PRODUCTION OF MICROALGAE BASED PROTEIN PRODUCTS

FROM ECONOMICS AND BIOPROCESSING PERSPECTIVES

A Dissertation

by

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ABSTRACT

The need for developing alternative protein sources to meet future food demand has driven microalgae to re-emerge as a promising biomass source. Considering its ability to produce quality protein, microalgae has been studied to produce protein concentrates, isolates, hydrolysates, and bioactive peptides. Despite its potential, current production methods still require optimization of processing conditions to ensure economic feasibility of algae products. Co-product extraction, such as high-value lipids, together with protein, has been proposed for increasing economic feasibility of the algae platform. Nevertheless, scalable methods for the sequential extraction of proteins and other high-value products are lacking. In this dissertation, we explore and optimize the processing of lipid-extracted microalgae into protein concentrates and hydrolysates for food applications. Chapter 2 reviews and analyzes the current methods for extraction and fractionation of protein products. Chapter 3 explores the technical and economic feasibility of a co-production platform of high-value lipids and protein concentrates from *Chlorella vulgaris*. Chapter 4 outlines and evaluates a process to produce a high-value protein hydrolysate from lipid extracted *Nannochloropis sp*. Finally, the data showed in the appendix gathers further optimization efforts on the production of microalgae protein hydrolysates, where an ultrafiltration and diafiltration process is used for the single-step clarification and purification of a higher quality product. The combined results from this work showed that the utilization of lipid extracted vs. whole microalgae significantly increased economic feasibility of the algae platform. The extraction by enzymatic

hydrolysis was shown to efficiently release most proteins, help remove chlorophyll from the extracts, and increase protein value, compared to mechanical extraction.

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DEDICATION

To my beloved country, Colombia.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

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NOMENCLATURE

LEA	Lipid extracted microalgae
Lysed LEA	Lysed (ball milled) lipid extracted microalgae
ProtConc	Protein concentrates from lipid extracted microalgae
IEX	Ion exchange
kDa	kilo Dalton
MW	Molecular weight
HPH	High pressure homogenization
w/v	weight by volume
w/w	weight by weight
%TP	percent of total protein

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1. INTRODUCTION*

Proteins constitute an important dietary nutrient for humans. They are also used in various industrial processes and products such as cleaning agents and textiles. Projections of increasing global protein demand in all areas, underscored by the need to fill the protein gap for a growing population's dietary needs, requires a correspondingly robust increase in supply to meet it. The current world population of 7.3 billion has a protein consumption demand of 202 million tons (MT). With projections of a 33% increase in population to 9.5 billion around 2050, the dietary protein demand has been predicted to increase to an upper level between 360 MT [1] and 1250 MT [2] to provide adequate food security. This dietary protein need does not include proteins used for feed and industrial purposes which puts further pressure on supply. The potential to meet the protein demand through increased production of animal and plant products alone is unrealistic due to the needs for feed and agricultural land which are already under pressure, and the potential ecological impact of high-density animal farming on climate change, antibiotic use, and waste management [1]. To meet the projected need in a sustainable manner, there must be a willingness to pivot to non-conventional sources to supplement current and future conventional animal and plant sources. Proposed alternative sources to meet the burgeoning need are diverse, and include cellular, concentrated, and purified proteins from bacteria, insects, and microalgae

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Microalgae have been used as nutritional supplements both historically and as supplements [3, 4]. They have documented use as food and feed in many cultures in Europe, Asia, North and South America and the Pacific Islands including the Aztecs of Laka Texcoco and the Kanembu people of Lake Chad [5, 6]. With high protein content (up to 50%) and high nutritional value based on amino acid composition [2, 7, 8], microalgae compares favorably to common sources of cell free protein such as soy (37%), milk (26%), meat (43%) and yeast (39%) [9-11].

When assessed for net protein utilization (NPU), a function of how digestible the protein is together with the biological value (BV) of the protein, whole-cell microalgae score higher than most plant sources, but lower than animal sources [2, 7]. The lower BV of microalgae protein, compared to animal sources, is most likely due to the presence of undigestible cell walls that reduce cell protein bioavailability [12].

While processing strategies such as cell lysis, protein isolation, and hydrolysis are proven to enhance digestibility and functionality of algae proteins [12] the current consumption of microalgae as a source of protein is limited to whole-cell products. The production of higher BV protein products, such as isolates, concentrates, and hydrolysates, from microalgae has been hampered by the high costs of the biomass, low protein extraction yields due to the presence of sturdy cell walls [13-16], and the limited protein purification processes for removal of contaminants from the protein extracts [17].

One strategy to reduce biomass costs is the co-extraction of protein along with other high-value lipids present in microalgae, including triglycerides containing essential

omega-3 unsaturated fatty acids, eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), and carotene antioxidants (astaxanthin, lutein and β-carotene) [4, 18-20]. Current lipid extraction processes, though, involve biomass drying and solvent incubation steps at high temperatures that tend to decrease protein extractability due to heat induced aggregation. Indeed, even in cases where mild temperature conditions are used, solubilization and extraction of protein from lipid extracted algae (LEA) can be much more challenging than from whole algae [21]. Understanding the behavior of the protein after exposure to solvents and/or drying, and finding ways to resolubilize it is critical for the development of efficient protein extraction processes from LEA [21, 22].

To produce protein concentrates and hydrolysates up to the standards of current products, the protein from LEA should not only be extracted but also concentrated to >70% protein content [23]. To our knowledge, only a few authors [23-25] have addressed the concentration of microalgae protein after its extraction, but either fail to report protein recovery and/or purity [23, 24], or show significant protein enrichment (75% protein) at the expense of recovery yield (<20%) [25]. Thus, the development of integrated bio-separation processes, that can selectively recover protein from LEA, while removing impurities such as salts and carbohydrates and provides a balance between protein recovery and purity is still required.

In this dissertation, we aim to understand and propose alternatives to overcome the barriers to the economic production of protein concentrates and hydrolysates from microalgae from a bio-processing and bio-separations perspective. In chapter 2, we

review and analyze the use of microalgae as an alternative protein source, current microalgae-derived protein products for human consumption and their properties, processing challenges for extraction and fractionation, and future trends for increasing their economic value. Then, in Chapter 3, we follow the recommendations for increasing economic value of microalgae protein by proposing a co-production platform of lutein and protein concentrates from *Chlorella* vulgaris and determining its economic feasibility. In Chapter 4, we develop a process for producing higher value protein hydrolysates from lipid extracted microalgae with potential applications in specialty foods and drinks. Lastly, the appendix gathers more recent data on an optimized ultrafiltration process for a single step clarification and purification of hydrolysates with enhanced pH and thermal stability properties.

2. SPECIFIC OBJECTIVES

- *Chapter 3:* To review and analyze the status of current extraction and fractionation processes for production of microalgae-based protein products and provide recommendations

- *Chapter 4:* To evaluate the technical and economic feasibility of a high-value lipids (lutein) and protein concentrates co-extraction platform from *Chlorella vulgaris*

- *Chapter 5:* To propose a protein hydrolysis, fractionation, and purification process for producing high-value protein hydrolysates from lipid extracted *Nannochloropsis sp.*

3. EXTRACTION AND FRACTIONATION OF MICROALGAE-BASED PROTEIN PRODUCTS*

3.1. Chapter summary

Microalgae are a valuable source of proteins that can be utilized as functional, nutritional, and therapeutic commodities. While process optimization and integration are still required to extend commercial applications, diverse approaches have been taken to process and characterize microalgae-derived protein products, such as protein concentrates, hydrolysates, and bioactive peptides. This review presents the current status of extraction and fractionation of protein products from microalgae and provides recommendations on: (1) processing factors (drying, cell disruption and enzymatic hydrolysis) that could affect protein release, functional properties, and extraction yield; (2) scalability and efficiency of fractionation processes for the production of protein concentrates, hydrolysates, and bioactive peptides; (3) techno-economic feasibility of algal-derived protein products; and (4) opportunities, challenges, and recommendations for further development of microalgal protein industry.

3.2. Introduction

Proteins are abundant macromolecules in all living organisms that constitute an important human dietary nutrient. With a current world population of 7.3 billion, protein demand is already 202 million tons (MT) annually and is projected to increase to 360-

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1250 MT by 2050 (Henchion et al., 2017; Ritala et al., 2017). The potential to meet the future protein demand through increased production of animal and plant products alone is unrealistic due to the also growing demand for feed and agricultural land (Henchion et al., 2017). Thus, alternative sources of dietary protein are needed to meet projected dietary needs. Proposed alternative sources are diverse and include bacteria, insects, and microalgae. High-protein content (~50%), favorable nutritional properties, and low allergenicity (Becker, 2007; Brown et al., 1997; Ritala et al., 2017) of protein products derived from microalgae result in the favorable comparison to common protein sources, such as soy (37% DW protein), milk (26% DW protein), meat (43% DW protein), and yeast (39% DW protein) (Barka & Blecker, 2016; Becker, 2004; Wells et al., 2017). Current lifestyle trends, such as the increased consumption of vegan, sustainable food products, and health-promoting nutraceuticals (Radnitz et al., 2015; Suleria et al., 2015) have brought microalgae to the forefront of non-animal protein sources.

Several microalgal species have documented historical use as food and feed in Europe, Asia, North and South America, and the Pacific Islands, (Heinis, 2010; Vonshak, 1997). More recently, certain microalgal species have earned the coveted “Generally Regarded As Safe” (GRAS) status under FDA regulations (Gong et al., 2011) and have been shown to have numerous health benefits (Kent et al., 2015). The use of microalgae as an alternative protein source, current microalgae-derived protein products for human consumption and their properties, processing challenges for extraction and fractionation, and future trends for increasing their economic value will be discussed in this review.

3.3. Microalgae protein products and properties

Current protein products obtainable from microalgae can be classified, based on their protein content and the degree of refining, as whole-cell protein, protein concentrates, isolates, hydrolysates, and bioactive peptides (Figure 1). Whole-cell protein has an intact tissue and cellular structure, is protein-dense, and is usually consumed directly (e.g. plant seeds, whole microalgae cells). Microalgae whole-cell protein contains approximately 40-50% protein, but percentages vary based on species and growth conditions. To obtain concentrated protein products (60-89% DW protein), such as protein concentrates, isolates, hydrolysates, and bioactive peptides, protein from microalgae cells should be extracted and proteins concentrated.

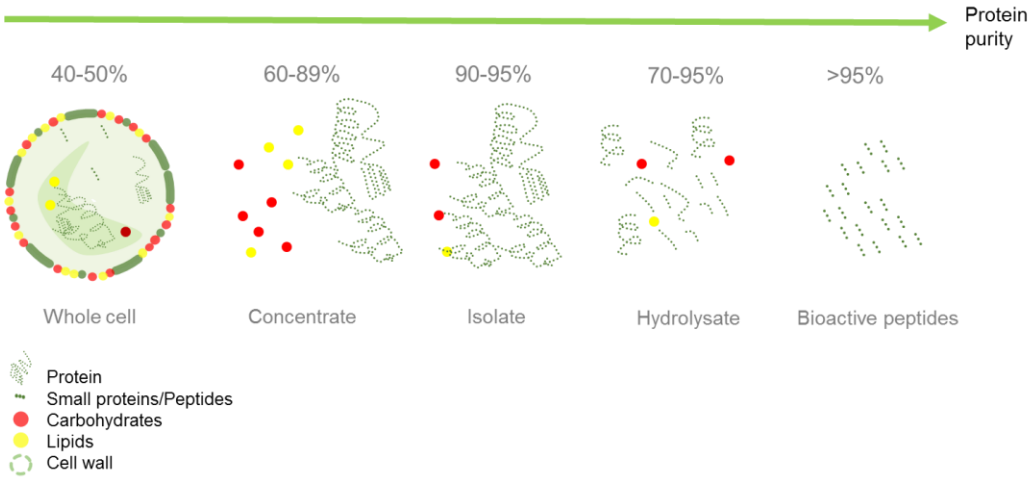

Figure 3.1 Classification of microalgae-based protein products based on their protein content[1]

[1] For this review, we will use the standard definition of commercial protein isolates (>90% protein concertation) and concentrates (>60%, <90% protein purity)

Relative to the target application, protein ingredients are also categorized based on their nutraceutical value and functional properties. The nutraceutical value of a protein product is related to the health and nutritional benefits that the product can provide with regards to digestibility, essential amino acids content, and bioactivity. These latter properties are desired in most food applications, especially when formulating fortified foods, nutrition shakes, and sports drinks.

Protein functional properties, on the other hand, contribute to the physical properties of the food product. The most common properties are emulsification, foaming ability, and capacity. These properties are determined by the protein structure and protein-protein interactions as well as protein interaction with other molecules in solution [26]. Emulsification refers to the protein holding capacity of oil and the stability of oil-in-protein emulsions. High emulsification capacity and stability are desired in food formulations such as soups and sauces. Foaming refers to the protein flexibility that allows the reduction of surface tension to keep air bubbles in suspension. Foaming ability is a property desired in creams, spreads, creamers, among others.

The extent of and the methods used for protein processing confer different properties in digestibility, bioactivity, emulsification, and foaming of the protein product. The end application should be considered when developing a downstream process for production of microalgae-based protein products. The following section will discuss the main protein products obtainable from microalgae and their nutraceutical and functional properties.

3.3.1. Whole-cell protein

Microalgae are a viable alternative to conventional protein sources because in addition to protein, they contain other valuable components such as healthy lipids, micronutrients, and dietary fiber [27]. Whole-cell protein is the most popular microalgae-derived product used for human consumption [7, 28, 29] because it has a high protein content (g-protein/DW). Compared to extracted protein, whole-cell protein, protected by cell wall and membranes, is less susceptible to drastic pH changes that might induce aggregation, denaturation and decrease its functionality [27]. However, there are still questions about digestibility and bioavailability parameters of proteins and amino acids in non-lysed and dried microalgae biomass, compared to conventional protein sources [9]. In most cases, the tough cell wall and internal membranes protect the cell and drastically reduce digestibility and bioavailability of microalgae cell protein. The digestibility of most common microalgal species, such as *Chlorella vulgaris,* can be significantly lower than traditional protein sources [30] due to inhibitory effects of cell wall polysaccharides on digestive tract enzymes (Wells et al 2017). Thus, algal cell walls with a high cellulose content *(C. vulgaris)* exhibit lower digestibility values than those with a thinner, easier to digest cell wall (*Spirulina platensis).* Furthermore, proteins trapped inside the cell and cell organelles may not display their full functional potential [27]. Therefore, a logical way to enhance the digestibility of algal protein is to disrupt the cell wall, which would allow access of digestive tract enzymes to intracellular proteins.

3.3.2. Protein concentrates and isolates

Algae protein concentrates and isolates are produced by concentration and/or partial purification of extracted host-cell proteins (Figure 3.1). Protein isolation allows an increase of the protein digestibility corrected amino acid score (PDCASS), a measure of a food source's amino acid content and digestibility [27]. Protein concentrates have been produced and evaluated from several algal species, including *Arthrospira sp*. [31], *Chlorella sp*.[12, 24, 27], *Scendenemus sp.*[17, 31], *and Nannochloropsis sp*.[31, 32]. Protein concentrates from *C. vulgaris* [24] and *C. pyrenoidosa* [23] had similar emulsification capacities and stabilities compared to commercial protein products such as sodium caseinate [23, 24]. Protein concentrates that were prepared under mild extraction conditions (neutral pH and temperatures less than 40^{o}C) and without the presence of denaturing or hydrolyzing agents had superior emulsification properties compared to proteins extracted under denaturing conditions [24, 33]. Because protein solubility is typically greater in alkaline than neutral pH conditions, a significant increase in foaming capacity and stability of algal protein concentrates has been observed between pH 10 and pH 11 [34].

3.3.3. Protein hydrolysates

Algae protein hydrolysates are obtained after subjecting either whole-cell or extracted protein to enzymatic hydrolysis, which breaks native algal proteins into smaller peptides. Protein hydrolysates typically exhibit improved biological value and certain bioactivities [12] compared to whole-cell protein and protein concentrates. They can be produced and marketed as heterogeneous mixtures of peptides of varying molecular weight (MW) or

as purified peptide fractions of a specific MW range with potential bioactivities. The in-vitro digestibility of *C. vulgaris* protein hydrolysates improves from 70% for whole-cell protein to 90-97% for protein hydrolysate [12, 35]. Furthermore, enzymatic hydrolysis of algal cells also reduces green pigmentation of microalgal protein, which would negatively affect marketing and consumer perception [36].

While partial enzymatic hydrolysis has been shown to improve emulsification and foamability of protein products properties, extended hydrolysis (<5 kDa peptides) significantly reduces their emulsification capacity and stability [37]. Thus, when good emulsification and/or foaming capacity and stability are desirable, protein hydrolysates containing peptides greater than >10-20 kDa in size should be targeted [38]. On the other hand, fractions containing small peptides ranging in size between 2 and 5 kDa usually exhibit bioactive properties and can be purified from algal protein hydrolysates and marketed as nutraceuticals (See section 2.1.4.).

Extended hydrolysis is recommended for hydrolysates used in specialized foods, such as protein drinks and supplements that benefit from the nutritional value of the amino acids and their high-water solubility. Most hydrolysates produced from microalgae that have a degree of hydrolysis ranging from 15% [12] to 25% [37] are desired for their biological value rather than their functional properties.

3.3.4. Bioactive peptides

Bioactive peptides are short peptides of around 3-40 amino acids that have a positive impact on human physiological functions. These protein fragments, which are usually inactive within the sequence of the parent protein, display bioactivity after being

released from polypeptide chain by enzymatic hydrolysis. The bioactive peptides are usually isolated from protein hydrolysates as pure peptides or a partially fractionated hydrolysate pool with enhanced bioactivity. Upon ingestion, the bioactive peptides, as a pure or partially purified product, reach the bloodstream to deliver a beneficial biological activity [39]. Reported benefits of algal peptides include antioxidant, [40, 41], anti-hypertensive [42] immune-modulatory [12], anti-cancer [43], hepatic-protective [44] and anticoagulant activities [45].

3.4. Current methods for production of proteins from microalgae

This section will focus on upstream and downstream processing steps involved in the production of main microalgae-based protein products discussed in the previous section: whole-cell protein, concentrates and isolates, hydrolysates, and bioactive peptides. As shown in the flow diagram below (Figure 3.2), microalgae cultivation (upstream) is the first step in the overall process that directly affects protein yield in subsequent downstream processing. Downstream unit operations consist of harvesting, drying, cell disruption, protein extraction, hydrolysis, and separation. Each step has a unique role as well as challenges and both will be discussed in the following sections.

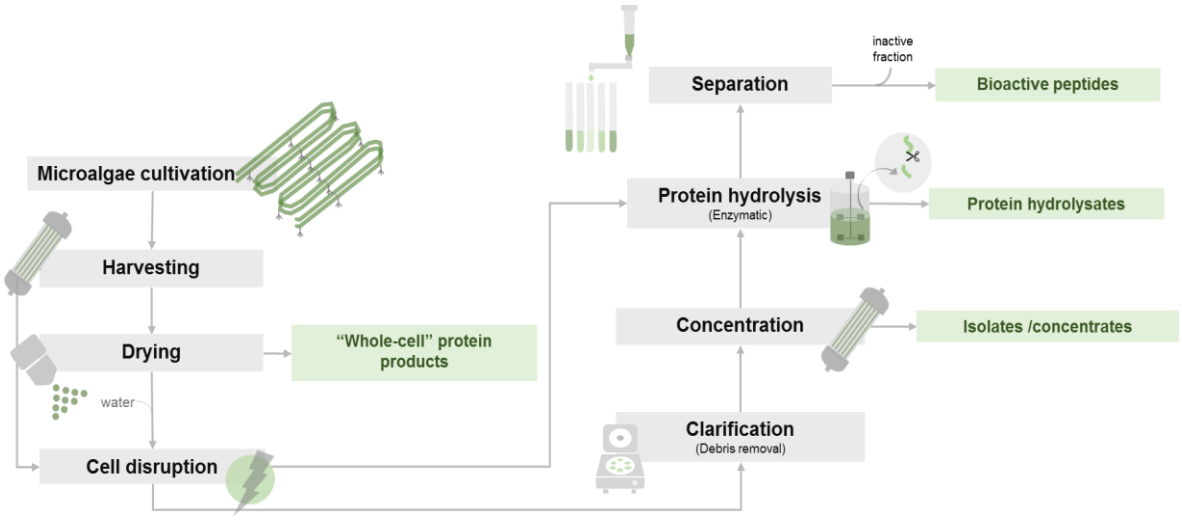

Figure 3.2 Flowchart indicating the main processing steps for the production of whole cell protein products, protein isolates and/or concentrates, protein hydrolysates, and bioactive peptides from microalgae

3.4.1. Cultivation

Classified based on the energy and carbon source used, microalgae have been cultivated under photoautotrophic, heterotrophic, and mixotrophic conditions (Table 3.1). To maximize the value of microalgae and reduce production costs, the goal of any cultivation process is to achieve both high protein yield (g protein/g biomass) and biomass productivity (g biomass /L culture). Key features of microalgae cultivation conditions and bioreactors that affect the overall protein productivity will be reviewed next.

Table 3.1 Cultivation conditions and productivities of selected algae species

Cultivation condition	Species	Nitrogen source	Carbon source	Biomass density (g/L)	Protein yield (g protein / g biomass)	Protein productivity (g/L)	Ref.
Photo-autotrophic	*Chlorella vulgaris*	1.25 g/L KNO_3	$NaHCO_3$	0.5	0.15	0.075	[46]
		1.25 g/L KNO_3	2% CO_2	4.5	0.20	0.9	[46]
		80 g/L $NaNO_3$	16 g/L $NaHCO_3$	0.2	0.51	0.102	[47]
		Nitrogen deprived	16 g/L $NaHCO_3$	0.2	0.13	0.026	[47]
		Nitrite	3.18 g/200mL $NaHCO_3$	0.2	0.41	0.082	[47]
	Dunaliella bardawill	1.0 g/L KNO_3	0.043 g/L $NaHCO_3$	0.8	0.45	0.36	[48]
	Tetraselmis	Yeast	Yeast plus F2 media	2.2	0.48	1.056	[41]
Heterotrophic	*Chlorella vulgaris*	1g Proteose peptone	10 g/L glucose	1.7	0.32	0.544	[49]
		1g Proteose peptone	10 g/L glycerol	0.7	0.45	0.315	[49]
		1g Proteose peptone + 0.25 g/L $NaNO_3$	10 g/L acetate	1.0	0.42	0.42	[49]
	Chlorella vulgaris	0.085 g/L $NaNO_3$	10 g/L Glucose	0.5	0.20	0.1	[46]
Heterotrophic fed-batch	*Chlorella regularis*	Urea concentration kept at ~5 g/L	Glucose kept at 0.5-10 g/L	84.0	0.62	52.08	[50]
	Chlorella vulgaris	9.14 g urea/100 g glucose	1.6 g glucose/g-cells	100-120	0.40	40	[51]

Table 3.1. Continued

Cultivation condition	Species	Nitrogen source	Carbon source	Biomass density (g/L)	Protein yield (g protein / g biomass)	Protein productivity (g/L)	Ref
Mixotrophic	*Chlorella ellipsoidea*	5:1 C/N	5:1 C/N	1.7	0.45	0.77	[52]
		20:1 C/N	Glucose feed at 24, 48, 72, 96 and 120 h	5.9	0.11	0.65	[52]
	Chlorella vulgaris	NR_2	CO_2 + Hydrolyzed dairy waste	3.6	0.64	2.30	[53]
		NR	CO_2 + Non-hydrolyzed dairy waste solution (10 g/L lactose)	2.0	0.55	1.10	[53]
	Chlorella pyrenoidosa	1.2 g/L Urea	Heterotrophic fed: 35 g/L glucose Autotrophic phase: 5%CO_2	15	0.64	9.60	[54]

2 NR: Non reported

3.4.1.1. Photoautotrophic cultivation

Under photoautotrophic cultivation, light and inorganic carbon (CO_2) are used as the energy and carbon sources for biosynthesis [55]. This cultivation method is advantageous in situations when the co-production of light-dependent products, such as photosynthetic pigments, is desirable [56]. Since light is the source of energy for microalgae grown photoautotrophically, light availability usually becomes a productivity-limiting factor [57].

Open-ponds and closed photobioreactors (PBRs) are the main systems for algal biomass production under photoautotrophic conditions. Most of the current large-scale cultivation systems are open ponds. The algae industry prefers open pond cultivation because they are easier to scale-up and require less initial capital investment compared to closed systems [58]. However, there are several limitations associated with open-pond cultivation, such as risks of contamination, water losses due to evaporation, and low biomass densities (0.5 g/L) [59]. Although enclosed photobioreactors (PBRs) allow better control of cultivation conditions [60], their scale-up is constrained by higher capital investment requirements (10 times higher than open ponds) and technical difficulties associated with bioreactor sterilization [61].

Protein accumulation in photoautotrophic systems depends on the nitrogen source and can range from 0.15 to up to 0.5 g-protein/g-biomass (Table 3.1). Because low cell densities are the main drawback of photoautotrophic systems, strategies for maximizing protein content per gram biomass DW, such as maximizing the nitrogen to carbon (N/C) ratio (Brennan & Owende, 2010), have been proposed and investigated (Section 3.1.4.)

3.4.1.2. Heterotrophic cultivation

Heterotrophic cultivation does not require light because organic carbon sources such as glucose and glycerol provide energy for biosynthesis [55]. Heterotrophic conditions often result in reduced protein yields and biomass productivities compared to photoautotrophic cultivation [46, 62, 63]. However, a fed-batch operation in heterotrophic bioreactors can be used to overcome this drawback (Table 3.1). The addition of carbon- and nitrogen-containing compounds to promote growth and protein accumulation have been proven to be successful strategies for maximizing cell density while preserving protein yield. Cell densities between 80 and 100 g/L and protein yields of 0.4 to 0.6 g/g (Table 3.1) have been achieved by the fed-batch cultivation of heterotrophic *C. vulgaris* cultures [50, 51, 64]. The nutritional properties of proteins isolated from heterotrophically-grown algae were comparable to those obtained from photoautotrophic cultures. Based on amino acid analysis reported by [51], heterotrophic cultivation of *C. vulgaris* yielded protein with a well-balanced amino acid profile, including a high content of the sulfur-containing amino acids, methionine, and cysteine. Thus, heterotrophic fed-batch cultivation appears to be an efficient method for maximizing protein productivity and delivering protein products for high-end food applications such as nutritionally enhanced drinks. Regardless of the potential of achieving high protein productivity under heterotrophic growth, one should be cognizant of the cost of the organic carbon (glucose). The glucose cost alone could amount to 80% of total cultivation medium costs [65] and would impact the economic viability of algal protein products [49].The utilization of cheaper acetates or recycled carbon sources, such

as glycerol from biodiesel industry and other industrial waste streams, could help to overcome this limitation [66].

3.4.1.3. Mixotrophic cultivation

Mixotrophic cultivation is typically conducted in PBRs with media supplemented with both, an organic and an inorganic carbon source [67, 68]. The presence of organic carbon provides substrate and energy flexibility to cells to circumvent light limiting the productivity of autotrophic systems [69]. Mixotrophic cultivation can be an effective strategy for maximizing light utilization for energy while not being restricted to it. The flexibility of mixotrophic cultures allows for improving the growth rate, shortening the growth cycle, reducing biomass loss during dark periods due to respiration, and enhancing lipid and protein productivity [65]. Mixotrophic growth is also amenable to a fed-batch operation, allowing for higher biomass productivities compared to a photoautotrophic operation (Table 3.1). Mixotrophic cultivation of certain microalgae, such as *Chlorella sp.*, has been shown to achieve a better balance between protein content and biomass density compared to heterotrophic and phototrophic cultures [54]. Biomass productivities of up to 10 g/L have been reached under mixotrophic fed-batch conditions for *Chlorella pyrenoidosa* [54] and *Haematococcus pluvialis [70]*. Regarding protein accumulation, Matos, Cavanholi [71] found that *Nannochlropsis gaditana* cultivated under mixotrophic conditions produced more protein (0.30 g/g) compared to photoautotrophic(0.23 g/g) and heterotrophic (~0.23 g/g) conditions. Salati, D'Imporzano [65] found as much as a 2.3-fold increase in protein productivity when *Chlorella sp.* was grown under mixotrophic conditions supplemented with whey waste,

compared to photoautotrophic growth. Regarding protein quality, protein expression patterns under mixotrophic conditions were very similar to those seen under photoautotrophic ones [72]. The presence of glucose does not apparently alter the expression of cellular proteins in *Chlorella sp.* [72].

