AQUEOUS ENZYMATIC EXTRACTION OF PROTEIN AND LIPID FROM THE MICROALGAE SPECIES CHLAMYDOMONAS REINHARDTII

by

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Abstract

Microalgae has potential as a biofuel feedstock and as a source of valuable bioproducts for a variety of food, feed, nutraceutical, and pharmaceutical industries. However, several challenges are associated with bioproduct extraction from microalgae. The complexity of microalgae cell necessitates use of energy intensive disruption methods but current chemical or mechanical techniques can degrade economically valuable bioproducts. Aqueous enzymatic extraction (AEE), is a non-solvent and environmentally friendly bio-product recovery method that provides an opportunity to design an integrated process for protein and oil fractionation while reducing industrial costs. Based on the mechanistic understanding of biomolecule distribution and compartmentation, an aqueous enzymatic treatment for the release of internally stored proteins and lipid bodies in wild type *Chlamydomonas reinhardtii* was developed. In this study, we optimized harvesting times that maximized lipid and protein yields in nitrogen depleted cultures of the microalgae *Chlamydomonas reinhardtii*. Furthermore, an aqueous enzymatic extraction (AEE) treatment was developed. First, four lytic enzymes were tested for their ability to permeate *C. reinhardtii* cell walls. After cells were permeable, another set of enzymes were tested for their ability to release internally stored bioproducts. Protein recovery and lipid characterization after enzymatic treatment indicated a 54% release of total soluble protein and a localization of lipids to the chloroplast. Additionally, the development of secondary enzyme treatment for chloroplast disruption achieved about 70% total lipids released into the supernatant. Taken together, results indicate the application of an enzymatic treatment scheme for protein and oil recovery as a promising alternative to traditional extraction processes.

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CHAPTER 1: INTRODUCTION

BIOMASS

The need for replacing fossil fuels, which are one of the main causes of Greenhouse gas emissions (GHG) (Schenk et al., 2008), has driven new research focused on exploring renewable energy sources such as biomass. Currently, biomass accounts for approximately 10 % of the global energy supply. Moreover, it can be utilized as a feedstock for the development of chemicals, building materials, paper, plastics, adhesives, and food and feed bioproducts. In general, biomass can be defined as *any organic matter that is available on a renewable or recurring basis (excluding old growth timber), including dedicated energy crops and trees, agricultural food and feed crop residues, aquatic plants, wood and wood residues, algae, animal wastes, and other waste materials* (Copeland, 2006). About 2.24×10^{11} tons of dry biomass is generated globally (Champagne, 2008). Today, forestry products and energy crops are the major feedstocks for bioenergy and bioproduct manufacturing.

ENERGY CROPS

Energy crops comprises oil crops (e.g. jatropha, oilseed rape, linseed, field mustard, sunflower, castor oil, olive, palm, coconut, groundnut, etc.), cereals (e.g. barley, wheat, oats, maize, rye, etc.), and lignocellulosic biomass (e.g. sweet sorghum, potato, sugar beet, sugarcane, etc.) (Maity, 2015). These crops are generally grown to produce vegetable oils and sugars. Cereal crops are the most important crop cultivated globally. The forecast production of cereals for 2016 reaches 2,544 million tons (FAO, 2016). They are the major feedstocks for food, bioplastics fermentation industry, and as an alternative energy source. Currently, wheat, corn, barley, and sorghum are the most relevant crops cultivated (Dunford, 2012). Their high content of starch and protein makes them a vital component of the human diet in many countries. Thus, only small fractions can be utilized in industrial products due to the high demand for food and feed applications.

Oilseeds are also major commodities grown globally. Among others, canola/rapeseed, soybeans, camelina, sunflower, are the most cultivated seeds. They provide useful functionalities such as lipid unsaturation with ester groups that can be utilized in a wide variety of products such as plasticizers, coatings, adhesives, polymers, and composites (Vijayendran, 2010).

Biomass from lignocellulosic material is another important source of fermentable sugars for significant industrial use. Lignocellulosic biomass can be used as feedstock for producing fuels, power, chemicals, and adhesives and bioplastics. Sugar is the main biomolecule utilized from lignocellulosic material. Other products such as lipids, proteins, and pectin are produced in very small quantities.

Even though obtaining biomass from crops sources appears to be a sustainable alternative to fossil fuels and the development of a wide variety of bioproducts, the increasing global demand for food production could be potentially competing for resources (Koning et al., 2008). For all the biomass sources mentioned above, increases in crop yields (about 2% per year) and resource use efficiencies (especially of nitrogen and water productivity in biomass production systems) would be necessary to meet the rapidly growing demand for food, feed, and industrial bioproducts over the next 20– 30 years (Spiertz and Ewert, 2009). Biomass from agricultural and forestry wastes has also been explored as an alternative biomass source. One advantage over energy crops is that it would not compete with food crops as residual biomass is utilized for energy and bioproducts development.

AGRICULTURAL AND FORESTRY RESIDUES AND WASTE

Agricultural residues are the by-products and waste streams produced during biomass processing and have substantial potentials as feedstock for biorefinery. The use of agricultural residues as biorefinery feedstocks is beneficial as it eliminates the need of sacrificing arable lands (Carriquiry & Timilsina, 2011). Examples include sawdust, bark, branches, and leaves/needles that are produced during processing of wood for bio-products or pulp. These products can be converted

to advanced biofuels or intermediates. Utilization of agricultural residues, forestry, animal and municipal solid wastes, and marine vegetation as feedstocks could ease the pressure on agricultural land needed to grow food. Nevertheless, limited accessibility to dense forests largely increases operation costs for logging/collection activities. There is also a growing concern regarding increases in gas and particle emissions which may be harmful to human health and ecosystems and risks of soil compaction due to removal of residues and an increase in the number of forest operations (Stupak et al., 2007). In general, there is a need for exploring and developing new biomass sources with higher economic value and less environmental and land impacts to meet the increasing demand of the biofuel and bioproducts industries.

ALGAE BIOMASS

One alternative to crop and forestry sources is the utilization of microalgae biomass as a feedstock for different bioproducts. Microalgae are photosynthethic eukaryotic microorganisms that use solar energy, nutrients, and carbon dioxide (CO_2) to produce proteins, starch, lipids, and other valuable compounds that can be used for several applications (Mendes et al., 2003). The oil content and biomass production from algae is far superior to that of terrestrial plants such as soybean and corn (Miao et al, 2004). Even the most widely used oil crops including oil, palm, and sugarcane cannot match the amount of lipids for biodiesel that algae can produce when compared on a percent dry weight basis (Rahman et al., 2014). Their cultivation has several advantages over other energy crops. They have a higher photosynthetic efficiency, higher biomass production, and faster growth rates (Mata et al., 2010). The theoretical oil yield can be 7–31 times higher than that achievable from other crops (Chisti, 2007). Algae's photosynthetic process also absorbs CO_2 emissions from fossil fuels sources, aiding CO_2 sequestration (Halim, et al., 2012) and reducing atmospheric air pollutants. Furthermore, lipids from algae are rich in saturated and unsaturated fatty acids such as oleic (18:1), palmitic (16:0), stearic (18:0), and linoleic (18:2) acids (Meng et al., 2009), making it ideal not only for fuel production, but also as a high value food product. To

ensure economic feasibility of microalgae as a biofuel and bioproduct feedstock, key engineering challenges must be addressed. Extraction of more than one bioproduct, minimization of energy requirements associated with the downstream processing steps and optimization of product quantity and quality are some of the few. Techno-economic evaluation for use of algae for biofuels production indicated that residual algal biomass (after oil extraction) must be either recycled back into the process or used as a feedstock for conversion to a marketable co-product (Gerken et al., 2013).

The microalgae specie Chlamydomonas reinhardtii

Chlamydomonas reinhardtii is known for producing lipids and other native proteins with potential applications in food and pharmaceutical industries. Induction of oil synthesis and accumulation in microalgae has increased interest among industrial and academic researchers (Siaut et al., 2011). Oil bodies are biomolecules of energy and carbon storage in many microalgae species. In the microalgae *C. reinhardtii*, cultivation in a nitrogen-depleted media has been shown to induce accumulation of TAGs enriched in palmitic, oleic, and linoleic acids. Siaut et al. (2011) obtained maximum oil accumulation at 48 to 72 h following nitrogen depletion. *C. reinhardtii* also accumulates high levels of native proteins, accounting for up to 48% of its mass. This microalgae species presents a unique opportunity to recover multiple bioproducts such as proteins and lipids.

Lipid accumulation & localization

C. reinhardtii is a model organism for the study of fundamental biological processes such as lipid metabolism (Moellering et al., 2009). Fan et al (2011) reported that the synthesis of fatty acids, the building blocks for triacylglycerides (TAG) and membrane lipids, occurs in their chloroplast. When starved from nitrogen, lipid droplets (LD) formation is stimulated. LD of nitrogen starved cells have been reported to engorge from the chloroplast stroma for some authors (Goodson et al., 2011) while others have reported that these LD bud from the endoplasmatic reticulum (ER) (Farese &Walther, 2009). Regarding LD size, they can be at least 10 times larger

(up to 3 μm) than the plastoglobules present in cells cultured in nitrogen replete media cells (Goodson et al, 2011). Each LD has a TAGs core surrounded by a layer of polar lipids (PLs) and structural proteins termed lipid droplet surface proteins (LDSP). LDSP might be attached to the polar surface of the LD and prevent them from coalescing (Huang et al., 2013).

Protein accumulation & localization

Microalgae are a unique source of non-allergenic proteins and other micronutrients (Plaza et al., 2009). In *C. reinhardtii*, proteins are mostly accumulated in the cytosol (15%) and internally stored inside the chloroplast. The chloroplast of *C. reinhardtii* can occupy up to 60% of cell volume, with a great capacity for endogenous protein accumulation (Franklin, 2005). A few thousand proteins are constantly exported to the chloroplast to function in photosynthesis and other processes (Inaba & Schnell, 2008). Some of the most canonical proteins include the small subunit of ribulose bisphosphate carboxylase (RuBisCO) and the light-harvesting complex II (LHCII) subunits, which are imported from the cytoplasm, and two proteins synthesized in the chloroplast: the D1 subunit, reaction core of the family of the multi-subunit photosystem II, which is located between the thylakoid membranes of chloroplasts (http://www.ebi.ac.uk/newt/) and the RuBisCO large subunit, which is targeted to the pyrenoid and sometimes co-localized in the eyespot (Uniake & Zerges, 2008).

Despite the promising potential for *C. reinhardtii* as a multiple bioproduct feedstock, challenges for the efficient recovery and purification of these products must be addressed. To date, very few studies have described the accumulation (Tsai et al., 2015; Duong et al., 2015) and the extraction (Cuellar-Bermudez et al., 2015) of both protein and lipid bioproducts from microalgae. The main challenge of extracting proteins and lipids from the algae biomass is to maximize extractable yield of each product separately while preserving the integrity of the others.

EXTRACTION

Extraction is a key processing step in recovering bioproducts from microalgae. It involves several processes that differ depending on the product to be extracted and determine the initial yield of the bioproduct. The first step in the extraction process is biomass cell disruption, which allows for the permeabilization of the cell wall so internally stored biomolecules such as proteins, lipids and starch, are more accessible and easier to extract.

MICROALGAE CELL WALL DISRUPTION

Current cell disruption processes involve the use of high temperature (>50°C) treatments, organic solvents, or highly acid or basic buffers that can potentially decrease protein solubility, thus decreasing extractability (Wilken & Nikolov, 2016). Moreover, other thermal (microwave, autoclaving, and freezing) and mechanical (bead-beating, milling, ultrasonication, high-pressure homogenization, and spray-drying) methods are energy intensive and require specialized equipment. Table 1 summarizes the general mechanism, advantages, and disadvantages of the most utilized cell disruption methods. In general, the high energy applied in these processes also induce non-specific degradation of the cell wall and other cell membranes that store lipids and proteins. Thus, all products are released simultaneously into the media, hindering the extraction of each product separately.

Mechanical disruption for lipid release demands a high energy input. Therefore, one challenge for efficiently extracting lipids and other products from microalgae is to release internally stored products, such as lipids, proteins, and starch from their intracellular compartments.

Table 1. Advantages and disadvantages of common cell disruption methods [1] Amos (1998), [2] McMillan et al (2013), [3]Schoenbach et al (2000), [4]Maskooki & Naghi (2012), [5] Sun (2014), [6] Mercer & Armenta (2011), [7]Dixon et al (2015)

Disruption method	Mechanism	Advantages	Disadvantages
Drying[1]	It involves a source of heat, method for removing the water, agitation to expose new material for drying	Improved extraction efficiency	Large heat requirements; Energy required for evaporating the water is dispersed into heating the biomass and heating the air
Microwaves[2]	Cyclic heating	Effective; Fast; Higher processingcapability	High energy requirements (746 kJ of energy for the 10 mL algae biomass)
Pulse electric field[3][4] [5]	Cell membrane electro-permeabilization	Non-thermal process; Higher yield, purity, and lower energy and time consumption than conventional thermal methods.	Scale up costs; Operational costs; Operational safety issues; Requires sufficient electric field strength
Ultrasound[6]	Cells are damaged and contents are released by cavitation	Reduced extraction time; reduced solvent consumption; greater penetration of solvent into cellular materials; improves the release	High power consumption; difficult to scale-up
Enzymes[7]	Cleaves specific types of bonds present in the cell wall	Mild process, GRAS; Green Extraction process; low energy requirements; highly specific; does not require drying	Low efficiency when enzyme is not specific to the substrate; enzyme prices; need of buffer adjustments

PROTEIN SOLUBILIZATION AND EXTRACTION

Cell disruption is one of the most relevant steps in a protein extraction processes. To purify proteins efficiently, they must be first released from internal cell compartments in a soluble form (Tan & Yiap, 2009). Protein extraction from algae can be challenging due to their stable cell wall (Greenwell et al., 2009) that requires cell wall disruption methods that are strong enough to disrupt the cell wall while preserving protein integrity. Common mechanical disruption methods, such as the French Press, glass beads, or sonication (Table 1) are used to break the cell wall, followed by detergent based total protein solubilization and extraction (Tan & Yiap, 2009). Among others, pH, temperature, and ionic strength of the aqueous solution can also affect protein extractability. For example, biomass subjected to intense heat, which rapidly denatures proteins, shows poor extractability (Smith, 1972). Contrarily, a slightly alkaline pH (~8) and temperatures between 25 to 37°C usually exhibit better protein extractability (Kinsella, 1979). Once proteins are extracted, they can be purified, used directly, or converted into valuable products.

OIL EXTRACTION

Current extraction methods for oil extraction require long processing times, petroleum-based solvents, or energy-intensive mechanical disruption treatments (Adam et al., 2012). Table 2 summarizes some of the advantages and limitations of currently used extraction methods.

In microalgae, traditional lipid extraction is performed with the aid of lipophilic extraction solvents. Currently, extraction using hexane is the most utilized at an industrial scale. For lipophilic extraction solvent systems, biomass drying or other high intensity cell disruption methods are required (Ranjan et al., 2010; Cravotto et al., 2008) as the immiscibility of unruptured cells do not permit solvent access to the internally stored lipid bodies. Cell disruption allows solvent to penetrate the cell and solubilize the lipids, propitiating a faster separation and recovery (Yap et al., 2014). When biphasic solvent systems are used, such Bligh & Dyer (chloroform-methanol-water), lipid recovery on unruptured cells is possible but requires long incubation times

due to passive diffusion of solvents and lipids across the cell wall (Ranjan et al., 2010). Thus, cell wall disruption is a key step to increase extractable yields. When cells are ruptured, the solvent (or solvent mixture) can rapidly diffuse and solubilize lipids into the hydrophobic phase while polar cell biomolecules remain in the aqueous (hydrophilic) phase (Figure 1).

Table 2. Advantages and disadvantages of common extraction methods Adapted from Mercer & Armenta (2011) & [1] Sierra et al (2016)

Extraction methods	Advantages	Limitations
Oil press	Easy to use, no solvent involved	Large amount of sample required, slow process
Solvent extraction	Solvent used are relatively inexpensive, reproducible	Most organic solvents are highly flammable and/or toxic, solvent recovery is expensive and energy intensive, large volume of solvent needed, usually involves biomass drying
Supercritical fluid extraction	Non-toxicity (absence of organic solvent in residue or extracts), 'green solvent' used, non-flammable and simple in operation	Often fails in quantitative extraction of polar analytes from solid matrices, insufficient interaction between supercritical CO_2 and the samples, high energy and equipment investment
Aqueous enzymatic extraction[1]	No organic solvents involved, environmentally friendly alternative, does not require drying of the biomass	High costs of commercial enzymes, it can require high incubation temperatures and buffer exchange steps

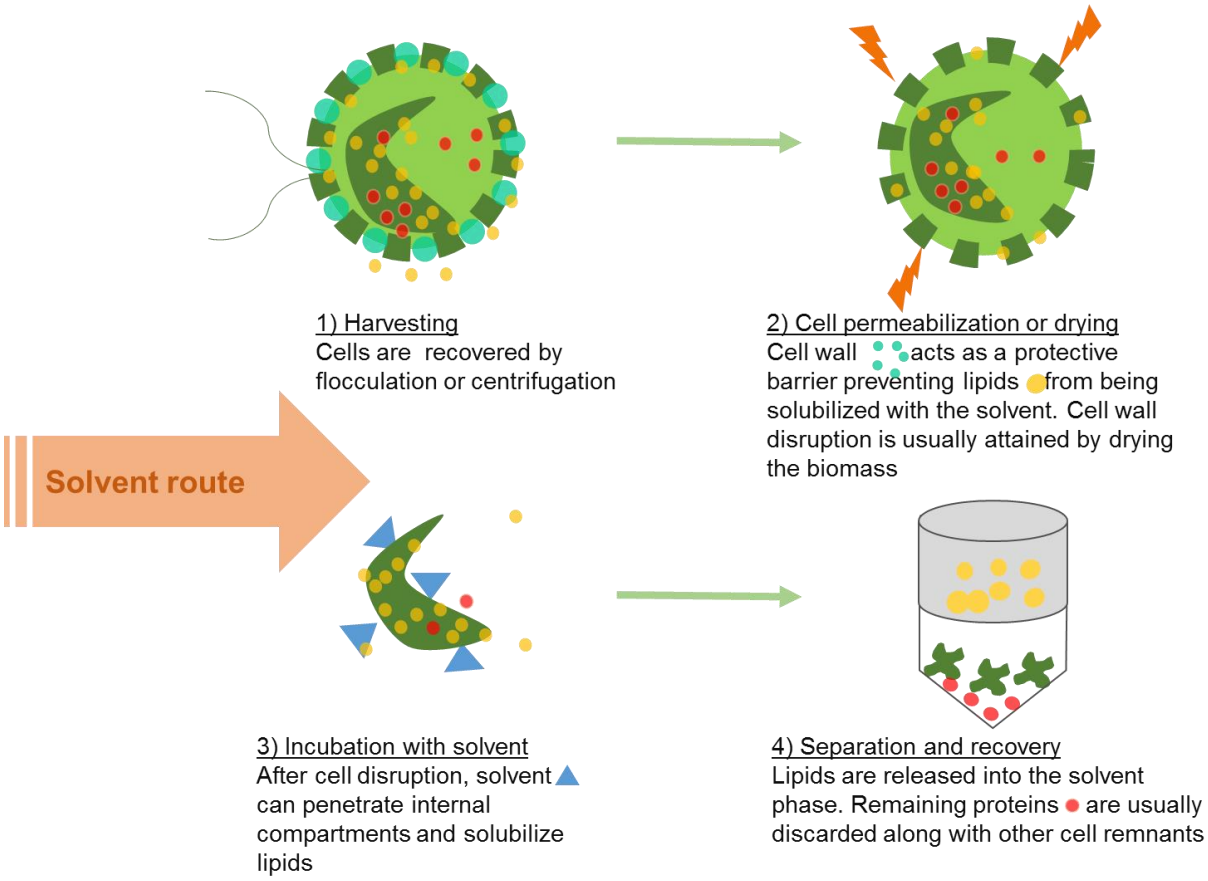


Figure 1. Solvent extraction process

Summarizing, to reduce production costs as well as environmental impacts, it is desirable to extract proteins and lipids without significant contamination by other cellular components (Scott et al., 2010) in an energy-efficient and environmentally friendly way, avoiding drying steps and the use large amounts of toxic substances. The development of Green Extraction processes should allow for the extraction of multiple bioproducts from microalgae in an energy efficient way, reducing negative environmental impacts of traditional extraction methods.

