BIOPROCESSING OF MICROALGAE FOR EXTRACTION OF HIGH-VALUE PRODUCTS

A Dissertation

by

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ABSTRACT

Microalgae are a rich source of various high-value biomolecules such as lipids, carbohydrates, protein and pigments. However, extraction of multiple high-value products is essential for sustainability and commercial feasibility of the microalgal production platform. Therefore, the first study of this dissertation was focused on optimizing processing parameters for extraction of pigments and functional proteins from *C. vulgaris*. A wet biomass-to-solvent ratio of 1:5 and 3 extraction stages were required to achieve maximum extraction yield of pigments. Effective protein release from wet biomass was achieved by high-pressure homogenization. Ethanol extraction of pigments affected protein solubility, and an alkaline pH was required to release the same total protein. Concentration of proteins was carried out using a two-stage membrane filtration process. Ethanol treatment and higher pH conditions did not negatively impact membrane filtration, nutritive value, nor the emulsification properties of protein concentrates. The effect of pulsed electric field (PEF) on imparting permeability and enhancing pigment extraction from *C. vulgaris* was also evaluated. PEF was successful in permeabilizing fresh cells and enhanced the lutein (2X) and chlorophylls yield (4X) at an electric field intensity of 6250 V/cm, pulse duration of 620 µs at a biomass concentration of 56 g/L, followed by a single-stage ethanol extraction for 45 minutes. Up to 14% of the total protein was released after PEF. Extraction of intracellular recombinant proteins from microalgae is usually accompanied by release of impurities such cell debris, nucleic acids, chlorophyll and host cell proteins. One of the major roadblocks in the purification of recombinant proteins is removal of impurities before

loading the extract onto the first chromatography column. Hence, the second study of this dissertation was focused on checking the effectiveness of chitosan precipitation on removal of impurities from *C. reinhartdii* extracts for the purification of a single chain antibody fragment (αCD22scFv) and comparing it to acid precipitation. Chitosan precipitation performed better than acid precipitation in terms of removal of impurities, reducing DNA by 91%, chlorophyll by 98% and host cell proteins by 81%. Capture chromatography (using a Capto Q column) performed on pretreated extracts resulted in 13-20 fold purification of αCD22scFv.

DEDICATION

To my beloved family for their unconditional love and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The literature review is divided into two main sections 1) high-value product extraction from microalgae which primarily focuses on growth and isolation of products from natural (non-recombinant) microalga *Chlorella vulgaris* and 2) Recombinant protein production in microalgae which focuses on expression and purification of therapeutic proteins from recombinant *Chlamydomonas reinhardtii.*

1.1 High-value products from microalgae

Microalgae are photosynthetic microorganisms and are rich sources of lipids, protein, carbohydrates, and other high-value compounds such as pigments, anti-oxidants and vitamins (Pulz & Gross, 2004). The versatile uses of microalgae for food, feed and energy have been identified decades ago (de la Noue & de Pauw, 1988). One of the major interests has always been to make use of microalgae for the production of biofuels (Chisti, 2007). Studies on algae for biofuels as a response to the energy crises of 1974 have been conducted in the 80s (de la Noue & de Pauw, 1988). Using the lesson learned from the 80s, algae-for-biofuels effort has been revived in past ten years focusing on increasing biomass productivity and oil accumulation by carrying out media optimization, novel bioreactor configuration, and molecular manipulation of metabolic pathways (Mercer & Armenta, 2011). In spite of significant engineering and scientific advances, life cycle and techno economic analysis published in the past few years still indicate that algae –for-biofuels production platform would not be sustainable unless

other high value products can be generated from algal biomass (Guedes et al., 2011; Perez-Garcia & Bashan, 2015; Safi et al., 2014c).