While heterotrophic fed-batch cultivation of microalgae can achieve higher biomass and protein productivities than mixotrophic cultivation, the utilization of organic and inorganic carbon in mixotrophic systems could alleviate the high-carbon costs associated with heterotrophic fed-batch cultivation. Mixotrophic cultivation appears to be an effective method that combines cultivation advantages of both photoautotrophic and heterotrophic systems. Like photoautotrophic systems, algae grown mixotrophically can also be subjected to co-extraction of photosynthetic products, such as pigments, along with the protein.

3.4.1.4. Carbon and nitrogen source effects on protein and biomass productivities

Whether mixotrophic, heterotrophic, or photoautotrophic conditions are used, protein accumulation depends both on overall biomass density and intracellular protein levels. As the data in Table 3.1 suggest, biomass and protein accumulation are also affected by nitrogen and carbon sources. While strategies for lipid [73, 74] and polysaccharide [75-77] accumulation are plentiful in the literature, there is relatively little published information related to increasing protein yield and productivity of microalgae cultures. Here, we briefly review the effects of nitrogen and carbon sources on protein productivity.

3.4.1.4.1. Nitrogen source

Nitrogen availability is the single most relevant factor in protein accumulation. Lourenço, Barbarino [78], estimated that nitrogen accounted for 63–88% of amino acid residues in exponentially growing and CO_2 - limited cultures. Depending on the source and amount of nitrogen in the media, total protein accumulation fluctuates within species. For *Dunaliella salina*, the total protein content was 2-fold higher when cells were grown with ammonia as a nitrogen source rather than nitrate [79], which is probably due to the metabolic control mechanisms for NO_3^- uptake, compared to the only partially controlled NH_4^+ influx. Mutlu, Isçk [47] reported ~10% decrease in protein yield in autotrophically grown *Chlorella sp*. cells when the nitrogen source was switched from sodium nitrate to nitrite. When nitrogen was completely removed from the media, the same authors [47] measured a 40% decrease in total protein. This is to be expected since most microalgae, under nitrogen starvation, degrade proteins to serve as a nitrogen source [80]. For some microalgal species, yeast extract has been shown to be a good source of nitrogen for efficient protein accumulation. For instance, *Tetraselmis sp.* utilizes organic nitrogen from yeast extract for both growth and protein accumulation. Kim, Mujtaba [41] showed that *Tetraselmis sp*. cells grown with yeast extract as the nitrogen source accumulated a significantly higher (up to 50% DW) amount of protein and biomass density (up to 10.4 g/L) when compared to ammonium, nitrate, nitrate, glycine, and urea. Yeast is a complex nutrient source, containing amino acids, peptides, and carbohydrates, which *Tetraselmis sp*. can use to enhance biomass and protein

accumulation. Generally speaking, cultivation media with low carbon to nitrogen ratios will favor protein accumulation [81].

3.4.1.4.2. Carbon source

Whether derived from organic or inorganic sources, carbon accounts for more than 90% of the microalgae nutrient media. The conversion yield of CO_2 to biomass is about 0.5–0.6 g per gram of carbon dioxide [72, 82]. Yeh and Chang [46] reported as much as 0.2 g/g of protein yield for *C. vulgaris* grown with CO_2 as the carbon source compared to using $NaHCO_3$ (0.15 g/g of protein) (Table 3.1); Protein (0.9 g/L) and biomass (4.5 g/L) productivities were also significantly higher [46] (Table 3.1). Under heterotrophic growth, glucose addition (10 g/L) yielded protein productivities (~1g/L) comparable to those of CO_2-photoautotrophic conditions [46]. Higher productivities with CO_2 or glucose as the carbon source were due to the significantly higher biomass accumulation compared to using $NaHCO_3$ as the carbon source. Thus, choosing the right carbon source is vital for achieving high biomass productivity, which usually translates into higher protein productivity.

3.4.2. Drying

Microalgae are usually harvested by centrifugation, flocculation-assisted settling or by tangential-flow microfiltration until ~11-23 wt% is reached [83, 84]. Biomass, which contains on average 90% residual water, can significantly increase handling, storage, and transportation costs if it is not immediately processed at the harvesting location [83]. In such cases, drying is used to reduce biomass volume and weight, extend the shelf life of algal biomass (Molina & Grima 2003) and minimize protein degradation. Even though

drying of biomass is a common practice in algae processing for biofuel production, the effect of drying on protein solubility and cost must be considered when developing protein products for food applications.

The drying temperature can significantly affect the biological value and functional properties of microalgal protein [9]. Temperatures between 55 and 60°C are optimal for recovering lipids, proteins, and vitamins, as higher drying temperatures (>60°C) can cause a significant denaturation of proteins [85].

The drying method affects protein extraction efficiency. Ansari et al. (2015) compared three different methods (sunlight, oven drying, and freeze-drying) for drying *Scenedesmus obliquus* biomass. The amount of extracted protein from the oven-dried (~50-60%) biomass was slightly higher compared to that recovered from sun-dried (40-50%), and freeze-dried (~45-55%) cells. Oven-drying at temperatures lower than 60°C appears to give better results in terms of protein extractability, dispersibility, and digestibility than freeze-drying and sun-drying [85].

Drying of algal biomass can also reduce protein extraction efficiency due to cell aggregation. Lin [86] reported that cell aggregation of freeze- or spray-dried microalgae biomass decreased protein extractability by lowering the cell surface area in contact with the solvent. This observation was confirmed by Safi, Charton [87], who commented that the freeze-drying of algal biomass samples hindered subsequent protein extraction. Thus, to maximize protein recovery, grinding of aggregated dry cells is usually required and practiced (Morris et al., 2008)

For the recovery of whole-cell protein products, drying is the key post-harvest unit operation performed before processing the dried biomass into powder for use as ingredients in food formulations [2]. When the desired product is either a protein isolate, concentrate, hydrolysate or bioactive peptide fraction, protein extraction is required. In this case, dried algal biomass must first be dispersed in the extraction solvent prior to cell disruption. Compared to the processing of freshly harvested wet biomass, the re-solubilization of dried cell material requires extra energy and water expense that can significantly increase production costs. [88]. If product or processing constraints requires cell drying, resuspension of dried biomass in water at 20% (w/v) dry should allow effective dispersion of cells [83].

3.4.3. Cell disruption

For a given intracellular product, an ideal disruption treatment is one that selectively releases the targeted product while using the least possible energy. The choice of cell disruption method depends on cell wall structure of algal species, product location, size, solubility, and applied energy (Table 3.2). Depending on the nature of the disruption force, disruption methods can be classified as physical (drying, sonication, and pulsed electric field), mechanical (bead milling, homogenization) and chemical/biological (acid, base, and enzymes) (Figure 3.2). In this review, we will use the term “permeabilized cell” to describe cells with partially ruptured cell wall and membranes and “lysed cells” to indicate completely disintegrated cells as depicted in Figure 3.3. The main barriers to microalgal protein extraction are the sturdy cell wall and the chloroplast’s thylakoid membrane, which encloses a significant fraction of microalgal protein [89].

Figure 3.3 Levels of cell disruption. From non-disrupted (left) to complete cell disruption-lysis (right)

3.4.3.1. Pulsed Electric Field (PEF)

PEF is a non-thermal cell permeabilization method (Figure 3.3, middle) that disrupts the lipid bilayer of cell membranes allowing molecules of certain sizes, such as small MW proteins, to enter into and/or diffuse out of the cells [32, 90]. A recent study by Coustets, Joubert-Durigneux [91] sheds some light on the relationship between applied PEF energy, microalgal species, and protein release. *H. pluvialis* treated with PEF (3 kV/cm) resulted in an increase in protein release compared to non-treated cells (Table 3.2). Based on the amount of protein released (10% of total protein), Coustets, Joubert-Durigneux [91] inferred that the extracted proteins were cytoplasmic proteins that diffused through permeabilized/disrupted plasma membrane. In agreement with previous studies [92], Coustets et al. (2015) observed that the field intensities needed for releasing cytosolic proteins from *H. pluvialis*, *C. vulgaris*, and *Nannochloropsis salina* were inversely proportional to cell size (Table 3.2). This is due to the close dependency between transmembrane pressure, and cell size. For instance, doubling cell diameter will double transmembrane pressure (for the same field intensity) which will then induce a

greater extent of cell membrane permeabilization [93]. Considering that *Haematococcus sp*. and *C. vulgaris* cells have ~5 times larger diameters compared to *Nannochloropsis sp.*, lower field intensity is required to permeabilize them via PEF. Earlier work by Toepfl, Heinz [94] indicated that protein release from *Chlorella sp.* increased to 15% of total protein by increasing voltage to 15 kV/cm. Other groups also tested protein release from microalgae with PEF and, similarly to previous studies, concluded that only 5% to10% of total proteins could be released from *C. vulgaris* [90] and *N. gaditana* [32] cells, respectively.

PEF could be used as a supplementary treatment, but it is not an efficient disruption method for complete protein extraction. When complete solubilization and extraction of internally stored proteins is required, energy-intensive cell disruption methods, or a combination of more than one treatment to induce cell lysis, is recommended.

Table 3.2 Effect of biomass cell disruption on protein extraction from different microalgae species

Cell Disruption/ Pretreatment	Biomass form (density)	Species	Process conditions	Extracted protein (% TP)	Comments	Ref
High-pressure homoge-nization	Freeze dried (20 g/L)	*Chlorella vulgaris*	2 passes at 2700 bar	53	High protein release compared to sonication, grinding, and chemical treatment (pH 12)	[15]
		Haematococcus pluvialis		41		
		Spirulina platensis		78		
	Frozen (100 g/L)	*Nannochloropsis gaditana*	1 pass at 1000 bar	49	Energy used: 0.32 $kWh.kg^{-1}$biomass Cost: \$0.2–\$0.3.kg^{-1} protein	[95]
	Fresh (10 g/L)	*Nannochloropsis sp.*	6 passes 1500 bar	91	Chlorophylls and carotenoids extraction Energy used: 0.422 $kWh.kg^{-1}$biomass	[96]
PEF	4°C (25 g/L)	*Chlorella vulgaris*	45°C at 17.1 kV/cm for 5 µs	4.4	Energy used: 1.11 kWh.kg−1biomass	[90]
	Fresh (~1 g/L)	*Chlorella sp.*	15 2 ms, at 4.5 kV	<10	Release cytoplasmic proteins	[91]
	Fresh (~1 g/L)	*Haematococcus pluvialis*	2 ms at 3 kV/cm	10	Release of cytoplasmic proteins	[91]
	Fresh (~1 g/L)	*Nannochloropsis salina*	2 pulses, 2 ms at 6kV/cm	>10	High field strength needed due to small cell size	[91]
	Frozen (60 g/L)	*Nannochloropsis gaditana*	10 pulses, 30 kV cm−1	10	Energy used: 10.44 kWh.kg−1biomass	[95]
Sonication	Frozen (120 g/L)	*Nannochloropsis sp.*	100% amplitude, 20 kHz, 4 min,300 W	20-50	Protein release higher (50%) for non-defatted biomass	[97]
	Dried defatted (30g/L)	*Spirulina platensis*	37 Hz, 30°C, 35 min , 50 min agitation	Up to 75	Sonication at pH 9	[98]
	Fresh (0.25 g/L)	*Chlorococcales, Chlorophyceae*	50W, 30kHz	~65[1]	Energy used: 1.6 $kWh.g^{-1}_{biomass}$	[99]
	Fresh (10 g/L)	*Nannochloropsis sp.*	1 pulse, 10 kV/cm	5	Almost no extraction of pigments	[96]

1 Assuming 50% protein content in cells
NR: Non-reported

Table 3.2. Continued

Cell Disruption/ Pretreatment	Biomass form (density)	*Species*	Process conditions	Extracted protein (% TP)	Comments	Ref
Bead milling	Stored at 4°C (>60 g/L)	*Chlorella vulgaris*	500s, zirconia beads	87.5	97% cell disintegration 25% less protein release when density increased from 25 to 145 g/L Energy used: 3 $kWh.kg^{-1}_{biomass}$	[100]
	Fresh, stored at 4°C (124 g/L)	*Chlorella sp*	0.6–0.8 mm zirconia beads, Dyno mill homogenizer	NR	~90% cell disintegration	[101]
	Frozen (77 g/L)	*Chlorella vulgaris*	40 min, 1- 1.6 mm Zirconium Silicate beads at 2500rpm	96	Total disruption after 30 min	[14]
	Frozen (100 g/L)	*Nannochloropsis gaditana*	0.5mm beads, Zirconium, 65% filling,20 min	>90%	Energy used: 0.43 $kWh.kg^{-1}_{biomass}$ Cost: \$0.5–1.2.$kg^{-1}_{Protein}$	[95]
Alcalase® enzymatic treatment	Frozen (NR)	*Nannochloropsis gaditana*	5h, 5% dry matter, pH 8.5	35	Energy used: <0.34 $kWh.kg^{-1}_{biomass}$	[95]
Autolysin	Frozen (1 g/L)	*Chlamydomonas reinhardtii*	5h, pH 7.5, 37°C	50	95% cell permeabilization	[102]
Protex XL	Microalgae meal (~108 g/L*)	*Chlorella fusca*	3h, 5% enzyme dosage	60	Microalgae had been previously defatted	[103]
Alkali treatment	Frozen (20 g/L)	*Chlorella vulgaris*	2h, pH 12	26	Lack of pigments in the aqueous phase	[14]
	Defatted microalgae meal (NR)	*Chlorella fusca*	55 mM NaOH	35	Increase in extraction yield by defatting biomass prior to alkaline extraction	[104]

3.4.3.2. Sonication

Sonication is a physical treatment based on bubble cavitation by ultrasound waves that promote a non-specific cell-surface barrier disruption [105]. Sonication of microalgae cells permeabilizes both the cell wall and the membrane, a key difference from PEF treatment, which permeabilizes only cell membranes [105]. When performed at high voltages, sonication induces non-homogeneous cell disruption [106]. Cells adjacent to collapsing cavitation bubbles get broken, whereas cells located farther away experience a smaller local energy flux and are killed but not lysed [105].

Few authors have explored the effect of sonication on cell lysis and solubilization of microalgal protein [97, 98, 102]. Due to the highly resistant cell wall and internal membranes of most microalgal species, sonication alone is not sufficient for complete extraction of proteins [102]. For example, sonication accompanied by a secondary cell disruption method, such as high-shear mixing, enzymatic cell-wall hydrolysis [102][102][102] or chemical treatment, have increased the release of soluble proteins from *Nannochloropsis sp*., *Chlamydomonas reinhardtii*, and *S. platensis* [97, 98, 102]. Considering high energy requirements for complete cell lysis by sonication (Table 3.2), the economic feasibility of employing a secondary treatment following sonication might be worth evaluating.

3.4.3.3. Bead milling

Bead milling is a high-intensity cell disruption method caused by the collision of high-speed spinning steel, zirconium, glass or ceramic beads [107] with microalgae cells [108]. This method is frequently used at the industrial scale because of its high

disruption efficiency (up to 98%), high-density biomass loading capacity (60-150 g/L), and relatively low energy input (0.43 kWh.kg-1biomass) (Table 3.2).

Cell density, feed flow rate, bead size, and grinding chamber volume are important variables that directly affect bead milling efficiency and energy consumption [101] (Table 3.2). As would be expected, disruption efficiency decreases with increasing feed flow rate and cell concentration (>150g/L). Disruption efficiency has been reported to be the highest when beads occupy ~65-85% of the grinding chamber [32, 95, 101]. For several microalgal species such as *Chlorella vulgaris, Neochloris sp., Tetraselmis sp.,* and *Nannochloropsis sp*., smaller bead sizes (0.3-0.4 mm) were optimal for achieving a balanced disintegration to energy input [100, 109]. With regard to protein release, bead milling is a more effective cell disruption method than sonication or PEF [14, 32]. Bead milling allows to extract up to 95% of total proteins at low to moderate energy consumption rates (0.43-3 kWh.kg-1 biomass).

3.4.3.4. High-pressure homogenization (HPH)

High-pressure homogenization is another mechanical disruption method where biomass under pressure is forced to pass through a narrow opening. The combination of intense shear force, cavitation, and turbulent flow [108] induce rapid cell disruption even of organisms with a highly resistant cell wall structures. In comparison to other cell disruption methods, HPH is one of the most efficient methods for releasing microalgal protein. Higher levels of protein solubilization were reported for *C. vulgaris, H. pluvialis,* and *N. gaditana* when disrupted using HPH (Table 3.2) compared with manual grinding, ultrasonication, and chemical (alkali) treatments [13-15].

Cell wall disruption efficiency was shown to be inversely proportional to cell wall rigidity. While HPH treatment on microalgae with less rigid cell walls (i.e., *P. cruentum, A. platensis*) resulted in 57% protein release, the same intensity treatment released about 10% less protein from microalgae with more rigid walls (i.e. *N. oculata, C. vulgaris*) [15, 32]. Besides cell wall structure, HPH treatment is highly dependent on the applied pressure and number of passes (Table 3.2). Three to six passes at 1000 to 1500 bar have shown to be effective for releasing 70-90% of microalgal protein from 1-2% biomass suspensions [21, 96]. HPH is a relatively energy efficient method (0.4 $kWh.kg^{-1}$biomass) that can achieve high disruption efficiency and high protein extraction yields (Table 3.2).

3.4.3.5. Enzymatic treatment

Enzymatic disruption methods are primarily used to improve extraction yields rather than for cell lysis alone and normally require a preceding cell permeabilization or disruption step [110]. Enzymatic treatment is generally performed under mild conditions and is an ecologically friendly, non-hazardous, and low energy alternative to mechanical and chemical techniques. In some instances, the enzymatic disruption may result in a more efficient protein extraction compared to mechanical and chemical cell disruption [111]. The success of an enzymatic treatment depends on the composition and complexity of the specific microalgae cell wall. The disruption of algal cells requires the application of more than one enzyme to break specific macromolecules constituting the complex cell wall. For example, Yamada and Sakaguchi [112] generated protoplasts for *C. vulgaris* strains using a combination of 4% onozuca, 2% macerozyme, and 1% pectinase enzymes. Soto-Sierra, Dixon [102] used gametolysin, an autolytic

metalloprotease produced by *Chlamydomonas reinhardtii*, to achieve cell permeabilization and up to 50% protein release. The combination of proteolysis of algal proteins with mechanical or chemical treatment has been successfully applied to increase protein extraction yields [103, 111]. Protein hydrolysis is not advisable if the functional properties of extracted protein hydrolysates are important for food applications. On the other hand, if the desired end product is a protein hydrolysate (Section 2.1.3.), then the application of proteolytic methods could be advantageous because both enzymatic hydrolysis and the release of protein fragments are accomplished in a single unit operation.

3.4.4. Further processing of microalgal protein products

Once microalgae cells have been disrupted, cell debris is removed by centrifugation or membrane filtration and the released soluble protein is further concentrated to produce protein concentrates or isolates via precipitation and/or diafiltration (DF) (Figure 3.2). Protein hydrolysates can be produced by proteolytic hydrolysis of crude cell lysates, protein concentrates or isolates. The few reports that detail extraction and production of protein concentrates, isolates and hydrolysates are discussed below.

3.4.4.1. Protein extraction and clarification

Protein extraction is usually achieved after cell disruption with any of the treatments reviewed in the previous section. A centrifugation step [17, 24, 32, 113] is usually performed at this stage to remove solids (debris) and clarify the protein-containing phase (supernatant). The typical centrifugation force required for clarification of algal lysates at industrial, and lab-scale range between 8,000 and 10,000 x g. [32, 113]. To improve

protein extraction yields, one could conduct cell disruption under alkaline conditions or incubate disrupted algae at pH >10 prior to centrifugation and clarification [24]. At pH conditions far from the isoelectric point of algae proteins (pI~6), proteins carry a higher net charge that induces their hydration and solubilization [114]. By changing the extraction conditions to >pH 10, protein aggregates are solubilized and released, resulting in improved protein extraction yield from 70% to almost 95% percent [24]. Additional processing and concentration of clarified protein extracts are usually required to achieve desired protein concentrations and purities.

3.4.4.2. Production of protein concentrates and isolates

Unit operations, such as membrane filtration [24, 32], protein precipitation, ion exchange chromatography, and dialysis [17] have been employed to concentrate extracted protein, remove chlorophyll pigments and other soluble non-protein molecules. Ursu, Marcati [24] compared the yield of protein concentrates prepared by ultrafiltration (UF) and precipitation. Lower protein recovery yields were observed when protein extraction was followed by acidic (isoelectric) precipitation (76%) compared to membrane filtration (95%) process using a 300kDa molecular weight cut off (MWCO) membrane [24]. With regards to protein purity, Safi, Olivieri [32] found that ultrafiltration (300 kDa MWCO) for protein concentration resulted in a protein content of less than 20% DW, which is still low for food ingredient applications, requiring at least 70% DW. When the same authors tested the performance efficiency of ultrafiltration using a higher MWCO membrane (1000 kDa) [32], no improvements on flux were found and additional protein losses, compared to the 300 kDa process, occurred. To increase the protein purity after

ultrafiltration, one could employ precipitation or ion exchange chromatography to remove polysaccharides (starch), pigments, and other extract impurities. The downside of added separation steps to increase the protein content of the concentrates is the reduced protein yield, which could be as low as 30% of initially extracted protein [17]. One of the few protein concentrates with higher than 70% DW protein content was produced via three-phase extraction. Waghmare, Salve [23] used three-solvent system to produce protein concentrates from *Chlorella pyrenoidosa.* After disrupting the biomass, the protein in the cell lysate was precipitated with ammonium sulfate and t-butanol at pH 6.0, resulting in the formation of a three-phase system. The precipitated protein migrated to the interface of the organic and aqueous phases. The interface fraction resulted in a high-quality protein concentrate with more than 78% DW protein content. Although the protein content obtained from this process was significantly higher compared to that produced by membrane filtration [24], the scalability of the three-solvent system should be evaluated as solvent costs could make the process economically impractical.

The only reported protein isolate, with 90-95% DW of protein content (Figure 3.1), was developed by Patinier [115]. This group extracted soluble proteins from *Chlorella protothecoides* by high-temperature and short-time treatment (140°C for 10 s) of the whole cell broth. After centrifugation, the protein (80% DW) in the cell-free liquid fraction was concentrated by acid precipitation (pH 4.5) for 8 h at 4^{o}C. The re-solubilized protein precipitate contained more than 90% DW protein. Nevertheless, the extracted cell biomass contained more than 58% of the initial protein, which indicates that only 40% of the total cell protein was extracted by the high-temperature treatment.

While high protein content (>90% DW protein) was achieved in this case, the low protein yield could make this protein isolate too expensive compared to conventional protein isolates.

3.4.5. Production of protein hydrolysates

The production of protein hydrolysates can follow two different routes: direct, *in situ*, protein hydrolysis of solvent-extracted cells (Table 3.3) and enzymatic hydrolysis of extracted protein concentrates. Most algae protein hydrolysates have been generated by *in situ* hydrolysis of the protein from solvent-extracted cells. *In situ* hydrolysis of algal biomass is an effective protein release method (Figure 3.2) that results in high protein extraction yields [12, 43, 103, 116, 117], and minimizes the extraction of insoluble carbohydrates and membrane-bound pigments [12, 36, 103]. The second route, enzymatic hydrolysis of protein concentrates, is the preferred method used for the production of commercial protein hydrolysates from whey (Guadix et al., 2006), soybean (Chiang et al., 2006), and pea (Li & Aluko, 2010) protein concentrates. The few reports in the published literature on hydrolysates from algal concentrates and isolates [113, 118] will be discussed next.

Table 3.3 Algae-derived protein concentrates and isolates

Species	Cell disruption and/or protein solubilization	Purification	Final protein purity (%)	Protein recovery yield (%)	Fold increase in purity	Ref.
Tetraselmis sp.	Zirconia bead milling for 30 min	Dialysis (1) EBA ion exchange adsorbent Streamline DEAE for washing away chlorophyll Dialysis (2) Depigmentation by acid (1 M HCl) precipitation at pH 3.5 and RT	64.4	7	1.8	[17]
Scenedesmus obliquus	Homogenization with glass beads for 30 min Incubation pH 10	Clarification RNase and DNase treatment, Precipitation (pH 4) Ethanol extraction of pigments and lipids	70	50	1.3	[119]
Nannochloropsis sp	Defatted by Chloroform- methanol Incubation pH 11	Clarification Acid precipitation	56	16-30	1.1	[97]
C. pyrenoidosa	Drying Ultrasonication Enzymatic treatment	TPP at pH 6 using butanol and 40% ammonium sulfate at solid load of 0.15 g/mL Protein concentrated in the middle phase	78.1	NR	1.6	[23]
Chlorella protothecoides	High temp extraction (50 to 150°C) for 5 min.	Microfiltration to separate soluble from cell debris 80% DW protein was concentrated by ultrafiltration using 1 or 5 kDa membrane	90	~20	2.0	[120]
Chlorella vulgaris	HPH at pH 12	Concentration/fractionation Precipitation in acid media (pH 4) or Ultrafiltration (300 KDa MWCO)	NR		NR	[24]

3.4.5.1. Hydrolysates from protein concentrates

Hydrolysis of microalgal protein from a concentrated protein fraction can be energy intensive considering the number of steps involved (Figure 3.4). Nevertheless, the few authors who have explored this approach were able to obtain protein purities of up to 80% DW. Velea, Vladulescu [113] (Table 3.3) patented a process for producing algal hydrolysates from a concentrated protein fraction. In this method, microalgae cells were first disrupted by enzymatic cell-wall hydrolysis augmented with sonication, and the released protein was separated from the cell debris by centrifugation. After removing the chlorophyll by addition of activated charcoal, the clarified lysate was subjected to ultrafiltration (1 kDa). The protein-containing retentate was hydrolyzed for 16 h at 60°C. The treatment resulted in a protein hydrolysate with a high degree of hydrolysis, containing soluble di- and tri-peptides, and more than 80% DW protein content. Patinier [118] also patented a scalable process to produce *C. protothecoides* hydrolysates with 80% DW protein content. The process started with bead milling of 30% by weight fresh biomass, followed by incubation of lysed cells with cellulases to maximize cell disruption and protein release. After hydrolysis of the protein slurry with an alkaline protease for 4 h at 60°C, the protein hydrolysate slurry was fractionated by three-phase centrifugation. The protein hydrolysate, recovered in the aqueous phase, was purified by ultrafiltration (<5kDa membrane) yielding a hydrolysate with 80% DW protein content [118]. While Patinier [118] did not report the final protein yield, it is expected to be higher compared to the non-disrupted biomass due to the combined effect of mechanical disruption and enzymatic hydrolysis. The hydrolysis of isolated microalgal protein is worth exploring further as

it might offer certain benefits, such as enhanced protein extraction yield, lower enzyme dosages requirements, and a more controlled degree of hydrolysis.

3.4.5.2. Protein hydrolysates from ethanol-extracted cell biomass

The protein hydrolysis of ethanol-extracted cells grants the selective release of peptides and other soluble compounds from whole-cells while avoiding high energy intensive disruption processes. The first step is the extraction of ethanol-soluble lipids (fatty acids, lutein, other carotenoids, etc.), followed by incubation of the extracted cells with a non-specific protease (Table 3.4), and separation of the peptide-rich supernatant via centrifugation. Protein hydrolysates from ethanol extracted biomass have high protein content (60% DW) [103], high digestibility (97%), and only 5% residual chlorophyll [12]. Several authors reported the improved efficiency of protein extraction yield when cells were incubated with an organic solvent to remove lipids and other hydrophobic molecules before enzymatic hydrolysis [12, 103, 117]. The observed benefit of solvent extraction was linked to the removal of hydrophobic molecules interacting with proteins, which permitted better enzyme-protein contact [103, 117].

3.4.5.3. Protein hydrolysates from deoiled cell biomass

Deoiled biomass (meal) in this review refers to the algal residue after hexane extraction of triglycerides. The deoiled algal meal is a cheaper feedstock and presents a logical strategy to reduce the production cost of microalgal protein hydrolysates. The use of deoiled microalgae instead of whole biomass has been investigated by several groups [43, 103, 104, 121]. The most relevant data have been generated from the hydrolysis of deoiled *Chlorella* species. Sheih, Wu [43] were able to release about 48% of total *C.*

vulgaris proteins by hydrolysis of deoiled biomass with pepsin for 15 h at 50°C (Table 3.4). The protein hydrolysate consisted of a mixture of small MW proteins (<20 kDa) and peptides with high anti-oxidative activity. Sari, Bruins [103] compared protein hydrolysates produced from hexane-extracted *C. vulgaris* meal and non-deoiled microalgae cells. The protein hydrolysate yield after the enzymatic hydrolysis *of C. fusca* with Protex 40XL was approximately 74% for the hexane-extracted meal and 60% for non-deoiled cell biomass [103].