GREEN EXTRACTION PROCESSES

A green extraction process is the one that utilizes renewable plant resources as feedstock, uses alternatives to solvents, reduces energy consumption, produces co-products instead of waste, reduces unit operations, and aims for yielding extracts without contaminants (Chemat et al., 2012). The development of green extraction processes and the use of renewable feedstocks has a central

role in environmental-friendly practices. A solvent-free extraction method is a more ecologically friendly and economically viable process that requires less energy to separate products in phases (Adam et al., 2012), relying on chemical properties of the materials, such as density or solubility differences, to promote their separation. Currently, research on bioproduct extraction from microalgae is largely focused on developing solutions that minimize the use of solvents, in a cost efficient manner (Chemat et al, 2012). Nevertheless, research addressing the implementation of solvent-free alternatives for extraction of bioproducts is scarce (Wang et al., 2015). Alternatives to the use of organic solvents include: mechanical disruption, supercritical CO_2, and aqueous enzymatic extraction. Aqueous enzymatic extraction processes are based on the selective decomposition of cellular components using enzymes. This process is considered a green extraction method that does not require prior drying of the biomass thus minimizing the energy involved in the process (Scott et al., 2010). Research involving the utilization of enzymes in extraction processes is limited to biomass pretreatment for cell wall disruption as a fully aqueous enzymatic extraction process of proteins and lipids from *C. reinhardtii* has not been developed yet.

AQUEOUS ENZYMATIC EXTRACTION

AQUEOUS ENZYMATIC ASSISTED EXTRACTION(AEAE)

AEAE has emerged as a promising alternative to assist multiple bioproduct extraction. The advantages of this process include highly selective disruption that allows for the extraction of targeted bioproducts, mild reaction conditions, such as neutral pH and incubation temperatures between 25°C and 37°C, and the absence of energy intensive drying steps. Aqueous enzymatic biomass pretreatments facilitate the release of internal products to the media while potentially avoiding bioproduct degradation (Demuez et al., 2015).

In a typical cell disruption process, a combination of enzymes is used to break the cell wall, release the lipid bodies from the cellular structure, and separate lipids from the protein/lipid matrix (lipoproteins). Aqueous enzymatic cell disruption consists of several key steps including: 1)

biomass harvesting, conditioning, and addition of enzymes; 2) incubation and churning for cell wall disruption, 3) addition of organic solvent for lipid extraction or secondary disruption treatment for protein solubilization if needed and 4) centrifugation and lipophilic phase recovery.

Potentially effective commercial enzymes for disrupting C. reinhardtii cell walls

AEAE from diverse feedstocks have recently been evaluated including soybean (de Moura et al., 2008, 2009a, 2009b, 2010; Kashyap, 2007; Rosenthal et al., 2001), sunflower (Latif and Anwar, 2011), and corn and corn fractions (Moreau et al., 2009; Wilken et al., 2016). Selection of enzymes is typically biomass-specific and based on composition and cell wall structure. Common enzyme classes selected for enzymatic extraction methods include cellulases, xylanases, proteases, amylases, and pectinases. The cell wall of *C. reinhardtii* is a cellulose-deficient structure that is primarily composed of proteins and hydroxyproline-rich glycoproteins (Imam and Snell, 1987). *C. reinhardtii* cell wall could potentially be degraded by certain lytic enzymes, such as lysozyme, due to its peptidoglycan hydrolase activity. The hydroxyproline-rich cell walls makes it also a possible suitable substrate for collagenase digestion. Finally, the highly active proteolytic enzyme, trypsin, could potentially cleave some of the protein-rich cell wall and promote cell permeabilization. Although these enzymes appear to be a good alternative for AEAE, there are some challenges associated with their utilization. For instance, applying commercial enzymes for AEAE can potentially increase extraction costs. When high purity and cleavage specificity are required, enzyme prices may exceed the value of the extracted bioproduct.

C. reinhardtii-produced autolysin for cell wall disruption

One solution for overcoming high enzyme prices is the exploitation of biological mechanisms for *in situ* enzyme production and utilization. The microalgae, *C. reinhardtii,* produces a cell wall-degrading protease induced by nitrogen deficient stress conditions during sexual reproduction (Jaenicke, 1981). One advantage of using this enzyme as a pretreatment is that only proline-rich residues contained in *C. reinhardtii* cell walls are suitable substrates for degradation, so recoverable

bioproducts, such as proteins would be expected to remain intact. Treating *C. reinhardtii* cells with autolysin could facilitate cell wall degradation and potential release of bioproducts while avoiding their degradation. This enzyme can be produced *in situ*, which would considerably decrease the cost of acquiring commercial enzymes. However, the utilization of autolysin as a biomass pretreatment for the extraction of bioproducts needs to be further explored.

In general, AEAE is used to improve extractable yields when traditional solvent extraction methods are used. As an alternative to solvent extraction, a secondary enzymatic treatment to complement enzymatic cell disruption can be developed. The secondary enzymatic treatment would aim to disrupt organelles and internal membranes so lipid bodies and other internally stored products can be released. Once released, lipids can be separated by coalescence. The development of a fully aqueous enzymatic extraction (AEE) process could potentially aid product fractionation and recovery due to the possibility of a selective enzyme degradation of targeted intracellular compartments following enzyme-mediated cell permeabilization. Furthermore, once internal products have been released, differences in solubilities and densities of the bioproducts to be recovered can be exploited for fractionation and recovery.

ENZYMATIC TREATMENT FOR LIPID RELEASE.

According to Thiam et al (2013), lipid droplets are a dispersed phase of an oil-in-water emulsion in the cytosol-aqueous media of cells. Lipid droplets form natural emulsions inside the cells with the help of certain emulsion stabilizers such as proteins and phospholipid surfactants (Leal-Calderon et al., 2007). An emulsion can prevent lipid droplets from coalescing into larger droplets, thus preventing lipid separation from the aqueous media. After recovering most of the proteins solubilized by the first enzymatic treatment, a secondary enzymatic treatment using a protease could promote 1) degradation of lipid droplet surface protein (LDSP) and 2) degradation other proteins present in the cell lysate, allowing for disruption of naturally occurring emulsions in the microalgae cell lysate. This could potentially promote lipid body release and detachment from

cell remnants. We choose four different proteases that could potentially cleave the lipid droplets surface proteins as well as other internal compartments entrapping the lipid bodies:

Trypsin: This general serine protease, can cleave a wide variety of protein substrates. The low acquisition cost and optimum activity under mild conditions (neutral pH and 37°C incubation temperature) of this enzyme makes it an attractive and economical option for cleaving *C. reinhardtii* chloroplasts. To release the lipid bodies, several chloroplast membranes would have to be cleaved. Besides LDSP, the chloroplast's outer and inner membrane and interconnected stacked thylakoid discs (Zerges & Rochaix., 1998) are possibly trapping the lipid bodies. Besides cleaving a wide variety of substrates, previous research on spinach chloroplasts (Jennings, 1980), showed that trypsin has a relaxing effect on thylakoid discs, promoting membrane unstacking. If this effect can be replicated in *C. reinhardtii* chloroplasts, trypsin could aid not only LDSP and inner and outer membrane digestion, but also thylakoid membranes relaxation and subsequent lipid body's release. Trypsin would also promote protein hydrolysis into smaller peptides, making digested proteins more soluble and suitable for food and feed applications as they are easier to absorb in the small intestine of mammals (Royston, 2009). Furthermore, trypsin is usually produced in mammal's digestive system and has been widely used in food processing industry, thus, if still present in the protein lysate after downstream processing, this protein would not be unsafe if ingested.

Alcalase: Alcalase is another serine endopeptidase of broad specificity that is suitable for the hydrolysis of a wide range of proteins, preferentially those containing aromatic amino acid residues. Alcalase is a food grade, low cost enzymatic preparation that has been successfully employed, among others, to produce detergents and soluble hydrolysates for soy protein and fish protein (Doucet et al, 2003). Alcalase has an optimum pH and incubation temperature for activity of 8.5 and ~60°C respectively, which makes it more energy intensive than trypsin treatment. Like trypsin treatment, alcalase would be targeting LDSP and chloroplast membranes. Even though its

effect on chloroplast stacking has not been explored yet, higher protein degradation and broader substrate specificity when compared to trypsin could make it a suitable enzyme for release of lipid bodies. Another challenge that needs to be further explored is the emulsifying effect of alcalase hydrolyzed proteins (Muzaifa et al., 2012), which could hinder fractionation of protein and oil as if released in an emulsified form.

DSM Metalloprotease (Maxipro): Maxipro is a food grade enzyme solution that contains an endoprotease enzyme with optimal activity at neutral pH. This bacterial protease can break down proteins of different sources into amino acids and oligopeptides to increase digestibility and improve functional properties (DSM, 2013). Metalloproteases have been also reported to serve as cell wall lytic enzymes (Wu, 2011). This might indicate that Maxipro could potentially digest different cell membranes. Based on its wide protein digestibility range, Maxipro would be simultaneously targeting LDSP and chloroplast membranes. Besides releasing lipid bodies, Maxipro could also enhance protein functionality at a later stage by improving protein solubility and decreasing its viscosity.

Glucanex: Glucanex is a lytic enzyme solution containing β-glucanase, cellulase, protease, and chitinase activities (Villetaz et al., 1984). It has a low cost and requires mild conditions for optimum activity (pH 6.0 and 25°C incubation temperature). Even though it has been mostly used for digesting yeast cell walls (Petit et al., 1994), the enzyme preparation could be effective in degrading certain components of the outer and inner chloroplast envelope such as carbohydrates and proteins. The protease activity could also be a potential digester of the LDSP.

THESIS OBJECTIVES

In this thesis, an aqueous enzymatic lipid and protein extraction process for the microalgae *C. reinhardtii* was proposed.

In chapter two, we asses 1) the feasibility of utilizing the microalgae *C. reinhardtii* as a lipid and protein production organism by selecting a harvesting time that optimizes yields of both

bioproducts, 2) the effectiveness of autolysin treatment on *C. reinhardtii* cell wall disruption and compare it with cell wall-degrading enzymes, 3) the level of cell disruption and product release achievable with the autolytic treatment by TEM imaging and cell counting, and 4) a combined autolysin-solvent treatment that facilitates lipid and protein recovery and compare yields obtained with traditional solvent or combined mechanical disruption-solvent extraction processes.

In chapter three, we 1) improved the previously cell disruption treatment developed to maximize protein solubilization, 2) developed a secondary enzymatic treatment for releasing intracellular products and promoting lipid droplets coalescence on the same microalgae species, and 3) evaluated its effectiveness by lipid quantification, staining, and visualization using TEM imaging.

CHAPTER TWO: ENZYMATIC CELL DISRUPTION

ABSTRACT

Microalgae has potential as a biofuel feedstock and as a source of valuable bioproducts for a variety of food, feed, nutraceutical, and pharmaceutical industries due to high yields of proteins, starch, and lipids. However, several challenges are associated with bioproduct extraction from microalgae. The complexity of microalgae cell walls necessitates use of energy intensive disruption methods, but current chemical or mechanical techniques can degrade economically valuable bioproducts. Therefore, disruption methods that target microalgae cell walls are essential, such as enzymatic biomass pretreatment for the release of specific biomolecules. Aqueous enzymatic pretreatment can preserve valuable bioproducts while permitting high levels of cell disruption. In this study, we optimized harvesting times that maximized lipid and protein yields in nitrogen depleted cultures of the microalgae Chlamydomonas reinhardtii. Furthermore, an aqueous enzymatic assisted extraction (AEAE) treatment was developed. Four lytic enzymes were tested for their ability to permeate C. reinhardtii cell walls. Once autolysin treatment was chosen as the preferred cell disruption method, treated cells were visualized by TEM imaging. TEM images and cell counts confirmed cell permeability (100%), further cell lysis (50%) and product release when cells were treated with autolysin for 24 h. Biomass was also subjected to lipid and protein extraction after autolysin treatment and yields were compared to other mechanical and chemical extraction methods. Protein extractability was significantly enhanced by the autolysin pretreatment when compared to sonication pretreatment. Solvent extraction accompanied with autolysin biomass pretreatment significantly enhanced lipid extractable yields as compared to only solvent extraction and solvent plus sonication extraction.

Keywords.

Microalgae, cell disruption, enzymes, autolysin, lipid, protein

MATERIALS AND METHODS

STRAIN AND CULTURE MEDIUM

Biomass production strain

Stock cultures of *Chlamydomonas reinhardtii* (CC-409 mt+) were obtained from the Chlamydomonas Resource Center, University of Minnesota. Cells were initially cultured in TAP media (Gorman & Levine, 1965). Once they reached the stationary phase (5-7 x 10^7 cells/mL), cells were re-suspended into TAP-N media (TAP media without the nitrogen source, NH_4Cl)

Mating strains

High efficiency *C. reinhardtii* strains CC-620 mt+ and CC-621 mt- were kindly provided by Dr. Bradley Olson from the Division of Biology at Kansas State University. Cells were grown in solid TAP media until high mating efficiency was achieved. After, cells were solubilized and suspended into liquid TAP media.

Algae biomass

C. reinhardtii cells were grown in TAP plates for 5 days under constant light conditions (27 $\mu M/m^2$-s) and then transferred to liquid TAP media. Once the lag phase was reached (~2 x 10^7 cells/ mL), biomass was centrifuged at 6,000 g for 5 min, washed, and re-suspended into the same volume of TAP-N media. Samples were collected at 24, 48, 72, and 96 h and stored at -80°C until used for analysis. All liquid cultures were shaken continuously at 122 rpm in an orbital shaker.

CHARACTERIZATION OF THE ALGAE EXTRACT

Total protein quantification

For each total protein determination, 10 mL of biomass was centrifuged at 6,000 g for 5 min. The supernatant was removed and the biomass was resuspended in a protein solubilization buffer containing 0.75 mM lithium dodecyl sulfate (Amresco), 2.5 mM glycerol (Amresco), 51.4 mM TRIS base (Biosciences), and 0.02 mM EDTA (Alfa Aesar). Each sample was sonicated four

times at 25% amplitude for a total of 2 min with 30 s cooling time in ice after each sonication cycle. Samples were centrifuged again under the same conditions and supernatant was recovered. Finally, lysates were diluted 10 times with phosphate buffer (pH. 9.6) and total protein was measured based on the method proposed by Smith et al.,(1985) using a BCA protein kit (Pierce^{TM}).

Lipid yield quantification

For total lipid quantification, a modified version of the Bligh & Dyer method (1959) was performed. Samples (90 mL) were centrifuged at 6,000 g for 5 min and the supernatant was decanted. Chloroform, methanol, and water were added to the pellet in a volume ratio of 1:2:1. Subsequently, samples were sonicated for 1 min at 25% amplitude, mixed overnight, and centrifuged at 6,000 g for 5 min. The bottom lipophilic layer was extracted and filtered into pre-weighed trays. Samples were evaporated and then dried in an oven at 95˚C for 1 h. Lipids remaining in the trays following solvent evaporation were weighed to calculate either total lipid content or recoverable lipid content by the following equation 1.

$$LY(\frac{g}{g}) = \frac{LP(g)}{BDW(g)} \qquad (1)$$

Where,

LY= Lipid yield or recoverable lipid content (lipid content/g of biomass)

LP= Lipid productivity (amount of extracted lipids)

BDW= Dry weight of the biomass (g)

AUTOLYSIN PREPARATION

To prepare autolysin, a modified protocol of that proposed by Jaenicke et al. (1987) was followed. High efficiency mating strains, CC-620 mt+ and CC-621 mt-, were cultured and placed under high intensity LED lights (35 µM/m^2-s). After three days of growing, each mating type was

independently transferred into TAP-N for a final cell concentration of 1 x 10^7 cells/mL. After 12 hours of constant mixing under high intensity LED lights, mating tests were performed to determine mating efficiency. For the test, 200 µL of each mating strain were mixed, allowed to mate for 5 min, and observed under the microscope (VWR® fluorescence inverted microscope). High mating efficiency was achieved and cells were ready to be mixed when approximately 95% of cells were mating. Both mating strains were mixed in a clear container, placed under high light for approximately 30 min, and then centrifuged at 6000 g for 5 min. Supernatants containing autolysin were filtered with a 0.45 µm PES membrane bottle-top sterile filter and stored at -80°C until use.