Depending on the species and cultivation, the protein and lipid content in microalgae can range from 20-70 % (Becker, 2007) and 10-54 % (Lari et al., 2016) of the dry weight (DW), respectively. However, the nutrient deficient conditions required to enhance lipid production are not compatible for protein accumulation (Vaičiulytė et al., 2014) Therefore, the choice of algal strains should be governed by their ability of produce significant amount of quality lipids or proteins under the product-optimal growth conditions. Out of the numerous microalgae with high protein content, a few have been chosen for large scale production which include, chlorophyceae Chlorella and Scenedesmus, and cyanobacteria Spirulina and Athrospira. Chlorella is a eukaryotic, spherical, unicellular algae with a diameter of 2-10 µm. Within the Chlorella genus, *C. vulgaris* is a promising candidate for extraction of multiple high-value products. Along with having a high protein content (42-58 % DW), it also a good source of pigments such as chlorophyll (1-2 % DW) and lutein (up to 3.7 mg/g DW), which have multiple therapeutic properties (Cha et al., 2009; Safi et al., 2014c).

The challenges associated with developing coproducts such as lipids, carotenoids, and proteins are incompatible cultivation conditions and extraction technologies that maximize accumulation and subsequent recovery of solvent-soluble lipids/triglycerides and water-soluble products (e.g. polysaccharides and proteins), respectively. Another process challenge of Chlorella is strong (rigid) cell wall, which offers excellent cell

protection and, also barrier to overcome for extraction of various products. Therefore, it is clear that compatible algal strains, cultivation conditions, and bioprocessing (recovery) strategies / technologies have to be developed for a shared production of these vastly different products. A possible alternative to alleviate the coproduction constraints is to utilize same algal bioreactor and downstream processing facilities for production of hydrophobic biomolecules (e.g, triglycerides and carotenoids) and hydrophilic macromolecules (e.g. carbohydrates and proteins) in separate product-optimized manufacturing campaigns.

1.1.1 Structure and components of Chlorella

C. vulgaris is a photosynthetic, eukaryotic unicellular microorganism with cell size ranging from 2-7 µm (Yamamoto et al., 2004). It has many structural elements similar to plant cells (Figure 1.1). Cell wall of *C. vulgaris* is very complex and rigid consisting of N-acetylglucosamine as the principal amino sugar present as a chitin-like glycan (Kapaun & Reisser, 1995). The cytoplasm consists of soluble proteins, minerals and water and holds internal organelles of *C .vulgaris* such as mitochondria, nucleus, vacuoles, Golgi body and a chloroplast (Kuchitsu et al., 1987; Safi et al., 2014c; Solomon et al., 1999). The chloroplast occupies a large fraction of algal cell. It houses a series of flattened vesicles or thylakoids, where primarily chlorophyll is synthesized along with other pigments such as β-carotene and lutein (Richmond, 2008). The chloroplast also contain an organelle known as pyrenoid, which typically consists of a matrix surrounded by starch sheath and is traversed by thylakoids (Engel et al., 2015).

The matrix contains high levels of ribulose-1,5-bisphophate – an enzyme which is essential to carbon dioxide fixation (McKay & Gibbs, 1991).
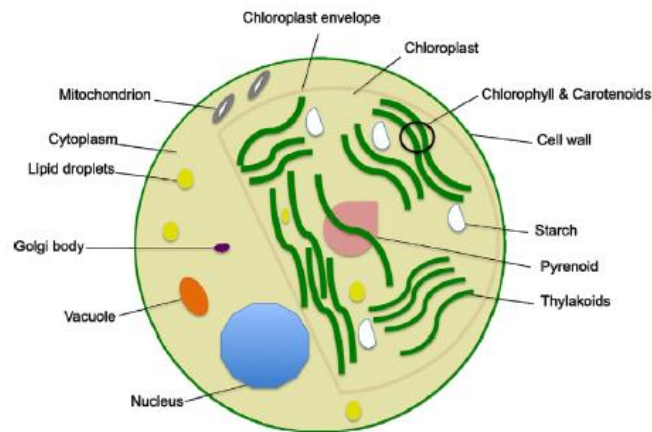
Figure 1.1 Schematic of *C. vulgaris* with different cell components. Reprinted from Safi et al., 2014.

