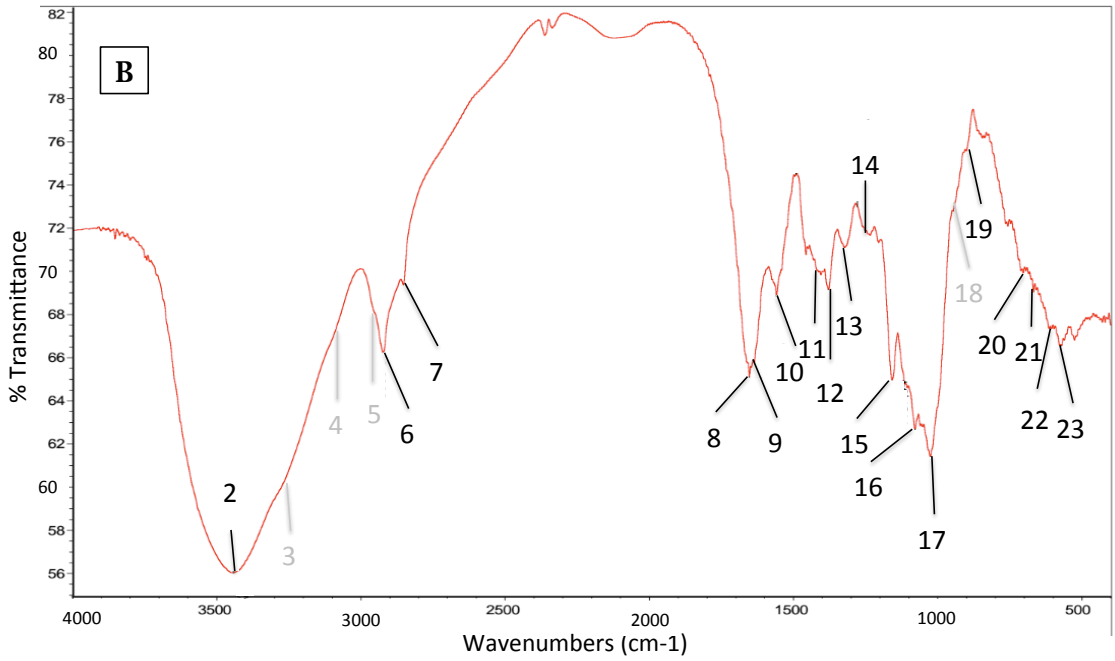


C. sorokiniana 2805

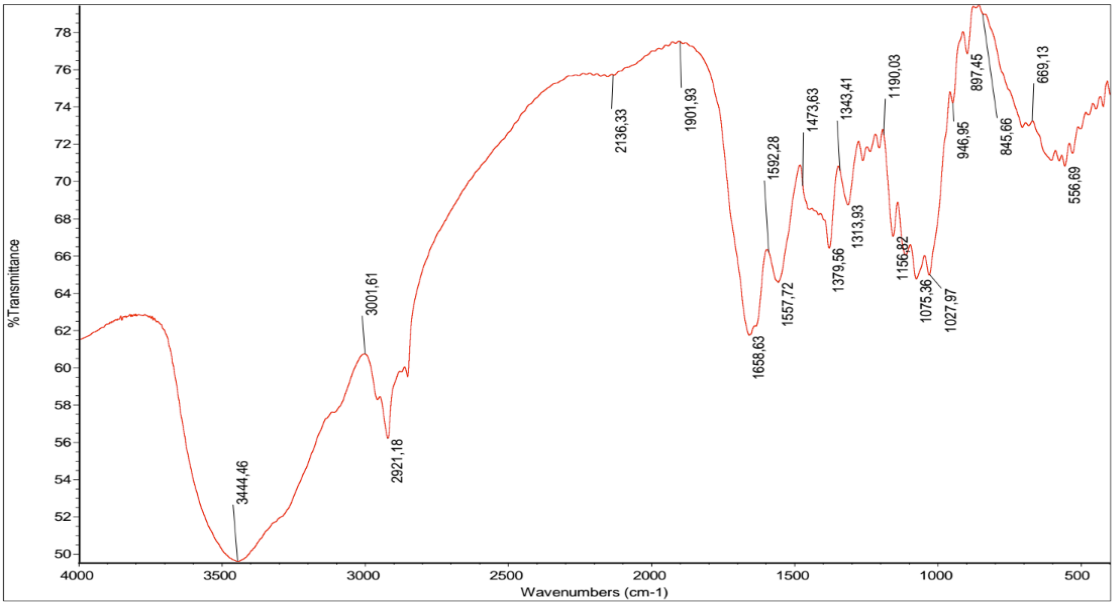


APPENDIX 4 – FTIR SPECTRA OF ALL THE MICROALGAL SPECIES SCREENED LOOKING FOR THE PRESENCE