AUTOLYSIN TREATMENT

Biomass was harvested and re-suspended in either autolysin buffer or TAP-N (nitrogen deficient) buffer as a negative control. Biomass was incubated at three different temperatures (25°C, 37°C, and 50°C) with constant mixing (250 rpm) for either 2, 4, or 24 h. Cell counts were taken before, during, and after treatment.

ENZYMATIC TREATMENT

To find the most suitable enzyme for cell disruption, various enzymes targeting the *C. reinhardtii* cell wall were tested (Table 3). For each treatment, biomass was harvested and re-suspended in enzyme buffer. Then, cell permeability percent was calculated at different incubation times.

Table 3. Description of enzymes used for cell permeabilization, cell wall target, compatible conditions and dosage guidelines for incubation. Adapted from [1] Wilken & Nikolov (2011) & Nakimbugwe et al. (2006), [2] Yoshida & Noda (1965), [3] Bergmann et al (1939) and [4]Jaenicke et al (1987).

Enzyme & Category	*C. reinhardtii* target	Compatible Conditions	Buffer used	Dosages	Source
Lysozyme[1] **Muramidase**	Glycoproteins. Peptidoglycan cell wall	pH range: 4.0-8.0 (optimum pH 6.24) Temp. range: 20°C-60°C (optimum 25°C)	66 mM Potassium phosphate +1mM EDTA pH 6.24	1-2 mg/mL	Amresco
Collagenase[2] **Metallo protease**	Hydroxyproline-rich cell wall	pH range: 5-11 (optimum pH 6.7) Temp range: 35°C-40°C (optimum 37°C)	100 mM Tris HCl pH 7.0	1 mg/mL	Sigma
Trypsin[3] **Serine endopeptidase**	Proteins in the cell wall, cleaving at the carboxyl side of lysine or arginine,	pH range: 7-9.5 (optimum pH 7.5) Temp. range: 20°C - 60°C (optimum 37°C)	100 mM Tris HCl pH 7.8	1 mg/mL	Amresco
Autolysin[4] **(Hydroxy)-proline metallo protease**	Proline rich proteins in the cell wall	pH range: 7-9.5 (optimum pH 7.5) Temp. range: 20°C - 40°C (optimum 35°C)	TAP-N pH 7.5	0.5 mL/mL	Produced *in-situ*

CELL PERMEABILITY ASSESMENT

For the quantitative analysis of cell permeability, samples (10 µL) were taken before, during, and after the enzymatic treatment and 10 µL of 1% NP-40 detergent was added to each sample. Cell suspension was loaded into an improved Neubauer hemocytometer (10 µL) and cell counts were performed. The percent of permeable cells was calculated with equation 2.

$$\%PC = \frac{CDA}{CB} * 100\% \quad (2)$$

Where,

%PC= Percent permeable cells

CDA = Cell count per mL of biomass during or after treatment

CB = Cell count per mL of biomass before the treatment

CELL LYSIS ASSESMENT

For the quantitative analysis of cell lysis, cell suspension was loaded into a hemocytometer (10 µL) and cell count was performed using an inverted microscope. Percent of lysed cells was calculated using equation 2.

TEM IMAGING

TEM pictures were taken throughout the enzymatic treatment using Tecnai™ G2 Spirit BioTWIN (FEI Company) at 80 kV acceleration voltages. Biomass samples were fixed in Trump's fixative overnight, post fixed with Osmium tetroxide, dehydrated in graded series of alcohol and embedded into spur resin. Ultra-thin sections were contrasted with uranyl acetate- lead citrate and observed under a FEI Tecnai 12 Bio-spirit Transmission electron microscope.

QUANTIFICATION OF PERCENT PROTEIN SOLUBILIZED

Biomass was first incubated with autolysin at room temperature for 4 h. After enzymatic treatment, biomass subjected to either no secondary treatment, sonication treatment (3 min at 35% amplitude or resuspension in protein solubilization buffer (1% LDS buffer (page 32) and supernatants collected. Total soluble protein was quantified using a BCA protein kit (Pierce ™). The percent protein solubilized was calculated based on a total extractable protein reference. Total extractable protein was calculated following the total protein quantification procedure described in page 32 and percent protein solubilized was determined using equation 3.

$$\% SP = \frac{TSP(\frac{\mu g}{mL})}{TEP(\frac{\mu g}{mL})} \qquad (3)$$

Where,

% SP= Percent protein solubilized when compared to a total extractable protein reference

TSP=Total protein solubilized (µg/mL)

TEP= Total extractable protein (µg/mL)

EVALUATION OF EXTRACTABLE LIPID YIELD AFTER AUTOLYSIN TREATMENT

Cell lysates from either autolysin treatment or control treatment (TAP-N) were subjected to a modified Bligh and Dyer (1959) and/or hexane extraction (Wang & Yuan, 2014).

Modified Bligh and Dyer extraction

Samples (90 mL) were centrifuged at 6,000 g for 5 min. Chloroform, methanol, and water were added to the pellet in a volume ratio of 1:2:1. Half of the samples treated with autolysin or control were sonicated for 1 min at 35% amplitude while the other half were not mechanically pretreated. In a rotary shaker, samples were mixed overnight at 100 rpm. Afterwards, lysates were centrifuged at 6000 g for 5 min and the bottom lipophilic layer was extracted and filtered into pre-weighed trays. In an air oven, samples were dried at 105°C for one hour and trays were weighed again. . The percent lipid content was calculated based on an extractable lipid yield reference. Extractable lipid yield was calculated following lipid yield quantification procedure described in page 33 and percent lipids released was determined using equation 4.

$$\% LC = \frac{TLC(g)}{ELY(g)} \qquad (4)$$

Where,

% LC= Percent lipid content when compared to an extractable lipid yield reference

TLC=Total lipid content (g)

ELY= Extractable lipid yield (g)

Hexane extraction

The solvent was added in a 1:1 v/v to either autolysin treated cells or control treated cells with or without sonication step. The tubes containing algal cells and solvent was shaken (150 rpm) overnight. After that, the tube was centrifuged at 2,000 g for 15 min to remove cell solids. The supernatant was carefully collected, evaporated, and then dried in an oven at 95°C for 1.5 h.

STATISTICAL ANALYSIS

One way analysis of variance (ANOVA) was conducted for statistical analysis of the experimental data using Graph-Prism 6 software. To compare significant differences between treatments, a Tukey adjustment was made for a family wise error rate of 0.05 (α_{FER}=0.05).

RESULTS AND DISCUSSION

KINETICS OF LIPID AND PROTEIN YIELDS

Evaluation of protein and lipid content and productivity is critical for determination of the optimum harvesting time for microalgae. Nitrogen stress conditions, which enhance lipid accumulation, can inhibit biomass growth, decrease total protein content, and alter the amount of certain proteins due to metabolic reprogramming (Wase et al., 2014). To determine the optimum harvesting time, protein and lipid composition and productivity kinetics were evaluated for *C. reinhardtii* growth in nitrogen depleted (TAP-N) and sufficient (TAP) cultures. For each harvesting time, cell density and protein and lipid yields were quantified, lipid droplets were visualized using Nile Red fluorescent dye, and protein molecular weight (MW) profiles were determined by gel electrophoresis. Figure 2(a) and 2(b) show lipid and protein accumulation in *C. reinhardtii* over time. TAP cultures did not have a significant increase in lipid accumulation over the 96 h period. No significant differences in lipid yields were found between TAP and TAP-N cultures harvested at 24, 72, or 96 h. A sharp decrease in lipid content was evident at 72 h under

nitrogen depleted conditions when compared to 48 h. Knowing that lipid bodies accumulate in the cell at a slower rate than the starch bodies, the decrease could have been caused by an over accumulation of starch bodies, induced by nitrogen depletion (Wang et al, 2009), that possibly saturated *C. reinhardtii* chloroplasts. Further studies should be performed to confirm these hypotheses and investigate the cause of the sharp decrease in lipid content. This tendency does not agree with lipid accumulation trends of other *C. reinhardtii* strains (Siaut et al., 2011) where a steady lipid yield is observed beyond 48 h of nitrogen depletion. Nevertheless, Siaut et al (2011) also reported dramatic differences in lipid accumulation between different *C. reinhardtii* strains.

In agreement with Wang et al (2009), higher lipid yields (~0.4 g/g) were found after 48 h under nitrogen depleted conditions (48 TAP-N) (Figure 2(a)). A 2.5-fold increase in lipid yield was observed when compared to TAP cultures and other harvesting times under nitrogen depleted conditions. According to Msanne et al. (2012), the increase in lipid accumulation at 48 h under nitrogen depletion is known to be attributed to a turnover of nitrogen-rich compounds such as proteins that may provide carbon/energy for TAG biosynthesis in the nutrient deprived cells. This could be why there is a significant decrease in protein content (Figure 2(a)) for cultures harvested at 96 h under nitrogen depleted (TAP-N) conditions when compared to TAP cultures at the same harvesting time. Fortunately, harvesting after 48 h under nitrogen depletion did not significantly decrease protein levels when compared to all the non-deprived cultures. For all the data points, variability (standard errors) in the protein content could be attributed to the wet extraction procedure that increased inconsistency between samples. To reduce variability, one option would be to dry and resuspend the biomass in buffer at a constant volume prior to protein extraction and analysis.

Protein molecular weight profiles were analyzed over time to reinforce protein BCA assay data. In agreement with protein contents (Figure 2(b)), Figure 3 also shows signs of protein degradation (box C) over time for TAP-N cultures as evidenced by the increased presence of low

molecular weight proteins (6 to 20 kDa) and a general decrease in higher molecular weight proteins (20 to 100 kDa) after 48 h under nitrogen depleted conditions. Furthermore, a specific decline in the amount of a ~50 kDa protein and a protein complex around 30 kDa as a function of time is evident for microalgae grown under nitrogen depleted conditions. The ~50 kDa protein (box A) is most likely RuBisCO, an abundant photosynthetic protein that is located in the chloroplast. Its degradation could be related to the mentioned decrease in protein synthesis and photosynthesis of nitrogen depleted cultures. The abundance of a 30 kDa protein complex (box B) seems to start decreasing at 72 h of nitrogen depletion as observed by decreasing protein band intensity. This protein appears to be the photosynthetic light harvesting complex (LHC) proteins. The decrease in RuBisCO and LHC proteins was previously reported by Zhang et al. (2002) when *C. reinhardtii* cells were sulfur depleted. Thus, as both nitrogen and sulfur deprivations are known to inhibit photosynthesis, the decrease in RuBisCO and LHC after nitrogen depletion is expected.

In general, protein band intensities of TAP cultured samples appear to be enhanced compared to those for the TAP-N cultures, indicating higher protein content for TAP cultures. Nevertheless, molecular weights protein profiles are mostly uniform among the TAP and TAP-N cultures between 24 and 48 h after nitrogen depletion. Based on this data, harvesting culture after 48 h in nitrogen depleted conditions seems to be adequate for lipid yield optimization while keeping total protein content and MW protein profile partially unchanged. Once harvesting times were chosen, the downstream cell disruption enzymatic treatment was designed. Biomass pretreatment was evaluated for its ability to achieve highest level of cell disruption.

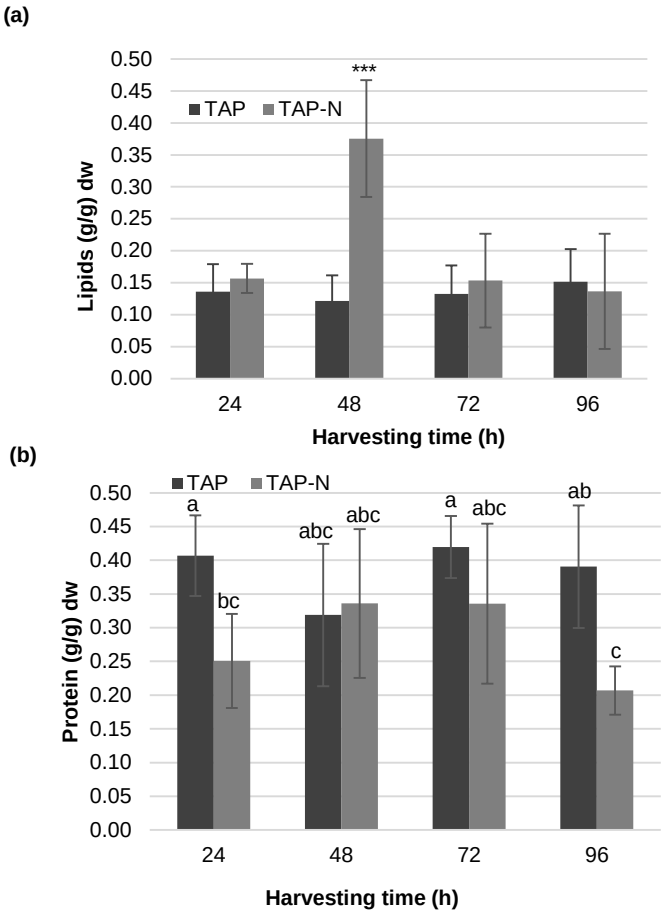


Figure 2. (a) Lipid and (b) total protein in gram per gram of dry weight biomass for cells grown in nitrogen sufficient (TAP) media or nitrogen depleted (TAP-N) media. Error bars represent standard error for n≥3. Significant differences were corrected for multiple comparisons with Tukey adjustment and a αFER =0.05. Different letters represent significant difference between treatments. Treatments with asterisks (***) are significantly different to those without asterisks. No letter or asterisks above SD bars represents no significant differences.

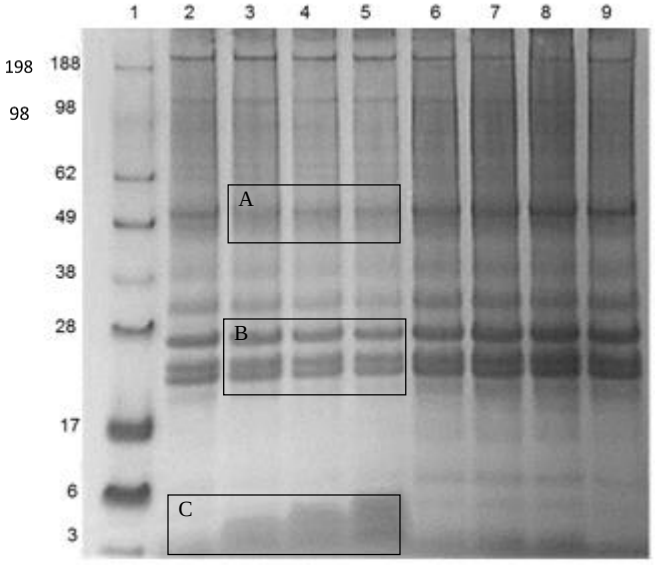


Figure 3. Coomassie-stained SDS-PAGE gel of molecular weight protein profiles for TAP and TAP-N *C. reinhardtii* cultures over time. Lane 1, Molecular weight marker; Lanes 2-5, protein profile of cultures 24 h (lane 2), 48 h (lane 3), 72 h (lane 4), and 96 h (lane 5) after nitrogen depletion; Lanes 6-9, protein profile of cultures 24 h (lane 6), 48 h (lane 7), 72 h (lane 8), and 96 h (lane 9) in nitrogen sufficient media, respectively. Apparent protein degradation is shown in the boxes. This gel is a representative sample of 3 replicates.

COMPARISON OF CELL LYSIS AMONG DIFFERENT ENZYMATIC TREATMENTS

Biomass pretreatment is usually employed to disrupt microalgae cell walls and allow bioproduct release into the media, thus facilitating the extraction process. To achieve high levels of cell disruption, an enzymatic treatment should be developed based on specific cell wall composition of the microalgae specie. To determine the most effective cell disruptive enzyme, microalgae biomass was treated with a set of potentially lytic enzymes including the autolytic protease produced by *C. reinhardtii, autolysin,* the glycoside hydrolase, lysozyme, the proline-cleaving enzyme, collagenase, and another less specific protease, trypsin. Cell permeability of *C. reinhardtii* cells was evaluated over time when treated with each enzyme. Microscopy analysis (Figure 4(a)) using NP-40 detergent indicated that after 2 h, biomass treated with autolysin had significantly higher levels of cell disruption (% permeable cells) when compared to either the control or the other enzymatic treatments. The difference in the extent of cell disruption is indicated by the significantly lower number of intact cells following NP-40 addition (Figure 4(a)). These results were confirmed by microscopy analysis with Sytox® green fluorescent dye as shown in Figure 4(b), which dyes nucleic acids of only permeable cells. Thus, if the selected enzyme treatment is effective in permeabilizing the cell wall, the dye will penetrate the cell, allowing nucleic acids to be stained and fluoresce. If not, the dye will not be able to enter the cell and stain nucleic acids. Biomass treated with autolysin (Figure 4(b)) showed higher number of fluorescent cells when compared to the control. Autolysin treatment resulted in more than 95% cell permeability when assessed with cell counting after NP-40 detergent addition or Sytox® green fluorescent staining (Figure 4(a) and (b)). Lysozyme also showed some lytic activity but to a lesser extent than autolysin. Regarding trypsin and collagenase, cell permeability was not significantly different when compared to the control. Given the results from this preliminary enzymatic pretreatment screening, autolysin was chosen as the most effective enzyme for permeabilizing *C. reinhardtii* cell walls.