By extracting lipids and proteins as hydrolysates from algae, not only can total revenue be increased but protein extraction yields are also enhanced (Table 3.4). Protein hydrolysis of deoiled or ethanol-extracted biomass offers several potential benefits, such as 1) energy intensive cell disruption methods (i.e., HPH), 2) higher initial protein concentration per unit dry biomass, 3) better economics of solvent-extracted algal biomass due to coproducts (triglycerides, other lipids, and pigments), 4) more efficient enzymatic hydrolysis of solvent extracted biomass, and 5) significant removal of chlorophyll (undesired color) by disruption of chlorophyll-protein interactions and selective peptide release. Nevertheless, further development of hydrolysate products with concomitant evaluation of purity, quality, and functional attributes is needed.

Table 3.4 Protein hydrolysates

Solvent extraction	Species	Cell disruption/ Storage	Hydrolysis method	Purification / processing	Protein recovery yield	Protein purity (%)	Fold increase in purity (compared to whole cells)	Ref.
None	*Chlorella fusca* (108 g/L)	None	Protex 40XL (5% w/w), T=60°C t=4h	Freeze drying	~50%	68	2.4	[103]
	NR (100 g/L)	Ultrasonication and enzymatic digestion with Glucanex at 4.5 pH, at 45°C	Alcalase and Flavourzyme (1% w/v each) T=40°C, t=16h, pH 7.0	Activated charcoal to remove chlorophyll Ultrafiltration (1kDa) Vacuum evaporation until 10% w/v Spray-drying	NR	83.4	1.7[5]	[113]
	Chlorella sp (220 g/L)	Bead milling at 80% filling	Basic protease T=60°C pH=8 t=~4h	Microfiltration Ultrafiltration (<5kDa) Evaporation (35%wt) Spray dried	80	NR	1.6	[115]
	Chlorella vulgaris (25 g/L)	Feeze dried	Pancreatin (8% v/w), T=45°C, pH, 7.5 for t=5h	Clarification	46.5	NR	NR	[35]
	Chlorella vulgaris (100 g/L)	Frozen (-20ºC)	Alcalase (5% v/w) T =50°C, t=4h, pH 8.5	Ultrafiltration Diafiltration (300 kDa)	24.8	NR	NR	[32]
	Chlorella vulgaris waste (100 g/L)	Dried	Pepsin (2% w/w), at 50ºC, pH ~3 for 15 h	Clarification	48.3	NR	NR	[43]
Ethanol	*Chlorella sp* and *Scenedesmus sp* mixture	Spray dried and physically disrupted	Subtilisin DY at 50º C pH 8, for 4 h	Clarification	70	66	1.32	[117]
	Chlorella vulgaris	Spray dried algae	Pancreatin (30 AU/g, at 37°C, pH= 7.5 t =4h	Clarification	~52	49.7	1	[12]

[5]Assuming 50% initial protein content in cells

3.4.5.4. Production of small peptides with bioactivity potential

The majority of reported bioactive peptides, which are typically 2 to 5kDa in size, have been purified from dairy [121-123], fish [80], soy [124], and cocoa bark [125] hydrolysates. Bioactive peptides from the abovementioned hydrolysates are typically isolated by a combination of two or more purification methods, such as selective precipitation, chromatography (ion-exchange, size exclusion, hydrophobic interaction, and reverse phase), ultrafiltration, and more recently, electro-membrane filtration [123, 125-127]. From an economic perspective, ultrafiltration is easier to scale-up, requires lower capital investment, and has lower operating costs than chromatographic methods. Because membrane filtration has a lower selectivity compared to chromatography methods, it is more suitable for concentration and size-based fractionation of hydrolysates with potential bioactivity than chromatographic purification of bioactive peptides. Cross-flow electro-membrane filtration combines two separations mechanisms, charge interaction and size exclusion, and seems to be a better alternative to either membrane filtration or ion-exchange chromatography. The presence of charge on the membrane surface has the potential to increase separation selectivity of charged peptides and peptide migration rate, and to reduce membrane fouling [123, 127].

There are few reports dealing with the purification of bioactive peptides from microalgal protein hydrolysates, and the majority of those utilized analytical methods for purification and analysis of isolated peptides [43, 128, 129] rather than scalable and economically viable techniques. Sheih, Wu [43] characterized angiotensin-converting enzyme (ACE) inhibitory peptide from *C. vulgaris* protease-digested biomass waste. The

lyophilized microalgal protein hydrolysate was fractionated by size exclusion chromatography using Sephacryl S-100 followed by anion exchange chromatography. Purified peptides (1.3 kDa) exhibited ACE inhibitory activity and were stable at a wide pH range between 2 and 12 and temperatures from 40^{o}C to 100°C. The bioactive peptides were also resistant to digestion by gastrointestinal enzymes. A similar purification approach was adopted by Vo, Ryu [129], who isolated two anti-inflammatory and anti-allergic peptides from the enzymatic hydrolysate of *Spirulina maxima*. The hydrolysate was first fractionated into >10, 5-10, 3-5 and <3 kDa fractions by membrane filtration. The <3 kDa fraction contained the highest anti-inflammatory activity and was further purified by anion exchange, gel filtration, and RP-HPLC. The end result was the isolation of two peptides consisting of 6 amino acid residues each with a size of ~686 Da, which exhibited anti-inflammatory activity. Nguyen, Qian [128] also isolated and characterized a peptide from deoiled *N. oculata* hydrolysate. To identify potential bioactivity, the protein hydrolysate produced using the protease, Alcalase, was fractionated and purified by two sequential HPLC columns. First, a size-exclusion column was used to collect different MW fractions and, then, the fraction that exhibited osteoblast differentiation activity was further purified on a C18- RP-HPLC column yielding a bioactive oligopeptide consisting of Met-Pro-Asp-Trp (529.2 Da). The separation methods using HPLC as described above are expensive, and high manufacturing cost could only be justified for bioactives that are FDA approved and sold as biotherapeutics.

3.4.6. Downstream processing options for microalgal protein products

The literature review of methods that have been used to extract and purify algal protein products reveals that the selection of unit operations, their sequence, and process conditions could easily affect product quality and yield. Therefore, in addition to yield and production cost, the process design should consider the required product attributes (solubility, functionality, purity, etc.) for the intended application (Figure 3.4).

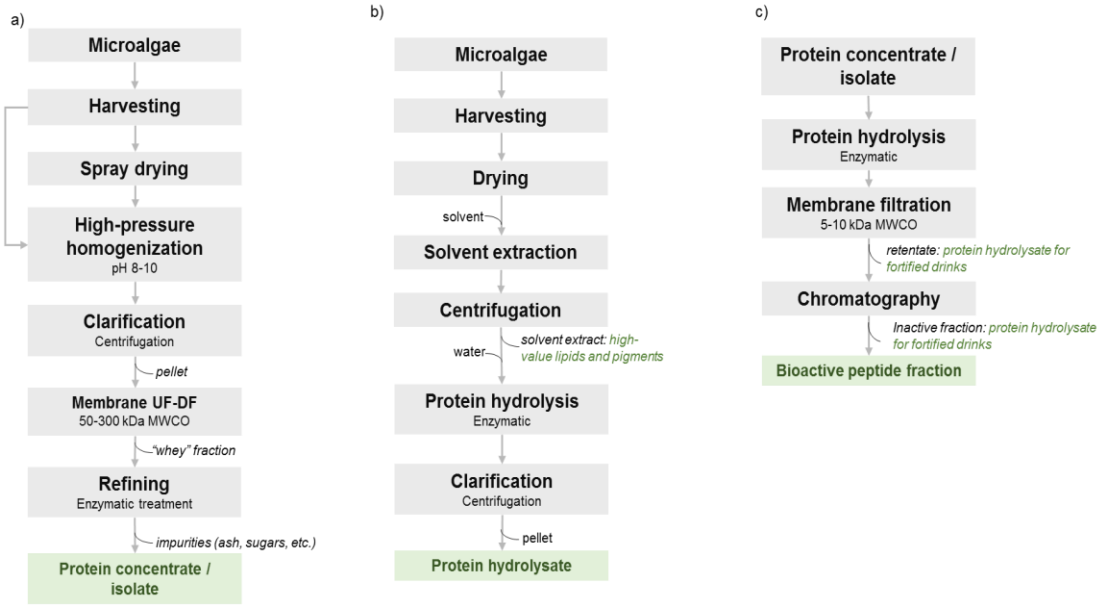

Figure 3.4 Suggested approaches to produce (a) protein concentrates, (b) hydrolysates, and (c) bioactive peptides

Protein concentrates or isolates are products that are expected to have water absorption, gelling, foaming, and emulsification properties for a variety of food applications (Figure 3.4 a). To make concentrates and isolates cost competitive, the downstream processing should allow for recovery of functional protein products at the highest possible yield and purity. Both factors are affected by the efficiencies of extraction and purification steps. To maximize protein extractability, cell disruption can be conducted by either HPH or

bead milling under alkaline conditions (pH 8-10), followed by clarification via centrifugation. At that point, protein concentration is expected to be around 50% soluble solids; thus, additional purification steps, such as ultrafiltration or protein precipitation, would still be required. Both methods seemed to yield the same amount (80%) of protein product with slightly different protein content. Protein concentration and purification of clarified extracts by ultrafiltration (300-1000kDa) and diafiltration resulted in less than 60% protein purity (Kulkarni and Nikolov, 2018) compared to 60-65% obtained by protein precipitation (data to be published) (Figure 3.4a). If protein precipitation is used, then a water wash of the precipitate could be applied to remove trapped salts and carbohydrates and increase the protein content [130]. To produce protein concentrates for food applications, which require preserving protein solubility and functionality, membrane filtration would be more suitable than precipitation (Ursu et al., 2014). Also, membrane filtration allows for "washing" of extracted protein by diafiltration and does not require centrifugation of the concentrated protein.

Additional purification steps will most likely be required to produce protein isolates with greater than 90% protein content. If most of the impurities present in the precipitate or membrane filtration concentrate (retentate) are polysaccharides (such as starch and cell-wall polysaccharides), an enzymatic treatment to solubilize those is likely worth exploring (as long as the enzyme cost is not overwhelming). Finally, spray-drying of the protein concentrates is recommended [130] as other forms of drying (e.g. drum drying) will decrease protein solubility [131].

Algae-derived protein hydrolysates are potential candidates for fortified and high-protein content beverages (Figure 3.5b). The protein products for fortified beverages require high solubility and stability at elevated temperatures (pasteurization) and acidic pH. Protein hydrolysates can be produced by a process outlined in Figure 4a, but a more elegant and potentially lower-cost option would be the direct protein hydrolysis of deoiled biomass as depicted in Figure 3.4 b [12, 103]. Solvent (ethanol) extraction of high-value lipids and pigments prior to protein hydrolysis has two merits. First, the removal of lipids increases the enzyme efficiency and extraction yield (section 3.4.3.2. and 3.4.3.3.), and second, the extracted lipids and/or pigments represent a potential revenue stream that can be used to offset the enzyme cost.

High-value nutraceuticals are other promising protein products from microalgae (Figure 3.4 c) that can be produced by purifying bioactive peptides from protein hydrolysates. While direct hydrolysis of deoiled biomass (Figure 3.4 b) could be used as a source of protein hydrolysates for purification of bioactives, protein concentrates are probably a better starting material for conducting controlled proteolysis (Figure 3.4 c). Production of bioactives from partially purified protein allows for a better control of the enzymatic process that will not be hampered by the presence deoiled biomass and loss of enzyme activity due to non-productive adsorption and/or inhibition. Optimization of the protein hydrolysis process would include the selection of optimal enzyme mixture and dosage, temperature, pH, and degree of hydrolysis that would maximize the yield of peptides with desired bioactivity. The hydrolyzed protein fraction containing bioactive peptides (less than 2 kDa in size) can be fractionated by tangential flow filtration using 5 or 10

kDa MWCO membrane. The peptide-rich fraction collected in the membrane permeate is further purified and concentrated by adsorption chromatography. The fraction of larger peptides (> 5 kDa) collected in the retentate could be used in fortified beverages or for other relevant protein hydrolysates applications.

Downstream processes to produce microalgae-based protein products outlined in this section are based on current technology and processes. There are several process variations and novel technologies that are likely to be implemented in the future. Regardless of the chosen downstream process options, challenges for process developers and manufacturers, like other bioprocess industries, are raw material (algal biomass) cost, product yield and quality, and manufacturing cost. Innovations, technological developments, and pilot-scale testing are vital for improving the efficiencies of current processes and meeting industrial benchmarks for protein products.

3.5. The projected cost of microalgal protein products

To estimate the potential production cost of protein hydrolysates, one has to take into account the cost of microalgae biomass cultivation and subsequent downstream processing operations, which may include cell disruption, clarification, protein concentration, fractionation and/or purification. Biomass cost estimates for several microalgal species and different bioreactor types and sizes are summarized in Table 3.5

Table 3.5 Biomass cost estimates for different bioreactor configurations and cultivation conditions and algae species

Cultivation	Bioreactor type and capacity	Species	Biomass Cost ($/kg DW)	Assumptions	Reference
Photoautotrophic	Open pond	*Chlorella vulgaris*	6.0	Heating, cooling and flue gas supplies, Netherland climate conditions, 4 crash related production restarts	[132]
		NR	4.95	Nederland climate conditions, 100 ha plant	[133]
		NR	1.28	Caribbean climate condition, CO_2 and medium are free. High photosynthetic efficiency	[133]
	Flat-panel PBR	*Chlorella sp.*	5.0	24% expenditure on energy, Nederland climate conditions, 100 ha plant	[134]
		Chlorella sp.	0.5	High photosynthetic efficiency, 2.4 % expenditure on energy, Caribbean climate condition, 100 ha plant	[134]
		Chlorella vulgaris	6.0	Heat and flue gas supplies, Netherlands location, 4 times/year cleaning	[132]
		NR	6.0	Nederland climate conditions	[133]
		NR	0.68	Caribbean climate condition, CO_2 free and medium are free. High photosynthetic efficiency	[133]
	Tubular PBR	NR	4.15	Nederland climate conditions. Biomass productivity: 41 ton ha-1 year-1	[133]
		Chlorella vulgaris	6.0	Heating, cooling and flue gas supplies, Netherlands location, 4 times/year cleaning	[132]
		NR	0.7	Caribbean climate condition, CO_2 free and medium are free. High photosynthetic efficiency	[133]
Heterotrophic	Fermenter	*Chlorella protothecoides*	1.4	~465 bioreactors occupying 7500 m^2	[135]
			1.2	Bioreactor expenses were optimized, 257 bioreactors occupying 5000 m^2	[135]
		Chlorella sp.	0.81	Carbon source (glucose) is completely converted to algal biomass. Only energy cost considered (electricity and glucose)	[136]

For photoautotrophic cultivation, using either raceway ponds or enclosed photobioreactors, biomass production cost can range from $0.35 to $6.0/kg DW. Interestingly, bioreactor configuration (i.e., open pond, tubular or flat-panel photo-bioreactors) does not impact the biomass cost as much as the externally supplied carbon source, energy consumption per m^2, biomass productivity of individual species, and growth conditions [132]. The most thorough evaluation of biomass cost as a function of cultivation conditions was provided by Wijffels and Barbosa [137] and Norsker, Barbosa [133]. Using conservative growth assumptions, such as the solar conditions of the Netherlands, yearly plastic replacement of the photobioreactors, and the cost of CO_2, Wijffels and Barbosa [137] estimated the cost of *Chlorella* sp. biomass at $5/kg DW. A different scenario with the same microalgal species but with flat panel bioreactors, which assumed a reduction of energy consumption by an order of magnitude, increase of photosynthetic efficiency from 5% to 7%, and relocation of microalgal cultivation to the Caribbean, resulted in an estimated biomass cost of $0.5/kg [137]. A similar 10-fold cost difference between optimized low-cost cultivation conditions and suboptimal ones was reported by Norsker, Barbosa [133]. Tabernero, Martín del Valle [135] modeled a hypothetical 10,000 ton/year biodiesel production plant that used heterotrophic cultivation of *C. protothecoides* in 465 continuous stirred bioreactors estimated a biomass cost of $1.4/kg DW. The authors correctly concluded that such scenario might not be economically practical because of the exceptionally high number of bioreactors and high-capital investment required (72% of total capital investment). Although the

estimates of $0.5 to $5 per kilogram algal biomass refer to future costs, current microalgae production costs are $10-50/kg DW (www.alibaba.com).

In addition to biomass cost, other factors that directly impact protein production costs include protein content of algal biomass and process yield. To get a sense of the impact of these factors on potential product cost, one could assume biomass costs of $10/kg DW, cell protein content of 0.5 g/g DW, and protein extraction yield of 70%. Using these assumptions, the extracted protein cost would be around $29/kg protein. In addition, protein yield losses of 15-20% are to be expected when producing protein concentrates or isolates by precipitation or membrane filtration. Therefore, without considering operating costs (i.e., labor, supplies, maintenance, etc.), the protein production cost, based only on biomass cost and process yield could easily end up being $34/kg protein. This estimate is far from current industry benchmarks for protein concentrates and isolates reported in Figure 3.5.

The use of deoiled biomass as a starting material for hydrolyzed protein products seems to be a reasonable strategy for cost reduction. Sari et al. (2016) developed a process for producing protein hydrolysates from deoiled algal meal. Taking into account the cost of deoiled algae meal ($0.2/kg) and process-related costs (chemicals, enzyme costs, energy for maintaining reaction temperature ~40ºC, and protein yield), Sari et al. (2016) estimated protein hydrolysate production costs at $1.4/kg. This example clearly demonstrates the impact of biomass cost on protein cost when offset by a co-product such as triglycerides (Sari et al., 2016).

Based on the presented cost scenarios and prices of current protein products summarized in Figure 3.5 below, it appears that at $0.2/kg biomass cost, protein hydrolysates and high-purity protein fractions (>90% protein content) could be competitively priced.

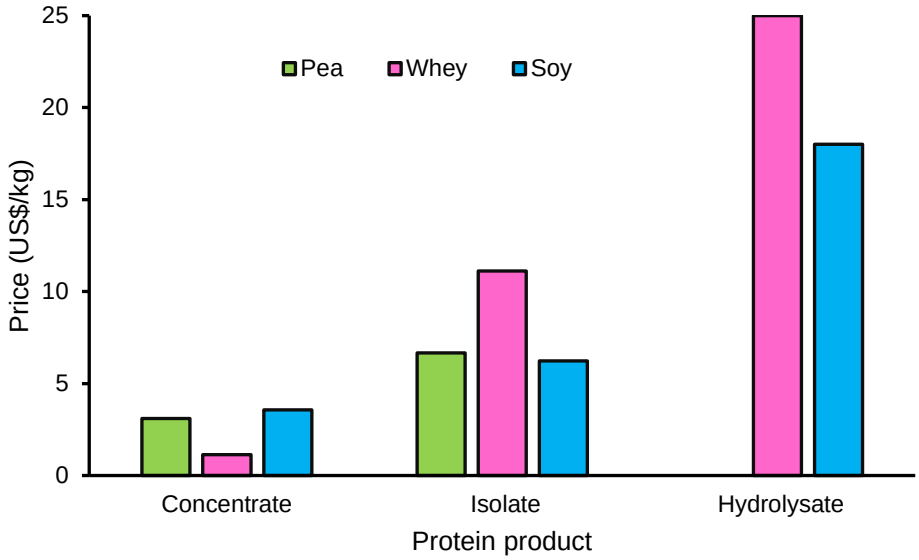

Figure 3.5 Current pricing of protein products from soy, whey, and peas[6] [55, 132].

3.6. Strategies for increasing economic feasibility of algae protein products

While there is an increasing interest in the production of protein products from microalgae, there are still several potential opportunities and strategies that can be used to improve the economic feasibility of microalgal-sourced products including optimization of biomass productivity, and process efficiency and extraction of multiple co-products.

[6] Pea hydrolysates were not found commercially

3.6.1. Optimization of biomass productivity and downstream process efficiency

The main issues affecting economic feasibility of the microalgae-based products are biomass productivity and cultivation costs, which at $10/kg-biomass are still an order of magnitude higher than desired. Cultivation of microalgae, as a land crop, would benefit from the implementation better agronomic practices to increase microalgae productivity. Reduction of input costs, such as CO_2, water, and nutrients, are critical as they constitute more than 50% of the cultivation costs [138]. Strategies to reduce such costs include water and nutrients recycling, CO_2 capture, and/or the use of alternative carbon sources, such as acetate.

The application of metabolic engineering for enhancing physiological properties of microalgal strains is another strategy for improving biomass and product yields that should result in reduced cultivation, harvesting and extraction costs. Proposed tactics include increased uptake of solar energy and higher photosynthetic yields, auto-flocculation at a certain growth stage to minimize harvesting costs, and development of larger cells with thinner cell walls, which would be easier to disrupt [134]. The incorporation of effluents from food processing or the wastewater treatment industry into microalgal cultivation is another strategic approach suited to maximize nutrient utilization and reduce environmental impact. The use of aquaculture wastewater [139] and textile effluents as nutrients [66] for the cultivation of *Chlorella* sp. are examples that have shown competitive biomass productivities and significant reductions in culture media costs.

While current research efforts have focused on evaluating and quantifying the extraction of microalgal protein, development of scalable protein fractionation and recovery options is lacking. In several instances, promising high-protein content isolates and hydrolysates have apparently been produced, but product composition, process yield or the reasons for incurred protein losses were not given. Rigorous tracking and reporting of protein yield and purity are important for identifying the future process improvement needs.

Because of low protein recovery yields and suboptimal process conditions, the current microalgal bioprocessing costs account for up to 50–60% of total product costs, while the industry benchmark for other protein feedstock is not higher than 30% [140]. Process optimization and integration of simpler, faster, and more efficient methods for protein extraction and purification are needed to reach the threshold of economic feasibility of the microalgal protein platform. The number of expensive and time-consuming unit operations required to produce protein concentrates, isolates and hydrolysates must be reduced in number and/or replaced with lower cost steps or multipurpose unit operations [140].

Multipurpose unit operations, such as three-phase partitioning, that can perform several functions, such as clarification, fractionation, and protein concentration, are a good example of process streamlining. *In situ* proteolysis of microalgal protein and the release of hydrolyzed proteins containing functional and/or bioactive peptides is another example of potential process rationalization. The choice to initially produce bioactive peptides and functional polypeptides, which have a greater market value than protein

concentrates [141], could be a good tactical prospect to increase the revenue from microalgae biomass. Ultimately, a techno-economic analysis must be carried out to determine cost/benefit factors for specific processes and product scenarios.

3.6.2. Extraction of multiple co-products

Based on the cost scenarios discussed in Section 4, it is clear that co-production of high-value products would benefit overall process sustainability and product economics [20, 142, 143]. Microalgae produce compounds that may be harvested as co-products to provide diverse revenue streams [81], and include triglycerides containing essential omega-3 unsaturated fatty acids, eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), carotene antioxidants (astaxanthin, lutein and β-carotene), and biogenic compounds such as novel antibiotics, drugs , and anti-fouling substances [4, 18-20]. The extraction of lipids, carbohydrates [66] and proteins [143, 144] for human consumption have the potential to significantly increase the profitability of microalgae farming [143]. The co-production of high-value nutraceuticals such as omega-3 from *Nannochloropsis* and food proteins [143], is expected to have greater bioproduct values than lipids for fuel and protein for feed applications [145]. Currently, EPA-rich oil and high-value protein products for human consumption appear to have the best economically sustainable appeal [143]. What is lacking to make this promising scenario viable is an integrated product-compatible process for extraction of lipophilic and hydrophilic products. Studies suggest possible pathways toward process integration and ultimate microalgal “biorefining” processes that rely on dried biomass that was first deoiled with an organic solvent and then subjected to enzymatic or alkaline

solubilization of proteins [12, 103, 104]. This specific process sequence is optimal for isolating lipophilic products, such as EPA, carotenoids, triglycerides, etc., but it is not for extracting functional microalgal protein for food applications.

The selection of an optimal extraction sequence is a key consideration for microalgae biorefining. Ansari, Shriwastav [145] proposed the extraction of proteins followed by lipids and carbohydrates as an optimal sequence for maximizing the value of the *Scenedesmus obliquus.* The conclusion reached by Ansari et al (2017) was based on the projected commercial value of protein concentrates (US $5/kg for food), lipids (US $0.5 /kg for fuels, US $2/kg for chemicals) and carbohydrates (US $1/kg) without considering process feasibility and cost. In other words, the demonstration of proposed criteria of the protein, lipids, and carbohydrates extraction sequence was conducted on an unrealistic scale and under unrealistic process conditions, such as gram biomass batches, microwave-mediated cell disruption, and exotic solvents. Nevertheless, developing a rationale for determining the optimal process sequence based on product stability, value, yield, and processing cost is the correct approach and merits further exploration.

3.7. Conclusions

The review of the status of microalgal protein production reveals several processing gaps and identifies advances necessary to close those gaps. Technological advances that need further attention include: 1) maximizing cell density and protein content; 2) development of protein-compatible process technologies for solvent extraction of high-value coproducts from wet biomass; and 3) development of scalable and cost-effective purification methods for production of bioactive peptides.

Current industrial cultivation of microalgae is done in open ponds, which are easier to scale-up and require significantly less capital investment compared to closed bioreactor systems. Major drawbacks of open-pond cultivation are water losses due to evaporation and relatively low biomass densities and protein content in harvested biomass. The options for maximizing protein content per gram biomass using organic nitrogen, such yeast extract, are too expensive for industrial scale operations. Nutrient recycling, more efficient CO_2 capture, improved agronomic practices and/or optimization of nitrogen to carbon ratio are more realistic venues to pursue. Closed systems, operating under mixotrophic or heterotrophic fed-batch conditions, can result in higher biomass and protein productivities than open ponds, but carbon source and capital investment are major cost barriers that have to be addressed before considering scale-up.

The challenges to protein-compatible bioprocessing are related to finding optimal solvent and extraction conditions for recovery of hydrophobic coproducts from water-laden biomass without irreversibly affecting protein extractability, functional properties, and process yield. Extraction of lipid coproducts (lutein, carotenoids, fatty acids, etc.) typically requires biomass drying, which affects protein functionality and/or protein extractability. On the other hand, solvent extraction of wet biomass limits the solubilization power of the solvent resulting in lower lipid coproduct yield. In addition, the exposure of wet biomass to organic solvents could cause partial denaturation of algal proteins, which is manifested in lower protein extraction yield and loss of functional properties (emulsification capacity).

The use of proteolytic enzymes to maximize protein extraction yield from dried and/or solvent extracted biomass (Sections 3.4.3.2 and 3.4.3.3) could be a good strategy if the goal is to generate protein hydrolysates for subsequent isolation of bioactive peptides. *In situ* proteolysis increases protein yield and results in protein hydrolysates that are relatively easy to separate from the cell residue by any solid-liquid clarification method. However, the enzyme cost and finding an efficient, low-cost separation method of bioactive peptides are potential barriers to market entry.

In conclusion, the integration of cost-efficient extraction and fractionation methods for multiple coproducts are the current bottleneck that hampers further advancement in this field. We believe that this review corroborates the potential of microalgae as a source of nutraceuticals and protein products. Once technical and economic barriers for the manufacturing of food products are surmounted, microalgae will reveal their true potential as a sustainable and renewable feedstock.

3.8. Acknowledgments

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3.9. Conflicts of Interest

The authors declare no conflict of interest.

4. PROCESSING OF PERMEABILIZED *CHLORELLA VULGARIS* BIOMASS INTO LUTEIN AND PROTEIN RICH PRODUCTS[*]

4.1. Chapter summary

Chlorella vulgaris species are rich in lutein, a high-value pigment known for its antioxidant properties. In this work, the effect of pulsed electric field (PEF) on enhancing the selective pigment extraction from fresh *Chlorella vulgaris* was tested. PEF treatment enhanced the lutein (2.2 ± 0.1-fold) and chlorophyll yields (5.2 ± 3.4-fold) compared to non-treated cells when using a single-stage ethanol extraction process. A process simulation model was used to assess the viability of the pigment extraction process. The cost estimates showed that the PEF treatment reduced the processing costs and enhanced the economic feasibility of the process. The extraction of algal protein from lutein-depleted biomass was also simulated to determine the benefit of using low-cost microalgae meal for production of proteins concentrates. The cost estimates revealed that unless extraction yield and protein content of the concentrate were improved, the estimated protein cost ($4.16 kg^{-1}) would not be competitive to existing similar products from soybeans and peas.