OF CHITINS AND CHITOSANS

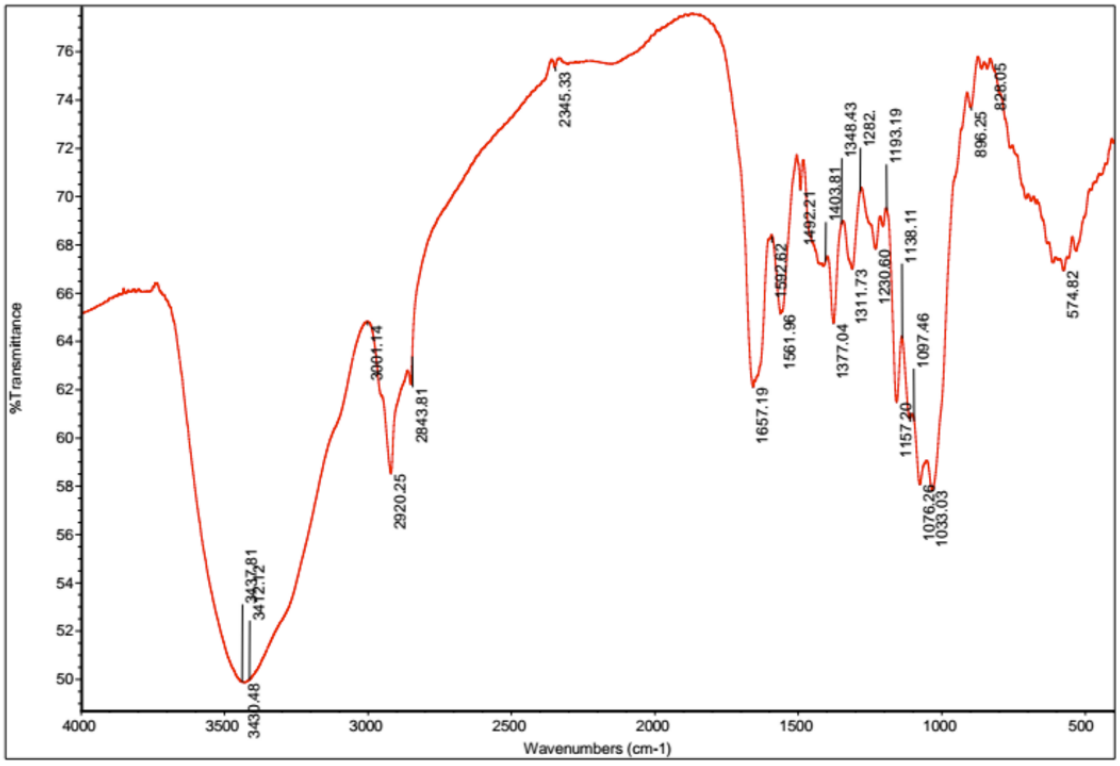
C. sp GAT-7



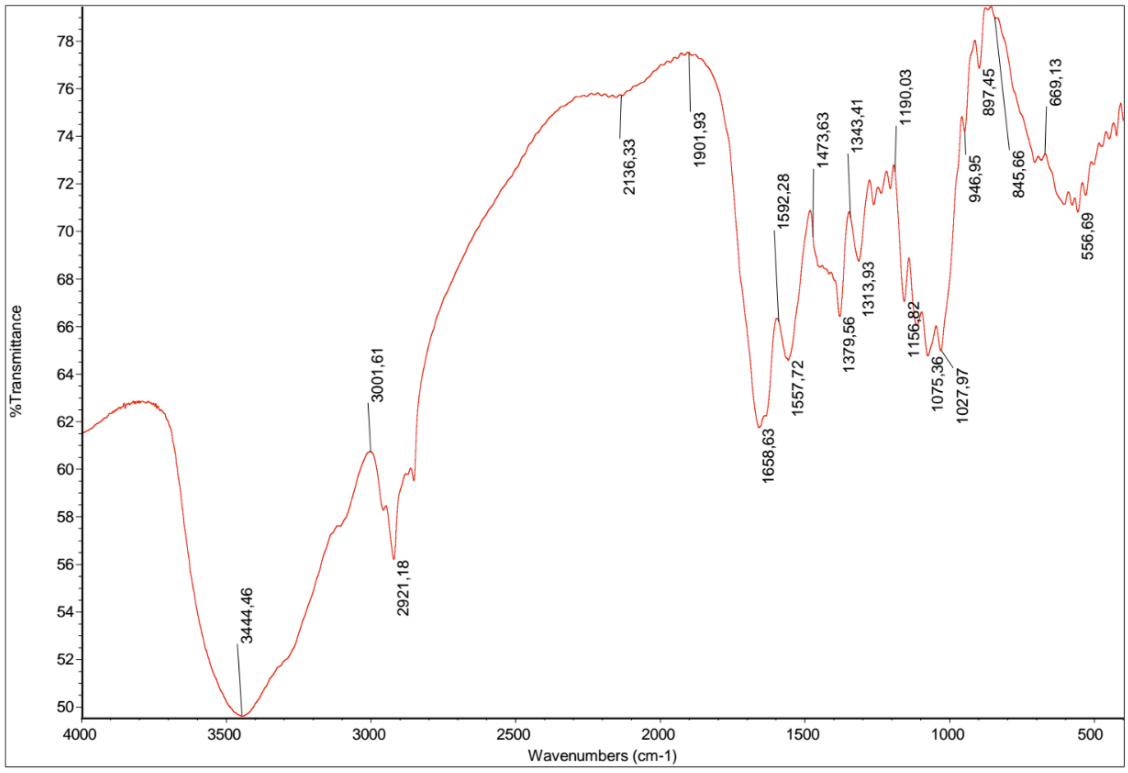
C. vulgaris H1993



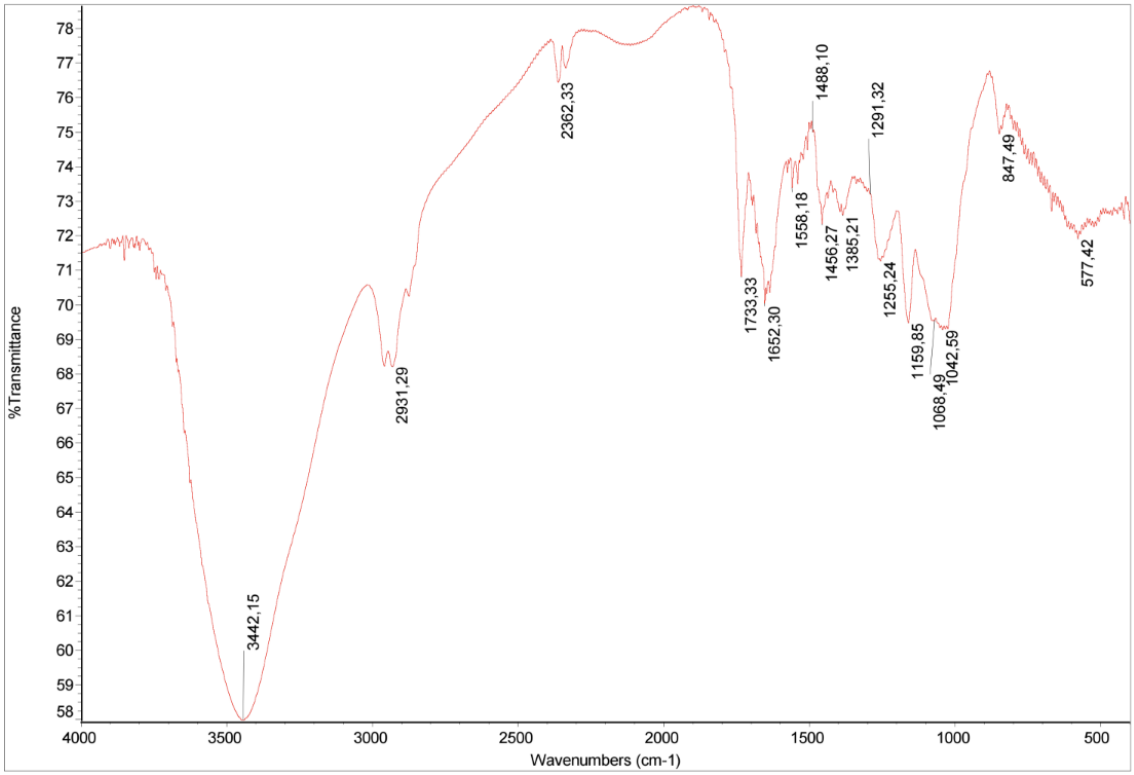