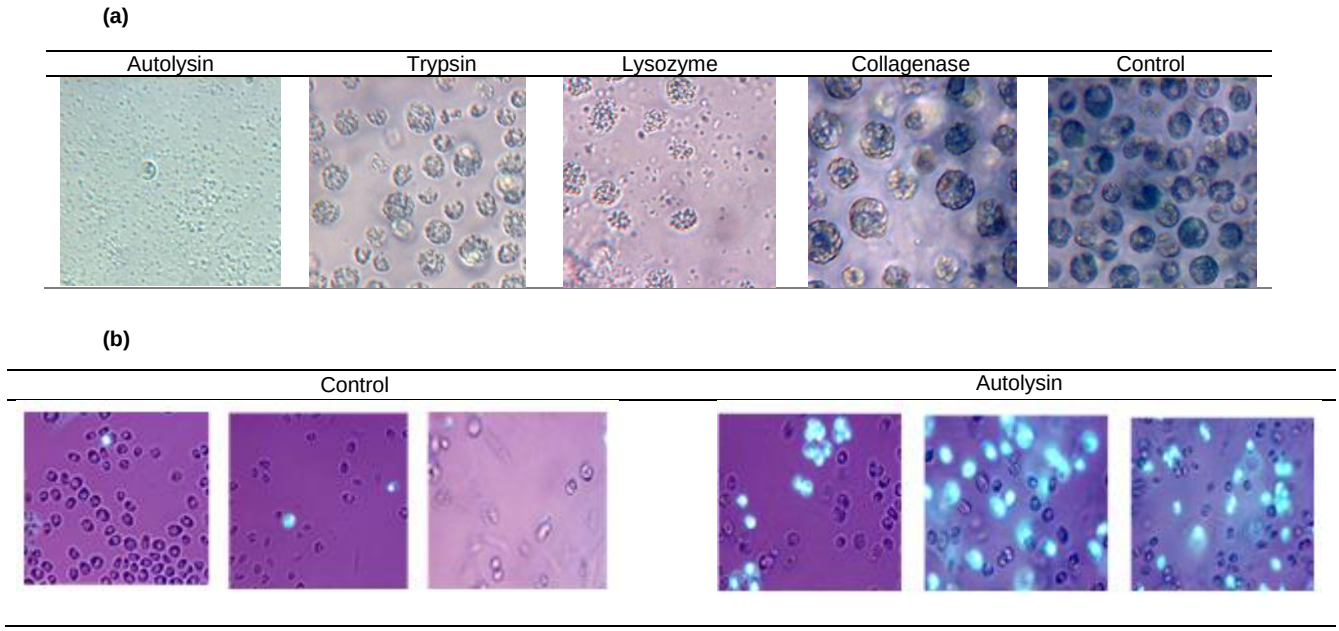


Figure 4. Cell permeability imaging assessment (a) using NP-40 to evaluate biomass treated with different enzymes for 2 h and (b) using Sytox® green under fluorescence microscope for control samples (biomass without autolysin) and autolysin-treated biomass was stained. Images are representative of >3 replicates.

ANALYSIS OF CELL DISRUPTION AFTER AUTOLYSIN TREATMENT

Incubation time effect on cell disruption

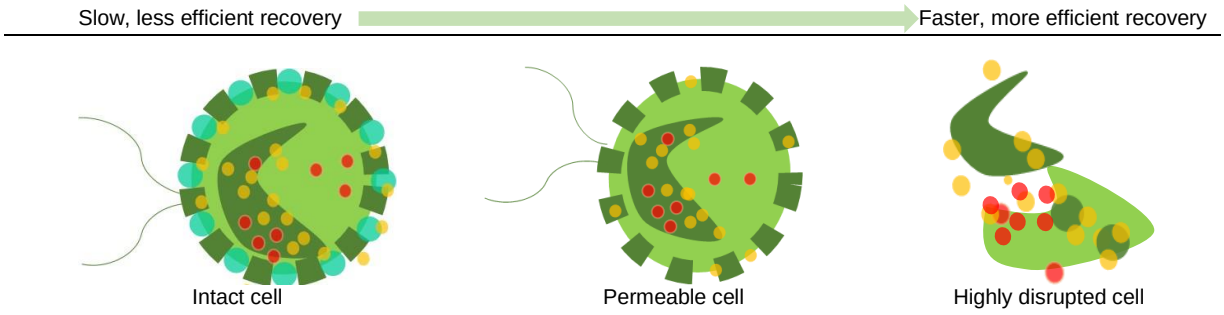
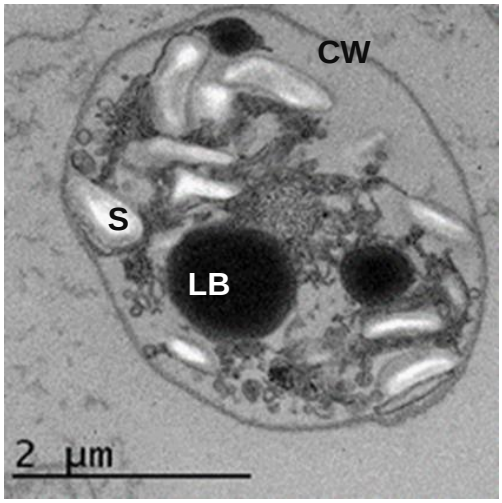
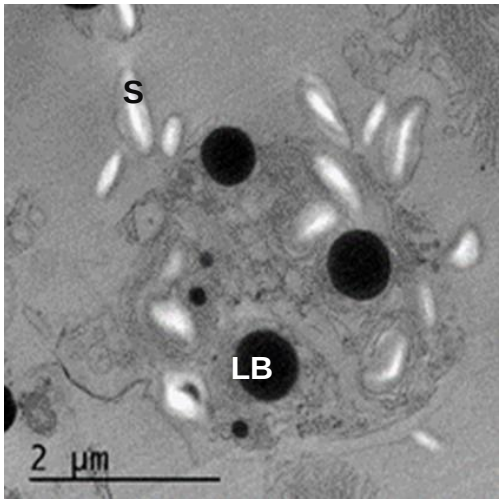
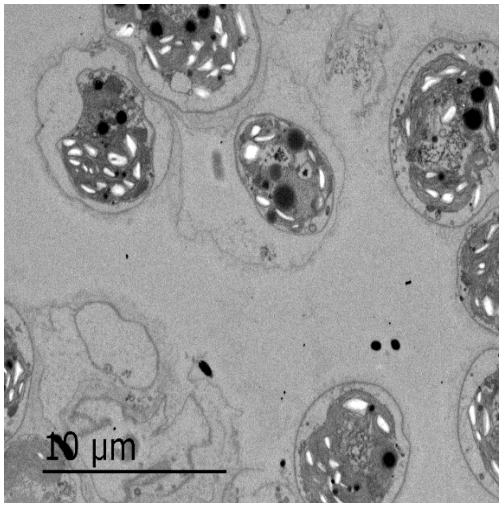
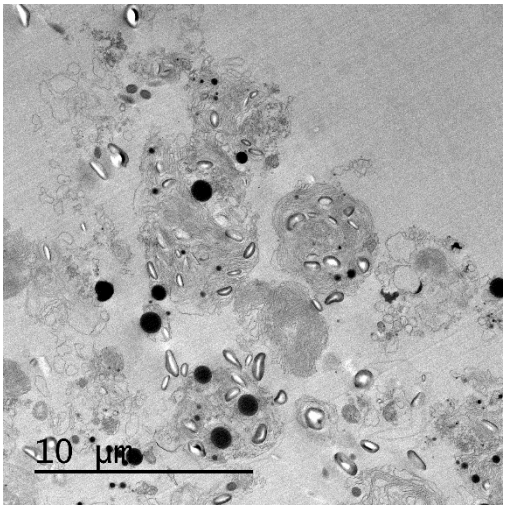
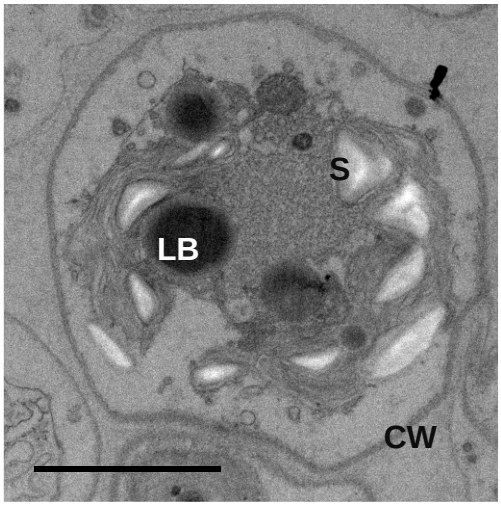
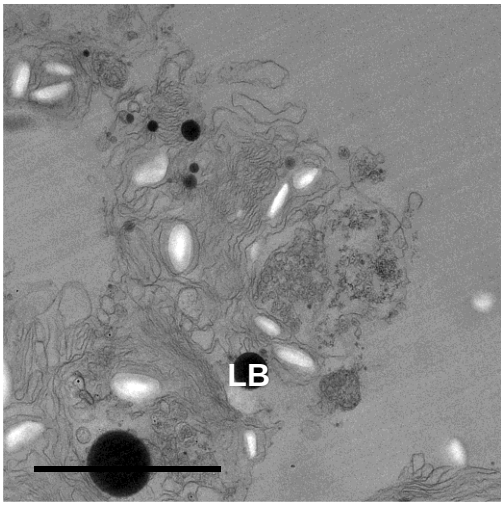


Figure 5. Schematic representation of different levels of cell disruption and their influence on lipid recovery. Adapted from Yap et al. (2014). Ovals represent cell wall proteins (blue) internally stored proteins (red) and lipid bodies (yellow).

In microalgae extraction processes, it is crucial to achieve high levels of cell disruption so the solvent can diffuse rapidly into the cells and the lipids can be released into the solvent phase. When cell walls are not disrupted (Figure 5, left) the solvent cannot access the lipids stored inside the cell, sometimes prolonging the recovery process or decreasing extractable lipid yields. When cells are permeable (Figure 5, center), the solvent can diffuse faster inside the cell, making the separation and recovery process more efficient. When cells are lysed (Figure 5(a), right) the bioproducts are directly available and diffusion into the cell is not needed, so the recoverable yield is maximized in a shorter time. In general, increasing levels of cell rupture increases the efficiency

of the lipid recovery process (Yap et al., 2014). Thus, once cell permeabilization was achieved, the effect of autolysin (Figure 5, center) on further cell disruption and internal product release (Figure 5, right) was evaluated by increasing the enzyme incubation period up to 24 h. To visualize differences in cell disruption between incubation times, TEM imaging was performed for cells treated for 2 h and 24 h with autolysin.

Confirming previous results, cells were permeable (Figure 6(b), right) and starch release was apparent after 2 h of autolysin treatment, while only intact cell wall was observed in the control treatment. Interestingly, after 24 h of autolysin treatment, several starch and lipid bodies were released (Figure 6(d), right) from the treated cells. Empty lipid and starch sacks were visible, providing evidence of product release as well as cell structure disruption (Figure 6(c), right). No intact cells were apparent after 24 h of autolysin treatment (Figure 6(c-d), right) while cell wall (Figure 6(a-d), left) remained intact and no bioproduct release was observed for the control cells throughout the entire treatment. TEM imaging indicates significant signs of cell integrity disruption after the autolysin treatment for the cells remaining after the 24 h of treatment (Figure 6(c), right). Lower magnification images (Figure 6(d), right) showed that even though cell disruption was high, lipid bodies were still attached to chloroplast remnants, which could prevent lipids from separating from the cell debris and coalescing.

Incubation Time		Control	Autolysin
2 h	(a)		
2h	(b)		
24 h	(c)		

24 h (d)

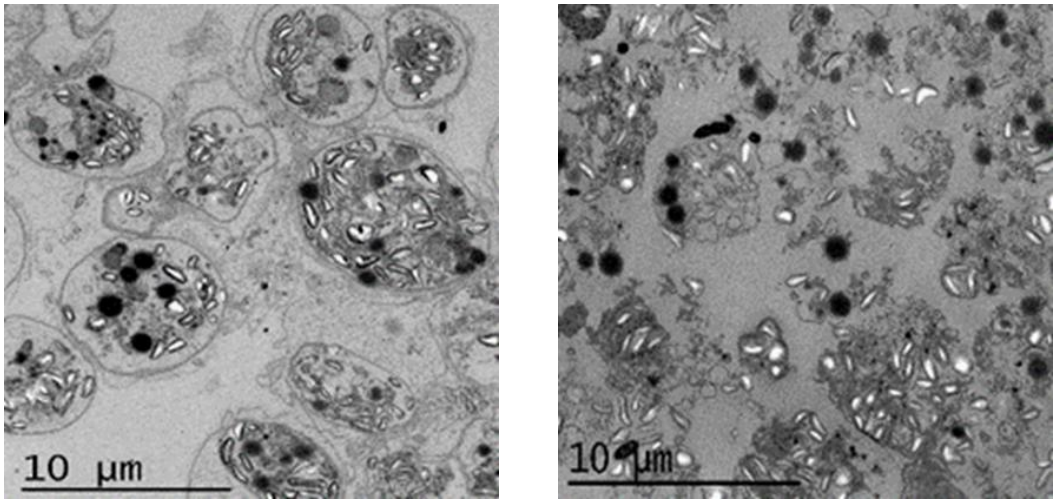


Figure 6. TEM images of *C. reinhardtii* cells incubated with TAP-N (control) or autolysin for 2 h and 24 h at 890x (a, c) and 2900x (b, d) magnification. Letters indicate cellular components: (S) starch bodies, (LB) lipid bodies (CW) cell wall. These images are representative of more than 2 replicates.

Temperature effect on cell lysis

From the previous qualitative findings, studies were conducted to further evaluate the ability of autolysin to catalyze not only cell wall permeation but also cell lysis when biomass was incubated with the enzyme for an extended time. The effect of increasing the temperature up to 35°C during autolysin treatment was also tested. Incubation and mixing of treated biomass was conducted at room temperature (25°C) and then increased to 35°C. Treatment effectiveness was determined by counting cells at pre-determined times (1, 4, and 24 h) during incubation and calculating the percent cell lysis. Results (Figure 7) showed that cell lysis could be significantly increased by increasing the incubation time from 2 h to 4 h. After 4 h of enzymatic treatment at room temperature, an average of 54 ± 7% were lysed. Furthermore, no significant differences were found between 35°C and 25°C treatment for 4 h of incubation. For the treatment at 35°C, percent of cell lysis was significantly higher at 2 h of incubation when compared to the treatment at 25°C.

Prolonging autolysin treatment for more than 4 h only showed a slight increase in about 10% cell lysis when comparing the treatment at 35°C for 24 h with 25°C for 4 h. To avoid larger incubation times and temperature treatments, incubation for 4 h at 25°C was selected over the treatment of 24 h at 35°C as the most efficient treatment conditions to optimize cell lysis. This

level of cell disruption should be enough to ensure a rapid and efficient rate of lipid recovery. With the cell disruption achieved, it should be possible to improve lipid extraction with biphasic solvent systems (such as hexane), which are immiscible in water and require high levels of cell disruption and monophasic-biphasic solvent systems (such as chloroform/methanol) which are miscible in water but diffuse at slower rate when cells are not permeable. For both temperatures, no significant levels of cell lysis were found in the control treatments for any incubation time or temperature (data not shown).

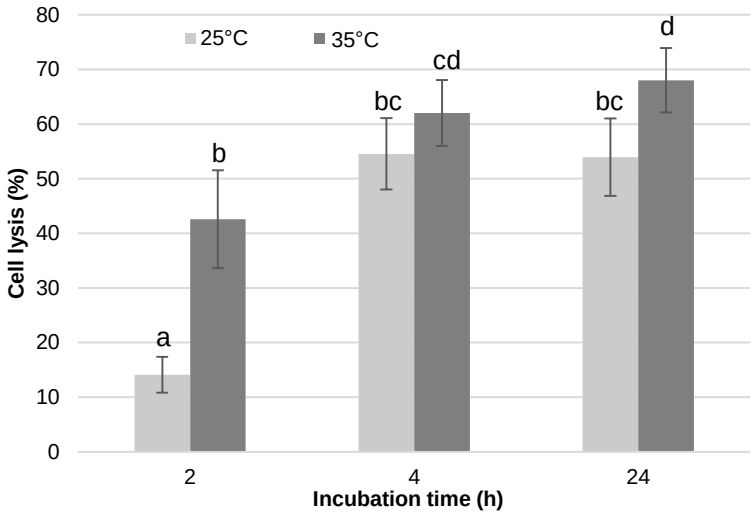


Figure 7. Percent cell lysis after autolysin treatment over time at different temperatures. Error bars represent standard error for n>3. Significant differences were corrected for multiple comparisons with Tukey adjustment and αFER =0.05. Different letters represent significant difference between treatments.

EFFECT OF AUTOLYSIN ON PROTEIN AND LIPID EXTRACTION

After treatment with autolysin, proteins in the slurry induced emulsion formation, preventing lipids from coalescing. Solvent addition was necessary to separate lipids from aqueous phase. If a solvent free system is desired, additional steps for further degrade cell debris and other proteins present would be needed. At this stage of the extraction process, solvent is still required to dissolve lipids, allowing them to separate from aqueous phase (proteins and cell debris) and coalesce in the solvent phase. Thus, once cell disruption and partial cell lysis was confirmed, the effect of the enzymatic pretreatment on recoverable lipid contents was evaluated. Biomass was pretreated with

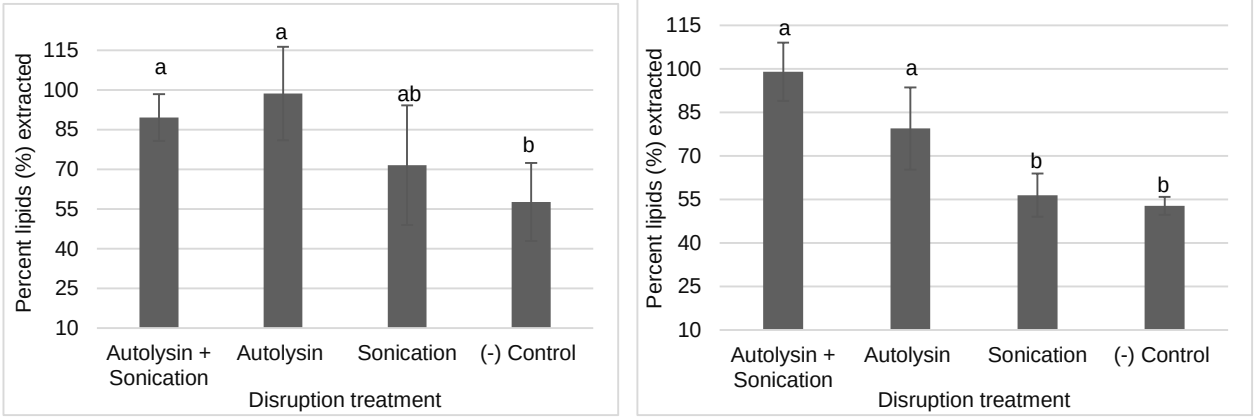
either autolysin, control buffer, autolysin plus sonication (1 min 35% amplitude), or TAP-N media (no autolysin) plus sonication. Following pretreatment, lipids were extracted using either Bligh and Dyer or hexane extraction to separate lipids from cell debris and other biomolecules. These two methods were chosen to represent two different mechanisms for lipid recovery: monophasic-biphasic (Bligh and Dyer) and biphasic solvent (hexane) extraction systems that are currently utilized for diverse applications (Yap et al., 2014). For both extraction methods, results showed a significant increase in extractable lipid yield for cells pretreated with autolysin when compared to cells with no pretreatment (Figure 8(a-b)). Addition of a sonication step following autolysin treatment did not significantly improve lipid recovery for either extraction method. When Bligh and Dyer extraction was performed (Figure 8(a)), no significant differences were found between autolysin plus sonication, autolysin, or sonication treatments. Nevertheless, extractable lipid yields for these pretreatments were significantly higher when compared to no pretreatment. When hexane was the extraction solvent, extractable lipid yield of biomass treated with autolysin (Figure 8(b)) was, on average, 30% higher when compared to either sonication pretreatment or a negative control where no biomass pretreatment was conducted. Improvements in extractable yields when autolysin treatment was performed were higher for the hexane extraction method probably because hexane *only* separates lipids from permeable feedstock material (Serrato, 1981) whereas solvents used in the Bligh and Dyer method can diffuse through non-permeable cells.