4.2. Keywords

Microalgae, lutein, PEF, protein, process economics

[*]Reprinted with permission from "Processing of permeabilized Chlorella vulgaris biomass into lutein and protein-rich products" by Soto-Sierra, Laura; Kulkarni, Sayali; Woodard, Susan L.; Nikolov, Zivko L., 2020. Journal of applied phycology, 32, 1697-1707, Copyright [2020] by Springer Link.

4.3. Introduction

Chlorella vulgaris is one the several microalgae species known to be a good source of proteins and pigments such as lutein and chlorophylls [146], which offer several health and nutraceutical benefits. Lutein content can reach concentrations as high as 7 mg per gram cell dry weight and total chlorophyll content can range from 10-30 mg per gram cell dry weight [15, 147] in *Chlorella sp*. Dietary intake of lutein prevents early atherosclerosis, decreases the rate of age-related macular degeneration [148], and ameliorates the onset or progression of cataracts [149]. Chlorophyll can also provide certain health-benefits, including immune system stimulation, blood and liver detoxification, and relief from sinusitis, fluid buildup, and skin rashes [150].

A typical downstream processing of microalgae starts with cell lysis using high-pressure homogenization or bead-milling, which results in extraction of a mixture of intracellular proteins, lutein, chlorophylls, and other pigments [14, 151]. The fractionation of potential protein and pigment co-products from complex algal cell lysates would be difficult and prohibitively expensive because it would involve multiple separation steps. A possible alternative to complete cell lysis is controlled permeabilization of cell biomass that would allow the development of sequential and/or selective extraction to generate multiple revenue streams. In our previous work [21], a co-extraction process of protein and carotenoids from a freeze-thawed, permeabilized biomass was developed. Although the freeze-thaw permeabilization enhanced the selective extraction of pigments from *Chlorella* sp., the freeze-thaw process is of a limited scalability due to the high energy requirements of the process. The aim of the present study was to develop a

scalable and economically viable permeabilization method that would enhance lutein extraction yield and be as selective as the freeze-thaw process.

Pulsed electric field (PEF) or electroporation is a phenomenon that causes the formation of temporary or permanent pores in cell membranes when biological cells are exposed to short pulses (μs or ms) of a high-intensity electric field (kV cm^{-1}) [152, 153]. While the effect of PEF on pigment extractability has been previously considered [154, 155], process parameters to maximize the selective extraction of lutein have not been investigated.

The objectives of this study were to 1) determine the effectiveness of PEF on cell permeabilization and subsequent extraction of lutein and chlorophyll from freshly harvested *C. vulgaris*, *2)* determine the effect of PEF permeabilization method on potential protein loss during lutein extraction, and 3) evaluate the economic feasibility of lutein extraction alone and lutein and protein co-extraction process.

4.4. Materials and methods

4.4.1. Cultivation of *C. vulgaris*

Chlorella vulgaris (UTEX 26, Austin, TX) was grown in Bold's Basal Media (BBM) [156]. Glucose was added at a concentration of 10 g L^{-1}. Inoculation of cultures was done in shake flasks (150-500 mL) with constant shaking (OrbiShaker XL, Benchmark, MA) at 115 rpm and then transferred to 5-10 L of fresh media grown in a 20 L carboy (Nalgene). Filtered air was bubbled (Whisper 100, Tetra) and biomass growth was undertaken at room temperature (22-25 °C) for 4-5 days, with a light/dark cycle of 12 hours each and light intensity of 34.5 μmol s^{-1} m^{-2}. Optical density was monitored at 750

nm and cells were harvested at the late exponential phase using centrifugation (Allegra 25R, Beckman Coulter, CA) at 7500 x g at 4°C for 10 minutes.

4.4.2. Dry weight determination

A cell suspension (V=15 mL) of known OD_{750} was filtered through a pre-weighed (W_1) Whatman 0.7µm glass fiber filter under vacuum. The filter containing the algal slurry was placed in a pre-weighed (W_2) metal tray. The tray was placed on an air oven for at least 2 h at 95°C or until reaching a constant weight measurement (W_3). The dry weight of the sample (DW) was determined using the following equation (1):

$$DW\ \left(\frac{g}{mL}\right) = \frac{W_3 - (W_1 + W_2)(g)}{V(mL)} \quad (1)$$

4.4.3. Pulsed electric field optimization for lutein and chlorophyll extraction from fresh *C. vulgaris*

Freshly harvested cells were re-suspended in 0.04 % (w/v) NaCl (1.1 mS cm^{-1}) solution. Pulsed electric field (PEF) was carried out using Gene Pulser Xcell ™ (Bio-rad) electroporation system and 4 mm electroporation cuvettes (Bio-rad). Control and PEF-treated cells were centrifuged at 7,500 x g, 10 min at 4°C to remove electroporation buffer within 30 min of PEF treatment. The electroporation buffer removed after the PEF treatment was used for protein analysis. Fifty milliliters of 95% ethanol were added per gram dry base cell biomass. Cells were removed by centrifugation and supernatants were analyzed for lutein and total chlorophyll content.

4.4.4. Kinetics of lutein and chlorophyll extraction from PEF-treated cell

Cell suspension of 14 g-DW biomass per L of water (OD_{750} = 20) were subjected to PEF treatment at an electric field strength of 6.25 kV cm^{-1} for 1 ms, followed by ethanol

extraction of pigments (50 mL ethanol g-DW^{-1}). Samples were collected for analysis during the 120 min kinetic study. In the first 20 min, sampling was done every 5 min and after that at larger time intervals ranging from 10 to 30 min. After collection, samples were centrifuged, supernatants collected, and lutein and chlorophyll in the extracts was determined via RP-HPLC (refer to section 2.3). To determine the percent release, the amount of extracted lutein and chlorophyll in each sample was compared to the total extractable lutein (5.2 mg g^{-1}) and chlorophyll (13 mg g^{-1}) value, respectively. The total extractable lutein and chlorophyll were defined as the respective amounts that could be extracted from PEF treated cells (1ms, 6.25 kV cm^{-1}) after 24h of incubation in ethanol (50 mL of 95% EtOH g-DW^{-1}). Percent released was calculated as follows (Equation 2 and 3):

$$\%\ lutein\ release = \frac{\frac{mg}{g}\ lutein\ at\ x\ time\ point}{Total\ extractable\ lutein\ (\frac{5.2\ mg}{g})} \qquad (2)$$

$$\%\ chloropyll\ release = \frac{\frac{mg}{g}\ chlorophyll\ at\ x\ time\ point}{Total\ extractable\ chlorophyll\ (13.3\frac{mg}{g})} \qquad (3)$$

4.4.5. One stage vs multi-stage extraction of lutein and chlorophyll at different biomass densities

Cell suspensions at 3 biomass densities- 14 g L^{-1} (OD_{750} = 20), 28 g L^{-1} (OD_{750} = 40) and 56 g L^{-1} (OD_{750} = 80) were subjected to PEF treatment at an electric field strength of 6.25 kV cm^{-1} for 1 ms. For multi-stage pigment extraction, biomass (50 mL g-DW^{-1}) was mixed with ethanol for 10 min followed by removal of supernatant. Ethanol extraction of the same biomass sample was repeated two more times - for a total of 3 extraction

stages. A single stage extraction was also carried out where biomass was mixed with ethanol for a total of 45 min. As previously reported [21], all the ethanol extraction processes were conducted at 50 mL-EtOH g-DW^{-1}.

4.4.6. Determination of minimum energy input for lutein extraction

Cell suspension at 56 g L^{-1} (OD_{750} = 80) was subjected to PEF treatment at an electric field strength of 6.25 kV cm^{-1} and varying total energy input by changing the duration of pulses (1 ms, 620 µs and 240 µs). Cells were mixed with ethanol for 45 min after PEF treatment to determine the amount of lutein, chlorophyll, and protein released as a function of the treatment time. The volumetric (W_v) specific energy input was calculated as previously described in the literature [157-159] (Equations 4 and 5):

$$W_v(kW\ h\ m^{-3}) = \frac{E^2.t.N.\sigma}{3600000} \qquad (4)$$

Where E is the electric field strength in V m^{-1}, t is the pulse duration in seconds, N is the number of pulses and σ is the electrical conductivity in S m^{-1} at room temperature. Subsequently and the mass specific (W_m) energy input was calculated as:

$$W_m\ \left(\frac{kWh}{kg}\right) = \frac{W_v}{C} \qquad (5)$$

Where C is the concentration of (dry) biomass in kg m^{-3}.

4.4.7. Analysis and quantification of lutein and chlorophylls

Concentrations of extracted lutein and chlorophyll in the cell-free ethanol extracts were determined by RP-HPLC as previously reported [21]. Briefly, 20 µL ethanol extract aliquots were injected onto a 4.6 x 250 mm, 5 µm Acclaim™ 120 C-18 column (ThermoFisher Scientific). Lutein and chlorophyll a and b were separated by a 0-40% linear gradient of dichloromethane in methanol over 25 min. The concentration of each

pigment was determined from the standard curves prepared with lutein and chlorophyll standards purchased from Sigma Aldrich.

4.4.8. Protein analysis

The protein content in *C. vulgaris* biomass was determined as described in Kulkarni and Nikolov [21]. Biomass at 15 g L^{-1} was centrifuged at 7,500 x g for 10 min, the supernatants were removed, and the pellets resuspended in 0.5 M NaOH solution and heated at 80 °C for 10 minutes. The suspension was then cooled to room temperature and centrifuged for 10 minutes at 15,000 x g, and the supernatant containing the solubilized protein was collected (F1). The pellet was re-extracted under the same conditions as the first step, sample was centrifuged, and the supernatant from the second extraction was also collected (F2). The resulting pellet was mixed with 0.5 M NaOH, heated for 10 minutes at 100 °C, the suspension was cooled to room temperature, sonicated for 1 minute at 50% output capacity (55 W, CL-188, Qsonica Sonicator), and centrifuged at 15,000 x g, for 10 minutes. The supernatant (F3) from the final extraction was collected. The protein content on each supernatant fraction (F1, F2, and F3) was determined using the Pierce bicinchoninic acid (BCA) assay kit (Thermo Fisher), bovine serum albumin was used as standard (working range from 50 - 2000 μg mL^{-1}), and absorption at 562 nm was measured using the VERSA max microplate reader (Molecular Devices, CA). The protein content (mg $mgDW^{-1}$) in the microalgae samples was calculated as the ratio between the sum of the protein contents (mg mL^{-1}) from each fraction (F1, F2, and F3), and the initial dry weight of the extracted sample.

4.4.9. Statistical Analysis

The statistical analysis was based on one-way analysis of variance (ANOVA) using JMP Pro 12 (SAS) software. Either one or two-factor design were performed to optimize process parameters. All experiments were done in independent triplicates. To compare significant differences ($p<0.05$) between treatments, a Tukey's adjustment was made for a family-wise error rate of 0.05.

4.4.10. Process simulation

The process modeling for this study was performed using SuperPro Designer, Version 10 (Intelligen, Inc., Scotch Plains, NJ; http://www.intelligen.com/), a software tool that supports performing mass and energy balances of a proposed process flowsheet and calculating equipment size, scheduling, capital investment, operating cost, and overall profitability analysis. This software has been used by others to determine the production cost of a variety of bioproducts, including biopharmaceuticals produced by fermentation [160], plant-made protein products [161-163], and biofuels from microalgae [164, 165]. The impact of the PEF treatment on the economic feasibility of the pigment extraction with ethanol was evaluated by modelling two scenarios. Case #1 considered production of lutein-rich pigment extracts from non-permeabilized biomass while Case #2 considered the same process with PEF-treated biomass. The economic viability of producing protein concentrates from pigment-depleted biomass was also evaluated. Process flow diagrams, specific unit operations, and equipment selection for the three case studies were based on bench scale data and supplemented with data from literature that were relevant to this work [21, 166, 167] (Table 4.1). The equipment costs were

obtained from previous estimates [106, 168], and direct quotes from industry. Technical parameters, such as extraction yields, EtOH requirements, protein concentration factor, and filtration parameters were estimated based on the data collected on this paper, previous research, and equipment capacity estimates [21, 166, 167] (Table 4.2)Main costs and prices, such as the costs of raw material, consumables, utilities, selling price of pigment extracts and protein concentrates, were estimated based on international prices (www.alibaba.com) and previous references [103, 166]. The cost of utilities, and other operating costs such as labor, were estimated based on Texas (USA) market prices.

For the downstream portion of the lutein and protein extraction and recovery manufacturing process, an annual operating time of 7,916 hours and ~1,500-1,800 batches were estimated. The cost estimates for lutein and protein products obtained by SuperPro simulation are direct production cost only, thus, do not include facility costs such as maintenance, depreciation, local taxes, insurance, and factory expenses.

Table 4.1 Major equipment costs

Type	Quantity	Size (Capacity)	Unit	Purchase Cost ($/Unit)	Case where was used		
					1	2	Protein
Biomass storage tank	1	5.6	m^3	67,000	x	x	
PEF	2	21.08	kW	500,000		x	
EtOH storage tank	1	61.11	m^3	118,000	x	x	
EtOH extraction tank	1	66.74	m^3	212,000	x	x	
Evaporator	1	11.87	m^2	131,000	x	x	
Decanter Centrifuge	2	15.0	$m^3 h^{-1}$	200,000	x	x	
Drum Dryer	1	16.1	m^2	153,000	x	x	
Storage tank prior to homogenization	1	8.1	m^3	33,000			x
Homogenizer	2	3.3	$m^3 h^{-1}$	509,000			x
Storage tank prior to membrane filtration	1	3.1	m^3	15,000			x
Diafiltration skid	1	114.70	m^2	775,000			x
300 kDa retentate receiver tank	1	1.6	m^3	12,000			x
300 kDa permeate receiver tank	1	4.52	m^3	23,000			x
Disk-stack Centrifuge	1	2.4	$m^3 h^{-1}$	660,000			x
Spray Dryer	1	0.9	m^3	120,000			x

Table 4.2 Input variables and process parameters

	Type	Value	Case where was used			Source
			Case 1	Case 2	Protein	Source
Process assumptions	Cell concentration at PEF treatment	56 g L^{-1}		x		This paper
	PEF treatment time and kV cm^{-1}	t = 0.650mS 6.25kV cm^{-1}		x		
	Lutein extraction yield	4 mg g^{-1} DW biomass		x		
	Lutein extraction yield	2 mg g^{-1} DW biomass	x			
	Ethanol to biomass ratio	50 mL EtOH g^{-1}biomass	x	x		
	Ethanol extraction time	60 min	x			
	Ethanol extraction time	45 min		x		
	Ethanol recycling	90%	x	x		Industry direct communication
	Max solids concentration after centrifugation	200 g L^{-1}	x	x	x	Centrifuge supplier
	HPH treatment	3 passes, at 15,000 psi			x	Kulkarni and Nikolov [21]
	Protein extraction yield	76%			x	Kulkarni and Nikolov [21]
	Membrane filtration	MWCO: 300 kDa Avg. flux:12-20 Concentration fold: 3X			x	Kulkarni and Nikolov [21]
	Concentrate protein content:	57%			x	Kulkarni and Nikolov [21]
Cost Assumptions	Labor cost (all-inclusive rate)	\$36 h^{-1}	x	x	x	Texas labor estimates for 2018
	Biomass cost	\$10 kg^{-1}	x	x	x	Soto-Sierra, Stoykova [166]
	EtOH cost	\$1.34 gal^{-1}	x	x	x	Markets insiders
	Membranes cost	\$1000-2000 h^{-1}-operation			x	Industry communication
	Selling price of extracted biomass	\$0.2 kg^{-1}	x	x		Sari, Bruins [103]

4.5. Results

4.5.1. Extraction kinetics of lutein and chlorophyll from permeabilized cells

(a)

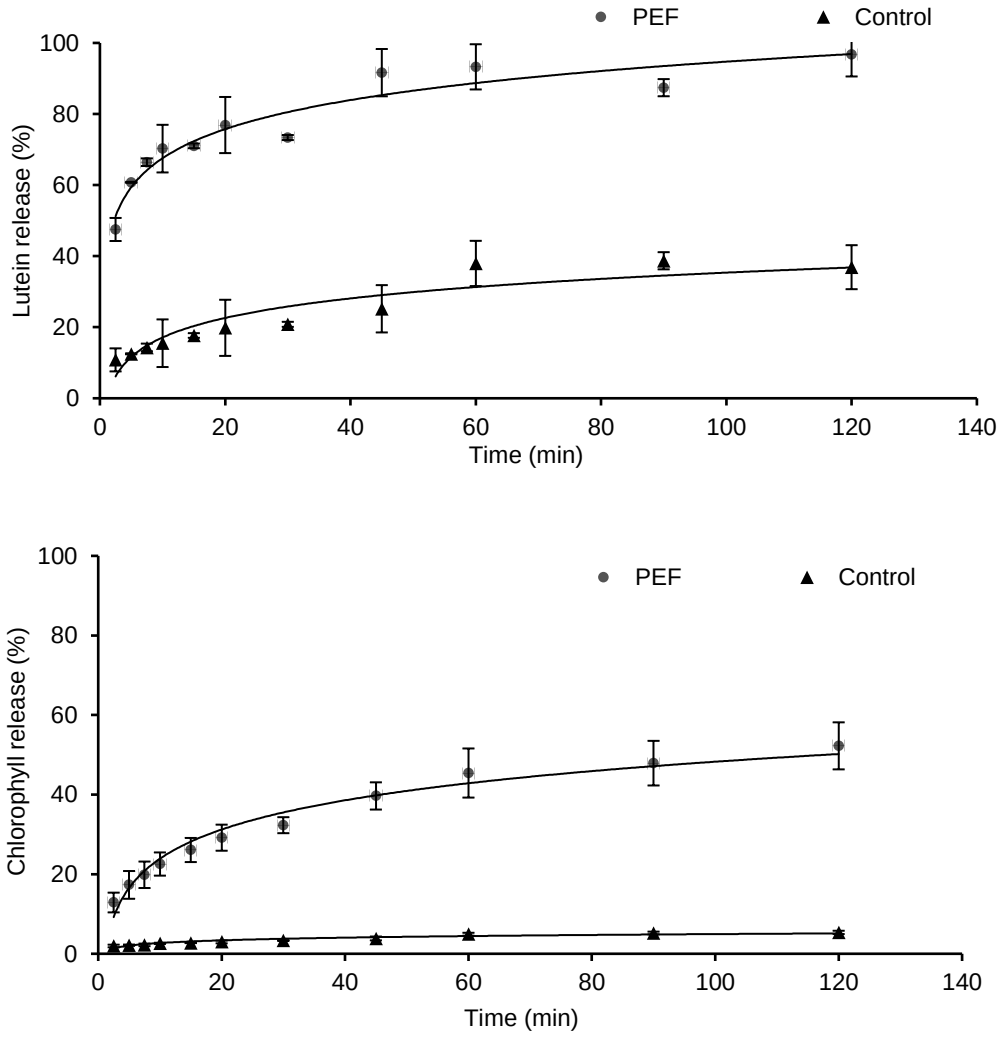


Figure 4.1 Kinetics of lutein (a) and total chlorophyll (b) extraction from control (non-PEF treated) and PEF-treated fresh *C. vulgaris* biomass[7]. Adapted with permission from Kulkarni and Nikolov, 2018

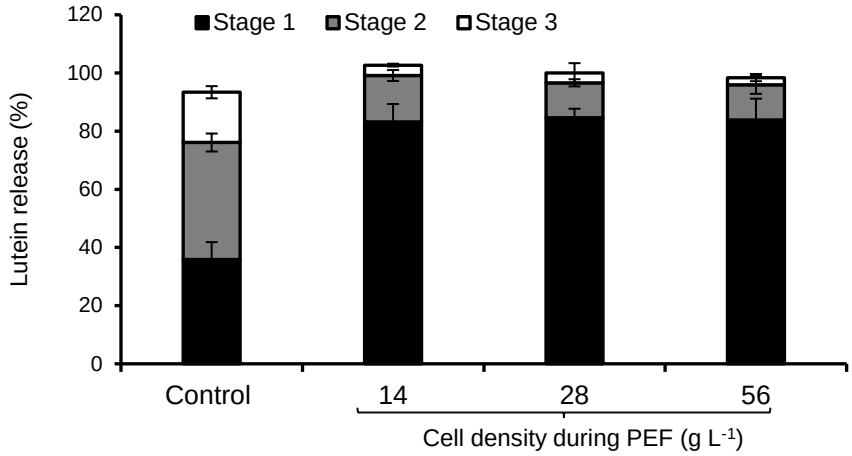
[7] 100% lutein corresponds to 5.2 mg g-1 DW in (a) and 100% chlorophyll correspond to 13 mg g-1 DW in (b). Values reported are averages of triplicates, error bars represent the standard deviation among replicates. Adapted from Kulkarni and Nikolov, 2018

The results of lutein release from PEF-permeabilized and untreated (control) biomass are shown in Figure 4.1a. Lutein release from PEF–treated cells followed a logarithmic trend ($y = 614.33 \ln(x) + 2104$, $R^2 = 0.92$) over the 120-min extraction period (Figure 4.1a). The extraction of lutein was faster during the first 10 min of extraction, and then rapidly plateaued to a concentration of 5 mg g^{-1} DW (96% of the total lutein). The lutein yield obtained by PEF permeabilization followed by ethanol extraction was within the range of reported yields (4.7-7.14 mg g^{-1} DW biomass) from *C. vulgaris* [147, 169]. The amount of extracted lutein from the control (Figure 4.1a) was significantly lower (2.6-fold), even after 120 min of mixing, indicating a much slower rate of diffusion of lutein from untreated cells. Since chlorophyll (a and b) is another major pigment of commercial interest present in *C. vulgaris*, the kinetics of chlorophyll extraction were also followed and plotted in Figure 4.1b. Similar to lutein, a logarithmic trend ($y = 1377 \ln(x) - 65.15$, $R^2 = 0.97$) of chlorophyll extraction was observed with PEF-treated cells. A rapid chlorophyll accumulation in the supernatant was detected during the first 10 min ($y = 164.13x + 1334$, $R^2 = 0.98$). The extraction rate, past the 10-min point, slowly decreased during extraction, but did not level off even after 120 min. The amount of chlorophyll released at the end of the 120-min extraction period reached 6.8 mg g^{-1} DW (51% of the total chlorophyll), which was 10-fold greater than the yield from the untreated (control) sample.

4.5.2. Effect of cell density and multi-stage extraction on recovery yields of lutein and chlorophyll

Lutein and chlorophyll extraction kinetics data in Figure 4.1 indicate that after the initial 10 min of extraction a relatively small yield gain can be achieved in the subsequent 110 min of incubation (Figure 4.1). For this reason, we investigated the potential benefit of conducting biomass extraction in three stages, 10 min each, to maximize the extraction yield and reduce the extraction to 30 min. Since the extraction kinetics in Figure 1 were performed at a suboptimal density for scale up (14 g L^{-1}), the effect of biomass density on extraction yield was also included in this study.

a



b

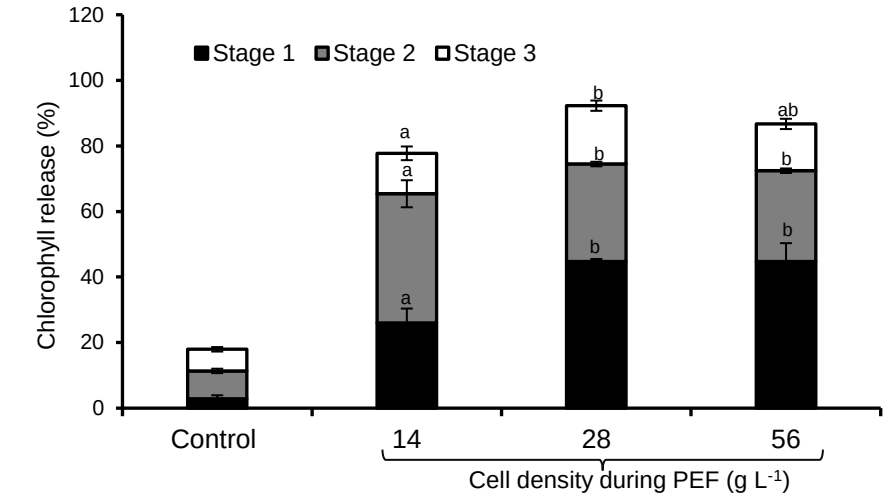


Figure 4.2 (a) Lutein extraction using a multi-stage extraction for PEF undertaken at different biomass concentrations[8]. (b) Extracted chlorophyll using a multi-stage extraction process after PEF[9]. The density of the control was 14g/L for both cases

[8] 100% corresponds to 5.0 mg g-1 DW

[9] 100% chlorophyll correspond to 13 mg g-1 DW

Values reported are averages of triplicates, error bars represent the standard deviation among replicates. For each graph, values not sharing the same letter are significantly different (α= 0.05). Control treatment was performed at the optimum conditions (14 g L-1) for pigment release

The results of the three-stage extraction of PEF-treated biomass at three different densities (14 g L^{-1}, 28 g L^{-1} and 56 g L^{-1}) are summarized in Figure 4.2. The data in Figure 4.2a indicate that the cell density did not have a significant effect ($p>0.05$) on lutein extraction yield at any of the three extraction stages. The same data indicate that a single-stage extraction could extract as much as 82% of the total lutein content in the biomass, and the subsequent two stages contribute approximately 15 and 2%, respectively. The untreated cell biomass (control) required three stages to achieve greater than 90% lutein recovery; 36% of the total lutein was extracted in the first stage, 40% in the second and 17% in the third stage.

Chlorophyll recovery from both, PEF-treated and untreated samples, did benefit more from the three-stage extraction than lutein (Figure 4.2b). After three extraction stages, the cumulative chlorophyll yield from PEF-treated biomass was ~88%, which is almost two times more than the amount reached after 120 min (Figure 4.1b). The control biomass yielded about 20% chlorophyll, a significantly lower amount ($p<0.05$) than PEF-treated biomass.

4.5.3. Effect of PEF permeabilization on potential protein losses during ethanol extraction

The objective of this experiment was to determine the optimal duration of the PEF treatment that maximizes lutein yield and minimizes protein losses. Protein and lutein release into ethanol phase were compared after PEF treatment with three different pulse duration times: 240, 620, and 1000 μs (Figure 4.3). The percent lutein release from chlorella cells treated for 240 μs was 70%, which is significantly lower ($p<0.05$) than for

620 µs and 1 ms (1000 µs). The protein loss was lower at 240 and 620 µs compared to 1 ms treatment, but it remained below 15% for all cases.