C. vulgaris CS41



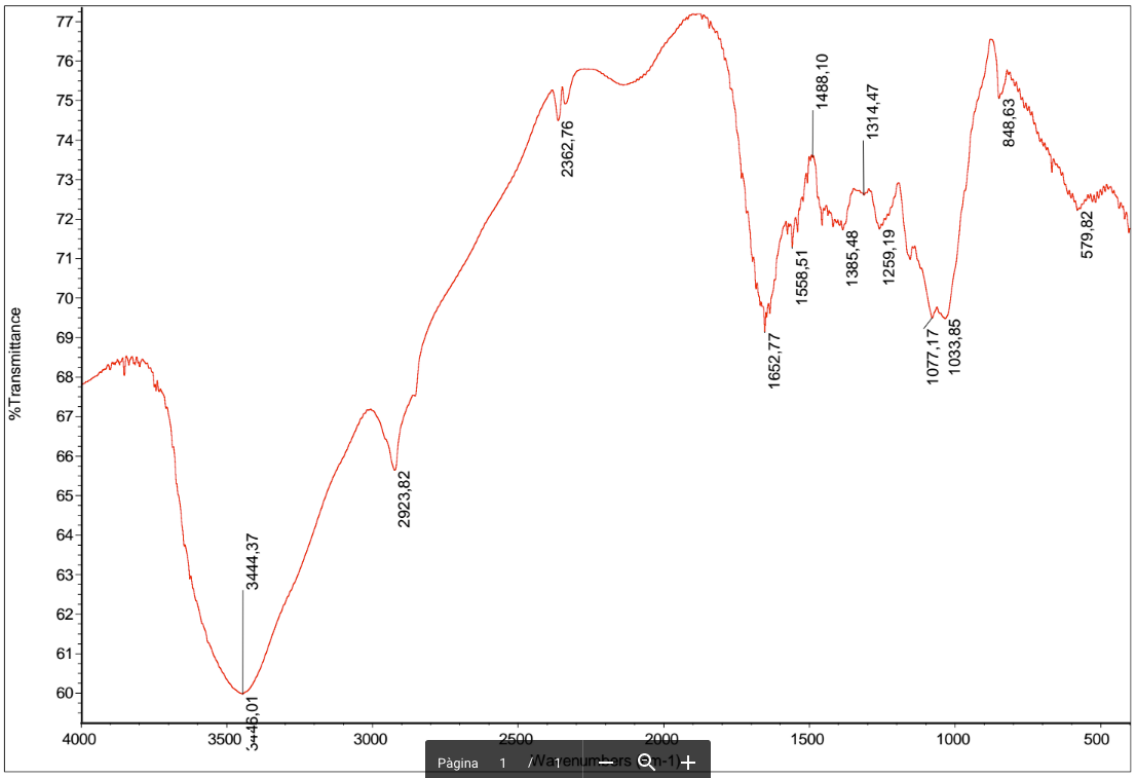
C. saccharophila 211/9A



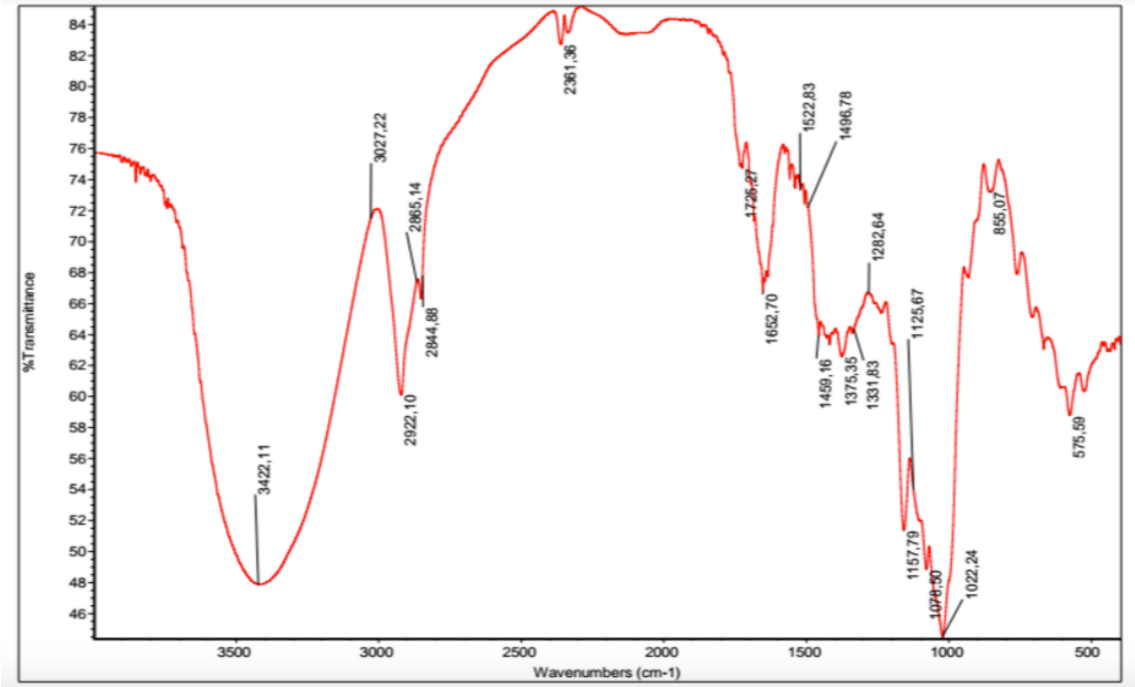
C. sp. GAT-3



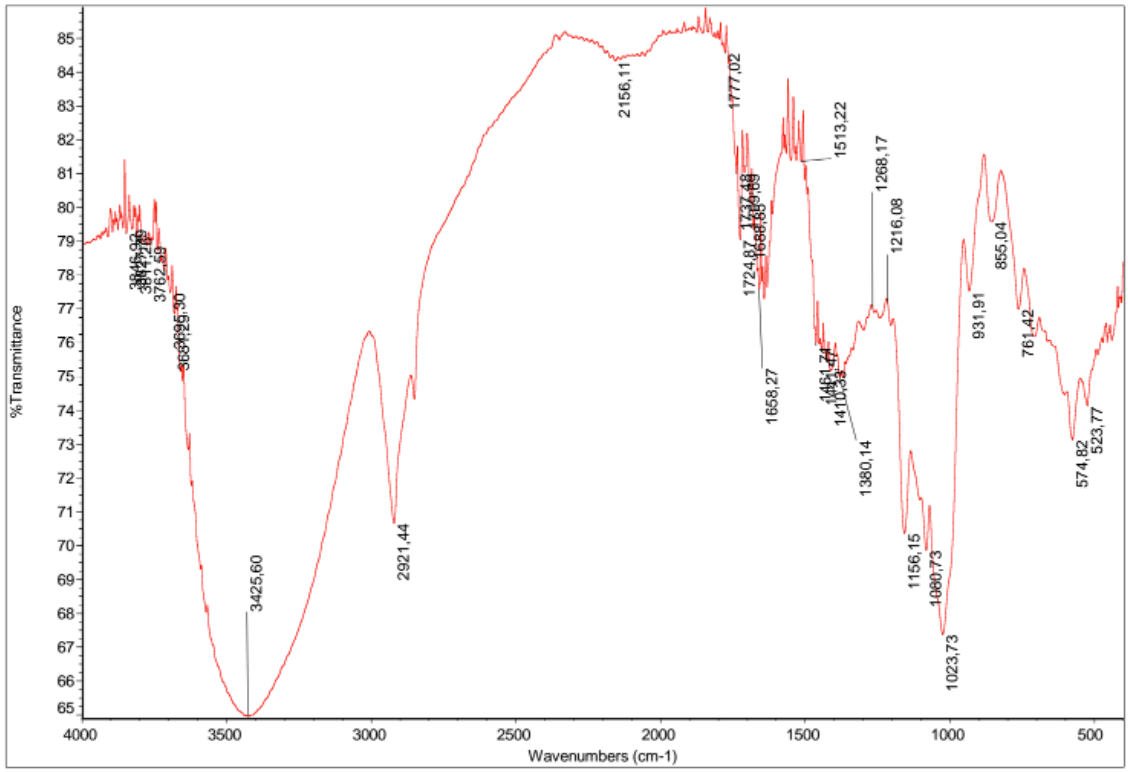
C. sp. GAT-4