1.1.2 Cultivation of Chlorella

Due to its high protein content and expression of other valuable products, Chlorella is produced on large scale in countries such as Taiwan, Germany and Japan (Spolaore et al., 2006). Chlorella is commercially produced by more than 70 companies, with Taiwan Chlorella Manufacturing and Co. being the largest producer of Chlorella (400 tons dried biomass/ year). In 2009, the annual production of Chlorella had reached 2000 tonnes (dry weight) (Spolaore et al., 2006). *C. vulgaris* has the ability to grow autotrophically, heterotrophically, and mixotrophically. In a lab scale study of *C. vulgaris*, mixotrophic growth resulted in increasing productivity (1.6 g/L DW) compared to heterotrophic growth (1.2 g/L DW). Both mixotrophic and heterotrophic conditions with glucose as

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the carbon source, were better than photoautotrophic growth (0.2 g/L DW) (Liang et al., 2009). In another study of the growth of *C. vulgaris* in 5 L photobioreactors with agricultural waste medium as carbon and nitrogen sources, maximum growth was achieved under mixotrophic conditions (2.6 g/L DW) which was 140 % higher than autotrophic conditions and 300 % higher than heterotrophic conditions (Mohammad Mirzaie et al., 2016). Usually, a higher productivity is obtained under mixotrophic growth than heterotrophic or autotrophic conditions. Utilization of CO_2 and organic carbon sources allows the mixotrophic regimen to shorten growth cycles, reduce biomass loss in the dark hours and since light is not a limiting factor for growth, reduce photo-inhibition (Andrade & Costa, 2007; Fernández et al., 2013; Park et al., 2012).

Various carbon sources such as glucose, glycerol, industrial wastes etc. can be utilized for heterotrophic or mixotrophic growth of *C. vulgaris* (Abreu et al., 2012; Liang et al., 2009). Glucose is the most commonly used carbon source for heterotrophic or mixotrophic growth because higher rates of growth are obtained with glucose compared to other substrate. This is because glucose has more energy content per mol than any other substrate (Griffiths et al., 1960). In a study comparing the effect of various carbon sources (glucose, glycerol, sodium acetate and sucrose) on the growth rate and biomass accumulation, maximum biomass production was achieved (2.08 g/L DW) with addition of glucose (Sharma et al., 2016). Other factors to consider for optimal growth of *C. vulgaris* are pH and temperature. The temperature range of 25-30 °C showed maximum growth of *C. vulgaris* and it decreased after 35 °C (Converti et al., 2009). In case of pH,

a range of 6.5 to 8.0 is acceptable for optimum growth, with the best growth achieved at the pH of 7.5 (Rachlin & Grosso, 1991).

Depending on growth media and conditions, the protein content in *C. vulgaris* can vary. For *C. vulgaris* grown in BG-11 medium, maximum protein content (42-45% DW) was achieved in nutrient (N and P) sufficient conditions (0-94 h). Under nutrient deprived phases (95-238 h), the lipid content in cells increased from 18 to 56 % with a consequential decrease in protein (24 %) (Vaičiulytė et al., 2014). Protein content was the maximum during the early log phase (40-60 %), carbohydrates content increased during late log phase while remaining constant during stationary phase (60%), and the lipid content steadily increased (up to 25%) as the biomass reached the death phase (Lv et al., 2010). When using different carbon sources for growth, to achieve maximum protein productivity, both protein content of cells and biomass density have to be considered. For example, when glucose was used to grow *C. vulgaris,* the protein content was 32% compared to 45% with glycerol as carbon source (Liang et al., 2009). But, because of higher biomass density achieved using glucose (1.7 g/L DW) compared to glycerol (0.7 g/L), the total protein productivity was 0.54 g/L in case of glucose versus that of 0.31 g/L with glycerol.