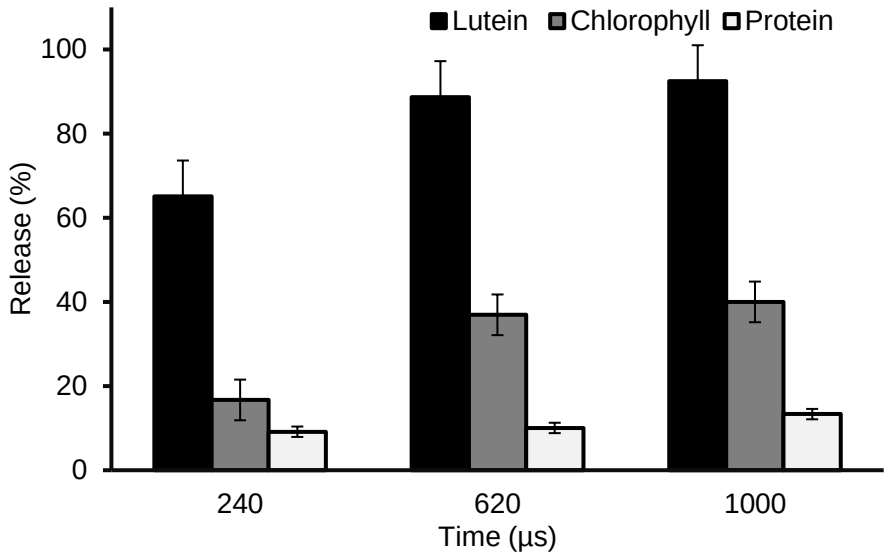


Figure 4.3. Lutein, chlorophyll, and protein release after PEF treatment of samples at 56 g L-1 and 6.25 kV cm-1[10]

[10] Values reported are averages of triplicates, error bars represent the standard deviation among replicates. Comparisons were made within same colored bars. Values with asterisk are statistically different (α= 0.05)

4.5.4. Process cost analysis

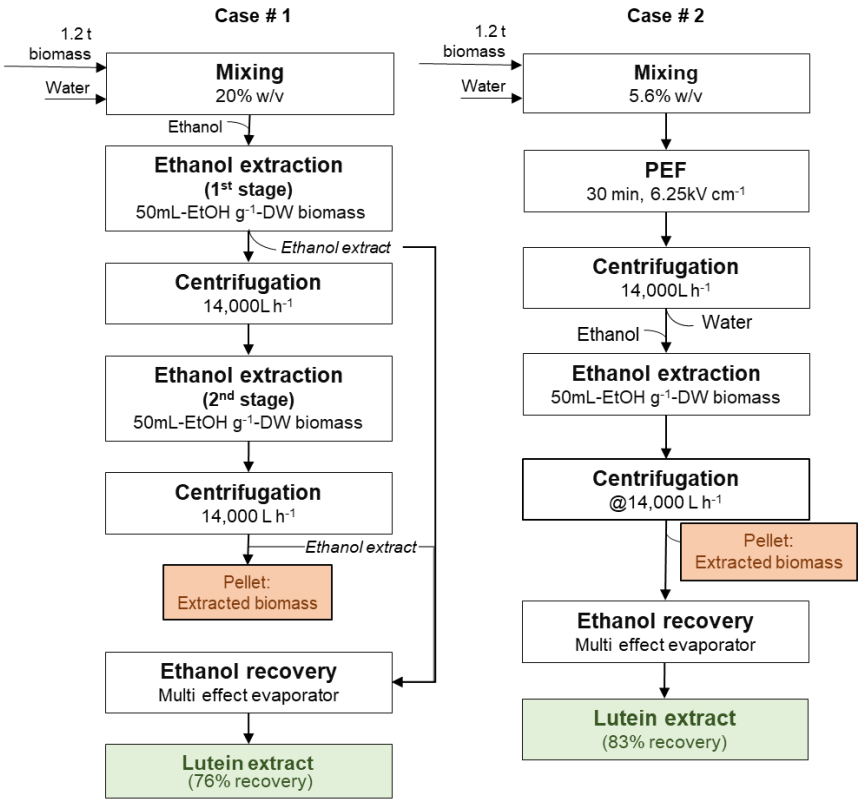


Figure 4.4 Lutein-rich extract production scenarios with and without PEF treatment

The process cost benefit of implementing the PEF treatment in the production of lutein-rich extract from microalgae was evaluated by comparing two scenarios (Case #1 and #2) outlined in Figure 4.4, and the SuperPro diagrams in Figure 4.5 and Figure 4.6.

The objective of the comparison was to determine the impact of PEF treatment on the direct manufacturing cost of the lutein-rich extract. Case #1 consists of two–stage EtOH extraction of untreated (no PEF treatment) microalgae. The biomass is incubated in ethanol, harvested by centrifugation, and then dispersed in fresh ethanol for re-extraction in the second stage. At the end of the second extraction, the biomass is separated by

centrifugation and the ethanol extract evaporated to recover the ethanol and produce a lutein-rich extract containing chlorophyll pigments. Case #2 consists of PEF biomass permeabilization followed by a single-stage ethanol extraction. Similarly, to Case #1, algal biomass after ethanol extraction is centrifuged and ethanol recovered by evaporation. The processing parameters for each case scenario were derived from data presented in the previous sections.

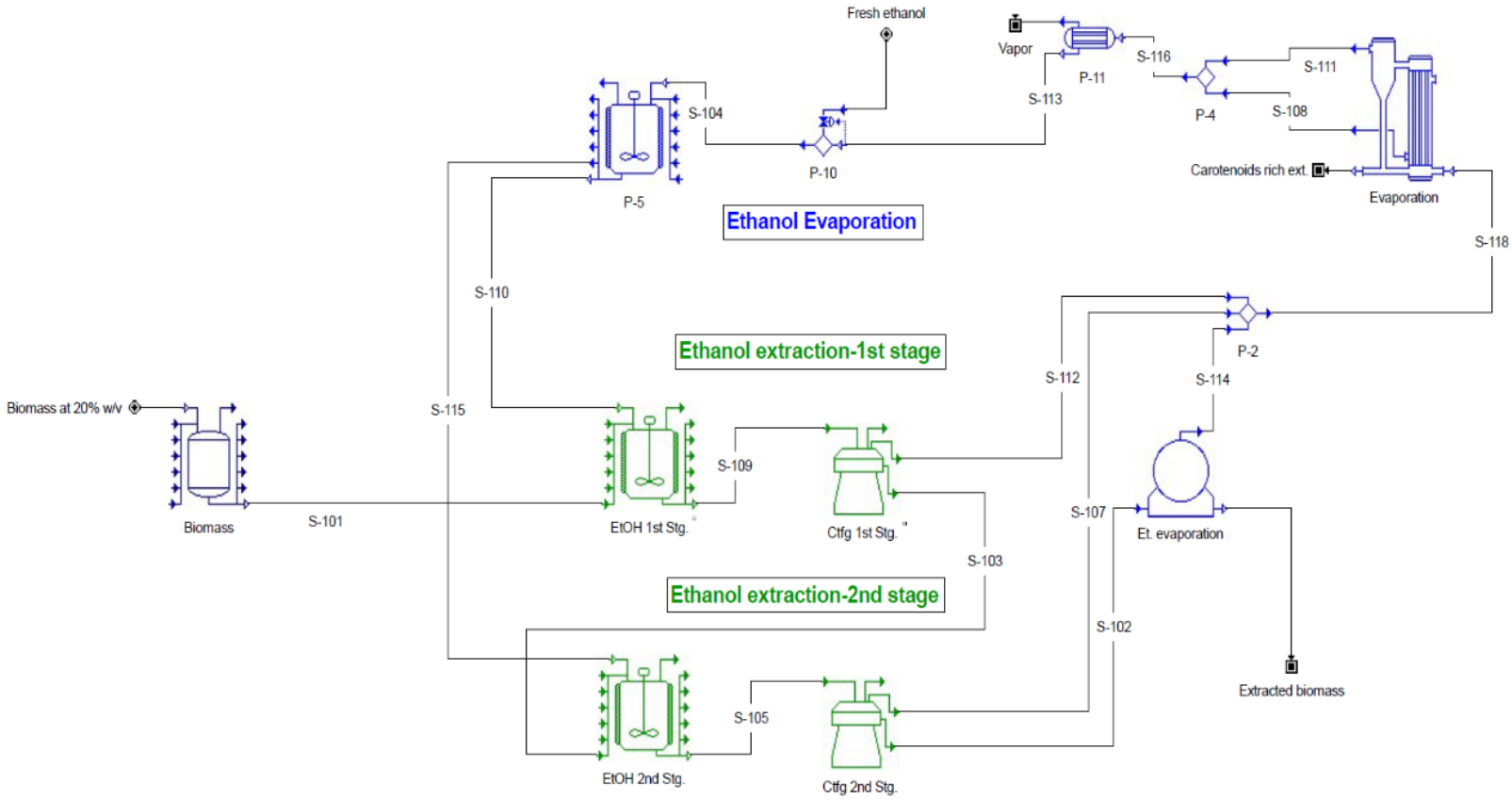


Figure 4.5 SuperPro process flow diagram of the two-stage ethanol extraction process (Case #1)

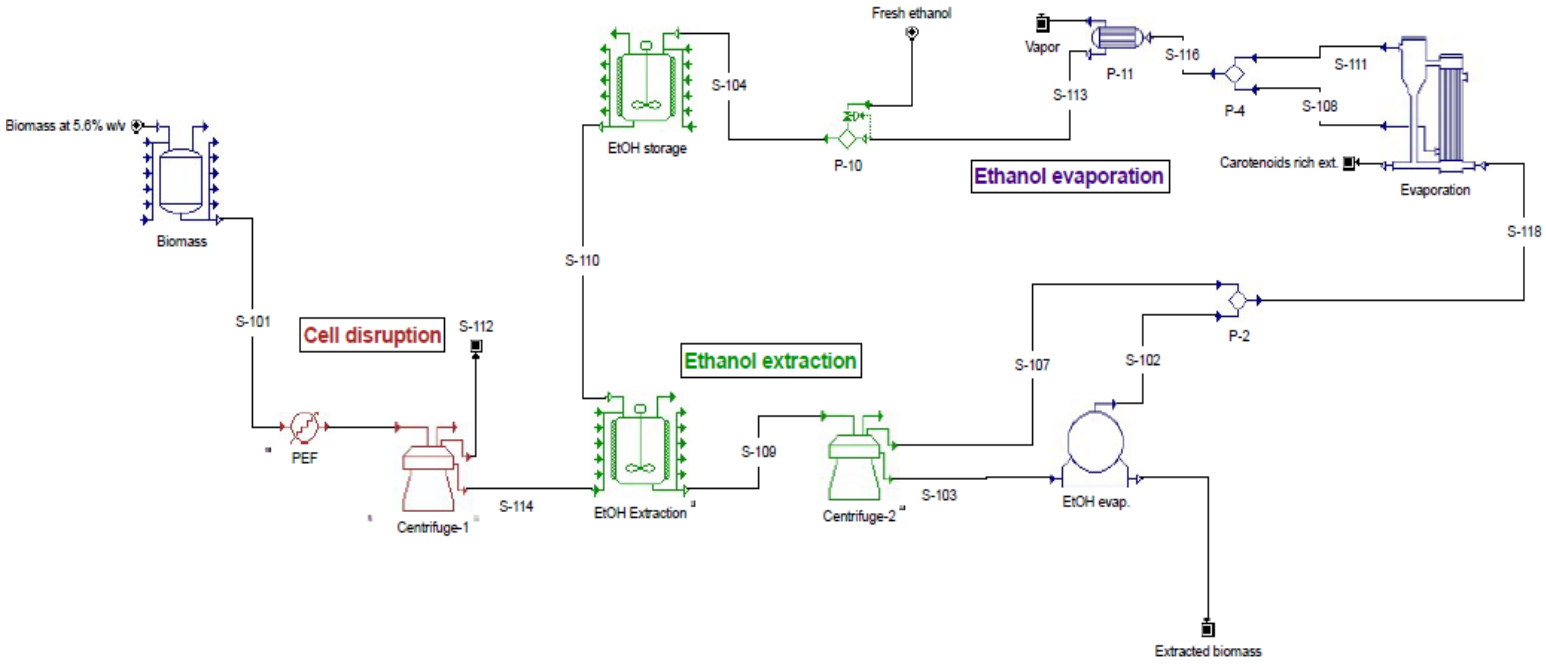


Figure 4.6 SuperPro process flow diagram of the ethanol extraction process from permeabilized biomass (Case #2)

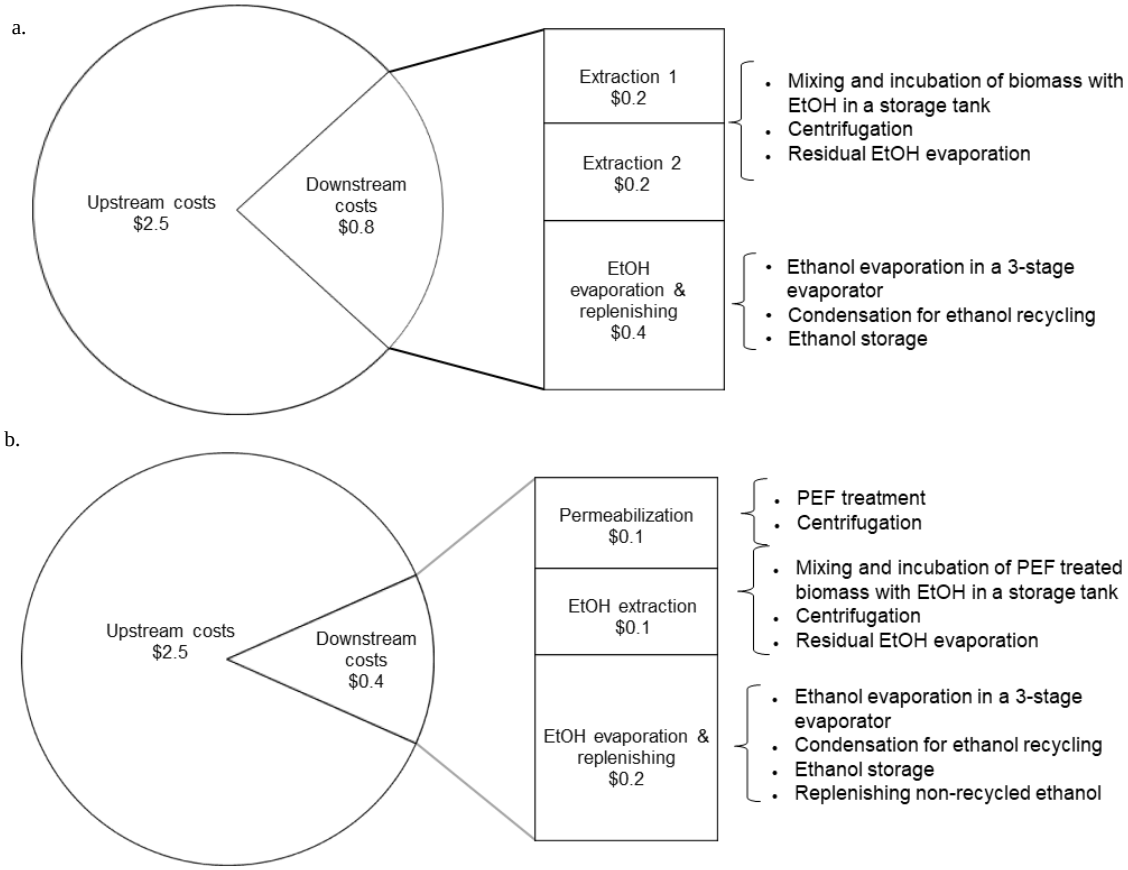


Figure 4.7 Direct cost distribution in dollars per g-lutein for (a) Case #1 and (b) Case #2

The results of downstream process simulation by SuperPro Designer are summarized in Figure 4.7. The contribution of each section to the annual direct manufacturing cost is expressed in \$ g^{-1} lutein. The upstream cost consists of process expenses associated with biomass cultivation in raceways ponds, harvesting, and concentration. Downstream processing cost in Case #1 accounted for the two-stage lutein extraction and ethanol recovery, and in Case #2 included biomass permeabilization, followed by the single-stage ethanol extraction and ethanol recovery. The reason for the overwhelming contribution of upstream processing (\$2.5 g^{-1}) to the total direct cost of lutein is the

current algal biomass production cost, which ranges from \$10-15 kg^{-1} dry biomass [166]. At \$10 kg^{-1} biomass cost, upstream expenses contribute 75% and 83% to the total direct production costs of Case #1 and #2, respectively.

The analysis of the downstream processing operations shows that the two-stage ethanol extraction (Case #1) has a higher cost compared to the single-stage (Case #2), primarily because of operating expenses associated with the additional extraction and centrifugation steps (Figure 4.7 a). The PEF treatment followed by centrifugation (Case #2), which increases the overall lutein extraction yield by 8%, and requires a single-stage extraction (Figure 4.7 b), reduced the extraction cost by \$0.3 g^{-1} compared to two-stage extraction in Case #1. The total (upstream plus downstream) direct production cost of \$3.0 g^{-1} lutein for Case #2 is still high given the current selling price of lutein-containing nutraceutical products from \$1.5 [170] to \$3.5 g^{-1} lutein [171].

4.5.4.1. Production of protein concentrates from lutein-depleted Chlorella biomass

The key assumption for this study was the potential price-tag value of ethanol-extracted biomass. For lutein–depleted biomass with a protein content between 20 and 40%, we assumed a biomass residue price in the range of \$0.2 to \$0.4 per kg [167], a value typical for protein-rich feed ingredients.

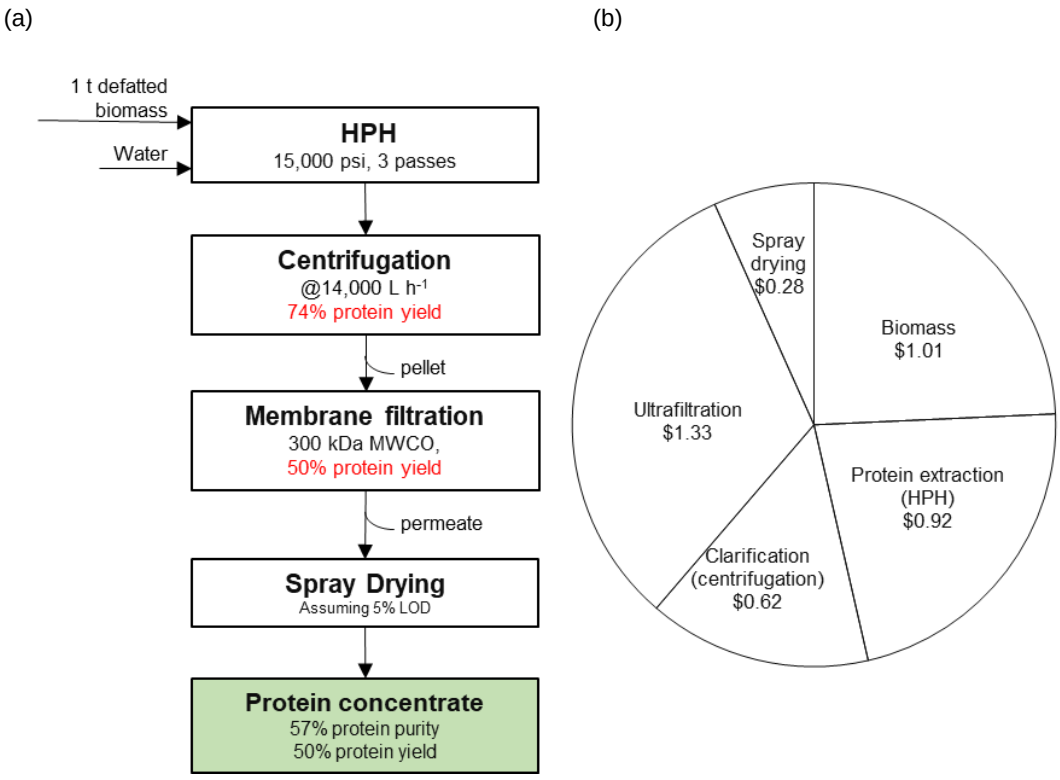


Figure 4.8 Process flow diagram (a) and cost distribution of each process step (b) on the production of protein concentrates from ethanol-extracted biomass[11]

To investigate this option, a previously developed protein recovery process [21] from ethanol-extracted *Chlorella vulgaris* biomass, shown in Figure 4.8, was modelled using ethanol-extracted biomass produced by Case #2 process (Figure 4.4b). The protein recovery process outlined in Figure 4.8a starts with dispersing the ethanol-extracted biomass in water at 1:5 weight-to-volume ratio and extracting the intracellular protein by homogenization at pH 12. The high pH extraction is required to overcome reduced (30%) protein solubility during ethanol extraction [21]. The homogenized biomass is

[11] Adapted from Kulkarni and Nikolov [105]

clarified by centrifugation and the protein extract concentrated by membrane ultrafiltration. The concentrated protein fraction consists of 57% protein, dry weight base. The protein yield is 50% of the initial biomass protein content. Specific process parameters for homogenization, protein extraction, and membrane concentration can be found in Kulkarni and Nikolov [21], the SuperPro flow chart (Figure 4.8), and Tables 1 and 2 under *Protein case*.

The use of ethanol-extracted biomass ($0.4 kg^{-1}) instead of non-extracted biomass ($10 kg^{-1}) for production of protein concentrates reduces the upstream costs from about $36 kg^{-1}-protein concentrate [166, 167], down to $0.95 kg^{-1}-concentrate.

The membrane ultrafiltration process that is used for protein concentration and removal of impurities, such as chlorophyll, sugars, and ash by diafiltration, is the most expensive step ($1.33 kg^{-1}, 35% production costs) mostly because of the labor and consumables (membrane replacement) cost. The protein extraction step by homogenization (HPH), which contributes $0.92 kg^{-1} (22%) to the total cost, is also high, but homogenization is currently the only option that could effectively release the protein from ethanol-extracted biomass.

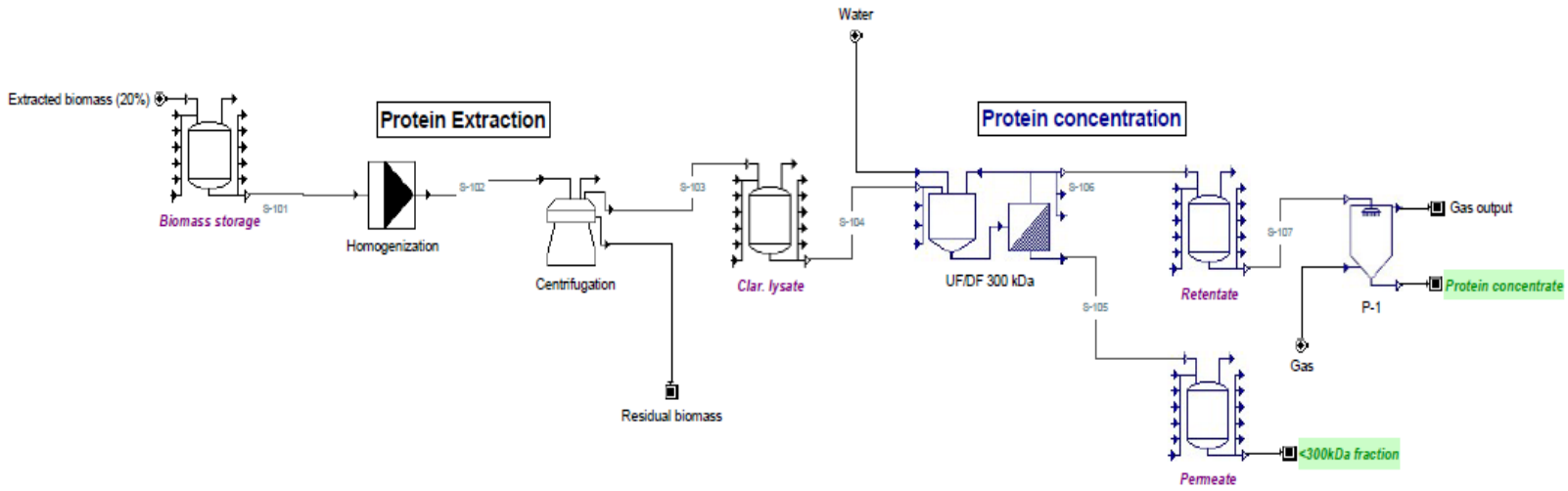


Figure 4.9 Super-Pro process flow diagram for production protein concentrates from ethanol-extracted biomass

4.6. Discussion

In our previous study [21] we compared ethanol extraction yields of lutein and chlorophyll from freshly harvested *C. vulgaris* to cells that were previously frozen at -80°C or treated with PEF. Both pretreatment methods significantly increased extraction yields of lutein (~2-3 fold) and chlorophyll (~10 fold) compared to extraction of freshly harvested cells without any pretreatment. Compared to freeze-thaw, lutein yield was slightly higher after exposing freshly harvested cells to PEF treatment. The opposite was observed for the chlorophyll extraction, which seemed to respond better to the freeze-thaw pretreatment than PEF. Since freeze-thawing is not easily scalable and would not be economical, we concluded that the pulsed electric field (PEF) technology is better suited for enhancing ethanol extraction of lutein and chlorophyll from freshly harvested cells. To better understand the effect of PEF treatment on the kinetics of lutein and chlorophyll extraction, we have examined their release from permeabilized cells into the ethanol phase for a period of 120 min (Figure 4.1). The lutein extraction rate from PEF-permeabilized biomass was fast in the first 10 min and then dropped drastically past the 45 min time point (Figure 4.1a). The highest lutein concentration was reached after 45 min of extraction. The results in Figure 4.1(a) confirmed that the PEF-treatment promoted permeabilization of the cell membrane, which enhanced the transport of smaller molecules such as lutein and chlorophyll across the cell membrane [172, 173]. A slower chlorophyll extraction kinetics compared to lutein for either treated or not PEF-treated biomass suggests that cell permeabilization may not be the sole reason for the observed difference. The 30-fold lower solubility of the chlorophyll in ethanol (5-10

mg/L) compared to lutein (300 mg/L) could be a factor affecting the slower release and lower than expected chlorophyll extraction (51 % of total) from permeabilized cells [14, 174]. Further examination of lutein and chlorophyll extraction kinetics in Figure 4.1 suggests that after approx. 45 min and 120 min, respectively, the extraction yields of lutein and chlorophyll have reached the maximum value. Because extraction yield and productivity (kg biomass processed per unit time) are key process variables relevant to extraction economics, two questions that merit further consideration were whether: 1) the extraction yield of lutein and chlorophyll after 45 min and 120 min contact time, respectively, was limited by the solid-liquid equilibrium; and 2) ethanol contact with cell biomass alone was sufficient to achieve cell permeabilization and release of lutein and chlorophyll. To address these two questions, the amounts (% of total) of lutein and chlorophyll released during three-stage extraction (Figure 4.2) were compared to the extracted lutein and chlorophyll after 45 min and 120 min incubation, respectively (Figure 4.1). The three-stage extraction data (Figure 4.2) confirmed the observations made in the kinetic experiments (Figure 4.1). Lutein was extracted at a faster rate than chlorophyll, and in 10 minutes, more than 82% of lutein had been extracted compared to 25% of the total chlorophyll. The subsequent two stages had a minor impact (about 17%) on the overall extraction yield of lutein, but they contributed to an additional 50% of the total extractable chlorophyll. Interestingly, the multiple extraction of PEF-treated biomass with fresh ethanol increased the chlorophyll yield almost two-fold compared to the single-stage 120 min incubation (Figure 4.1).

The effect of ethanol alone on membrane permeabilization and extraction of the two pigments is demonstrated by examining the respective controls in Figure 4.2. For example, most of the lutein (>90%) could be extracted in three stages by providing fresh ethanol at each stage to control (not PEF treated) biomass. Exposing the *chlorella* biomass from the first stage to fresh ethanol in the following two stages enhanced the concentration difference between the solvent and lutein to drive the diffusion of solubilized lutein out of the cell. The chlorophyll extraction did have a similar extraction pattern as lutein, but different endpoints. In the first stage, a very small fraction (< 5%) was extracted, followed by a higher amount (9%) in the second stage, and about 7% in the third. The results of this comparison show that ethanol penetrates the cell membrane to reach lutein and chlorophyll in the chloroplast, but only lutein can be extracted without PEF-pretreatment. The difference in extracted chlorophyll between treated and untreated samples suggests that ethanol diffusion alone into the chloroplast is not enough for releasing chlorophyll molecules from the thylakoid membrane. Therefore, application of PEF or another cell disruptive method to break chlorophyll–protein complexes associated with thylakoid membrane would be necessary if chlorophyll and/or chloroplast protein are desired products.

Experimental data presented in the previous sections indicated that the main advantage of the PEF treatment is the reduction in processing time, i.e., lutein extraction could be performed in a single stage with less than 1h of contact time with ethanol and less than 15 % protein loss to the ethanol extract (Figure 4.3).

While implementation of the PEF-treatment reduces downstream costs by almost 40%, the total (upstream plus downstream) direct production cost of \$3.0 g^{-1} lutein is still high given the current selling price of lutein-containing nutraceutical products from \$1.5 [170] to \$3.5 g^{-1}-lutein [171].

Considering the current high biomass production cost, there is little incentive for further improvement of downstream processing efficiency because the latter contributes to less than 40% (20-40%) of lutein production cost. However, it is quite clear that the reduction of the upstream cost i.e. biomass cost, should be a target for delivering cost-competitive nutraceutical products from microalgae.

Lowering the cost of algal biomass is also an important target for developing protein products from microalgae. Therefore, combining the production of both lutein and protein from the same starting algal biomass might be a reasonable strategy of distributing the cost burden of the starting material over the two co-products.

The use of lutein-depleted microalgae biomass has a lower market value (\$0.2-0.4 kg^{-1}) compared to whole algae (\$10 kg^{-1}). The process cost scenario presented in Figure 4.8 and section 3.4.1 suggests that the production cost of protein concentrates of \$4.2 kg^{-1} would fall within the bulk price range of protein concentrates produced from whey, soybeans, and peas (\$3-5 kg^{-1}). The current bottleneck of algal-derived protein concentrates is the marginal product purity of 57± 5% that can be achieved with the process described in Figure 6 [166]. At this purity and direct production cost of \$4.2 kg^{-1}, algal protein concentrates would be difficult to market against the established soy and whey concentrates. At least a 1.3-fold increase in protein purity would be needed for

algal protein products to compete with current soy and whey protein concentrates (≥ 70% protein content). Although the integrated lutein and protein co-production process requires improvements and optimization, we demonstrated that the utilization of previously extracted biomass could increase economic feasibility for developing protein products from microalgae.