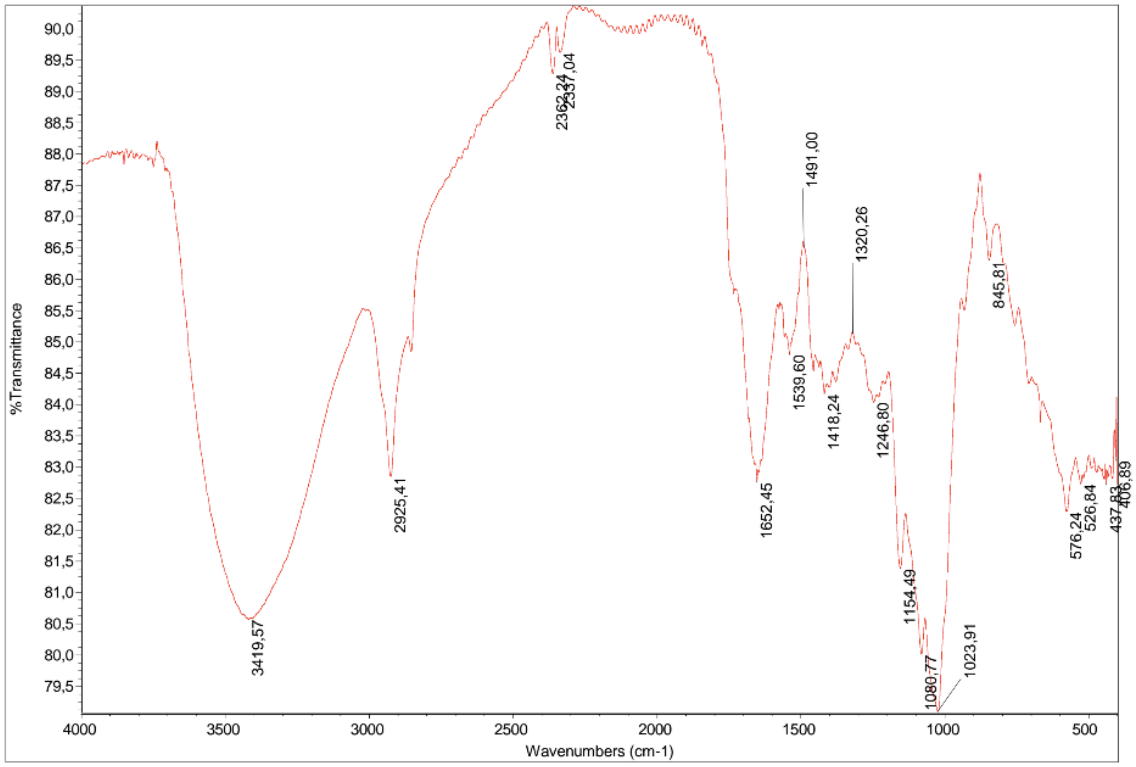
S. sp. GAT - 8



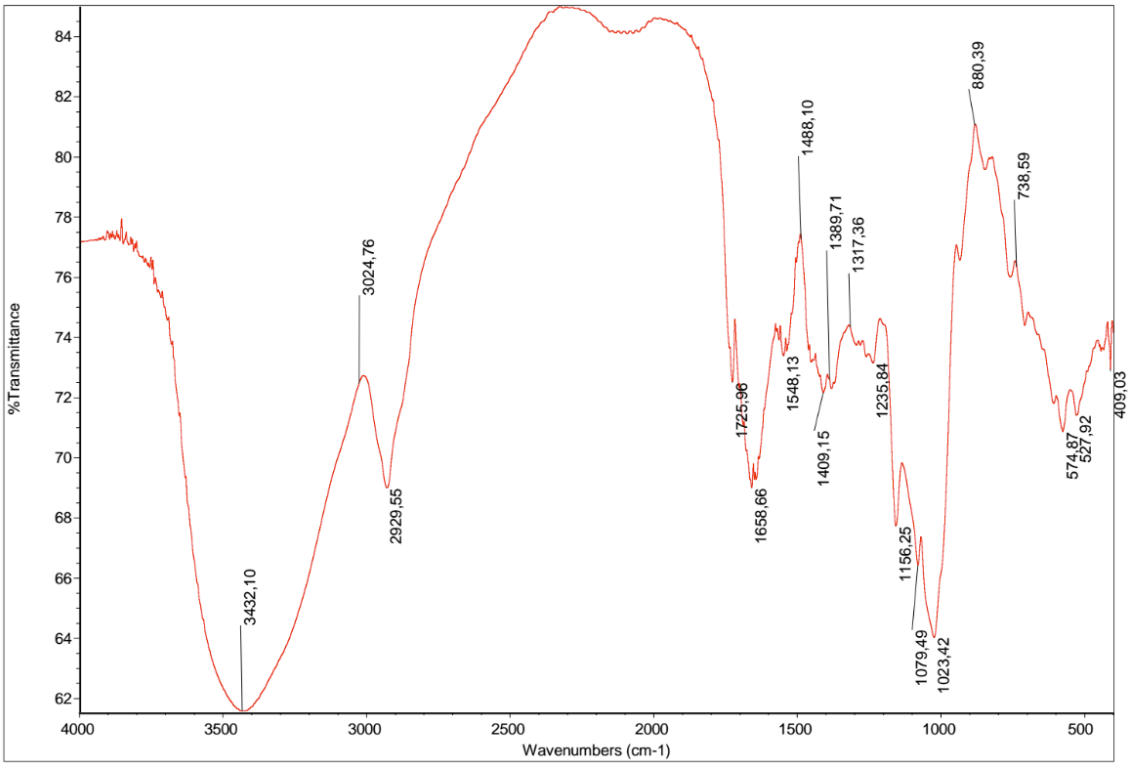
S. sp. GAT - 9



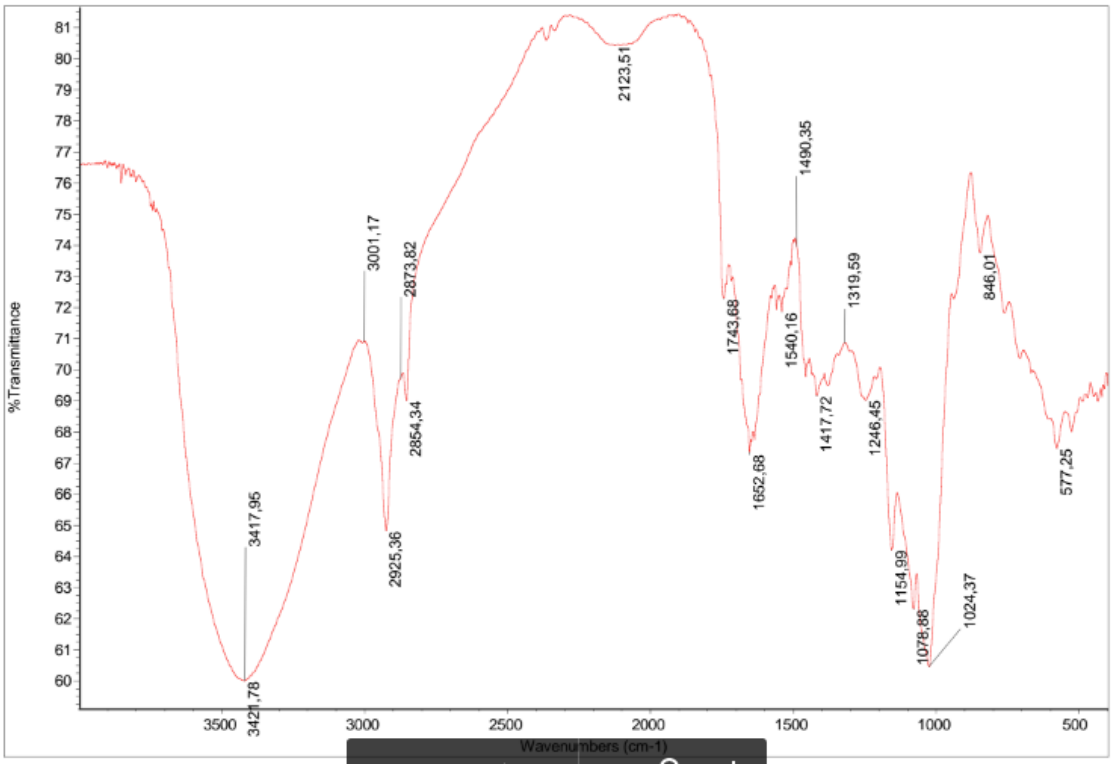
C. sp. GAT - 1