In case of pigments such as lutein, factors such as temperature, pH, and irradiance have an impact on pigment accumulation. Maintaining growth temperature of *Chlorella protothecoides* at 32 °C instead of 28 °C resulted in 20 % increase in lutein content but 14 % decrease in biomass concentration (Shi & Chen, 2002). Lutein content increased at extreme pH (6 and 9), but maximum lutein productivity was achieved at pH 6.5 due to

increase in biomass production (Sánchez et al., 2008). The lutein content in *Chlorella*

sorokiniana decreased slightly with mixotrophic conditions (1.07 mg/g DW biomass)

compared to autotrophic conditions (1.76 mg/g DW biomass), but the biomass growth

rate was higher for mixotrophic cultures (1.67 day^{-1}) compared to autotrophic cultures

(1.01 day^{-1}) (Van Wagenen et al., 2015). Irradiance usually has a positive effect on lutein

productivity. Increasing irradiance from 90 µmol photon m^{-2} s^{-1} to 920 µmol photon m^{-2}

s^{-1} lead to rise in lutein productivity (from 17 mg/L to 25 mg/L), largely due to the

increase in biomass accumulation (Del Campo et al., 2004). In case of some organisms

such as *C. protothecoides,* for accumulation of chloroplast (and eventually majority of

the proteins and pigments), photo-autotrophic conditions were required (Xiong et al.,

2010). In conclusion, a mixotrophic regimen for growth seems to be the best avenue for

achieving maximum biomass productivity and support biochemical pathways that lead to

the formation of proteins and pigments (Perez-Garcia & Bashan, 2015).

1.1.3 High-value proteins from Chlorella

C. vulgaris is known for its high protein content which ranges from 42 to 58 % of the

dry weight (Safi et al., 2014c). Amino acid profiles of proteins extracted from *C.*

vulgaris compare well to the reference pattern recommended by WHO/FAO (Becker,

2004; FAO & WHO, 1973). However, when equated to egg, minor deficiencies are

observed among sulfur-containing amino acids cysteine and methionine which are

common amongst other plant-based proteins (Becker, 2007). Nutritive quality of

proteins can be measured by various methods on top of the amino acid composition

(Becker, 2004; Ursu et al., 2014). One of them is biological value (BV), which is the measure of nitrogen retained for growth or maintenance, and another parameter is digestibility coefficient (DC). The third parameter is known as net protein utilization (NPU) is equivalent to the calculation DC x BV and measures both biological value of amino acids absorbed from the food and digestibility of the protein. Another factor used to determine the quality of protein is called the protein efficiency ratio (PER), which is expressed in terms of weight gain per unit protein consumed by a test animal in feeding trials. The BV, DC, NPU and PER values for drum-dried Chlorella were 77, 89, 68 and 2 respectively (Becker, 2004) and were on the higher side of the range for other algal species (67-78 for BV, 72-89 for DC, 52-68 for NPU and 1.14-2.10 for PER). However, qualitative parameters of protein from Chlorella fell short of established protein sources like egg and casein. Due to the rigidity of the cell wall and consequently the inability of digestive enzymes to reach intracellular components, qualitative parameters for Chrorella aren't comparable to traditional protein sources. Hence, it is vital that effective treatments be carried out to disrupt the cell wall and make proteins and other intracellular components accessible to digestive enzymes.

1.1.4 Lutein and other pigments in Chlorella

Light is converted into chemical energy for CO_2 fixation in oxygenic photosynthetic organisms. Two pigment-binding protein complexes called photosystem I and II (PSI, PSII) are part of the photosynthetic machinery located in the chloroplast thylakoid membrane (Ballottari et al., 2013). Light harvesting is performed by chlorophyll and

carotenoids mainly attached to peripheral antenna proteins called Lhca (in PSI) and Lhcb