4.7. Conclusions

In this work we demonstrated that PEF-treatment of harvested algal biomass was a successful strategy to enhance extraction rates and yields of lutein and chlorophyll and reduce their production costs. To maximize the recovery of chlorophyll, which has a lower solubility in ethanol than lutein, a multi-stage extraction would be required to maintain the extraction driving force i.e. solid-liquid concentration gradient. Contrarily, most of the lutein (>80%) can be extracted from PEF treated cells in one single stage. Process conditions that were optimum for ethanol extraction of lutein resulted in less than 15% loss protein loss. We have also shown that protein remaining in the lutein-extracted biomass can be further processed to generate protein concentrates and additional product revenue. While the high cost of algal biomass can be overcome by using the less expensive lutein-depleted algal biomass, an increase of extracted protein content is needed to make protein concentrates or isolates economically viable.

4.8. Acknowledgments

This work was supported in part by Dr. Nikolov's Dow Professorship, Texas AgriLife Research Bioenergy Initiatives Program, Texas A&M University.

4.9. Conflicts of Interest

The authors declare no conflict of interest.

5. PROCESS DEVELOPMENT OF ENZYMATICALLY - GENERATED ALGAL PROTEIN HYDROLYSATES FOR SPECIALTY FOOD APPLICATIONS

5.1. Chapter summary

Lipid-extracted microalgae is a cheaper feedstock alternative to whole cell microalgae for production of protein products. Pre-extraction of high-value lipids significantly reduces biomass costs and concentrates protein in the de-lipidated biomass residue. Protein hydrolysates are one of the most valuable products that could be obtained from lipid-extracted microalgae. Their high solubility makes them particularly suited as supplements in high-value sport and nutritional drinks. Additional advantages of protein hydrolysates over other protein products include enhanced digestibility and potential bioactivity. Still, the development of feasible downstream processes for the economic production of hydrolysates from lipid-extracted microalgae is at an exploratory stage. Previous work has studied the protein hydrolysis reaction but major processing factors such as extraction yield, protein purity, and appearance of the final product are yet to be optimized. This study aimed to develop a process for the production, recovery, and purification of protein hydrolysates from lipid-extracted microalgae. Results showed that mechanical lysis of the lipid-extracted microalgae was necessary to maximize the protein hydrolysis rate and extraction yield. Once hydrolyzed and released, a combination of acidic precipitation, centrifugation, and depth filtration, followed by an ion exchange step for salt removal yielded a final protein recovery and purity of 64 and 72%, respectively. To our knowledge, this is the first study reporting such high purity and recovery for an algae protein product. The hydrolysate produced was free of chlorophyll

and consisted mostly of 1-20 kDa peptides with an amino acid distribution comparable to that of soy protein.

Keywords:

Microalgae, protein, hydrolysates, food.

5.2. Introduction

Microalgae are biomolecule factories that have the potential to supplement the nutritional and energy needs of the population [175]. Their chemical diversity makes them extremely attractive for exploiting a wide range of biomolecules such as proteins, lipids, and antioxidants [21]. Currently, the commercial production of microalgae products is mostly focused on niche markets of high-value lipids such as antioxidants (astaxanthin, phycocyanin, and lutein) as they have shown to provide multiple health benefits such as reducing the risk of coronary disease, cancer, and psychiatric disorders [176].

Microalgae have recently gained interest as an alternative source of protein to meet projected dietary needs of the growing population [20, 177]. Diverse nutritional and functional properties, low allergenicity, and high-protein content (~50% DW) [2, 7, 8] make microalgae a promising commercial source comparable to soybeans (37% DW protein), milk (26% DW protein), meat (43% DW protein), and yeast (39% DW protein) [9-11].

The development of traditional protein products such as whole-cell protein [2], protein concentrates [17, 22], and hydrolysates [12, 167, 178] from microalgae have been investigated in the past decades but economically feasible processes are yet to be

developed. Lower value products such as protein concentrates and whole-cell protein are relatively expensive to produce and their cost cannot compete with current selling prices from traditional sources ($3-5/kg) [22, 166]. Protein hydrolysis is an alternative way to valorize the biomass [177, 179], as the final product has high solubility, digestibility, and bioactivity properties [39, 180, 181] that increases market value. While there is potential for developing hydrolysates from microalgae, the high costs of the microalgae biomass ($10/kg) and the lack of scalable downstream processing strategies for production has hindered commercialization.

The use of lipid-extracted algae (LEA) as a feedstock could be 5-10 times less expensive than whole-cell biomass ($10/kg) and favor the profitability of microalgae-derived protein hydrolysates [166, 167]. Although the use of LEA is a key factor in improving the profitability of hydrolysates [166], biomass drying and solvent exposure steps used in lipid extraction processes tend to decrease protein solubility due to heat- and solvent-induced denaturation and aggregation [182]. One strategy to overcome low protein extractability from the LEA is to use enzymatic hydrolysis as a tool to increase protein solubility by reducing denatured protein to smaller fragments. Several studies provided evidence that the open-structure of denatured protein in lipid-extracted microalgae provides enzymes easier access to peptide bonds and increases the rate of the hydrolysis reaction [12, 103, 117, 183]. While the latter might be the case for proteins in solution, intact or partially permeabilized cell wall and membranes within LEA impose physical barriers, which may limit enzyme accessibility and hydrolysis rate. Hydrolysis of solvent-extracted algal biomass [12, 103, 111, 117] has previously been reported, but

results from those studies cannot be broadly applied to any LEA biomass due to differences in cell wall composition, physical pretreatment of cell biomass before the solvent extraction, de-lipidation solvents, and extraction temperatures.

Previous studies on protein hydrolysis from microalgae are mostly focused on the optimization of the hydrolysis reaction and characterization of the peptides obtained [12, 103, 117] rather than downstream process development for purification of the hydrolysates. The purification usually only involves a single centrifugation step for removal of the cell debris. While this is probably enough for hydrolysates intended for food applications where colored and diverse macromolecular products are acceptable, the product obtained would not be suitable for formulation of drinks and other applications where well-dispersed, free of contaminants, and stable protein solutions are desirable. Thus, we also investigated methods to increase the protein purity and stability of hydrolysates.

To elucidate factors impacting process design, product quality and cost, we compare enzyme-facilitated protein release from three types of LEA-sourced starting material: LEA prepared by ethanol (EtOH) extraction lysed LEA, and a protein concentrate (ProtConc) produced from homogenized LEA (Figure 5.1). Furthermore, we propose a fractionation and purification process to enhance the quality of the protein hydrolysates. The objectives of the study were to: 1) Compare process advantages (i.e. greater hydrolysis rate, higher protein recovery yield) of protein hydrolysis using lysed LEA, LEA, or ProtConc (Figure 5.1); 2) Understand molecular changes in the cell and organelle structure after hydrolysis via TEM imaging; 3) Develop a protein recovery and

purification process from lysed cell material; and 4) Evaluate the quality of the protein hydrolysate generated.

5.3. Materials and Methods

5.3.1. Substrate preparation

5.3.1.1. Lipid-extracted algae (LEA)

Nannochloropsis sp. biomass was grown in open ponds by our industry collaborator Qualitas Inc., concentrated down to 20% solids, and stored at -20°C until use. The frozen paste was thawed at room temperature prior to processing. EtOH extraction was conducted following a modification of the protocol developed by Kulkarni and Nikolov [21] by extracting the biomass in 50 mL EtOH/g-DW fresh algae and re-extracting under the same conditions (2 stages) with an incubation time of 45 min/stage. An incubation temperature of 60°C was used to maximize carotenoids, chlorophyll, and fat extractability. At the end of the process, a green extract containing 40% of the starting solids was removed from the biomass. The LEA obtained contained approximately 40% protein, 19% ash, 10% lipids, and 16% carbohydrates on a dry weight basis.

5.3.1.2. Lysed and lipid extracted algae (lysed LEA)

LEA were subjected to ball milling at pH 11 using a planetary mill from MSE Supplies with 0.5 mm diameter zirconia beads. The chamber was filled with beads 50% as per manufacturer recommendations. A ball-milling time of 180 min was selected for maximizing protein release (Supplementary Figure 1).

5.3.1.3. Protein concentrates (ProtConc)

Protein concentrates from LEA were obtained using a modified protocol of the one proposed by Soto-Sierra, Kulkarni [22]. Lipid extracted microalgae were suspended in 2.5% water and pH adjusted to 12. The slurry was then subjected to 3 passes through a high-pressure homogenizer at 15,000 psi using the M-110P Microfluidizer®, followed by centrifugation at 9,000 x g for 10 min at room temperature for removal of insoluble material. The pH of the clarified extract was dropped to 4.5 using 1M HCl and mixed on a stir plate at 500 rpm for 30 min to precipitate protein. The protein precipitate was recovered by centrifugation at 9,000 x g for 10 min at room temperature and the supernatant was discarded. The final protein purity of the concentrate, determined by methods given in section 3.4.4, was 70.5% and the overall protein recovery, determined by the method described in section 3.4.3., was 41.1%.

5.3.2. Enzymatic hydrolysis

Each hydrolysis substrate (LEA, lysed LEA, ProtConc) was suspended in water at two different solids concentrations: 67 g-biomass/L and 178 g-biomass/L for LEA and lysed LEA cells and 41 g-solids/L and 110g-solids/L for ProtConc to achieve a final protein concentration in the suspension of 30 g-protein/L and 80 g-protein/L, respectively. The enzyme dosage of 3.5 mL Alcalase preparation (Sigma Aldrich CAT# 126741) per 100 g biomass protein (3.5% v/w) and the reaction conditions (45^{o}C and pH 9.5) were selected based on previous studies [183, 184]. Peptide release during the 3 h reaction course was followed by taking samples every 5 min for the first hour of reaction followed by sampling at 2 h and 3 h. The degree of hydrolysis (DH) and protein release were

monitored at each time point and the rates of reaction were compared for the three substrates (LEA, lysed LEA, and ProtConc).

1.1.1. Protein release

Yields were calculated following the protocol for the recovery of soluble peptides proposed by Olsen and Adler-Nissen [184]. Samples of the hydrolysates were taken after 2, 3, and 4 h of hydrolysis and resuspended in 0.1 M citrate buffer at pH 4.2 and then incubated at >90°C to deactivate Alcalase. Samples were centrifuged at 9,000 x g for 5 min and supernatants and pellets were collected separately. The pellets were washed with the pH 4.2 buffer to recover any trapped peptides in the insoluble fraction. Each sample (pellets-P-, supernatant-S_1-, washed supernatant-S_2-) was digested in 6 M HCl for complete hydrolysis and total amino nitrogen (AN) was determined using an N-OPA [185]. The protein/peptides distribution was calculated by performing a mass balance between the amino nitrogen in the pellet (Equation 6) and the supernatants (Equation 7).

$$\%\ protein\ in\ pellet = \frac{AN\left(\frac{mg}{L}\right)\ in\ sample}{Total\ AN\ (P+S_1+S_2)\left(\frac{mg}{L}\right)} * 100\% \qquad (6)$$

$$\%\ soluble\ protein = \frac{AN\ in\ ext.1 + AN\ in\ ext.2\ \left(\frac{mg}{L}\right)}{Total\ AN\ \left(\frac{mg}{L}\right)} * 100\% \qquad (7)$$

5.3.3. Hydrolysis scale-up and hydrolysates purification

The lysed LEA was hydrolyzed at 50°C using an enzyme dosage of 0.9 mL Alcalase / 100 g (0.9% v/w) and pH of 9.5, which was maintained using 2 M NaOH. After 2 h, the pH of the hydrolysate was dropped to 4.5 using 1 M HCl and the mixture was incubated

on a heat plate at 95℃ for 5 min [186, 187] to deactivate Alcalase enzyme and precipitate insoluble protein/peptides.

5.3.4. Fractionation

A modified protocol of the one proposed by Adler-Nissen, Poulsen [187] was followed to clarify the hydrolysates. The slurry was centrifuged at 9,000 x g for 9 min and the supernatant collected (S1). The pellet was resuspended in water at pH 4.3 and mixed thoroughly to solubilize trapped proteins in the precipitate. The slurry was centrifuged again under the same conditions and supernatant (S2) was recovered.

5.3.4.1. Depth filtration (clarification)

S1 and S2 were subjected to depth filtration using the Supracap 50 Pall® Depth filtration capsule (SC050PDD1) with a 0.2-3.5 µm retention rating and 22 cm^2 filtration area to remove unwanted debris / insoluble protein. The drop in debris/insoluble protein was confirmed by measuring the turbidity of the supernatants before (S1 and S2) and after filtration (FS1 and FS2) at 750 nm using a Molecular Devices SpectraMax® plate reader and SoftMax Pro software for analysis.

5.3.4.2. Ion exchange treatment

The AmberLite® MB20 H/OH mixed bed ion exchange resin (hydrogen and hydroxide form) form Supelco was used to remove excess salt from the hydrolysates. First, the resin was washed 5 times with 25 mL water/mL resin. Then, a protein hydrolysate solution containing ~12 mg-protein/mL was added to the resin for a final ratio of 7 g-resin/g-protein or 14 g-resin/g-protein. The resin-protein solution was then mixed until reaching equilibrium, approximately 30 min. To keep track of the salt removal

efficiency, the conductivity of the solution was measured before, during, and after the treatment. Finally, the mixing was stopped to allow the resin to settle by gravity. The desalted hydrolysates were pipetted away from the resin and collected separately. To determine protein losses in the resin, a sample of the desalted hydrolysates was subjected to protein quantification following procedure described in section 3.4.3.

5.3.5. Analytical methods

5.3.5.1. Degree of hydrolysis (DH)

The DH was measured following a modified protocol of the one proposed by Mat, Cattenoz [188]. Free protons are released during the enzymatic reaction, causing a decrease in the pH of the reaction mixture, and the addition of base is required to maintain the pH. The amount of base required has a direct relationship to the number of hydrolyzed peptide bonds and can be used to estimate the DH. The amount of base added to maintain the pH at 9.5 was recorded at each time point. The degree of hydrolysis (Equation 10) was calculated by finding the number of hydrolyzed peptide bonds (Equations 8 and 9) over the total number of peptide bonds per g-protein:

$$h = \frac{V*N}{m\alpha_{NH^2}} \qquad (8)$$

Where,

$h = number\ of\ hydrolyzed\ peptide\ bonds\ per\ gram\ protein$

$V = volume\ of\ titrant\ (mL)$

$N = normality\ of\ the\ titrant\ in\ \left(\frac{meq}{mL}\right)$

$m = mass\ of\ protein\ (g)$

$\alpha_{NH^2} = mean\ degree\ of\ dissociation,\ calculated\ as\ follows$:

$$\alpha = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} \quad (9)$$

$$DH\% = \frac{h}{h_{tot}} \quad (10)$$

Where,

$h_{tot} = number\ of\ peptide\ bonds\ in\ the\ substrate\ per\ gram\ protein$ (~8)

5.3.5.2. Initial rate of reaction

The initial rates of reaction (linear range) were estimated for every combination of substrate and solids concentration tested at a fixed enzyme (Eo) to substrate (So) ratio of 3.5 mL enzyme/100 g protein. Significant differences among initial reaction rates were found by modelling the data (Adj R^2 = 0.88) using a full-factorial model, Tukey's adjustment for multiple comparison, and an α_{FAM}=0.05.

5.3.5.3. Protein extraction yield

Samples from hydrolysis and fractionation experiments were diluted 2-fold with 12 M HCl for a final concentration of 6 M HCl. They were then incubated for 24 h at 100℃ for complete protein hydrolysis. The hydrolyzed samples were centrifuged and diluted at least 10-fold. Total amino nitrogen was measured following N-OPA protocol [189]. The protein content was calculated by using a 6.25 nitrogen-to-protein conversion factor, obtained by correlating total protein from amino acid analysis with the amino nitrogen result and comparing it to previous factors from literature [190, 191].

5.3.5.4. Purity

Purity after fractionation, clarification, and IEX was measured by freeze-drying the resulting samples using a benchtop Labconco FreeZone Benchtop Freeze Dry System. A sample of known weight (~10 mg) was extracted in 1 mL 6 M HCl for 24 h at 100℃ for

complete hydrolysis. Protein content was calculated following the method described in section 3.4.3. Purity of the sample was found by finding the protein-to-dry weight ratio.

5.3.5.5. Size exclusion chromatography

Characterization of the extracts was done using a TSK gel 2000swxl (Tosoh Bioscience) size exclusion analytical column, the AKTA purifier system, a UV detector at 220 nm for peptide detection, and a VIS detector at 649 nm for chlorophyll detection. The MW of the hydrolysates was compared to a defined protein standard mixture (Cat # 1511901) from Bio-Rad.

5.3.5.6. TEM

Transmission electric microscopy was used to understand the enzymatic reaction at a microstructural level. The substrates before and after hydrolysis were subjected to TEM imaging. The samples were fixed in Trump's fixative (1:1), stained with 1% osmium solution, and dehydrated through several acetone washes. Finally, samples were embedded in a Spurr resin and curated at 50°C. The cross-linked polymer block containing the samples were subjected to thin slicing. The slices were laid down in a grid and observed under the TEM. The process of embedding, slicing, and microscopy was conducted at the Microscopy and Imaging Center at Texas A&M University.

5.3.5.7. Amino acid (AA) analysis

The protein hydrolysate extract was further hydrolyzed along with the internal standard by incubation in 6 M HCl at 110°C for 20 h, then diluted 12 X in 0.4 M Borate buffer. One microliter of the diluted sample was injected on a Hypersil HPLC column and the intensity of the AA peaks at 340 nm was detected and compared to the internal standard.

Sample processing and analysis was conducted by the Protein Chemistry Lab at Texas A&M University.

5.3.6. Statistical analysis

The JMP® statistical software was used for statistical analysis. Significant differences among multiple treatments were determined using Tukey's adjustment for multiple comparisons and an α_{FAM} of 0.05. For all the experiments, at least 3 independent replicates ($n \geq 3$) were conducted to determine significant differences.

5.4. Results

5.4.1. Understanding the impact of biomass pre-processing on enzyme kinetics and protein release

One of the objectives of this study was to compare enzyme-facilitated protein release from three types of protein-rich algal material: LEA, lysed LEA, and Protein Concentrates (ProtConc) (Figure 5.1). The comparison of process steps needed to produce LEA, lysed LEA and ProtConc immediately shows the attractiveness of making hydrolysates from LEA. The hydrolysis of LEA (Figure 5.1) compared to ProtConc allows circumventing extraction and fractionation steps and potentially reducing processing cost. The direct use of proteases, such as Alcalase, with lipid extracted *Chlorella sp.* [103, 111, 117, 192-194], *Scenedesmus sp.* [111, 117, 193], *Dunaliella salina*, *Spirulina platensis*[195] biomass (meal) have resulted in protein extraction yields ranging from 50 to 70%. The reason for this range (as low as 50%) is not clear but potential causes include non-productive binding of the enzyme to cell walls and debris, internal accumulation of hydrolyzed protein leading to product inhibition when whole

cells are used, and restricted access of the enzyme resulting in lower yield. To circumvent those potential limitations and increase the extraction yield, a mechanical (bead mill) lysis of algal biomass slurry prior to hydrolysis was performed. The production of the ProtConc, in addition to cell lysis by homogenization, required lysate clarification and protein precipitation.

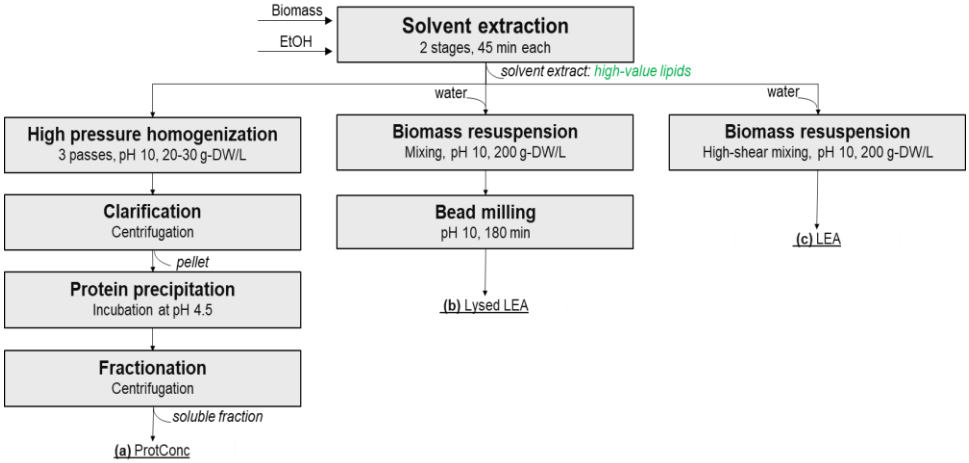


Figure 5.1 Biomass processing steps used to generate (a) protein concentrates - ProtConc-, (b) lipid-extracted and lysed biomass (lysed LEA) and (c) lipid-extracted biomass (LEA) prior to hydrolysis.

The LEA, lysed LEA, and ProtConc suspensions (Figure 5.1) were incubated with Alcalase using a fixed enzyme dosage (3.5% v/w) within the enzyme-to-protein ranges recommended elsewhere [183, 187]. The kinetics of hydrolysis were monitored via DH. The protein yields after 120-240 min of hydrolysis were estimated by measuring the protein concentration of centrifuged slurries.

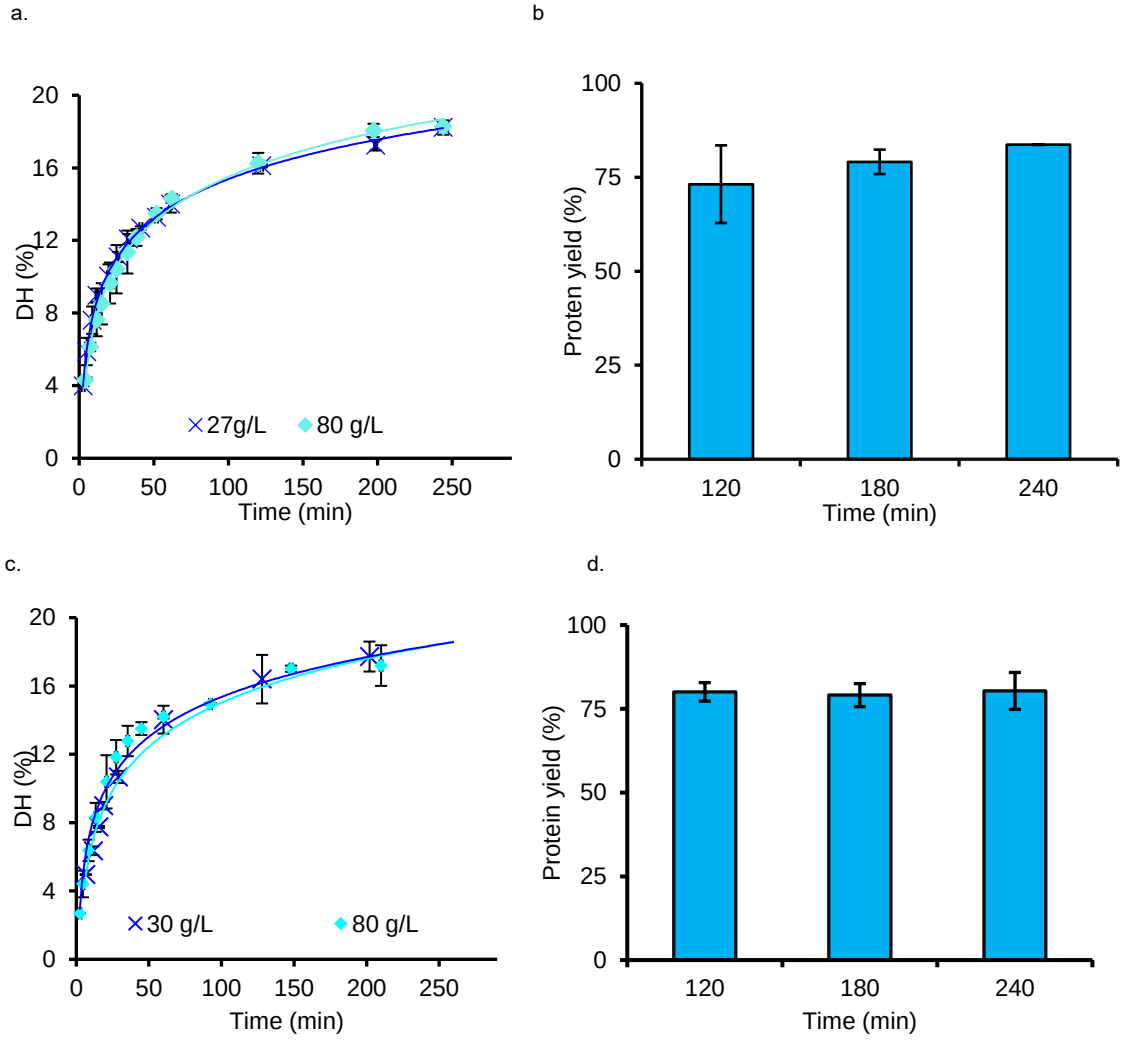


Figure 5.2 Degree of hydrolysis (DH) and protein yield (%) as function of incubation time and biomass concentration (g/L) at constant enzyme dosage of 3.5 mL enzyme/100 g biomass protein (3.5% v/w). (a) ProtConc hydrolysis; (b) yield of hydrolysates from ProtConc, (c) lysed LEA hydrolysis; (d) yield of hydrolysates from lysed LEA; (e) LEA hydrolysis, (f) yield of hydrolysates from LEA[12].

e. f.

[12] Results shown are the averages of at least 3 independent replicates (n≥3). Error bars represent standard deviations within replicates.

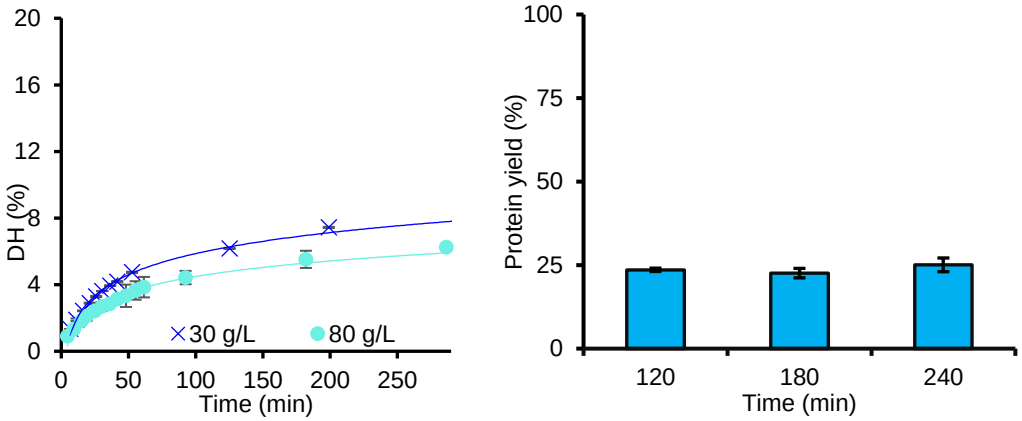


Figure 5.2. Continued

Table 5.1 Initial rates of hydrolysis using enzymatic dosage of 3.5 % (v/w) and constant Eo/So ratio of 0.045[13]

Substrate	Substrate concentration (g/L)	Initial reaction rate (dDH/dt) (min^{-1})	Confidence intervals	
			Lower 95%	Upper 95%
ProtConc	30	0.90[a]	0.80	1.00
	80	0.80[a]	0.70	0.90
Lysed LEA	30	0.75[a]	0.61	0.88
	80	0.89[a]	0.75	1.02
LEA (whole cells)	30	0.16[b]	-0.01	0.32
	80	0.16[b]	0.03	0.30

The estimates from Table 5.1 revealed that the initial hydrolysis rates for ProtConc and lysed LEA substrates at constant enzyme-to-substrate ratio (E_o/S_o) were not significantly different (α_{FAM}=0.05). The entire hydrolysis reaction course (Figure 5.2 a and 2c.) was also similar and independent of the substrate concentration for ProtConc and lysed LEA.

[13] Different letters correspond to significant differences among the initial reaction rates. Results shown are the averages of 3 independent replicates (n=3).