Hexane is the most utilized solvent for lipid recovery at an industrial scale. It can be an energy intensive extraction method as it can only separate lipids from permeable cells, which is usually achieved by drying biomass. By using autolysin as a pretreatment, energy expenses in the drying step could be avoided as autolysin can permeate cells with no need of pre-drying. In the case of Bligh and Dyer, the utilization of autolysin could potentially reduce incubation times with the solvent as the products are ready to be dissolved in the solvent phase and slow diffusion processes through the cell wall are avoided. Summarizing, for both extraction methods, autolysin appeared

to be an efficient pretreatment, significantly improving extractable yields up to 100% of recovery. After the enhancing effect of autolysin on lipid recovery was confirmed, its effect on protein recovery was explored.

To recover proteins, they must be solubilized and released from internal compartments into the media. To analyze the effect of autolysin on protein solubilization, autolysin treatment was compared to detergent and sonication treatment. We chose detergent treatment as it is one of the most common chemical solubilizing agents in the isolation and purification of membrane proteins (le Maire et al., 2000), allowing the release of membrane and internally stored proteins. Results showed that protein was completely solubilized (Figure 8(c)) by both autolysin plus detergent and autolysin plus sonication treatment. Neither sonication, detergent nor autolysin treatment by itself could solubilize protein completely. Interestingly, autolysin itself solubilized 20% (w/v) of the total protein content. Autolysin treatment significantly improve protein solubilization for both sonication and detergent treatment by approximately 15 and 20%, respectively.

(a) **(b)**



(c)

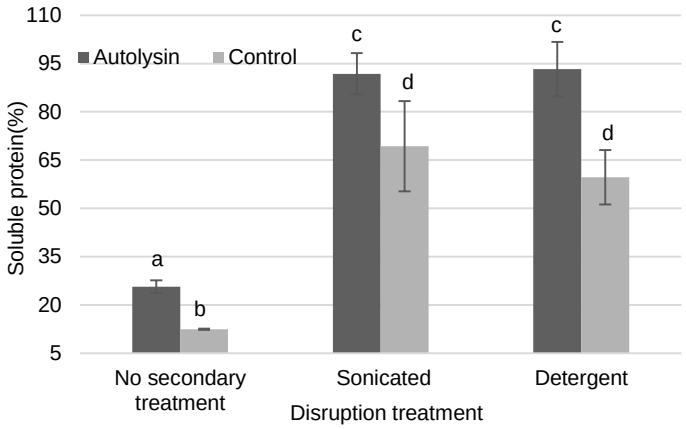


Figure 8. (a) Percent lipids extracted by (a) Bligh and Dyer and (b) hexane extraction for different biomass pretreatments (c) Percent of total protein solubilized over time of extracted supernatants under different cell disruption treatments. Soluble protein percentages were calculated based on a total extractable protein reference. Extractable lipid yield percentages were calculated based on a total extractable lipid yield reference Error bars represent standard error for $n \geq 3$. Significant differences were corrected for multiple comparisons with Tukey adjustment and $\alpha FER = 0.05$. Different letters represent significant differences between treatments.

Based on these results, autolysin pretreatment appears to be more efficient when compared to sonication treatment for lipid and protein extraction. A combined sonication plus autolysin treatment completely solubilized total protein from the biomass. In the case of lipid extraction, the sonication step was not necessary when autolysin treatment was performed. In general, autolysin appears to be a highly effective cell disruption treatment alternative. Autolysin effectiveness can be attributed to the selective degradation of cellular membranes rather than the deliberated disruptive energy applied in a sonication pretreatment. Autolysin selectively degraded cell membranes (Figure 9(b)) while preserving internal products. For all the different treatments tested, total protein and lipid recovery was only achieved when autolysin pretreatment was performed (Figure 9 (c & d)). Presumably, sonication treatment could lyse the cells only if sonication energy (amplitude) is significantly increased, but only autolysin can completely permeabilize cells with no need of additional energy input. Hence, autolysin pretreatment can facilitate and ensure recovery of internally stored products, while minimizing the energy invested in the process. Autolysin treatment has the potential to improve bioproduct recovery, reduce energy consumption,

buffer exchange steps, enzyme conditioning and acquisition costs as well as increasing lipid and protein recovery yields.

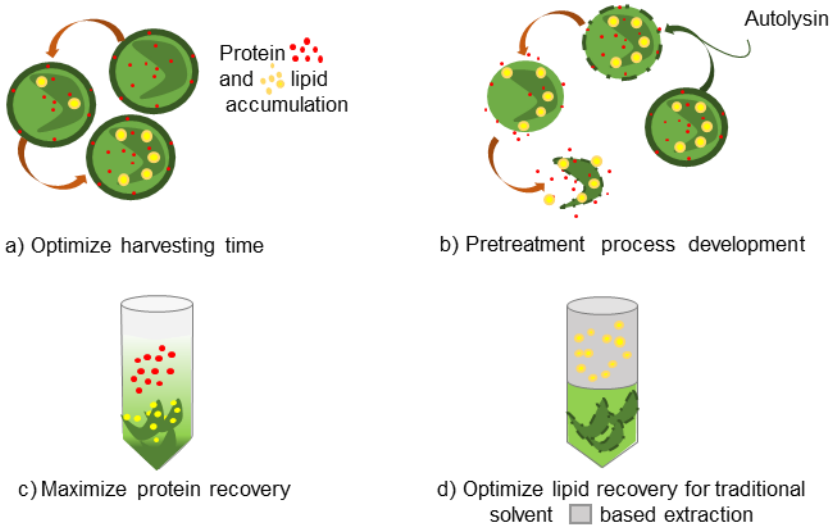


Figure 9. Schematic representation of the aqueous enzymatic assisted extraction process developed

CONCLUSIONS

This study has explored the possibility of maximizing lipid and protein accumulation and recovery in the microalgae *Chlamydomonas reinhardtii* at optimized harvesting times. Results showed that after resuspension in nitrogen deficient media, a harvesting time of 48 h had the highest protein and lipid yields, while preserving the protein profiling partially intact. Furthermore, biomass incubation with autolysin produced by *C. reinhardtii* was shown to be a feasible enzymatic pretreatment to degrade algae cell walls. Aqueous enzymatic autolysin pretreatment can potentially reduce costs associated with drying steps, mechanical cell disruption, enzyme acquisition, buffer exchange, and temperature and pH adjustments.

Autolysin treatment exhibits complete cell permeabilization when compared to the other enzymatic treatments. Interestingly, not only cell permeabilization but also cell lysis was achieved with autolysin treatment when incubation time was increased. Further research should be conducted in order to determine the effect of temperature increase on protein solubility and lipid release in *C. reinhardtii* cells during autolysin pretreatment.

Autolysin appears to be an effective biomass pretreatment for the extraction of proteins and lipids, allowing the elimination of energy intensive disruption processes and significantly increasing extractable protein and lipid yield when compared to either no disruption treatment or sonication pretreatment. The utilization of autolysin coupled with sonication can be a good alternative for extracting proteins from *C. reinhardtii* that will be further converted into food products or other applications that forbid the use of harmful solvents.

Further research should focus on designing a protein solubilization process that could optimize extractable protein yield after autolysin treatment with no need of additional mechanical nor chemical treatments. Due to the lytic effects of the enzyme, longer incubation times and temperatures could potentially increase the percent of total protein solubilized by autolysin. Lipid extraction still appears to require the utilization of a coupled enzyme-solvent system possibly because lipid bodies are still attached to cell remnants and surrounded by a lipid droplet surface protein that prevents them from coalesce. Nonetheless, biomass incubation with general proteases, such as trypsin, after protein extraction, could facilitate lipid droplet surface protein degradation and coalescence. Additionally, it can promote general degradation of other proteins that favor emulsion formation and prevent lipids from coalesce, acting as surfactants. Further research should be conducted in order to develop an economically feasible, solvent free process for the extraction of both bioproducts.

Optimization of autolysin activity and production should be addressed for this particular application. Currently, no major research efforts have been made to optimize autolysin activity due to the nature of its current application (cell transformation in the biology field). In general, enzymatic lysis of microalgae cell via autolysins, predator-secreted enzymes, or any exploitable enzyme secretion mechanism could potentially overcome enzyme prices and increase efficiency of current enzymatic treatments. The utilization of *in situ* produced enzymes has been demonstrated to be successful in other microalgae species, such as bacterial lysis for *Nannochloropsis sp.*

(Wang, 2014). Nevertheless, applying these autolytic mechanisms for different biomass sources should be further explored.

CHAPTER 3: AQUEOUS ENZYMATIC PROTEIN AND LIPID RELEASE

ABSTRACT

The use of aqueous enzymatic extraction (AEE), a non-solvent and environmentally friendly bioproduct recovery method, provides an opportunity to design an integrated process for protein and oil fractionation to reduce bioenergy and bioproducts industrial costs. A study was conducted for the establishment of an enzymatic treatment and extraction process for oil and native proteins from wild type Chlamydomonas reinhardtii. This microalgae has a demonstrated potential to serve as an expression platform for a variety of bio-products. Advances in biochemical and molecular manipulation have led to increased biomass productivity and oil accumulation in C. reinhardtii. Based on the mechanistic understanding of biomolecules distribution and compartmentation, an aqueous enzymatic treatment for the release of internally stored lipid bodies was designed. Application of a C. reinhardtii-produced protease, autolysin, for lysis of the microalgae cell wall was followed by a secondary treatment with trypsin for chloroplast disruption and lipid body release. Protein recovery and lipid characterization after autolysin treatment indicated a 54% release of total soluble protein and a localization of lipids to the chloroplast. Additionally, the development of secondary enzyme treatment (trypsin) for chloroplast and oil body lysis demonstrated a higher percent of total lipids released into the supernatant. Taken together, results indicate the application of an enzymatic treatment scheme for protein and oil recovery as a promising alternative to traditional extraction processes.

MATERIALS AND METHODS

STRAIN AND CULTURE MEDIUM

Algae biomass

Stock cultures of *Chlamydomonas reinhardtii* (CC-409 mt+) were obtained from Chlamydomonas Resource Center, University of Minnesota. *C reinhardtii cells were* grown in

TAP plates for 5 days under constant light conditions (27 μM/m^2-s) and then transferred to liquid TAP media. Once the lag phase was reached (~1 $X10^7$ cells per mL), biomass was centrifuged at 6,000 g for 5 min, washed, and re-suspended for 48 h into the same volume of nitrogen depleted (TAP-N) media. After depletion period, biomass was harvested and stored at -80°C until use.

Mating strains

High efficiency *C. reinhardtii* strains CC-620 mt+ and CC-621 mt- were kindly provided by Dr. Olson from the Biology Department at Kansas State University. Cells were grown in solid TAP media until high mating efficiency was achieved. After, cells were solubilized and suspended into liquid TAP media.

CHARACTERIZATION OF THE ALGAE EXTRACT

Total protein quantification

For each total protein determination, 10 mL of biomass was centrifuged at 6,000 g for 5 min. The supernatant was removed and the biomass was resuspended in a protein solubilization buffer containing 0.75 mM lithium dodecyl sulfate (Amresco), 2.5 mM glycerol (Amresco), 51.4 mM TRIS base (Biosciences), and 0.02 mM EDTA (Alfa Aesar). Each sample was sonicated four times at 25% amplitude for a total of 2 min, with 30 s cooling time in ice after each sonication cycle. Samples were centrifuged again under the same conditions and supernatant was recovered. Finally, lysates were diluted 10 times with phosphate buffer (pH. 9.6) and total protein was measured using a BCA protein kit (Pierce™).

Lipid yield quantification

For total lipid quantification, a modified version of the Bligh & Dyer method (1959) was performed. Samples (90 mL) were centrifuged at 6,000 g for 5 min and the supernatant was decanted. Chloroform, methanol, and water were added to the pellet in a volume ratio of 1:2:1.

Subsequently, samples were sonicated for 1 min at 25% amplitude, mixed overnight, and centrifuged at 6,000 g for 5 min. The bottom lipophilic layer was extracted and filtered into pre-weighed trays. Samples were evaporated and then dried in an oven at 95˚C for 1 h. Lipids left in the trays without solvent were weighed to calculate either total lipid content or recoverable lipid content by the following equation 5.

$$LY(\frac{g}{g}) = \frac{LP(g)}{BDW(g)} \qquad (5)$$

Where,

LY= Lipid yield or recoverable lipid content (lipid content per gram of biomass

LP= Lipid productivity (amount of extracted lipids)

BDW= Dry weight of the Biomass (g)

AUTOLYSIN PREPARATION

To prepare autolysin, a modified protocol of the one proposed by Jaenicke et al. (1987) was followed. High efficiency mating strains, CC-620 mt+ and CC-621 mt-, were cultured into Bio One culture plates and placed under high intensity LED lights (35 µM/m^2-s). Three days after growth, each mating type was independently transferred into TAP-N for a final cell concentration of 1 x 10^7 cells/mL. After 12 h of constant mixing under high intensity LED lights, mating tests were performed to determine mating efficiency. For the test, 200 µL of each mating strain were mixed, allowed to mate for 5 min and observed under the microscope (VWR® fluorescence inverted microscope). If approximately 95% of cells were mating, high mating efficiency was achieved and cells were ready to be mixed. Both mating strains were mixed in a clear container, placed under high light for approximately 30 min and then centrifuged at 6000 g for 5 min. Supernatants containing autolysin were filtered with a 0.45 µm PES membrane bottle-top sterile filter and stored at -80°C until use.

PRIMARY ENZYMATIC TREATMENT USING AUTOLYSIN

Biomass was harvested and re-suspended in either autolysin buffer or TAP-N (nitrogen deficient) buffer as a negative control. Biomass was incubated at three different temperatures (25°C, 37°C, or 50°C) with constant mixing (250 rpm) for different time periods. Cell counts were taken before, during, and after treatment.

TEM IMAGING

TEM pictures were taken at the Nannotechnology Innovation Center of Kansas State (NICKS) using Tecnai™ G2 Spirit BioTWIN (FEI Company) at 80 kV acceleration voltages. Biomass samples were fixed in Trump's fixative overnight, post fixed with Osmium tetroxide, dehydrated in graded series of alcohol and embedded in spur resin. Ultra-thin sections were contrasted with uranyl acetate lead citrate and observed under FEI Tecnai 12 Bio-spirit transmission electron microscope.

QUANTIFICATION OF PERCENT PROTEIN RELEASED AFTER AUTOLYSIN TREATMENT

Biomass was first incubated with autolysin at room temperature for 4 h. Incubation with autolysin was then continued at 3 different temperatures (25°C, 37°C, or 50°C) for 20 h.

After enzymatic treatment, biomass was centrifuged at 7000 g and supernatants collected. Total soluble protein was quantified using a BCA protein kit (Pierce ™). The percent protein solubilized was calculated based on a total extractable protein reference. Total extractable protein was calculated following the total protein quantification procedure described in page 55 and percent protein solubilized was determined using equation 6,

$$\%SP = \frac{TSP(\frac{\mu g}{mL})}{TEP(\frac{\mu g}{mL})} \qquad (6)$$

Where,

%SP= Percent solubilized protein when compared to a total extractable protein reference

TSP=Total protein solubilized (μg/mL)

TEP= Total extractable protein (μg/mL)

QUANTIFICATION OF LIPID RELEASE AFTER AUTOLYSIN TREATMENT

Samples after enzymatic treatment were centrifuged at 6,000 g for 5 min. Pellet was discarded and hexane was added to the supernatants in a 1:1 v/v. The tubes containing algal cells and solvent was shaken (150 rpm) overnight. The tube was then centrifuged at 2,020xg for 15 min to remove cell solids. The supernatant was carefully collected, evaporated, and then dried in an oven at 95˚C for 1.5 h.

QUANTIFICATION OF LIPID CONTENT ON AUTOLYSIN TREATED- ISOLATED CHLOROPLASTS

To determine the amount of lipids remaining in the chloroplasts after autolysin treatment, biomass was treated with autolysin for 24 h. Cell lysate was centrifuged and remaining chloroplasts were isolated following the protocol published by Mason et al (2006). After isolation, chloroplasts were re-solubilized in storage buffer (50 mM HEPES-KOH pH 8.0 + 0.3 M sorbitol) and divided into two samples of the same volume. One sample was used to calculate dry weight of the isolated chloroplasts and the other sample was subjected to lipid extraction using a modified Bligh and Dyer method. Lipid content was calculated using equation 5.

$$\frac{DWL}{DWB} * 100\% \qquad (5)$$

Where,

DWL= Total lipid dry weight in the isolated chloroplast sample (g)

DWB= Chloroplast sample dry weight (g)

SECONDARY ENZYMATIC TREATMENT

After cells were treated with autolysin, biomass was centrifuged and supernatants were collected for further protein recovery. The remaining pellets were subjected to a secondary enzymatic treatment to release lipids from internal compartments and dissociate them from proteins, facilitating coalescence. To find the most suitable enzyme for product release, different enzymes targeting *C. reinhardtii* internal compartments were tested (Table 4). For the treatment, cell lysate was centrifuged and re-suspended in enzyme buffer. After incubation, cell lysis percent and lipid release was calculated.