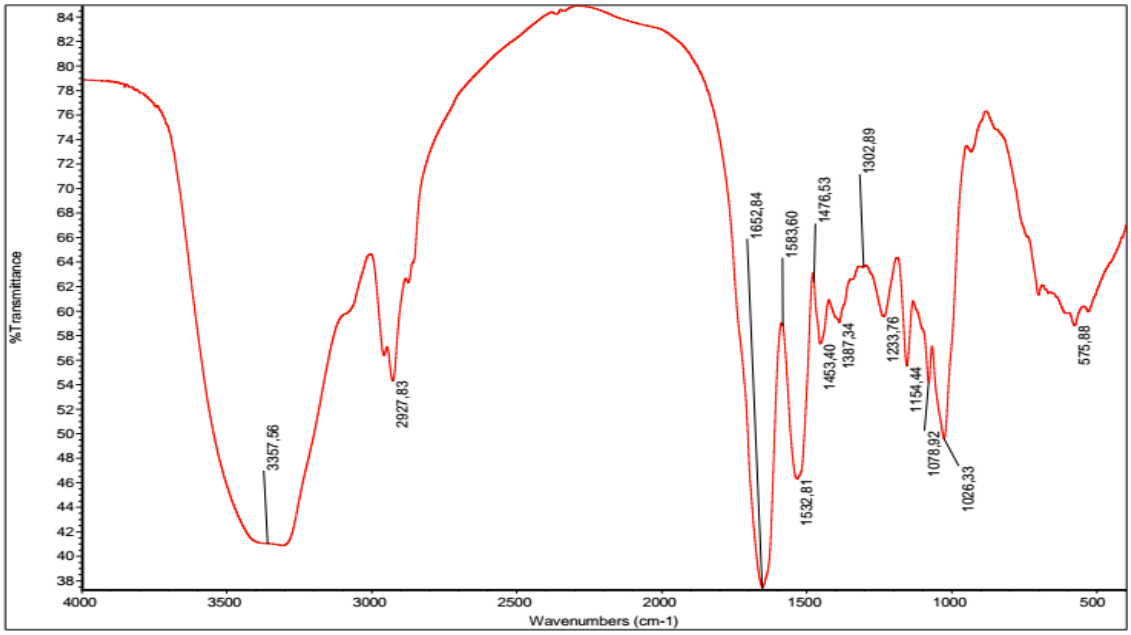
C. zofingiensis B32



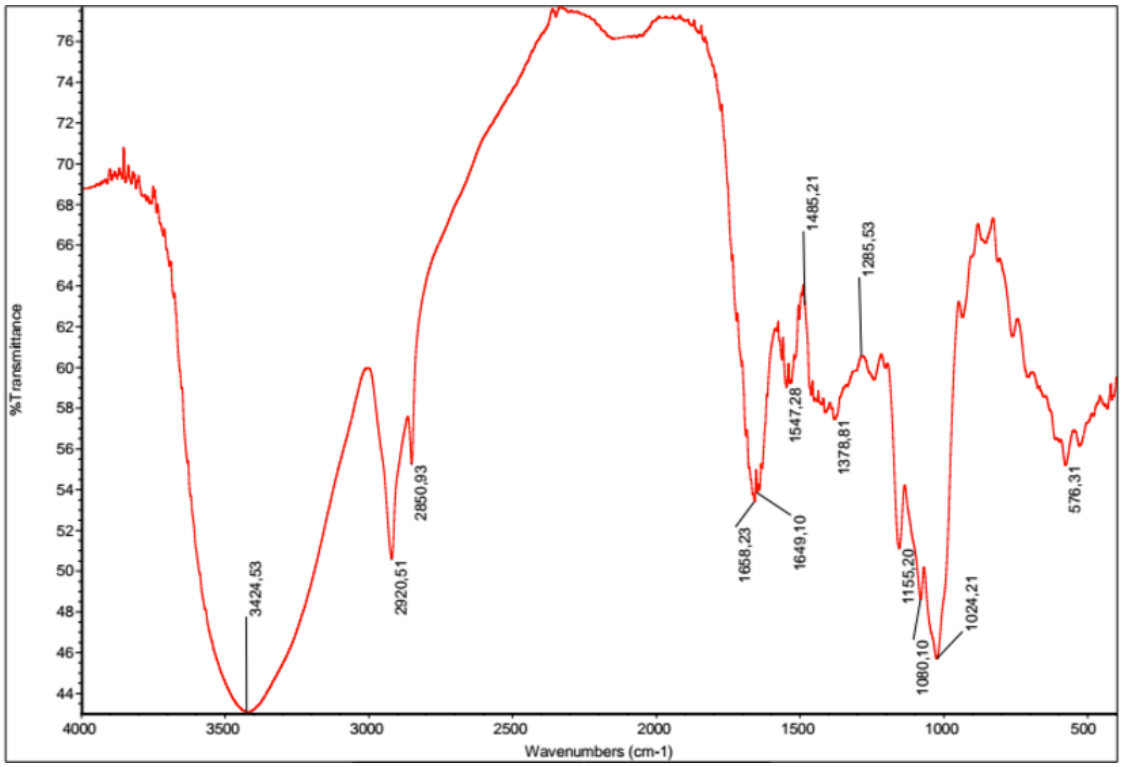
C. sp. GAT-2



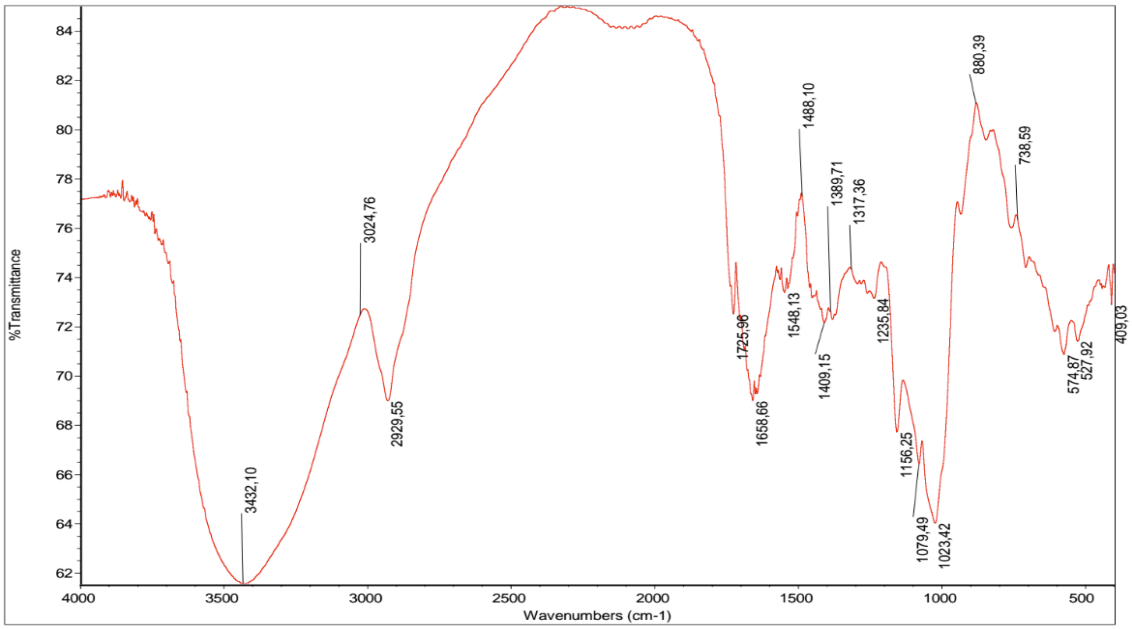
C. reinhardtii CC124



S. subspicatus AC139



C. sorokiniana 2805



APPENDIX 5 – GLYCOSIDIC LINKAGE COMPOSITION OF CELL WALL FRACTIONS AND RESIDUAL FRACTIONS OF THE CHITOSAN EXTRACTION PROCESS OF DIFFERENT MICROALAGAE

Note: 'tr' and 'nd' means 'trace amount' (mol%<0.1) and 'not detected', respectively. The sum of percentages presented may not be precisely 100.0 due to rounding. Experiments were performed in duplicates.