For both substrates, the hydrolysis curves gradually plateau as large molecular weight (MW) algal proteins are being degraded into peptides. Since we did not observe a drastic difference between the hydrolysis curves at 80 and 30 g/L substrate concentration, we assume that the reaction was not significantly affected by substrate or product inhibition. The yields of hydrolyzed protein for both substrates, lysed LEA and ProtConc, agreed with the later observation, as they were not significantly different, irrespective of the initial substrate concentration or the method of the substrate source/preparation (Figure 5.2 b and 2d).

The similar results obtained for lysed LEA and ProtConc in Figure 5.2 (a-d) also indicate that Alcalase is capable of hydrolyzing the insoluble protein attached to the cell debris in lysed LEA at a similar rate as the soluble, extracted protein from ProtConc. The latter is also evidenced by the high protein extraction yield obtained from lysed LEA (Figure 5.2 d) vs. non-hydrolyzed, lysed LEA (Figure S1) (~75 vs. 30%). Altogether, these results suggest that cell debris present in lysed LEA does not appear to delay or inhibit the progress of the enzymatic hydrolysis reaction.

The presence of intact cell walls (Figure 5.2 e), on the other hand, did have a significant impact on the enzymatic reaction rate. The initial hydrolysis rate was ~6-fold lower for LEA compared to the ProtConc and lysed LEA substrates (Table 5.1). Most likely, the presence of intact cell walls (Figure 5.3 a) hindered enzyme access to the chloroplast protein [196] resulting in significantly slower protein hydrolysis and yield compared to the readily available substrate in lysed LEA (Figure 5.3 c). Similar results were reported by Akaberi, Gusbeth [196], and García, Fernández [193] who observed an 1.5-fold

increase in the DH by permeabilizing microalgal biomass with PEF and 4-fold by lysing the biomass by bead milling prior to enzymatic hydrolysis. These results, together with our findings, confirm the need for mechanical cell disruption prior to enzymatic hydrolysis to overcome the recalcitrant biodegradation of microalgae cells.

To further understand the impact of substrate disruption on the hydrolysis rate, we also examined the microstructure of the whole LEA and lysed LEA cells before and after enzymatic hydrolysis via TEM. For whole LEA, results in Figure 5.3 (a and b) show there is very little difference in the cell structure, the chloroplast and protein complex inside the chloroplast after (1 h) hydrolysis of whole LEA. This indicates that the hydrolyzed protein that was measured in supernatant at 120-240 min of hydrolysis (Figure 5.2 f) was probably derived from extracellular material (Figure 5.3 a and 3b, yellow circles) and from smaller MW proteins that had diffused from the cell apoplast through the cell wall into medium [197].

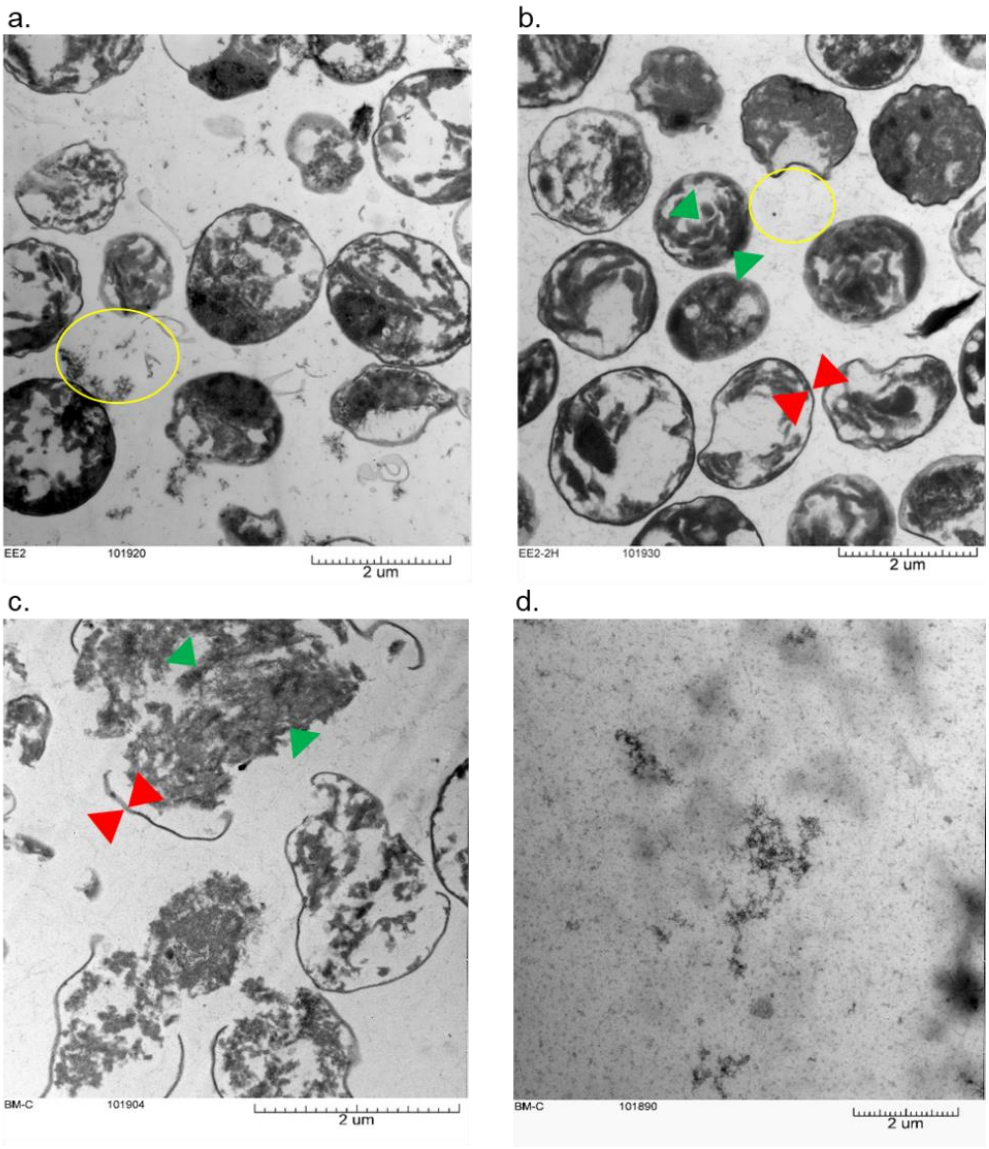


Figure 5.3 TEM images of (a) LEA before enzymatic hydrolysis, (b) LEA after enzymatic hydrolysis (c) lysed LEA before enzymatic hydrolysis (d) lysed LEA after enzymatic hydrolysis[14]

Contrary to whole LEA, the cell walls of lysed LEA (Figure 5.3 c, red arrows) were

partially fragmented and the chloroplast membranes (Figure 5.3 c, green arrows) had

[14] Hydrolysis was performed with 3.5 mL Alcalase/ 100 g protein (3.5% v/w). Red and green arrows show cell wall and chloroplast/chloroplast remnants, respectively. Yellow circles show extracellular material/cell debris.

been disrupted and exposed to the extracellular environment. The cell disruption, indeed, allowed for access of the hydrolytic enzymes to the chloroplast protein. At the end of the hydrolysis reaction (Figure 5.3 d), most subcellular structures (i.e. cell wall, membranes, chloroplast) originally visible in Figure 5.3, c (red and green arrows) had been degraded. The latter observation is consistent with the conclusion presented above that ball-mill disrupted cells allow further hydrolysis of chloroplast-associated protein.

From the results obtained (Figure 5.2 and Figure 5.3) the hydrolysis of lysed LEA biomass (Figure 5.1 b) appeared to be the best process route for producing hydrolysates from microalgae and was selected for further processing optimization. Whole LEA yielded significantly less hydrolyzed protein compared to the other two substrates due to the hindered access of the enzyme to the substrate. Furthermore, the multiple process steps (Figure 5.1 a) needed to generate protein concentrates from microalgae [22, 198] suggested that the ProtConc process option (depicted in Figure 5.1 a) was less economically favorable [22, 166], compared to lysed LEA.

Given the rapid leveling of the enzymatic reaction curves (Figure 5.2 c) and the protein yield graph (Figure 5.2 d) for the lysed LEA substrate, testing lower enzyme dosages that could achieve the same extraction yield seemed warranted. The results of enzyme dosage testing and optimization are summarized below (Figure 5.4).

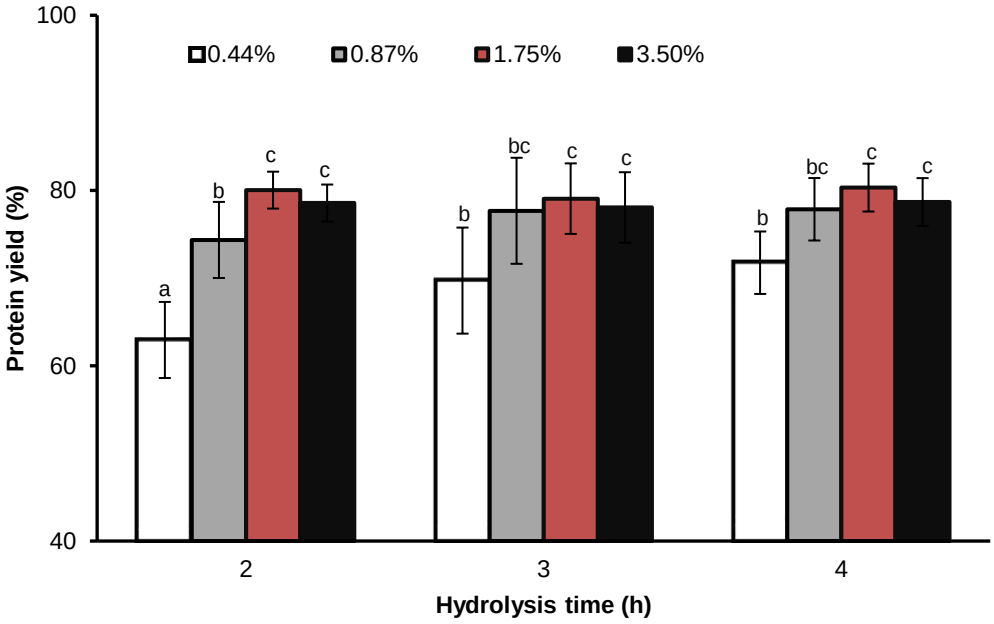


Figure 5.4 Impact of time - 2, 3, and 4 h- and enzymatic dosage-0.44, 0.87, 1.75, and 3.5 mL enzyme per 100 g-biomass protein (% v/w) - on the yield of hydrolyzed extracted protein[15]

To determine the critical enzyme concentration, protein release after 2, 3, and 4 h of hydrolysis was tested at the dosages indicated in Figure 5.4. Results showed no significant differences in the achieved extraction yields at 3 and 4 h of reaction with enzyme dosages from 0.87 to 3.5% v/w (Figure 5.4). At the lowest enzyme dosage (0.44%) evaluated, though, the protein extraction yield seemed to drop considerably, specially at ≤ 2h incubation time. Most likely, the enzyme was no longer saturated at 0.44% concentration and required additional time to hydrolyze the substrate.

[15] Protein yield (%) corresponds to the percent of protein in the supernatant after hydrolysis and centrifugation of lysed LEA at each time point and dosage. Results shown are the averages of at least 3 independent replicates (n≥3). Error bars represent standard deviations within replicates.

An enzyme dosage of at least 0.87% and 3 h of incubation time were required to achieve >75% protein yield. A similar trend was observed by Sari, Bruins [178], who found that a 1% (v/w) dosage of an alkaline endoprotease (Protex 40XL) and hydrolysis time of 3-5 h were sufficient for extracting ~70% of the protein from lipid-extracted microalgae meal. Slightly higher yields and lower enzyme dosage found in the present study could be attributed to the cell disruption step implemented before hydrolysis and the different enzyme cocktail used. To further increase the economic feasibility of algal-derived protein hydrolysate, we decided to apply the enzyme dosage of 0.87% to produce the protein hydrolysates described below.

5.4.2. Clarification and fractionation the protein hydrolysates from lysed LEA

Following enzymatic hydrolysis of lysed LEA, the soluble peptides remained in the slurry with insoluble debris, cells, and unhydrolyzed proteins. To prepare protein hydrolysates for food applications where purity and stability matter, we evaluated the fractionation and polishing steps described in Figure 1.5. An acidic precipitation step was used to precipitate the algal cell debris and the residual, unhydrolyzed protein complexes, followed by centrifugation, and depth filtration. The acidic precipitation followed by centrifugation fractionated soluble peptides to the supernatant. Peptides remaining in the supernatant after the pH 4.5 precipitation step [23, 27, 30] are expected to be stable and appropriate for supplementation of liquid formulations, such as energy drinks and protein shakes, usually formulated at low pHs [40, 41].

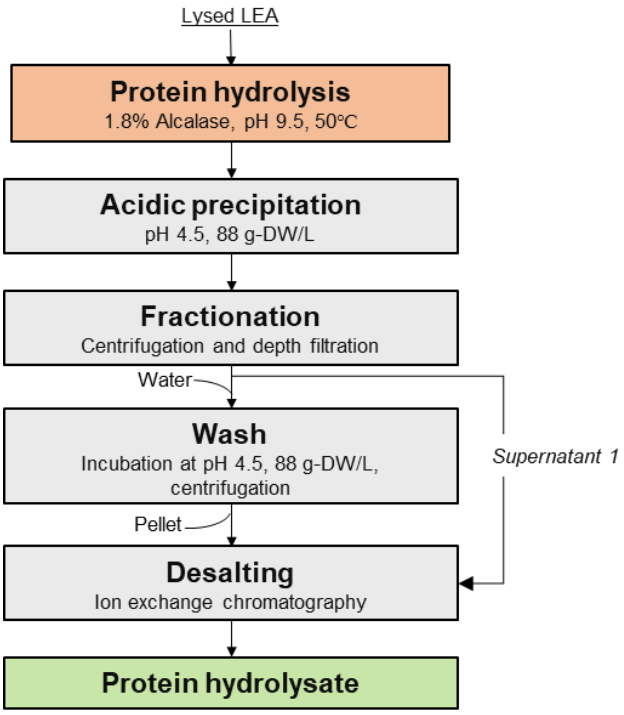


Figure 5.5 Process flow diagram for production of crude protein hydrolysates from lysed LEA

Table 5.2 Protein recovery and purification table of hydrolysates produced by the process shown in Figure 5[16]

Sample	Protein recovery (%)	Protein content (% DW)	Fold increase in purity (vs. homogenized material)	Salt (mg/mL)	mg-salt/ mg-protein	mg-salt / mg-hydrolysate
Lysed LEA	100	40 ± 2	1	2.1	0.1	0.04
Precipitated hydrolysate	100	35 ± 2	0.9	5.9	0.2	0.07
Clarified hydrolysate	64 ± 1	63 ± 2	1.6	5.5	0.5	0.32
IEX hydrolysate	60 ± 4	72 ± 3	1.8	2.6	0.2	0.14

[16] Protein recovery and content shown in the table represents the average of at least 3 independent replicates (n≥3) followed by their standard deviations. Precipitated hydrolysate sample shows 100% protein recovery as there was no fractionation involved in that step (only pH drop and mixing).

The precipitate (pellet) consisted of chloroplast remnants and the chlorophyll associated with the insoluble protein. Depth filtration removed the leftover suspended material and aggregates that were not removed by centrifugation. The results in Table 5.2 show that up to 64% of the initial protein can be recovered in the soluble hydrolysate fraction with a final protein content of 63 ±2 %. While most of the impurities were successfully removed, the estimated protein content of the hydrolysate was only 1.6-fold greater than the protein amount in the starting homogenized biomass (40%). A significant protein loss (~34%) in the centrifugation and filtration steps, accompanied by an increase in salt content during hydrolysis and acidic precipitation steps (from 0.1 in the homogenized biomass to 0.5 g-salt/g-protein after precipitation) were the main reasons for the marginal protein enrichment from 40% in the homogenized biomass to 63% in the clarified lysate. Salt alone, which was generated by multiple pH adjustments during hydrolysis and acidic precipitation, caused the purity of the slurry to drop from 40% to 35% (Table 5.2).

To remove the excess salts, the suitability of a demineralization step using a mixed-bed IEX resin was tested. The data in Figure 5.6 show that a ratio of 7 g-resin/g-protein removed approximately 50% of the salt with less than 4% protein loss. The 50% reduction in salt content resulted in the increase of protein purity from 63 to 72% (Table 5.2). Although a higher resin-to-protein ratio (14 g/g) further enhanced salt removal (95% removal), it bound significantly more protein resulting in 14% protein loss without an enhancement in protein content, compared to the purity at 7g-resin/g-protein. Using a 7 g-resin/g-protein ratio reduced the total salt concentration in the hydrolysate from

0.03% w/w to 0.01% w/w, which is comparable to the estimated nutritional composition of ready-to-drink protein beverages [199].

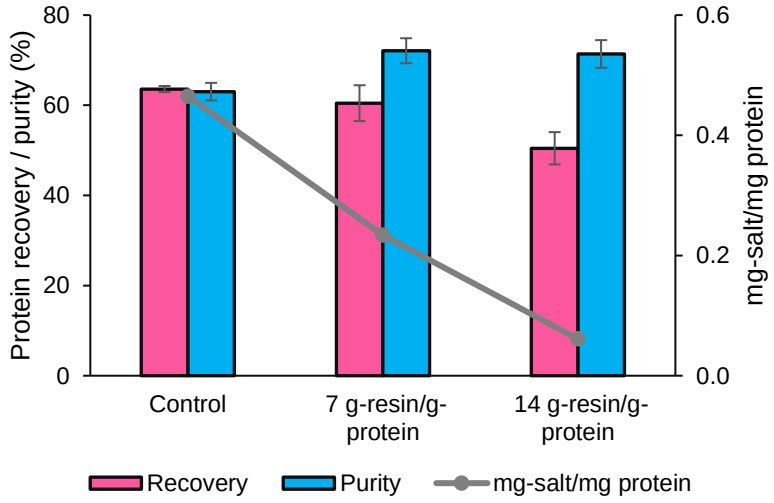


Figure 5.6 Effect of desalting step on protein purity, recovery, and salt content of protein hydrolysates[17]

The implementation of an ion exchange step appears to be an efficient way of removing excess salt generated during the hydrolysis process and enhancing the purity of the final product. Ion exchange mix-bed resins have been commonly employed for water demineralization [200] as they are a cheaper alternative to reverse osmosis. The resin beads can be easily sedimented by gravity or filtered out as the hydrolysates are recovered in the liquid fraction. The resin costs can also be mitigated by washing, regenerating, and reusing the resin beads [201].

[17] Desalting was performed by incubating the hydrolysate with a mixed bed ion exchange resin for 30 min. Results shown are the averages of at least 3 independent replicates (n≥3). Error bars represent standard deviations within replicates.

5.4.3. The effect of protein hydrolysis on product quality

Once the desired purity and yield was obtained, we proceeded to evaluate the quality of the hydrolysate by determining its MW distribution, the presence of chlorophyll in the final product, and its AA distribution.

5.4.3.1. Color and MW distribution

The color and MW distribution of the LEA protein hydrolysates were analyzed by size exclusion chromatography. The elution profile of a ProtConc was included for comparison with a non-hydrolyzed counterpart. Absorbance at 220 and 649 nm was used to monitor the elution of protein and chlorophyll, respectively (Figure 5.7). Chlorophyll was monitored as the green color and potential degradation products from chlorophyll, such as pheophorbides, are not desired in the final protein product [202].

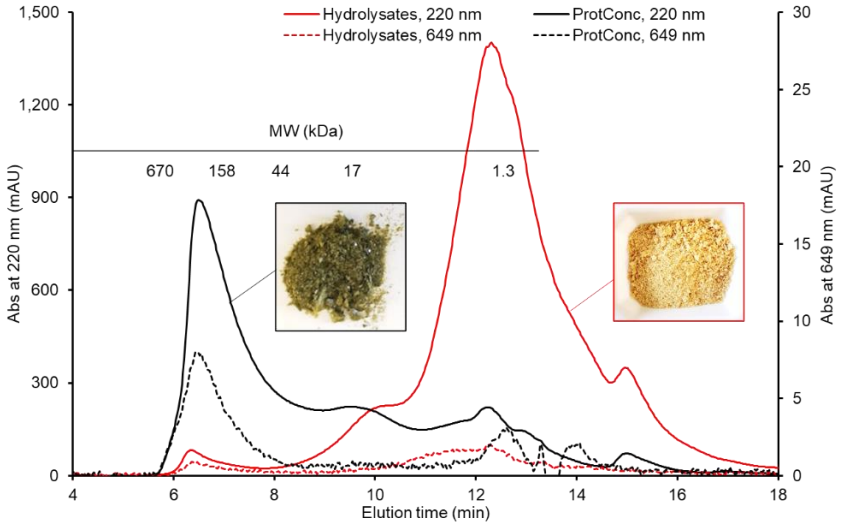


Figure 5.7 Size exclusion chromatograms of non-hydrolyzed LEA protein concentrates -ProtConc- (black lines) and hydrolysates from lysed LEA (red lines). Solid lines show the protein (A280), and dotted lines show the chlorophyll (A649) elution profiles[18]

[18] Results shown are representative of at least 2 independent replicates.

The black solid line in Figure 5.7 shows the MW profile of a non-hydrolyzed ProtConc. The protein extract consisted mostly of large photosynthetic protein complexes from 100 to 600 kDa, along with a wide range (~1-50 kDa) of smaller proteins. The MW profile of the hydrolyzed protein (Figure 5.7, red solid line) showed that most peptides were between 2 and 10 kDa in size, with a few in the larger MW range (from 17 to 200 kDa). Proteins with MW in this lower range are expected to have improved solubility and digestibility and reduced antigenicity [12].

Results in Figure 5.7 show that the larger MW fraction (150-670 kDa) of non-hydrolyzed ProtConc (black line) co-eluted with chlorophyll (dotted black line). The co-eluted large protein and chlorophyll peaks (6.5 min) most likely correspond to chloroplast protein-pigment complexes which are tightly bound through hydrogen bonding in plant and algae systems [203]. Indeed, the protein-pigment interaction is what hinders chlorophyll (green color) removal from algae protein [204] (Figure 5.7, picture outlined in black).

Compared to the ProtConc, the 220 to 649 nm absorbance ratio of the hydrolyzed protein is significantly higher, suggesting that most of the chlorophyll (Figure 5.7, dotted red line) was removed after hydrolysis and subsequent fractionation steps discussed previously (Figure 5.6). This observation agrees with results published by Morris, Almarales [12], where pigment could not be detected after clarification of a protein hydrolysate from *Chlorella vulgaris* biomass. It appears that photosynthetic complexes in the chloroplast were disrupted during enzymatic hydrolysis, and the chlorophyll was no longer bound to the hydrolyzed protein and thus, recovered in the supernatant of

centrifuged precipitate. This hypothetical explanation is supported by TEM analysis and observed disappearance of subcellular structures in Figure 5.3. Furthermore, chlorophyll removal from hydrolyzed protein is evident by the beige color of freeze-dried hydrolysates (picture outlined in red) rather than the green-colored of the ProtConc (picture outlined in black) shown in Figure 5.7. Hydrolysates that are visibly free of green pigments would be highly desirable for liquid food and drink applications [205].

5.4.3.2. Nutritional value of the hydrolysate

To determine whether the hydrolysis and fractionation processes impacted the nutritive value of the microalgae protein, an amino acid analysis was conducted on extracted algal protein and protein hydrolysate samples. The amino acid profiles of the protein hydrolysates were compared to that of LEA, soy, and whey proteins. Results in Table 5.3 demonstrate that essential AA content of microalgae protein is similar to whey and higher than that from soy. However, the percent lysine was lower in the hydrolysates compared to the other 3 protein samples.

Table 5.3 Essential AA distribution of the hydrolysates vs. soy and whey [206][19]

AA	Lysed LEA hydrolysates	LEA	Soy	Whey
HIS	1.4±0.1%	1.5±0.0%	2.6%	1.3%
LYS	4.8±0.0%	5.6±0.0%	6.0%	9.7%
MET	2.7±0.0%	2.3±0.0%	1.3%	1.7%
PHE	6.0±0.1%	5.9±0.0%	5.2%	2.6%
THR	6.2±0.0%	5.8±0.0%	3.6%	7.9%
ILE	5.3±0.0%	5.1±0.0%	4.8%	5.6%
LEU	9.9±0.1%	9.4±0.0%	7.7%	10.3%
VAL	6.2±0.0%	5.7±0.0%	4.7%	5.9%
Essential	42.6±0.4%	41.3±0.2%	35.9%	45%

The lysed LEA hydrolysates and the soy and whey protein extracts had a comparable amino acid profile and distribution of essential amino acids, indicating that our final product has a similar nutritional value compared to traditional protein sources. There was only a slight decrease in lysine (Table 5.3), which could had been caused by oxidation due to Maillard reactions [207]. We suspect the presence of Maillard products as there was some browning detected in the hydrolyzed protein samples. The Maillard reactions could have been induced during the protein hydrolysis under alkaline conditions, and/or the high-temperature enzyme deactivation treatment [208]. Compared to soy, the protein hydrolysates exhibited a higher ratio of essential-to-total amino acids. The amount of lysine in the hydrolysates, though, was about 2- and 1.3-fold

[19] Table shows the average percentage by weight composition of amino acids plus minus the standard deviation between two replicates (n=2).

lower than that in whey and soy, respectively. Lysine is one of the most valuable amino acids in the sports and nutritional drinks industry due to its limited availability in diets high in cereals [209]. Future work, thus, should focus on preventing lysine degradation due to Maillard reactions in the hydrolysates production process. For instance, low pH is known to inhibit Maillard reactions even at high temperatures [208]. Besides a reduced lysine content, the nutritional value of the algal protein hydrolysates is comparable to that of whey protein, the primary choice for the energy and sports drink industry.

5.5. Conclusions

The results provided in this manuscript show that cell lysis by ball milling can enhance the enzymatic reaction rate by overcoming enzyme concentration limitations in *Nannochloropsis sp.* LEA. We also found that processing of LEA into protein concentrates prior to hydrolysis did not further improve the reaction rate nor the extraction yield, compared to lysed LEA. After hydrolysis of lysed LEA, high recovery yields as well as chlorophyll removal were obtained via acidic precipitation followed by centrifugation and depth filtration. An IEX mixed bed resin was shown to successfully remove salts and increase the protein purity of the hydrolysates. To our knowledge, this is the first integrated process of hydrolysis and fractionation of chlorophyll free LEA protein with greater than 60% and 70% recovery yield and protein purity, respectively. Future work should focus on minimizing Maillard reactions during protein hydrolysis and enzyme deactivation steps to prevent lysine degradation. Further evaluation of the quality and suitability of the protein hydrolysates for formulation of specialized protein drinks is also required. Testing their temperature and pH stability as well as their

solubility is important to determine the shelf stability of the hydrolysates. Finally, estimating the production and capital costs of the proposed process is required to determine its economic feasibility.

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5.7. Author Contributions

All authors made substantial contributions in conceptualizing, drafting, developing and reviewing the manuscript, and All authors commented on previous versions of the manuscript. The paper was reviewed and approved by all authors prior to submission for peer review.

5.8. Conflicts of Interest

The authors declare no conflict of interest.

5.9. Statement of Informed Consent, Human/Animal Rights

No conflicts, informed consent, human or animal rights applicable

6. CONCLUSIONS

In this work we identified and addressed the main barriers to high-value protein product development from lipid extracted microalgae (LEA) with the aim of valorizing microalgae biomass waste. During the course of my dissertation research, we found that selecting optimal lipid extraction conditions that do not compromise the protein quality or solubilizing denatured LEA protein via enzymatic hydrolysis was crucial to ensure high extraction yield and quality of the protein from LEA. The production of protein concentrates from LEA (dry algal biomass), showed that drying and high temperature treatments of the biomass during the lipid extraction stage must be avoided to efficiently extract the protein via mechanical means (high-pressure homogenization). In cases where mild lipid extraction is not practical, a proteolytic treatment of LEA could be applied to enhance protein extraction and produce protein hydrolysates. The intended application of the protein product should determine the processing strategy i.e. with or without enzymatic intervention.