(in PSII) (Figure 1.2). For photosynthetic organisms, the presence of carotenoids is

required for protecting the cells against the harmful effects of O_2 and light.
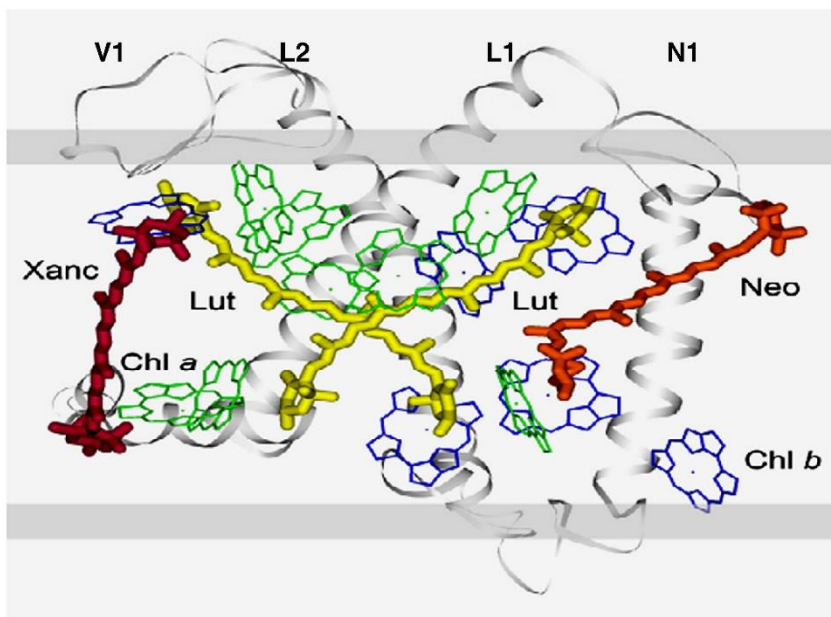
Figure 1.2 Structure of Lhcb and location of pigments. The protein backbone with
the three transmembrane helices (gray) is shown in the background, the pigments
are shown in color. Reprinted from Jahns & Holzwarth, 2012.

Over 700 carotenoids have been identified so far and they are widely distributed in

nature in a range of colors (Lorenz & Cysewski, 2000; Zhang et al., 2014). Amongst

carotenoids, lutein has gained special attention in academic and industry research

because of its bioactivities, the most notable one being its antioxidant activity. Lutein

has the ability to be rapidly oxidized, by reducing the availability of free radicals to react

with cellular components such as protein, DNA and unsaturated lipids (Woodall et al., 1997). Like other carotenoids, the conjugated double bond system of the molecule contributes to the antioxidant property (Figure 1.3). Studies have shown that lutein inhibited oxidation of low-density lipoprotein which can suppress progression of cardiovascular diseases (Chopra & Thurnham, 1994). Lutein is one of the few carotenoids that can be absorbed in the blood stream after ingestion (John et al., 2002). It can accumulate in the human retina and can have a protective effect due to its ability to filter out blue light. Lutein intake is effective in preventing early atherosclerosis (Dwyer et al., 2001) and is also known to ameliorate the onset or progression of cataracts (Shao et al., 2008). Lutein is prescribed to patients suffering with age-related macular degeneration (AMD) (Dwyer et al., 2001; Fernández-Sevilla et al., 2010). Due to its antioxidant and therapeutic properties, lutein supplements are sold in the market in the form of soft gels and dried powder. In the eastern world, there are over 100,000,000 potential patients at risk of suffering from AMD, which indicates that there's a large market for lutein. Pure lutein obtained from marigold flowers in crystalline form is susceptible to oxidation, so it is usually sold in form of crystal suspensions in oil (corn or sunflower). This formulation can contain up to 20 % weight lutein or lutein esters. In order to make softgels, these lutein concentrates can be of different formulas or diluted more in oils to concentration of 10-20 mg/ml (Fernández-Sevilla et al., 2010). Lutein is also used as a food colorant (E 161b) in a range of food products such as fine bakery wares, decorations and coatings, edible ices, desserts including flavored milk products,

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sauces and seasonings, edible cheese rind and edible casings, and soups (EFSA, 2012)

amounting to sales of $150,000,000 in the US alone (Fernández-Sevilla et al., 2010).