Linkage	I. galbana LB2307	H. pluvialis K0084	Chlorella sp. Gat-7	Chlorella sp. OP
t-Ara*f*	1,6	0,3	2.7	1,6
2-Ara*f*	2,3	1,0	nd	nd
3-Ara*f*	0,6	nd	nd	nd
5-Ara*f*	0,6	nd	nd	nd
2-5-Ara*f*	1,0	nd	nd	nd
t-Gal*p*	2,4	0,5	2.1	1,8
3-Gal*p*	1,9	0,4	1.2	1,2
6-Gal*p*	2,8	0,2	3.8	1,1
2,3-Gal*p*	1,3	nd	nd	nd
2-6- Gal*p*	1,1	nd	nd	nd
3,6-Gal*p*	0,7	nd	0.4	1,8
4,6-Gal*p*	0,7	nd	nd	nd
t-GalA*p*	0,3	0,1	tr	0,2
t-Glc*p*	4,1	0,5	1.4	1,8
3-Glc*p*	1,1	0,2	14.5	1,6
4-Glc*p*	30,7	16,1	13.9	24,0
6-Glc*p*	6,3	nd	11.7	2,4
3,6-Glc*p*	0,4	nd	0.2	0,2
4,6-Glc*p*	1,0	0,3	0.5	0,7
t-GlcA*p*	0,7	0,1	0.4	0,6
4-GlcA*p*	nd	nd	4.7	11,7
2,3-GlcA*p*	0,6	nd	nd	nd

Continues from previous page				
2,4-Glc*A*p	0,6	tr	2.9	0,8
3,4-Glc*A*p	0,5	nd	1.6	1,7
t-GlcNAc	0,3	tr	0.6	1,3
4-GlcNAc	1,3	0,1	7.6	5,5
t-Man*p*	1,8	1,1	1.5	2,9
2-Man*p*	nd	3,0	nd	0,7
3-Man*p*	4,5	nd	0.5	0,4
4-Man*p*	12,3	62,8	5.4	7,3
6-Man*p*	0,8	7,5	nd	nd
2,3-Man*p*	nd	0,3	nd	nd
2,4-Man*p*	1,5	0,8	nd	0,4
2,6-Man*p*	0,2	0,1	nd	nd
4,6-Man*p*	1,0	0,6	0.5	1,2
t-Rhm*p*	0,8	0,1	0.4	0,8
2-Rhm*p*	nd	nd	9.2	2,9
3-Rhm*p*	nd	nd	3.5	2,0
2,3-Rhm*p*	nd	nd	1.9	2,2
t-Xyl*p*	1,9	1,1	2.3	2,4
3-Xyl*p*	0,4	0,9	tr	0,5
4-Xylp	6,6	1,6	4.3	16,3
2,4-Xyl*p*+3,4-Xyl*p*	0,7	nd	nd	nd
2,3,4-Xyl*p*	0,4	nd	nd	nd

Table 21 - Monosaccharide linkage analysis of the cell wall of four different microalgae (*I. galbana LB2307, H. pluvialis K0084, Chlorella sp. GAT-7 and Chlorella sp. OP.*).

Linkage	I. galbana LB2307	H. pluvialis K0084	Chlorella sp. Gat-7	Chlorella sp. OP
t-Ara*f*	1,2	nd	0,2	0,4
2-Ara*f*	nd	tr	nd	nd
3-Ara*f*	nd	nd	nd	nd
5-Ara*f*	0,9	nd	nd	nd
2-5-Ara*f*	1,8	nd	nd	nd
t-Gal*p*	1,3	0,1	0,1	0,2
3-Gal*p*	2,0	0,1	nd	nd
6-Gal*p*	1,7	nd	nd	nd
2,3-Gal*p*	1,4	nd	nd	nd
2-6- Gal*p*	nd	nd	nd	nd
3,6-Gal*p*	0,9	nd	nd	nd
4,6-Gal*p*	nd	nd	nd	nd
t-GalA*p*	0,3	0,1	tr	0,1
t-Glc*p*	1,7	1,2	4,5	1,2
3-Glc*p*	0,2	nd	0,3	0,6
4-Glc*p*	45,6	13,5	76,6	63,4
6-Glc*p*	1,1	nd	0,1	nd
3,6-Glc*p*	0,3	nd	nd	nd
4,6-Glc*p*	1,0	0,3	3,1	0,4
t-GlcA*p*	1,7	0,1	tr	0,3
4-GlcA*p*	nd	nd	nd	nd
2,3-GlcA*p*	1,0	nd	nd	nd
3,4-GlcA*p*	0,3	nd	0,2	0,2
t-GlcNAc	nd	nd	tr	0,1
t-GlcNAc	nd	nd	tr	0,1

Continues from previous page				
4-GlcNAc	0,3	tr	12,5	13,1
t-Man*p*	1,9	0,8	0,2	1,2
2-Man*p*	nd	0,5	nd	nd
3-Man*p*	7,1	nd	1,0	nd
4-Man*p*	16,5	74,5	nd	9,8
6-Man*p*	1,0	6,8	nd	nd
2,3-Man*p*	nd	nd	nd	nd
2,4-Man*p*	2,7	0,2	nd	nd
2,6-Man*p*	nd	nd	nd	nd
3,6-Man*p*	0,2	nd	nd	nd
4,6-Man*p*	0,8	0,7	nd	nd
t-Rhm*p*	nd	0,1	tr	0,4
2-Rhm*p*	nd	nd	0,4	1,0
3-Rhm*p*	nd	nd	0,2	1,4
2,3-Rhm*p*	nd	nd	0,1	1,2
t-Xyl*p*	0,4	0,2	0,1	1,8
3-Xyl*p*	0,7	tr	tr	0,2
4-Xylp	3,9	0,8	0,5	1,4

Table 22 - Monosaccharide linkage analysis of the residual fraction of the chitosan extraction process of four different microalgae (*I. galbana LB2307, H. pluvialis K0084, Chlorella sp. GAT-7 and Chlorella sp. OP.*).

APPENDIX 6 – COBALT PHYLOGENETIC TREE OF CHLORELLA AND FUNGAL CDAS