The literature review presented in Chapter 2, reveals that protein hydrolysates are more soluble and digestible than protein concentrates, but have lower emulsification, foaming, and gelling capacity. The lack of secondary and tertiary structure of the peptides present in the hydrolysates limits their functional properties required for some food applications. Thus, hydrolysates might not be well suited for the formulation of vegan meats, whipped creams, and sauces, but could be ideal for fortification of high-value drinks, and protein and baby formulas. Protein concentrates derived from microalgae, on the other hand,

often have a green pigmentation due to the presence of chlorophyll-protein complexes and are more likely to precipitate at lower pHs. As a result, algal protein concentrates are better suited for formulation of baked products, green sauces, and vegan meats than fortified protein beverages.

The co-production of protein concentrates and high-value lipids from microalgal biomass can be challenging. The extraction of high-value lipids (lutein, carotenoids, fatty acids, etc.) typically requires biomass drying before incubation with organic solvents. Thermal drying negatively affects protein functionality and extractability but provides solvent access to the lipids and enhances their extractability. On the other hand, the lipids extraction from wet biomass results in suboptimal lipids yield due to low cell permeability and the presence of water. One strategy to overcome low lipid extractability from wet biomass would be to treat microalgae biomass with pulse electric field (PEF) prior to EtOH extraction. By pretreating the microalgae with PEF, high-value lipids, such as lutein, were extracted to up to 80% recovery yield in one single extraction stage. Given the mild lipid extraction conditions used, the protein remaining in the lutein-extracted biomass could be efficiently extracted and concentrated via high pressure homogenization (HPH) and ultrafiltration/diafiltration (UF/DF) to generate protein concentrates and additional product revenue. We found that the protein concentrates from LEA could be produced at a cost comparable ($5/kg-concentrate) to the protein concentrates from traditional protein sources (soy, whey) ($3-5/kg). Nevertheless, a further increase of the final protein purity, reduction of production costs, and

valorization of the protein product is still needed to ensure the economic feasibility of the protein product from LEA.

The production of protein hydrolysates demonstrated that the hydrolysis of protein to small peptides increases the protein product value and enhances the extraction yield from the LEA. The hydrolysates were produced via enzymatic hydrolysis of mechanically disrupted LEA followed by centrifugation, filtration, and purification via ion exchange. The process developed, yielded a chlorophyll free protein hydrolysate of higher market value ($10-20 vs $3-5/kg) and protein purity (72 vs 57%), compared to the protein concentrates discussed in Chapter 3. The achieved protein purity of 72% is similar to current protein concentrates on the market, and higher than previously reported data for algal protein products.

Future work in food protein development from microalgae should aim to optimize two key processing parameters: protein recovery yield and purity. During this research, we observed that lower recovery during the protein extraction stage was usually accompanied by lower protein purity, higher production costs, and lower value of the final product. Thus, further development and optimization of protein extraction technologies from recalcitrant microalgae cells is required not only to reduce processing costs, but also enhance the quality of the protein product. There is also a need to implement bio-separatation technologies, i.e. ultrafiltration and chromatography, to increase the purity of the protein from LEA once it has been extracted. While we intended to optimize the production of protein concentrates and hydrolysates from LEA, there is still potential for improving the protein purity up to the standards of whey and

soy isolates (>80% protein content) without severely compromising the protein recovery yield or drastically increasing costs.

To better understand the implications of the results presented in this dissertation, future studies should determine the scalability and economic feasibility of the proposed processes for production of protein products from LEA. Furthermore, a more in-depth evaluation of the quality of the protein concentrates and hydrolysates generated should be conducted. Determining their shelf stability, palatability, safety, and bioactive properties should help finding their potential applications and value in the food and/or nutraceutical industry.

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APPENDIX

Membrane ultrafiltration for single-step clarification and purification of soluble protein hydrolysates for high-value drinks

Introduction

The use of de-lipidated biomass is key to improving the profitability of protein products from microalgae. The processes involved in lipid extraction, however, can induce protein denaturation and decrease its extractability [185]. An effective extraction and purification process are therefore required for the extraction of proteins from lipid extracted microalgae (LEA). In the past years, enzymatic protein hydrolysis has emerged as a useful and versatile technology for processing protein-rich feedstocks [212], increasing overall protein value, and overcoming low protein extractability and solubility [169]. Previous research has shown that protein from lipid extracted microalgae can be extracted and solubilized through enzymatic hydrolysis, obtaining high extraction yields in short incubation times [34, 106, 170, 213]. In fact, the protein hydrolysis rate from LEA has been shown to be significantly faster, compared to that from non-extracted microalgae [34, 106, 120, 186]. Enzymatic treatment with proteases, such as Alcalase, on lysed LEA can hydrolyze and release 64-70% with no need of protein extraction prior to hydrolysis [106, 170, 213].

After hydrolysis, lysed LEA debris, non-hydrolysable substrate, and non-protein contaminants must be removed from the protein hydrolysate to ensure its solubility and purity [213]. In our previous study, we found that isoelectric precipitation followed by depth filtration successfully fractionated the protein hydrolysates away from large

contaminants [213]. Depth filtration and other dead-end filtration configurations are advantageous for most biotechnological processes requiring removal of large particulates [214]. However, the relatively large pore size of dead-end filters (0.2 µm – 1.0 µm) is not suitable for removing soluble protein membrane complexes and unhydrolyzed protein, which are more prone to aggregate than small peptides. The presence of large protein fragments (i.e. >200kDa) might be challenging for specific end-use applications, such as high-value drinks, as they might precipitate and create unwanted settling during storage.

Membrane ultrafiltration has been previously used to remove unhydrolyzed protein away from soluble, low MW peptides [125, 215, 216]. The inclusion of an UF step in addition to centrifugation and depth filtration for purification of the algal protein hydrolysates may significantly increase processing costs and could render the product economically unfeasible. One potential strategy to minimize processing costs is to use ultrafiltration not only to remove unhydrolyzed protein, but also to clarify the hydrolyzed slurry while omitting centrifugation and depth filtration operations.

Cell lysis and hydrolysis of LEA will release intracellular biomolecules of wide MW ranges, that could limit the performance of an ultrafiltration membrane [217]. Thus, it is critical to find the optimum hydrolysis and ultrafiltration parameters that grant permeation of the soluble peptides while retaining unwanted molecules without severely fouling the UF membrane.

In this study, we evaluate the use of a hollow fiber ultrafiltration membrane for clarification and purification of protein hydrolysates from lysed microalgae slurry and compare the quality of the product to clarification by centrifugation followed by dead end filtration (0.2 µm). The specific tasks in this investigation were to: 1) Find the MWCO and enzyme dosage that allows a higher protein recovery yield, while maintaining a suitable flux during UF 2) Determine the impact of dosage and MWCO on the MW distribution of the hydrolysates. 3) Remove residual color from the hydrolysates 4) Compare the heat and pH stability of the ultrafiltered vs. centrifuged plus depth filtered hydrolysates 5) Evaluate the economic viability of the protein hydrolysate.

Methods

Substrate pre-processing

Lipid extraction

Cells will be extracted following a modified protocol of the one proposed by Kulkarni and Nikolov [21]. Fresh biomass will be extracted in 50 mL EtOH/g-DW fresh biomass and re-extracted under the same conditions (2 stages), with an incubation time of 45 min /stage. An incubation temperature of 60°C was used to maximize carotenoids, chlorophyll, and fat extractability.

Cell disruption

The de-lipidated whole cells were subjected to ball milling at pH 11, using a planetary mill from MSE Supplies, and 0.5 mm diameter zirconia beads. The chamber was 50% filled with beads as per manufacturer recommendations. A ball milling time of 120 min was selected for maximizing cell disruption.

Protein hydrolysis

The lysed LEA slurry was brought to pH 9.5 and 50°C in a heat plate under continuous mixing. The hydrolysis reaction was started by adding Alcalase at the selected enzyme dosage (0.9, 1.8, or 3.5 % v/w-protein) and continued for 3h while maintaining the pH at 9.5 by constant addition of NaOH. At the end of hydrolysis, the pH was brought down to 4.5 by addition of HCl and the enzyme was deactivated by incubation at 95°C for 5min.

Fractionation

Centrifugation plus depth filtration

The protocol for clarification of protein hydrolysates developed by Soto-Sierra, Wilken [210] was followed. After hydrolysis and deactivation, the slurry was centrifuged at 9,000 x g for 9 min and the supernatant collected (S1). The pellet was resuspended in water at pH 4.3 and mixed thoroughly to solubilize trapped proteins in the precipitate. The slurry was centrifuged again under the same conditions and supernatant (S2) was recovered. S1 and S2 were subjected to depth filtration using the Supracap 50 Pall® Depth filtration capsule (SC050PDD1) with a 0.2-3.5 µm retention rating and 22 cm^2 filtration area to remove unwanted debris / insoluble protein.

Membrane ultrafiltration

Based on previous literature and the MW distribution of the hydrolysates [210], a 50 (D02-E050-10-N) and a 100 (D02-E100-10-N) kDa Spectrum® hollow fiber filter modules of 1 mm I.D. were selected for purification of the hydrolysates. After hydrolysis and deactivation, the slurry was subjected to ultrafiltration in concentration mode until the concentration of solids in the retentate reached ~ 200 g-DW/L, or the

pressure in the feed exceeded 10 psi. Then, the UF unit was operated under continuous diafiltration mode for 2 diafiltration volumes (DV). Samples were taken before diafiltration and after each DV (DV1 and DV2) and the protein yield was determined at each point. The flux, TMP and g of permeate were tracked over time using the KF Comm real-time data collection software.

Hydrolysates yield

The yield was calculated following the protocol for recovery of soluble peptides proposed by Olsen and Adler-Nissen [184]. Each sample was digested in 6N HCl for 24 h until hydrolysis. Total amino nitrogen was determined using a N-OPA[185] and a nitrogen-to-protein conversion factor of 6.25 was used to determine protein content in the samples.

Size exclusion chromatography

Characterization of the extracts will be done using a TSK gel G2000SWxl size exclusion analytical column, the AKTA pure system, and an UV detector at 206 nm.

Heat stability studies

Hydrolysates samples (10 mg/mL) prepared by both fractionation methods were adjusted to the selected pH (4, 6, and 8). In a heat plate, half of the samples, were subjected to thermal treatment at 95°C for 10 min. Then, 1 mL of each, heated and non-heated samples were loaded on a cuvette and inserted in the Zetasizer Nano ZS sample chamber (1mL) for dynamic light scattering (DLS) measurements at 25°C and a 173° scattering angle. The DLS data was automatically converted to intensity and volume distribution by particle size.

Results

Optimizing protein recovery from UF DF process

The lysed LEA was disrupted at different Alcalase dosages (0.9-3.5%), followed by enzyme deactivation and protein precipitation at pH 4.5, and immediate clarification / purification by ultrafiltration (Figure 6.1). The objective was to find the right hydrolysis and ultrafiltration conditions that allowed passage of small, hydrolyzed peptides while removing potentially insoluble protein aggregates, chloroplast remnants, cell debris, among other impurities.

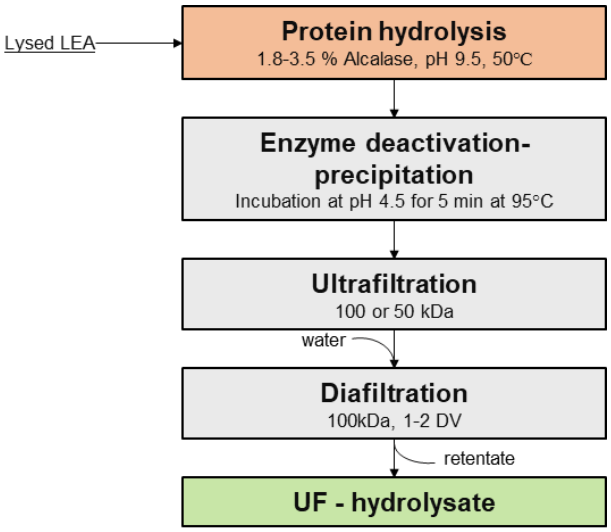

Figure 6.1 Flow diagram of the process for production of UF-hydrolysates

To determine the optimum conditions for protein recovery, we first pre-screened several variables (pH, enzyme deactivation temperature, and MWCO) that could potentially impact the flux and protein recovery in the permeate side. From statistical analysis (data not shown), we found that enzyme dosage, MWCO, and their interaction were the only significant factors affecting protein recovery and flux. Optimization of both factors was conducted by testing protein recovery and flux at the conditions shown in Table 6.1.

When using a 50kDa MWCO membrane, we observed an enhancement of the protein recovery from 56 to 70% as the enzymatic dosage during hydrolysis was increased from 0.9 to 3.5%, respectively. The highest flux was achieved with 50 kDa and 3.5% enzymatic dosage.

Table 6.1 Impact of MWCO and enzyme dosage on flux and protein recovery in the permeate and after one (1 DV) and two (2 DV) diafiltration volumes

			Cumulative protein recovery		
Alcalase Dosage (%v/w)	**Membrane MWCO (kDa)**	**Average flux (LMH)**	**Permeate (%w/w)**	**1 DV (%w/w)**	**2 DV (%w/w)**
3.5	100	25 +-2	50 +-5	63 ± 4	73 ± 2
1.8	100	25 ± 1	53 ± 5	65 ± 4	73 ± 3
3.5	50	32 ± 6	46 ± 4	61 ± 4	70 ± 3
1.8	50	20 ± 0	41 ± 5	57 ± 2	66 ± 2
0.9	50	16 ± 3	33 ± 7	48 ± 6	56 ± 6

The average filtration flux also increased as the enzyme dosage increased, which could indicate higher concentration polarization or gradual pore fouling of the membrane when filtering slurries hydrolyzed at lower enzyme dosages. The hydrolysis at 1.8% v/w enzyme dosage and clarification of the slurry thorough a 100kDa hollow fiber filtration membrane exhibited the highest protein recovery in the permeate site at an average flux of ~ 25 LMH. Results from the 100kDa UF runs indicated that a further increase in the enzyme dosage (to 3.5%) did not improve protein recovery nor the flux. For all the experiments conducted, we observed a rapid feed pressure buildup, which we attributed to the constant increase in solids content in the retentate side. Future experiments should focus on understanding the impact of viscosity in the purification of the protein

hydrolysates and on optimizing variables, such as temperature, affecting the transmembrane pressure and flux [211].

After ultrafiltration, 47% of the protein still remained in the permeate site ($C_{s,o}$ = 100-53% from Table 6.1, 2nd row), and diafiltration of the retentate was required to further increase the peptides recovery in the permeate. To find the optimum number of DV, we determined experimentally the protein recovery after 1 and 2 DVs and estimated a peptide retention coefficient of 0.65. Then, estimated potential peptides recovery to up to 4 DVs using equation (Equation 11).

$$n = ln\left(\frac{C_S}{C_{s,o}}\right)^{-\frac{1}{1-r_S}} \qquad (11)$$

Table 6.2 Diafiltration volumes optimization

Diafiltration volumes (DV)	Peptides concentration in the retentate (Cs) (%)	Estimated protein recovery in the permeate (%)	Actual protein recovery in permeate (%)	Estimated solids content (mg/mL)
0	47.0	47.8	53.0	39.1
1	33.1	63.2	65.0	34.5
2	23.3	74.1	73.0	30.3
3	16.4	81.7	--	26.8
4	11.6	87.1	--	23.8

If we assume a constant retention coefficient for all the peptides, there is potential to increase protein recovery from 73% up to 81.7% (7.6% max based Table 6.1) if we increase the number of DVs from 2 to 3. Nevertheless, previous studies [212-214] on ultrafiltration of hydrolyzed protein isolates and concentrates have found that UF-DF usually retains between 30-40% of total protein in the retentate and that more than 60%

of such protein corresponds to unhydrolyzable peptides and enzyme. Thus, there is a high chance that only marginal improvement in protein recovery can be obtained after 2 DVs.

Another consideration in maximizing hydrolysate recovery by DF is the dilution of protein concentration in the permeate. One has to perform cost-benefit analysis of DF process as the over-dilution of hydrolysates would require greater energy input for drying the final product and longer processing (DF and drying) times [212]. The estimates given in Table 6.2 show the solids content decreases from 35 down to 24 mg/mL, approx. 4 mg/ml per each additional DFV. Based on the results from Tables 1 and 2, we decided to use two DFV to minimize the dilution effect and increase protein recovery to 70% with 50 or 100 MWCO membranes.

Product analysis

The product potential applications depend on pH and T stability, which in turn are related to the MW profile composition of the final product. The purpose in the study was to compare the hydrolysates clarified by ultrafiltration (UF-hydrolysates) vs. the ones made by centrifugation and depth filtration (F-hydrolysates) as reported in our previous work[210] (Figure 6.2). The MW distribution was determined by size exclusion chromatography using a TSK G2000swxl column.

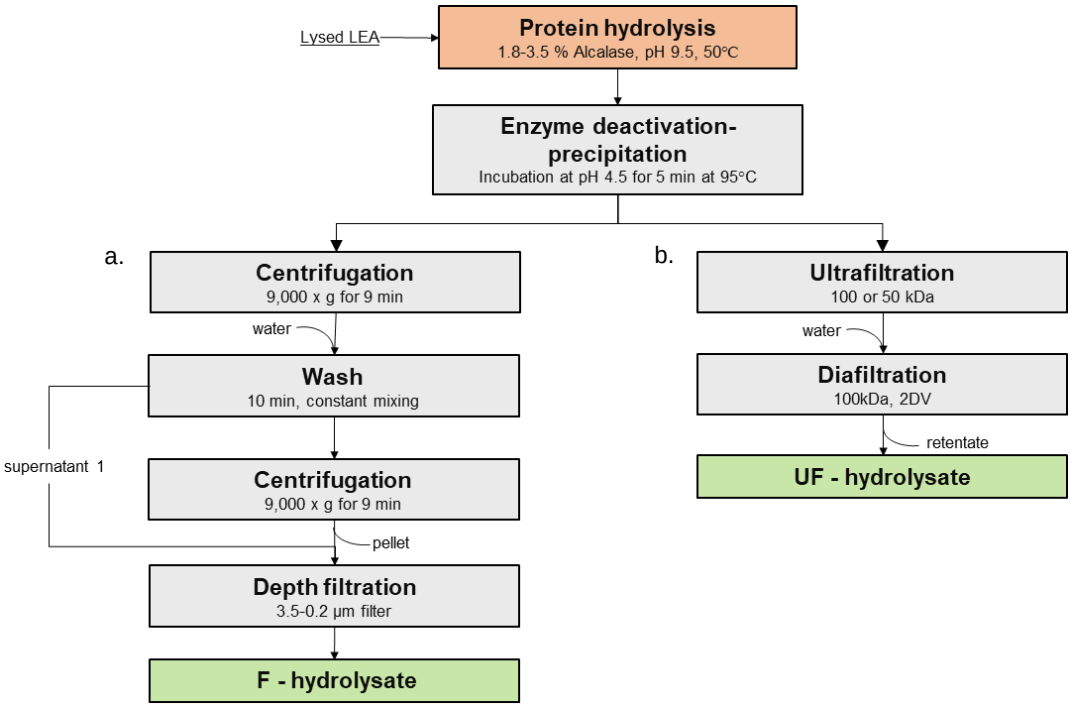

Figure 6.2 Production routes to protein hydrolysates: a. Protein hydrolysates by centrifugation and depth filtration (F- hydrolysate) vs. b. Protein hydrolysates by ultrafiltration and diafiltration (UF- hydrolysate)

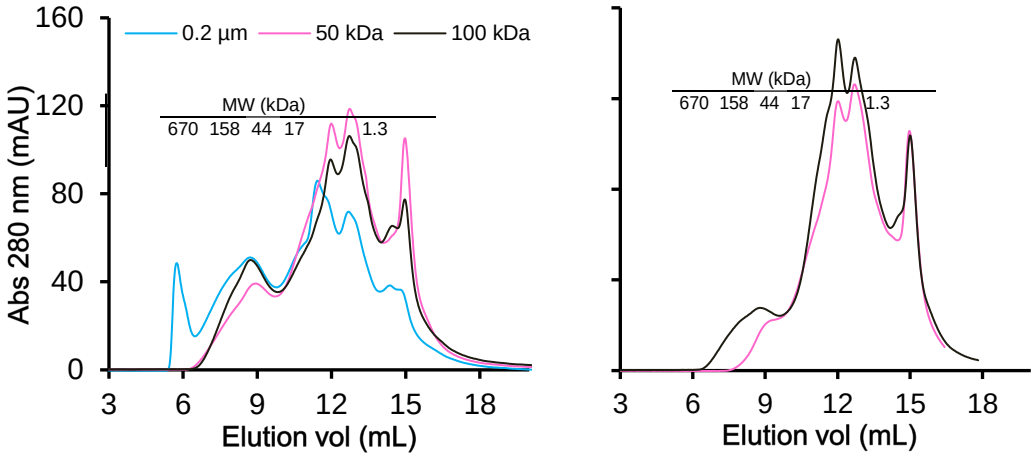

Figure 6.3 MW distribution of filtered protein hydrolyzed at 1.8 (left) vs 3.5% (right) enzyme dosage

The molecular weight profiles showed that the F-hydrolysate contained a much larger fraction of high MW proteins of ~200 to 600 kDa that was no longer present in the UF-hydrolysates at all the dosages and MWCO tested. The later indicated that centrifugation and depth filtration process were not selective enough towards small peptides and co-fractionated larger proteins. Indeed, precipitation is limited by the solubility characteristics of the molecules present (i.e. hydrophobicity and charge) in the slurry at the precipitation pH [215], rather than their MW. These results agree with previous studies [117] on production of protein hydrolysates from microalgae where a 100-200 kDa MW peak is also observed during size exclusion analysis.

On the other side, the ultrafiltered samples consisted mostly of peptides ranging from 1-50 kDa that eluted from 8 through 14 mL, and probably some smaller di, tripeptides, and free AA, that eluted in a sharp peak at 15.5 mL. We also observed a decrease in the height of a small peak (at ~8.5 min) corresponding to 50-100 kDa MW proteins as the enzyme dosage increased and the MWCO of the membrane decreased. The later agrees with results from Table 6.1, where we observed a higher protein recovery (73 vs 66%) after hydrolysis at 1.8% enzyme dosage when a 100kDa instead a 50kDa membrane was used (Table 6.1). For future process scale up, one should consider that the 50kDa MWCO membrane could potentially suffer more severe pore plugging, as the 50-100 kDa proteins are being forced through the pores, and lower fluxes due the smaller pore size, compared to the 100kDa membrane.

Based on results from Table 6.1 and Figure 6.3, a 1.8% enzyme dosage, and a 100kDa MWCO filter appears to be optimum for enhancing the ultrafiltration performance and

the protein recovery yield, while efficiently removing large proteins for the UF-hydrolysate. We suspected that removing the 200-600kDa MW proteins via ultrafiltration could help enhancing the pH and heat stability, and solubility of the UF-hydrolysates, compared to the F-hydrolysates. To further investigate this, the particle size distribution of both, F- and UF-hydrolysates was compared via dynamic light scattering (LDS) after heat treatment (10 min at 95°C) at different pHs (4, 6, 8).

Impact of depth filtration vs. ultrafiltration on heat stability

Results showed that most of the F-hydrolysates (Figure 6.4a) had a wide size distribution, which changed drastically upon pH adjustment and/or heating. The DLS graphs of the pH 4.0 and 6.0 samples, heated and not heated, showed peaks between 100 and 1000 nm, which are an indication of particle aggregation [216]. At pH 8.0, though, the size distribution changed upon heating but remained between 0.1 and 10 nm.

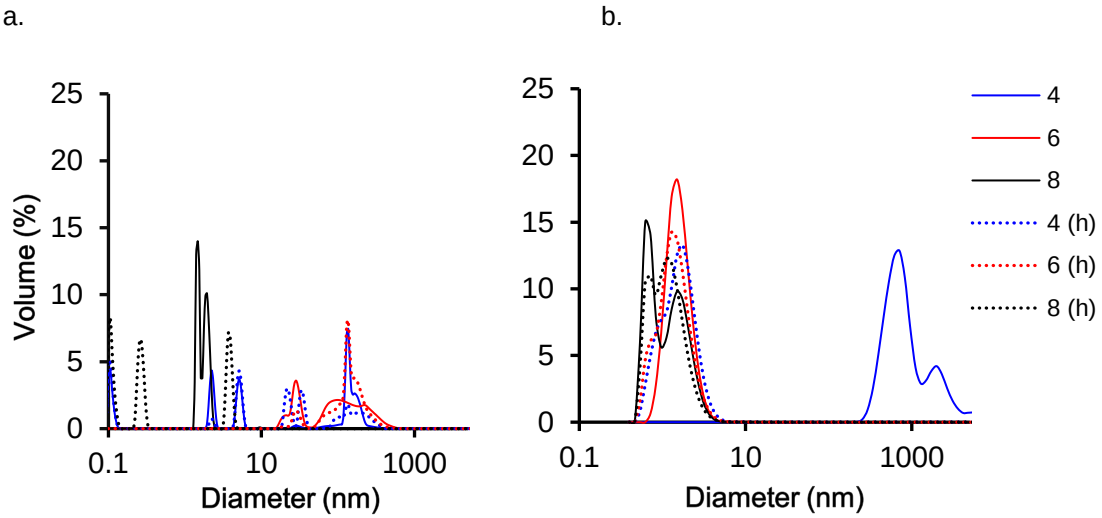

Figure 6.4 Particle size distribution of F- (a) vs. UF- (b) hydrolysates treated at different pH (4, 6, 8) with (h) or without thermal treatment.

The UF-hydrolysates (Figure 6.4b), on the other side, were more stable at the pHs tested before and after heating as evidenced by the similar size distribution profiles. The diameter of the particles in the ultrafiltered mixture ranged from 0.2-10 nm for most samples tested except for the non-heated ones at pH 4.0. The non-heated samples at pH 4.0 appeared to be significantly more aggregated than their heated counterparts. We hypothesize that pH 4.0, which is very close to the average pI of algae proteins (pH 4.0-5.5) [24], induced the aggregation of protein fragments and peptides present in the hydrolysate [216], and that the heating might have helped disrupting the molecular interactions causing such aggregation. Since the peptides cannot form complex molecular structures, the heating might have just dissociated week intramolecular bridges [216] and dissipated the aggregation. These results agree with the behavior of the depth filtered samples at pH 4.0 (Figure 6.4b), where a peak at 100 -1000 nm seems to shift to 10-100 nm after the thermal treatment. From the results obtained, the ultrafiltered samples seem to be more stable and better suited for formulation of drinks at pHs between 6 and 8 as they will be less prone to aggregate.

To further explore the impact of heating on the hydrolyzed samples, we looked at the browning of freeze dried F-vs UF- hydrolysates and compared them to a protein concentrate from soy (Figure 6.5). While the soy protein concentrate did not show any signs of browning, we observed some darkening on the UF-hydrolysates sample, and a more notorious browning in the F-hydrolysates. A higher content of carbohydrates, together with the presence of small peptides might have caused the browning, a likely consequence of Maillard reactions [207, 217].

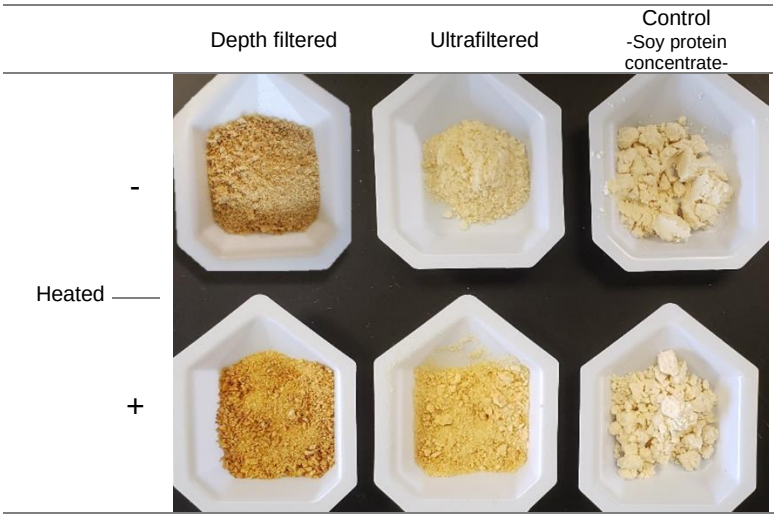

Figure 6.5 Color change upon heating dried and depth filtered vs. ultrafiltered hydrolysates.

Conclusions

The implementation of an ultrafiltration process for single step clarification and purification of protein hydrolysates step appears to be a good strategy to minimize purification steps as well as maximize the quality of the final product. UF-hydrolysates appear to be more heat and pH stable than the F-hydrolysates, thus might be better suited for formulation of high value protein drinks. The end application and the estimation of the processing costs should determine the feasibility of the UF vs. F hydrolysates.