Table 4. Description of enzymes used for internal compartment disruption, cell target and compatible conditions for incubation. Adapted from [1] Bergmann et al (1939), [2] Wu & Chen (2011), [3] Baillely et al (2000) [2] Kim & Chang et al (2004)

Enzyme & category	*C. reinhardtii* target	Conditions	Buffer	Source
Trypsin [1] Protease	Proteins associated with lipid bodies, thylakoid membranes, inner and outer chloroplast membranes	pH range: 7-9.5 (optimum pH 7.5) Temp. range: 20°C -60°C (optimum 37°C)	100 mM Tris HCl pH 7.8	Amresco
DSM metallo protease ® [2] Endopeptidase	Proteins associated with lipid bodies, inner and outer chloroplast membranes	pH range: 5.0-9.0 (optimum pH 7.5) Temp. range: 25°C -70°C (optimum 40°C)	100 mM Tris HCl pH 7.8	DSM Food Specialties B.V.
Alcalase ® [3] Serine endopeptidase	Proteins associated with lipid bodies, inner and outer chloroplast membranes	pH range: 6.5-11.0 (optimum pH 8.5) Temp. range: 40°C -80°C (optimum 70°C)	100 mM Tris HCl pH 8.5	Sigma Aldrich Co.
Glucanex ® [4] β-glucanase, cellulase, protease, and chitinase	Intracellular matrix in the chloroplast and proteins associated with lipid bodies	pH range: 5.0-8.0 Temp. range: 25°C -40°C	100 mM Tris HCl pH 6.5	Sigma Aldrich Co.

CELL LYSIS

For the quantitative analysis of cell lysis, cell suspension was loaded into a hemocytometer (10 µL) and cell count was performed using an inverted microscope. Percent of lysed cells was calculated with equation 7.

$$\%CL = \frac{CDA}{CB} * 100\% \qquad (7)$$

Where,

%CL= Percent cells lysed

CDA= Cell count per mL of biomass during or after treatment

CB=Cell count per mL of biomass before the treatment

EVALUATION OF LIPID RELEASE AFTER SECONDARY ENZYMATIC TREATMENT

After secondary enzymatic treatment, cells were subjected to a modified Bligh and Dyer (1959) and/or hexane extraction (Wang & Yuan, 2014).

Modified Bligh and Dyer extraction

Samples after secondary enzymatic treatment were centrifuged at 6,000 g for 5 min. The supernatants (lysate) were collected and chloroform and methanol were layered on top in a ratio of 1:1:2 (lysate: chloroform: methanol). Samples were mixed overnight at 100 rpm in a rotary shaker, centrifuged at 6,000 g for 5 min and bottom lipophilic layer was extracted and filtered into pre-weighed trays. Finally, samples were dried at 105°C for 1 h in an air for oven and trays were reweighed. The percent lipids released was calculated based on an extractable lipid yield reference. Extractable lipid yield was calculated following lipid yield quantification procedure described in page 55 and percent lipids released was determined using equation 8,

$$\%\ LC = \frac{TLC(g)}{ELC(g)} \qquad (8)$$

Where,

% LC= Percent lipid content when compared to an extractable lipid yield reference

TLC=Total lipid content (g)

ELC= Extractable lipid content (g)

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was conducted for statistical analysis of the experimental data using SAS & Grap-Prism 6 software. To compare significant differences between treatments, a Tukey adjustment was made for a family wise error rate of 0.05 ($\alpha FER=0.05$).

RESULTS

To design a process for protein and lipid recovery, loss or degradation of either product should be minimized. Proteins are more vulnerable to temperature treatment and shear induced degradation. Furthermore, when associated with lipids, they promote emulsion formation, impeding lipid separation from the aqueous media. A sequential extraction where most of the protein is released first is preferable as it can minimize protein degradation as well as prevent emulsion formation that could hinder lipid recovery. Thus, the first step was to solubilize and recover proteins.

EFFECT OF TEMPERATURE AND TIME ON CELL DISRTUPTION AND PROTEIN AND LIPID RECOVERY

Prior work (Sierra et al., 2016) indicated that autolysin is an effective enzymatic pretreatment for complete cell disruption of *C. reinhardtii* cell walls. Complete cell wall disruption and ~20% protein recovery by centrifugation was achieved after cells were incubated with autolysin for 4 h at 25°C. To evaluate and optimize protein recovery after the enzymatic treatment, biomass incubation with autolysin was performed at different temperatures (25, 35 and 50°C) and extended incubation times (8, 17 and 24 h) and total protein solubilized was compared among treatments. Biomass was

incubated with either autolysin or control buffer at each temperature. At each time point, biomass was centrifuged and the supernatants were collected and total soluble protein was calculated. Results (Figure 10(a)) showed that at 24 h of autolysin treatment, protein solubilization for all temperatures was significantly higher when compared to the control buffer. Nevertheless, a significant increase in protein solubilization of approximately 10% was observed for the control treatment at 50°C when compared to 25°C whereas no significant increase was observed after 24 h of incubation with autolysin between treatments at 25°C and 50°C. This indicated that at 50˚C there was protein being solubilized by the high temperature treatment rather than by autolysin. The treatment at 50˚C solubilized a significantly lower amount of protein when compared to treatments at 25˚C and 35˚C for all time points(data not shown). Reduced protein extractability at 50˚C could be attributed to a decrease of autolysin activity, protein denaturation, or a decrease in protein solubility characteristic of high temperature treatments (Wilken & Nikolov, 2015). Protein solubilization was significantly higher at 35˚C when compared to autolysin treatments at 25˚C and 50˚C. On average, 54.2% ± 1.1 of the protein was solubilized after autolysin treatment at 35˚C. Regarding incubation time, the amount of protein solubilized was ~15% higher for samples incubated 24 h when compared to 8 and 18 h of incubation (Figure 10(b)). Based on this results, autolysin pretreatment for 24 h at 35°C was chosen as the optimum condition for cell wall disruption and protein release. The remaining proteins are possibly still stored in internal compartments along with the lipid bodies and their recovery can be completed at a later stage. Once most of the protein was solubilized, lipid release was evaluated.

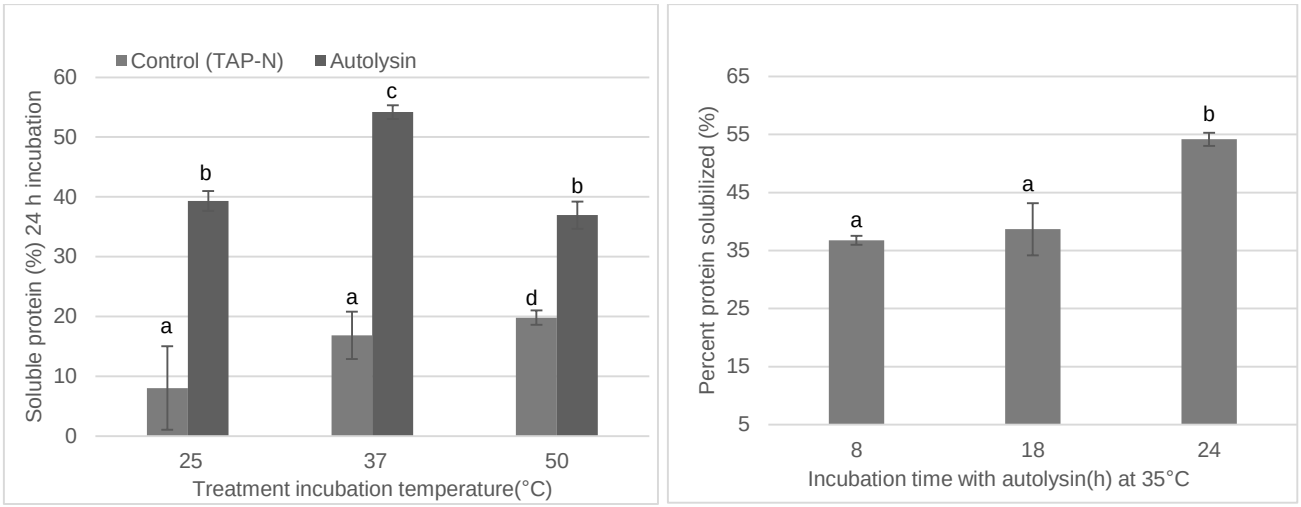


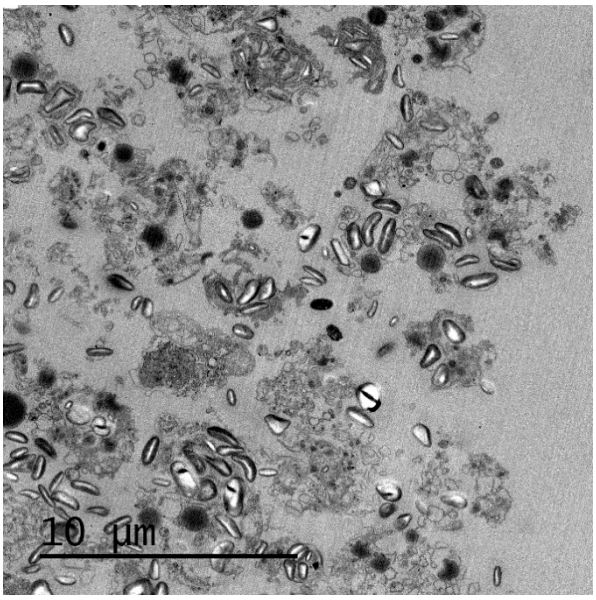
Figure 10. (a) Percent of total soluble protein released after 24 h of autolysin treatment at 3 different incubation temperatures and (b) Percent of protein released at 35˚C at 3 different incubation times. Percentages were calculated based on a total extractable protein reference. Error bars represent standard error for n>3. Comparisons were made within and between groups and significant differences were corrected for multiple comparisons with Tukey adjustment and an αFER =0.05. Different letters represent significant difference between treatments.

EFFECT OF ENHANCED AUTOLYSIN TREATMENT ON LIPID RECOVERY

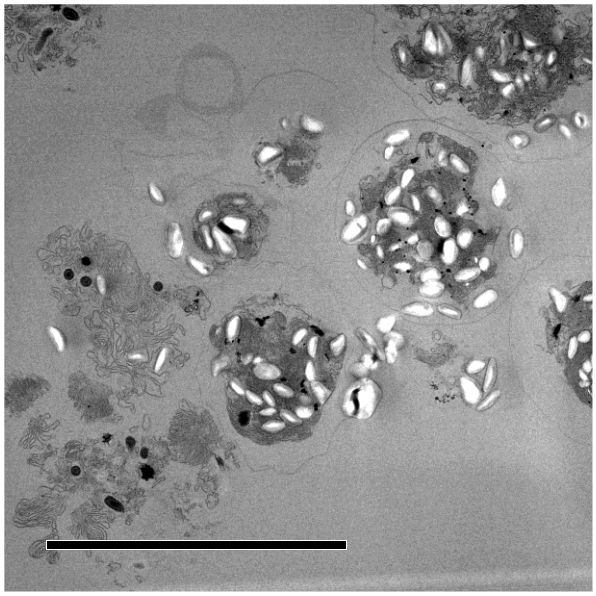
In previous research, Sierra et al (2016) reported that autolysin treatment at 25°C achieved complete cell disruption while keeping lipid bodies in the solid fraction still attached to cell remnants. To determine the effect on lipid release of increasing temperature and incubation time, samples were treated with autolysin for 24 h at 35˚C. Samples were centrifuged and supernatants were separated from cell pellets. TEM images of cell pellets were taken after autolysin treatment at 25°C and 35°C. Results (Figure 11(a)) showed that for both temperature treatments, the majority of lipid bodies were still trapped in the solid fraction (pellet) of the cell lysate. Presumably, lipid body surface proteins and phospholipids were associating with other proteins and polar biomolecules, preventing TAG from being released. Even though most of the lipid bodies were still contained in the solid fraction, TEM images also show an apparent reduction of lipid body size for the biomass treated at 35°C when compared to the treatment at 25°C.

(a)

25˚C



35˚C



(b)

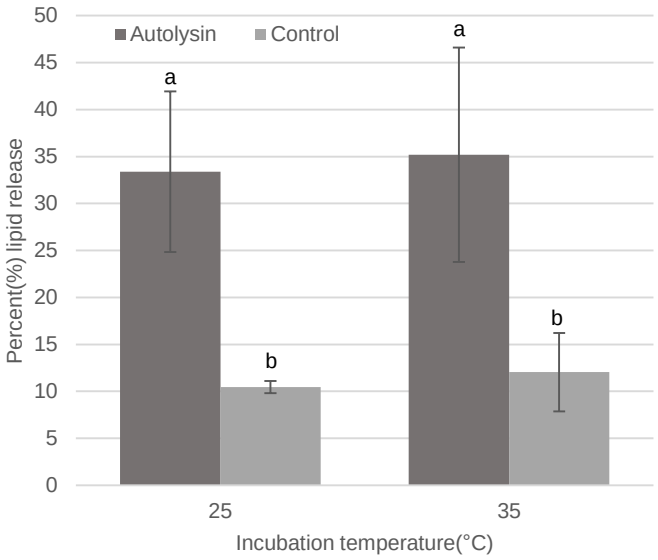


Figure 11. (a) TEM images of solid fractions after autolysin treatment at 25°C (left) and 35°C (right). (b) Percent lipid released to the supernatants of biomass treated with or without autolysin at 25°C and 35°C. Percentages were calculated based on an extractable lipid yield reference. Significant differences were found using a P-Value of 0.05

To further explore these results, lipid release into the supernatants after 24 h of autolysin treatment at 35°C was quantified by hexane extraction. We chose hexane extraction as this non-polar solvent *only* separates lipids from permeable feedstock material (Serrato, 1981). It allows for separation of free lipid bodies into the solvent phase, whereas lipid bodies still contained in the cell are not immiscible in the solvent and stay in the aqueous phase. This way, we were ensured that the amount of lipid quantified only belongs to free oil.

Results (Figure 11(b)) exhibited that an average of ~33% of lipids were released into the media. At this stage of the enzymatic extraction process, release of a significant amount of lipids into the liquid stream it is not ideal. Nevertheless, by applying autolysin, we were able to extract ~54% total proteins while ~67% of the lipids still remained in the solid fraction. If optimized, this sequential extraction process will save energy intensive steps involved in the separation of both bioproducts. Further studies should focus on optimizing incubation time, pH, buffer conductivity and enzyme dosage in order to maximize protein solubilization while minimizing lipid release.

LIPID CONTENT ON ISOLATED CHLOROPLASTS

To develop a solvent free extraction system, a secondary treatment that promoted lipid body release and oil demulsification was needed. First, we aimed to understand why the majority of the lipid bodies were not being released after the cells were disrupted. Based on research regarding lipid body accumulation of *C. reinhardtii* cells (Fan et al., 2011) and previous TEM images (Figure 11(a)), lipids can be stored in the ER and/or inside the chloroplast. If stored in the chloroplast, the previously characterized (Moellering & Benning, 2009) lipid droplet surface proteins (LDSP), could be associating with other polar biomolecules inside this organelle, preventing lipids from coalescing. Determining where lipid bodies are attached after the enzymatic treatment would provide additional information regarding which cell structures need to be cleaved to release the lipids. Thus, the next step was to confirm if the lipid bodies were enclosed in the remaining chloroplasts and chloroplasts remnants. To do so, chloroplasts remnants and thylakoids were isolated after autolysin treatment (24 h at 35°C) based on a modified protocol of the one proposed by Mason et al. (2006) and lipid content in the intact chloroplast plus thylakoids fractions were calculated.

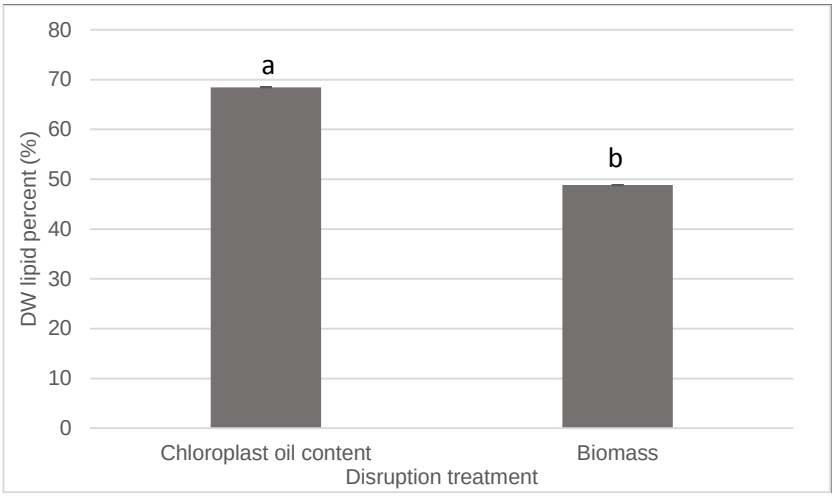


Figure 12. Percent lipid content of isolated chloroplasts and whole cell biomass. Error bars represent standard error for $n \geq 3$. Significant differences were found based on an $\alpha = 0.05$. Different letters represent significant difference between samples.