Figure 1.3 Structure of lutein.

Currently, lutein is commercially produced from petals of marigold flowers (0.5 -1.2 %

of DW). (Šivel et al., 2014). However, the drawback of lutein production from marigold

petals is that process is labor intensive and land demanding. Various micro-algal species

such as Muriellopsis, Scenedesmus, Chlorella, Chloroccum and Neospongiococcus are

known to produce lutein. Lutein content in Chlorella can amount to 3.4 – 4.6 mg/g DW

(Del Campo et al., 2007). The combination of biomass productivity and lutein content

make microalgae like *C. vulgaris* a very good candidate for commercial lutein

production. However in order to compensate the high harvesting cost of microalgal

biomass and the high energy demand of cell disruption, it is necessary that other

important by-products are extracted (Lin et al., 2015).

Another valuable pigment that can be of commercial use from algae is chlorophyll.

Chlorophyll a (Figure 1.4) is the chief photosynthetic pigment which helps in transfer of

light energy to a chemical acceptor. Accessory pigments like chlorophyll b (Figure 1.4),

which are primarily present in higher plants and algae, aid the transfer of light to

chlorophyll a (İnanç, 2011). Chlorophyllin, a derivative of chlorophyll is used as a food

coloring agent (natural green 3). Therapeutic properties of chlorophyll are summarized

by Ferruzzi & Blakeslee (2007). Chlorophyll stimulates immune system, provides relief

against sinusitis, fluid buildup and skin rashes, helps combat anemia, cleans toxins from

blood and detoxifies the liver (Ferruzzi & Blakeslee, 2007).
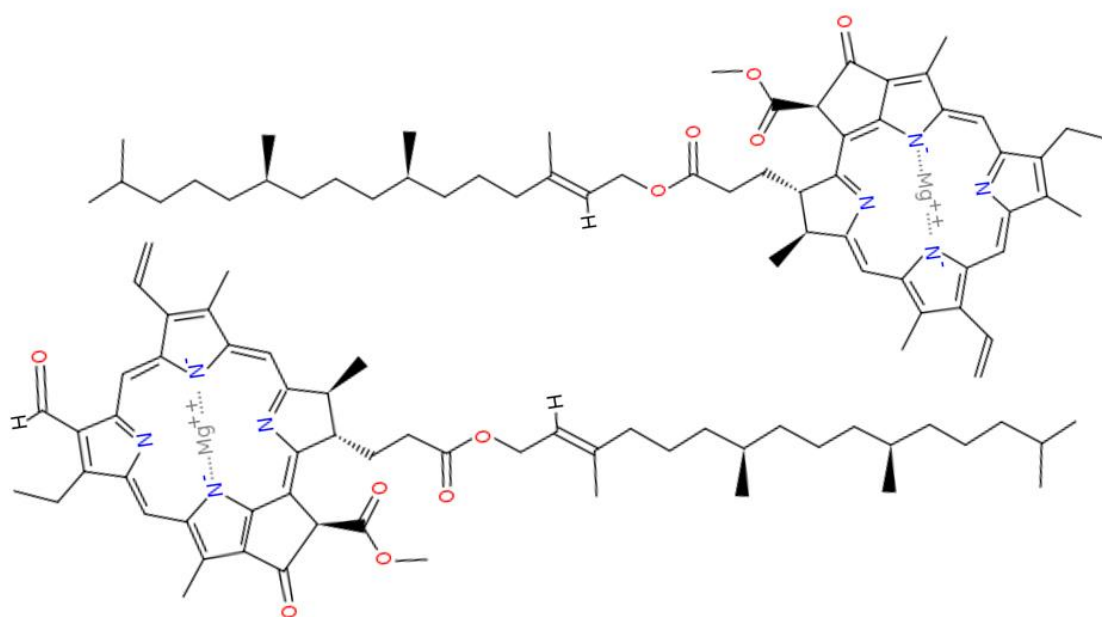
Figure 1.4 Structure of chlorophyll a (upper diagram) and chlorophyll b (lower diagram).