The increase in DW lipid percent indicated that chloroplasts and the disrupted thylakoids concentrated the majority of the lipids still trapped in the solid fraction. Results (Figure 12) showed that the lipid content of chloroplasts plus thylakoids was almost 70% (60% total lipids in *C. reinhardtii* cells). This is a 1.5-fold concentration when compared to whole cells. Furthermore, the gram dry basis sum of chloroplast lipids plus lipids released after autolysin treatment was approximately ~ 0.38 g lipids/g which is about 90% of the total lipid content in a *C. reinhardtii* cell after 48 h of nitrogen depletion (Sierra et al., 2016). This indicated that the majority of the lipids that were still trapped in the solid fraction were stored in the chloroplasts. Possibly, stacked membranes in the chloroplasts were trapping lipid bodies. Furthermore, the amphiphilic nature of the chloroplasts could be reducing the interfacial tension between the aqueous solution and the lipid bodies, contributing to the stabilization of dispersed droplets and avoiding their association. The attachment is possibly made between lipid droplet surface protein (LDSP) and proteins or other polar molecules in the chloroplast. Consequently, the next treatment to be designed should target not only the LDSP (Moellering & Benning, 2009) but also proteins and other molecules present in the chloroplast. Thus, the next step was to design an aqueous enzymatic treatment to disrupt chloroplast remnants and lipid droplet surface proteins (LDSP), so attached lipids could be released

EFFECT OF A SECONDARY ENZYMATIC TREATMENT ON LIPID RELEASE

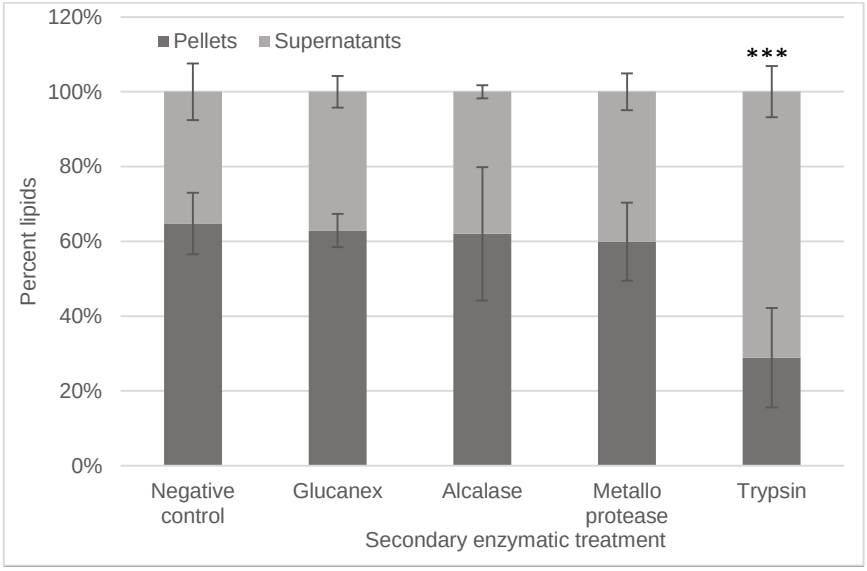
Thus far, autolysin treatment was able to permeabilize the cells and solubilize most of the proteins by extending incubation time. To release lipid bodies from internal compartments, in this case, the chloroplasts, LDSP need to be cleaved so the lipid bodies can be released from the disrupted chloroplasts remnants. To design an efficient AEE treatment, it is crucial to make sure that proteins are being cleaved by the protease chosen. Based on previous data, serine and other general proteases were selected as the best fit for cleaving LDSP and other chloroplast proteins. Alcalase and DSM-metalloprotease are widely used in the food industry and capable of cleaving several proteins into peptides. The DSM-metalloprotease was also chosen due to the mild conditions (pH 7.5 and 40°C) required for optimum activity. It is expected that Alcalase and DSM-metalloprotease will be able to break inner and outer chloroplasts membranes, if still present, and digest LDSP. Trypsin was selected as it can approximately cleave the ~260 amino acid chain of the *C. reinhardtii* LDSP about 20 times based on the primary structure and cleavage specificity. Trypsin treatment could also promote the release of lipid bodies attached between thylakoids by disrupting membrane stacking as it was reported in chloroplasts of plants such as spinach (Jennings, et al., 1980). Finally, the enzyme mixture present in Glucanex was expected to be able to target multiple components of the chloroplast membranes at relatively mild conditions (25°C and pH of 6.0).

To test the ability of these enzymes to release lipid bodies, biomass was treated with autolysin and protein was recovered as specified in pages 61-62. After, biomass was re-suspended and incubated in a solid/liquid ratio of 18 mg/mL with buffer only (negative control) or enzyme saturated buffer at optimum conditions. After 24 h of incubation, biomass was centrifuged and supernatants and pellets were collected separately. Finally, lipid extractions were performed on each fraction (supernatants & pellets) of each enzymatic treatment and lipid percent was calculated based on an extractable lipid yield. If lipids were being released from the chloroplasts, then percent lipids was expected to be higher in the supernatant samples when compared to the pellet

fraction of the same sample. On the other side, if the enzymatic treatment was not efficiently releasing lipids from chloroplasts remnants, most of the lipids were expected to be trapped in the pellet fraction.

Results indicated a significant increase in percent of oil released only for samples incubated with autolysin plus trypsin treatment. Figure 13(a) shows that more than 70% of lipids still trapped in the solid fraction (pellet) after autolysin treatment were released by trypsin treatment while no significant differences were found for the remaining enzymatic treatments when compared to the negative control. Figure 13(b), confirms the effectiveness of trypsin treatment on lipid release. When supernatants of the trypsin treatment were treated with Nile Red (yellow florescence), lipid bodies were only visible in the trypsin treated samples whereas no lipid bodies were found in the control treatment. With autolysin plus trypsin treatment, we were able to release more than 80% of total lipids stored in *C. reinhardtii* cells.

(a)



(b)

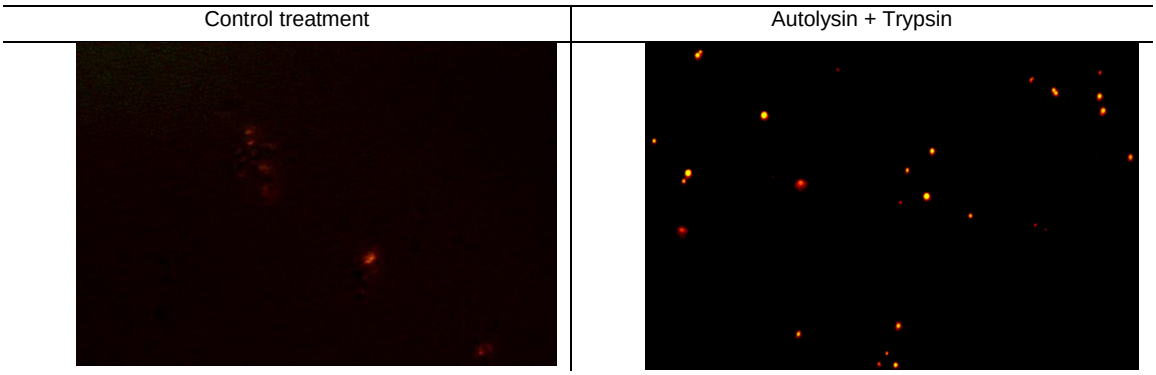
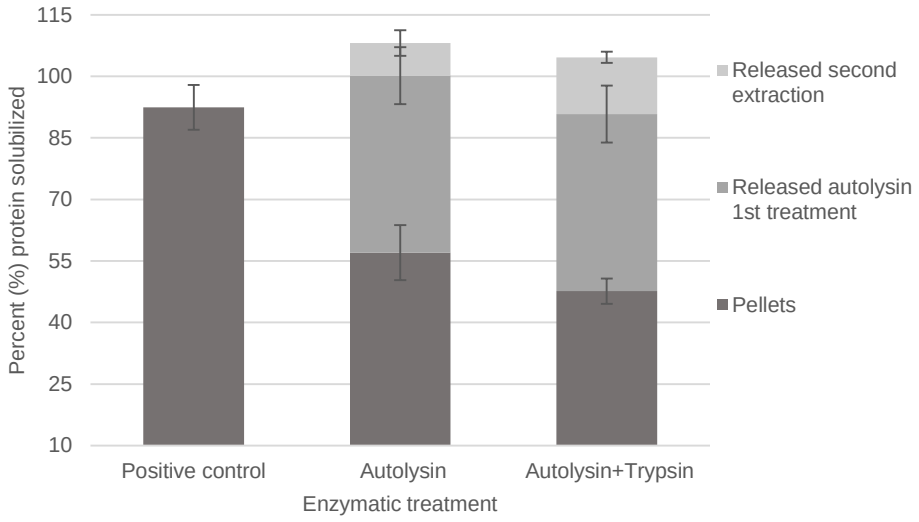


Figure 13. (a) Total lipid content (g/L) of the supernatants and pellets after incubation with TAP-N (control), autolysin and autolysin plus trypsin. Percentages were calculated based on an extractable lipid yield reference Fluorescence microscopy imaging (b) of lipids (yellow fluorescence) stained with Nile Red. Error bars represent standard error for n>3.Comparisons were made within and between groups. Significant differences were corrected for multiple comparisons with Tukey adjustment and an αFER =0.05. Asterisks represent significant difference between treatments.

PROTEIN RELEASE THROUGHOUT THE EXTRACTION PROCESS

Once lipid release was achieved, protein localization and solubilization at each stage of the extraction process (Figure 14(a)) was followed. Total protein release after autolysin and autolysin followed by trypsin treatment was quantified. Furthermore, molecular weight (MW) protein profiles (Figure 14(b) were analyzed to identify the proteins that were either being degraded or solubilized throughout the enzymatic treatment.

(a)



(b)

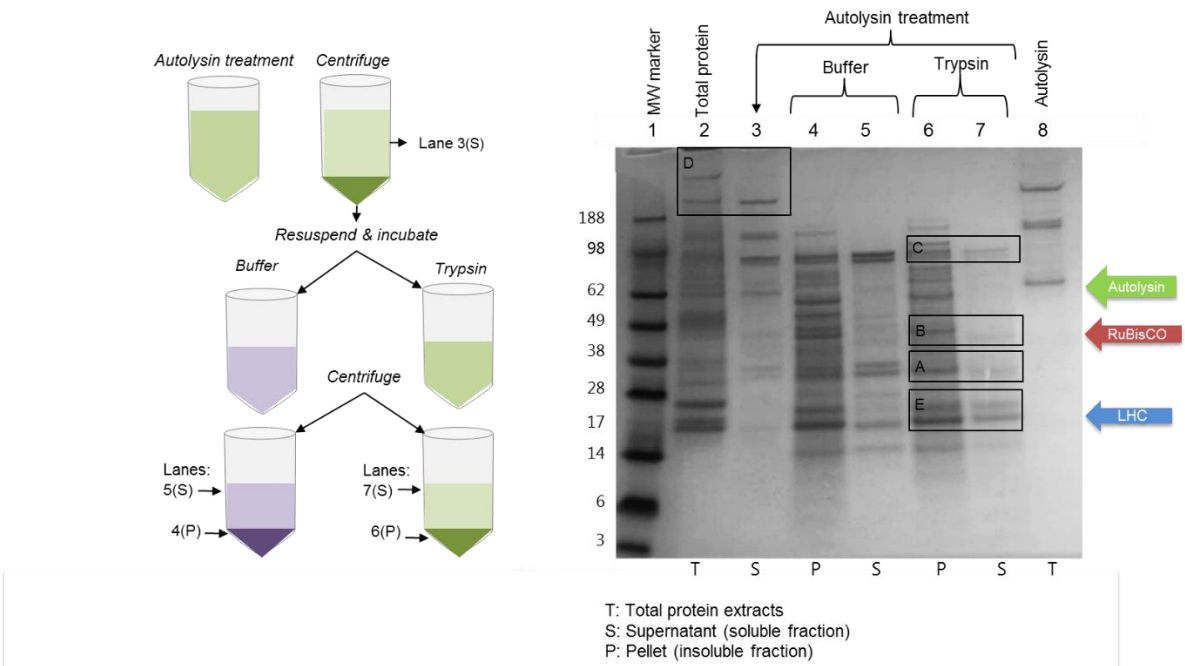


Figure 14. (a) Percent protein content (%) of the supernatants and pellets after first incubation with autolysin and posterior incubation either with autolysin or autolysin plus trypsin. Percentages were calculated based on a total extractable protein reference Error bars represent standard error for n>3.Comparisons were made within and between groups. Differences were corrected for multiple comparisons with Tukey adjustment and an αFER =0.05. Different letters represent significant difference between treatments. (b)On the left, diagram showing sample collection process for gel electrophoresis analysis. On the right, MW protein profile. MW marker (Lane 1); Total protein-10X concentration- (Lane 2); Supernatant after autolysin treatment-5X concentration (Lane 3); Pellet-10X concentration- (Lane 4) and supernatant-22.5X concentration (Lane 5) after incubation in buffer at 35 C for 24; h no trypsin 5; Pellet -10X concentration (Lane 6) and supernatants-22.5X concentration- (Lane 7) after autolysin treatment and resuspension in buffer plus trypsin; Lane 8, Autolysin-17X concentration.

Results showed that after ~55% protein solubilization induced by the autolysin treatment, trypsin treatment only increased protein solubilization ~5% on average (Figure 14(a), which was not significantly higher when compared to the control treatment (autolysin followed by incubation in buffer at 35°C, no trypsin). Even though only small fragments of the protein stored in the chloroplast were solubilized by trypsin, the specific digestion was enough to release lipids stored between the thylakoid membranes. Figure 14(b), lane 6, pellet after autolysin plus trypsin treatment, showed a decrease in band intensity of a complex of proteins of MW ~17 to 30 kDa, which is characteristic of the light harvesting complex, indicating that a portion of them were being cleaved or solubilized. Consequently, the band intensity of this proteins is increased in lane 7, corresponding to solubilized protein after autolysin plus trypsin treatment (supernatants). Solubilization of these proteins was possibly induced by the trypsin digestion. After trypsin, a

slight decrease in proteins of ~35 kDa, ~45kDa and ~98 kDa is also apparent (Figure 14(b), lanes 6 & 7, boxes A to C). Moreover, the gel shows that after autolysin treatment (Figure 14(b), lane 3) high molecular weight proteins (box D) are completely solubilized. These proteins can potentially be the glycosylated cell wall proteins, which are characterized by a high molecular weight, and were being solubilized early on after autolysin treatment. Proteins that have not yet been solubilized can be recovered from the solid fraction using a mechanical, chemical, or a tertiary enzymatic treatment. One advantage of preserving the proteins in the solid fraction is that it allows for the selective recovery of lipids from the liquid phase while keeping most of the proteins in the solid fraction (pellet). The separation caused by the density difference between both products, could potentially decrease steps and energy involved in the extraction process allowing for recovery of each product at higher purities.

EFFECT OF TRYPSIN TREATMENT ON CELL STRUCTURE AND BIOPRODUCT RELEASE

To better understand why trypsin treatment was promoting lipid release while keeping proteins in the solid fraction, the effect of autolysin plus trypsin with autolysin treatment only on lipid release was analyzed and compared by TEM imaging. Results showed a high level of cell disruption after autolysin treatment (Figure 15(a), A, B) and disruption of chloroplast envelopes was also apparent. Nevertheless, when samples were only incubated with autolysin, numerous lipid bodies were still attached to the internal portion of large and still highly compacted thylakoid membranes (Figure 15(a), D).

For the autolysin plus trypsin treated samples, Figure 15(b), G-I shows an apparent decrease in membrane stacking and relaxation of thylakoid (T) membranes when compared to samples only treated with autolysin (Figure 15(a), D). This effect was previously reported (Jennings, et al., 1980) when treating spinach chloroplasts with trypsin. According to Grebanier et al. (1979), the main effect of trypsin on chloroplast membranes is to digest a small fragment from the light-harvesting protein complex. This digestion was also visible on the gel showed previously (Figure

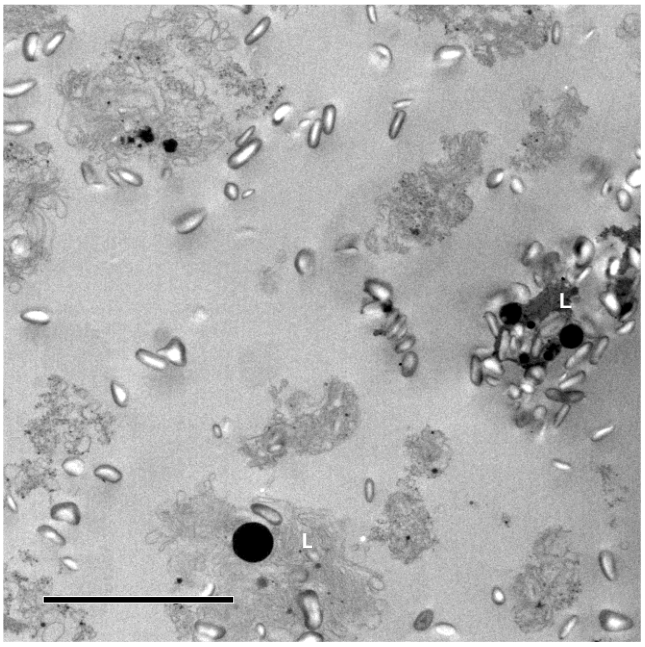
14(b), box E) where the band intensity of LHC proteins appeared to decrease in the solid fraction sample after trypsin treatment (lane 6) and increased in the soluble fraction (lane 7). Possibly, the relaxation of the thylakoid membranes accompanied by the disruption of lipid body proteins induced lipid bodies' release. A reduction in the amount of lipids still attached in the pellet when trypsin treatment was performed confirms this effect.

Interestingly, the autolysin plus trypsin treated samples (Figure 15(b), E &F) showed large amounts of free starch granules in some of the TEM sections. Possibly, starch released from the chloroplasts was precipitated at the bottom of the pellet after the centrifugation steps involved in sample preparation, therefore, TEM sections of the bottom of the pellet exhibit significant starch accumulation. This is most likely due to the higher density of the starch granules (~1.5 g/cm³) when compared to the thylakoid fragments (~1g/m^3) and lipid bodies (~0.9 g/m^3). If starch is one of the products to be recovered, the difference in density when compared to other cell components will allow this product to accumulate at the bottom of the solid phase, facilitating its recovery and further purification. Finally, empty lipid and starch body sacks are visible throughout the images after treatment with trypsin (Figure 15(b), J). Possibly, sacks were permeabilized by trypsin digestion, allowing both bioproducts to be released.

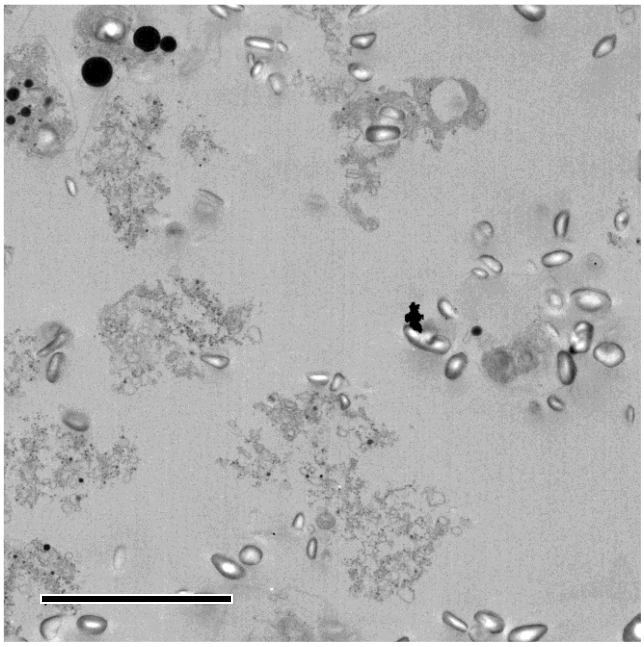
Certainly, the AEE treatment designed not only facilitates lipid and protein extraction, but also propitiates starch recovery. Further research should aim to optimize the fractionation and extraction of these three products after the enzymatic treatment.

(a) Pellet after 24 h autolysin treatment

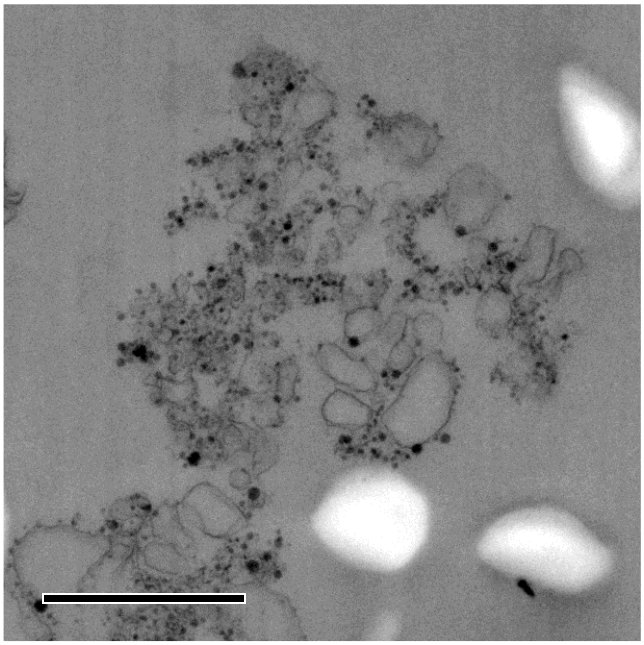
A.



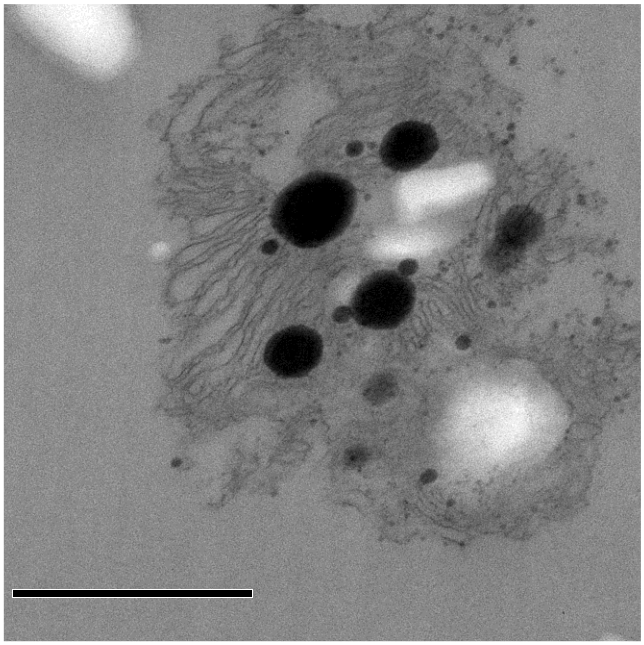
B.



C.

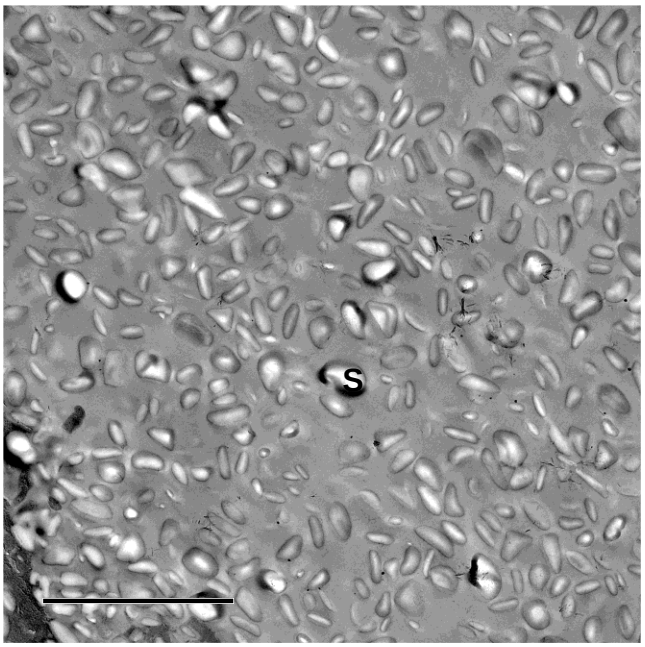


D.

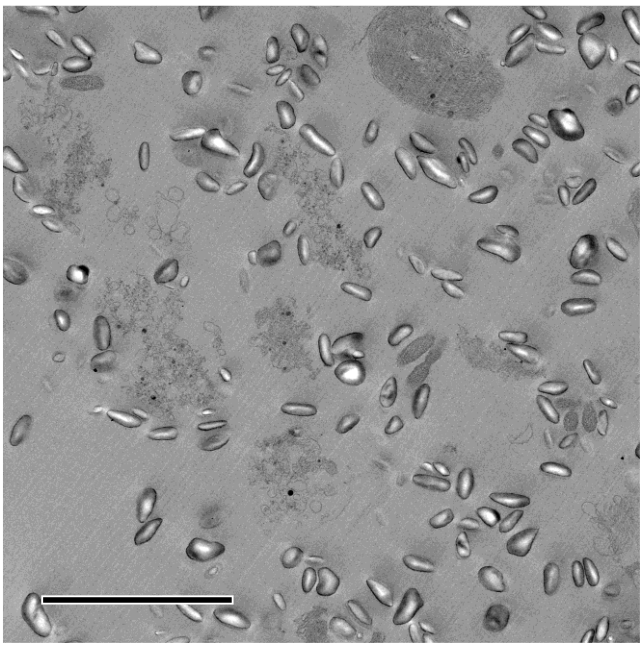


(b) Pellet after 24 h autolysin treatment plus 24 h trypsin treatment

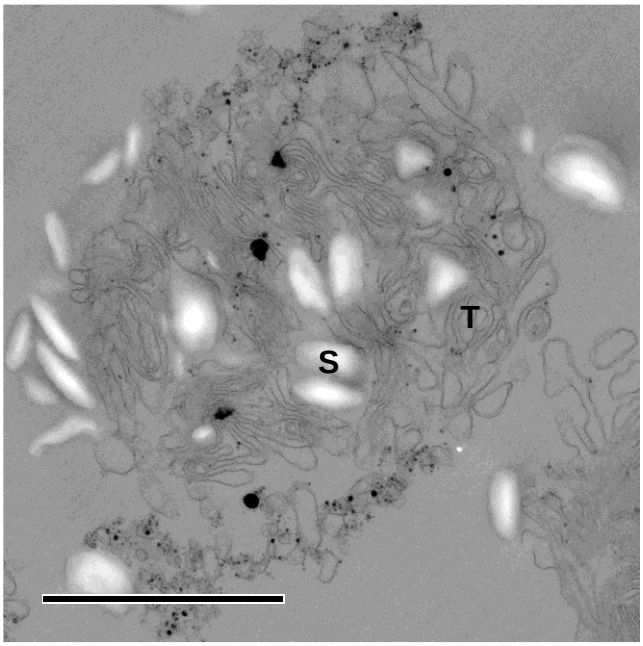
E.



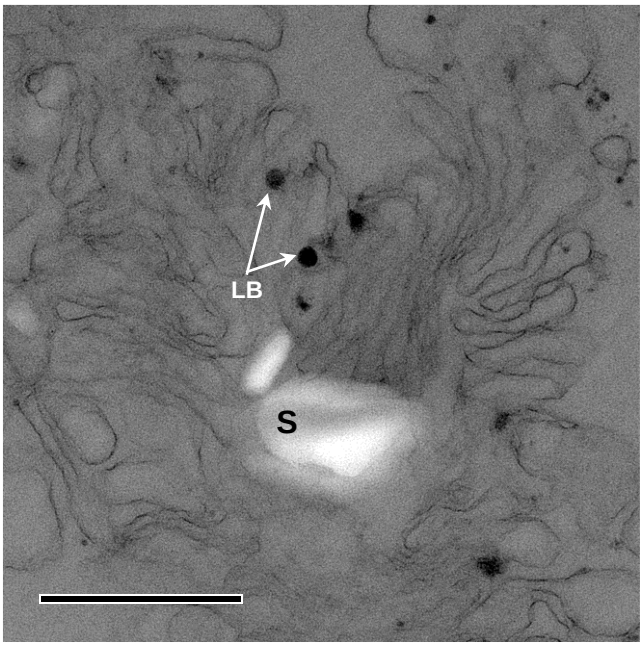
F.



G.



H.



I. J.

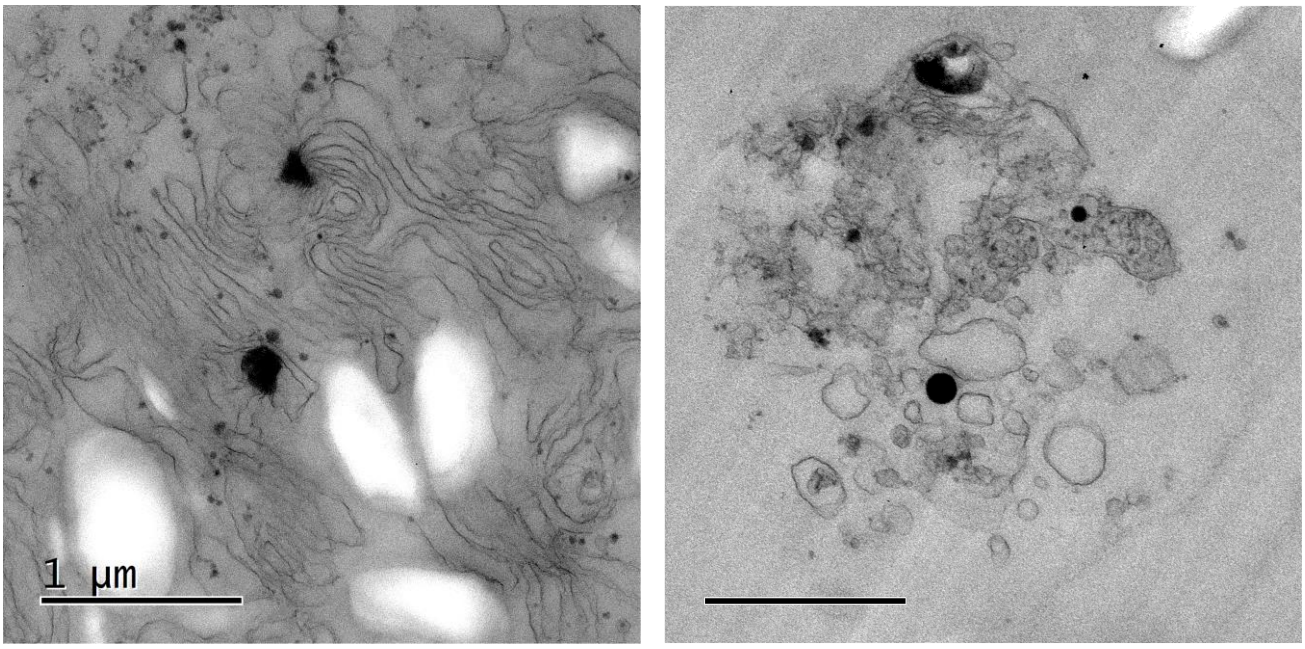


Figure 15. TEM images of C. reinhardtii cells incubated with autolysin-control-(a) or autolysin plus trypsin(b) at 1200x ((a) A, B and (b) E, F.), 2900x ((b) G) and 6400x ((a) C, D and (b) H-J)) magnification. Letters indicate cellular components: (S) starch bodies, (LB) lipid bodies, (T) thylakoids, (LBS) lipid body sacks. These images are representative of >2 replicates.

Summarizing, with our primary and secondary enzymatic treatments we were able to transform cells with intact cell walls, into highly disrupted cells (Figure 16 left), and finally, into partially fractionated bioproducts (Figure 16 right).

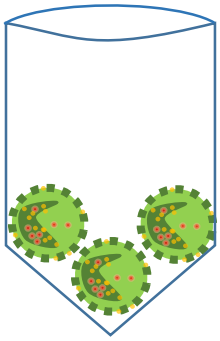
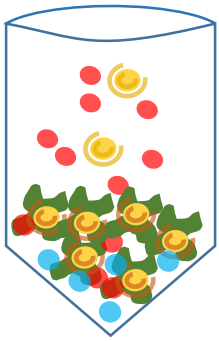
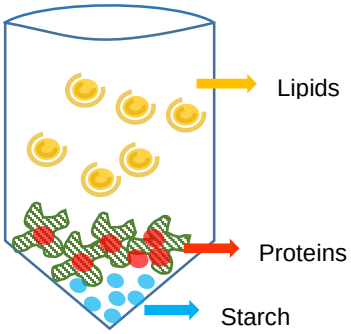
Autolysin treatment (4 h at 25°C)	**Extended autolysin treatment (24 h at 35°C)**	**Trypsin treatment**
		
Highly permeable cells. Products haven't been released to the supernatant	Highly disrupted. More than half of total protein has been solublilized (red dots), but lipid bodies (yellow dots) are still attached to chloroplasts remnants (filled green crosses) and trapped in the solid fraction	Protein digestion induced lipids (yellow dots) release. Chloroplasts remnants are unstacked (green crosses) and remained in the solid fraction). Starch accumulated at the bottom

Figure 16. Schematic representation of the enzymatic treatment

AQUEOUS ENZYMATIC VS SONICATION EXTRACTION

To evaluate effectiveness of trypsin, lipid release was compared between samples subjected to autolysin followed by trypsin, autolysin only, and sonication for 3 min at 35% amplitude with and without the aid of autolysin. For all the samples, lipids were quantified by the Bligh and Dyer method and the percent of lipids released were calculated and compared to an extractable lipid yield reference. Figure 17 shows that after trypsin treatment, there was a significant increase on lipid release of more than ~60% when compared to all the other treatments. Sonication and autolysin treatments by itself only solubilized about 10% of total lipids. When sonication was performed in combination with the autolysin treatment, a significant increase in lipid release was observed when compared to either autolysin or sonication only. This increase, however, was not even half of the amount released by the autolysin plus trypsin treatment. The high increase in lipid release can be attributed to the relaxation of thylakoid membrane as well as the targeted digestion of LDSP that most likely were holding chloroplasts remnants and lipid bodies together. Sonication is a relatively strong disruptive treatment but it could be dispersing the disruptive energy among all the biomass while some specific biomolecules that needed to be cleaved remained undisrupted. In this case, cell disruption was not sufficient for lipid release from biomass as degradation of proteins, specifically those associated with lipids, was also needed. Once lipids have been detached and released, they can be easily recovered from the slurry (such as cellular residue, enzyme, by-products, etc.) by coalescence or centrifugation (Lindell & Reddy, 2011).

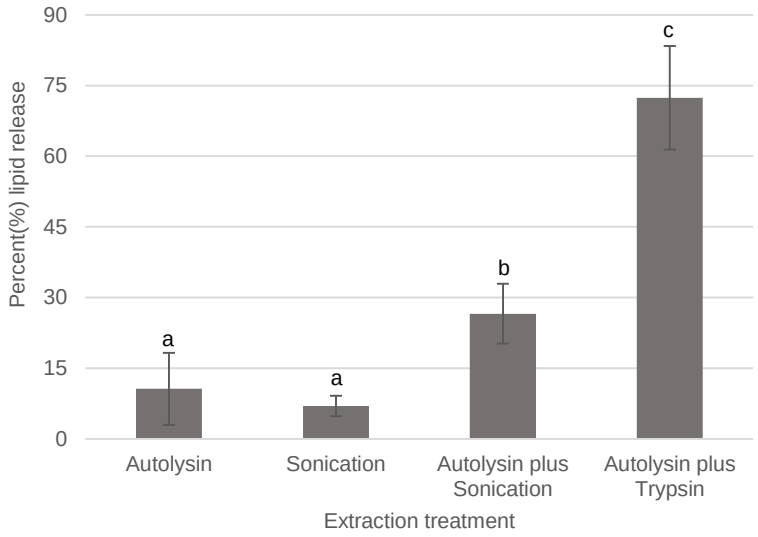


Figure 17. Lipid release by different disruption treatments. Tukey adjustment for multiple comparisons. Error bars represent standard error for n>3. Percentages were calculated based on an extractable lipid yield reference Comparisons were made within and between groups. Differences were corrected for multiple comparisons with Tukey adjustment and an αFER =0.05. Different letters represent significant difference between treatments.

CONCLUSIONS

The AEE process described utilized nitrogen-deprived *C. reinhardtii* as a feedstock for native protein and lipids. Microalgae was initially treated with an *in situ*-produced autolysin that specially targeted the composition of the *C. reinhardtii* cell wall. Based on TEM imaging, not only was the cell wall disrupted but after 24 h of incubation with autolysin, only chloroplast membranes remained partially intact. Protein content in the supernatant following autolysin treatment was maximal at 54% TSP with a temperature of 37°C for the last 20 h of incubation in autolysin. Even though cells were highly disrupted, further degradation of polar material surrounding the lipid bodies was necessary to separate lipids from the solid fraction.

Chloroplast isolation confirmed that the remaining lipids to be released were located inside internal thylakoid membranes, which was expected as the metabolic pathway for producing lipids takes place in the chloroplasts. A secondary enzymatic treatment with trypsin resulted in the release of ~73% of the lipids within chloroplasts. Trypsin-mediated lipid release was possibly achieved by relaxing thylakoid staking and cleaving LDSP, which connect the lipids and other

chloroplast residues and/or by breaking emulsion formation promoted not only by LDSP but also other proteins present in the cell lysate. Evidence of starch accumulation at the bottom of the solid fraction indicated that this one could potentially be recovered with no need of additional treatment. Further work should focus on optimizing starch, lipid, and protein yields.

With this research, we are one step further into designing a cost and energy effective process for separating and extracting microalgae bioproducts such as protein and lipids. When compared to traditional extraction processes, the enzymatic treatment developed has the potential to save energy costs and increase economic feasibility due to extraction of multiple bioproducts. Furthermore, it can potentially be implemented for the recovery of recombinant proteins due to the targeted degradation of specific organelles, while keeping other proteins intact. Finally, this treatment could avoid the utilization of toxic solvents or chemicals, thus products extracted could be utilized in the food industry.

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