1.1.5 Isolation of products from Chlorella

As with any other microorganism the release of intracellular cell components requires

cell lysis (cell wall and membrane disruption). The cell wall of Chlorella is composed of

two major constituents: alkali soluble hemicellulose and alkali-insoluble rigid wall

(Takeda & Hirokawa, 1978). The former is composed of neutral sugars, glucose,

mannose, rhamnose, and arabinose, while the latter glucosamine as the main constituent.

In another study, it was determined that cell wall of *C. vulgaris* is composed of 24-74 %

neutral sugars, 4-24 % uronic acids, 6-15 % glucosamine and 4-5 % protein

(Blumreisinger et al., 1983). Data identifying the rigid cell wall component of *Chlorella*

Pbi indicated the presence of glycosaminoglycan which can be regarded as a chitin-like

glycan (Kapaun & Reisser, 1995). Depending on the component(s) of interest

(intracellular location, MW, solubility in aqueous buffers or organic solvent) and end

application, different strategies for extraction of intracellular products have been used.

1.1.5.1 Extraction of lutein and other pigments from Chlorella

Lutein along with other pigment such as chlorophyll a, chlorophyll b, and β-carotene

have extracted from Chlorella with the help of various solvents such as ethanol,

dichloromethane, ethyl acetate and methanol. Extraction conditions along with the

pigment yield and productivity have been summarized in Table 1.1.

Table 1.1 Current pigments extraction process from Chlorella.

Microalgae, biomass pretreatment	Cell disruption, extraction and purification	Main compound yield and productivity	Reference
C. vulgaris spray-drying	Cell disruption: none	Lutein 2.5 mg/g DW[+]. Other compound: chlorophyll 8.5 mg/DW, β-carotene 0.02 mg/g DW	Kitada et al. (2009)
	Extraction: supercritical CO_2 extraction at 2.5 ml/min with 7.5 % ethanol as entrainer, 30 MPa, 60 °C		
C. vulgaris spray-drying	Cell disruption: none	90-98 % lutein purity, 85-91 % lutein yield	Li et al. (2002)
	Extraction: treatment with 10 M KOH with 2.5 % ascorbic acid, 60 °C for 10 mins, addition of 50 ml dichloromethane, multiple extractions till biomass was colorless		
	Purification: 30 % ethanol wash to remove polar components, vacuum evaporation to dry lutein in organic phase, re-dissolving in 85 % ethanol, fat soluble impurities then removed by hexane extraction, heavy phase diluted to 8.5 % ethanol to precipitate pure lutein		
C. vulgaris, wet biomass storage at - 80°C	Cell disruption & extraction: Viscozyme treatment (2 h, enzyme concentration 1.23% (v/w), pH 4.5, temperature 50°C) followed by ultrasound extraction (35 kHz, 56.58 W/cm^2), 37.7 °C, 162 min, 1:30 biomass to ethanol ratio	Lutein yield: 3.36 mg/g wet biomass	Deenu et al. (2013)

Table 1.1 Continued.

Microalgae, biomass pretreatment	Cell disruption, extraction and purification	Main compound yield and productivity	Reference
	Purification : 6 % KOH at 50 °C for 30 mins, followed by liquid-liquid extraction with ethyl acetate and silica gel column		
C. zofingiensis, wet biomass	Cell disruption & extraction: mortar and alumina and multiple acetone extractions, evaporation and dissolving in ethyl acetate (2:1 v/v) followed by treatment with 1:1 KOH in methanol (2% w/v) for 15 min at 0°C in darkness, reaction stopped by adding (1:2) 10 % NaCl	Lutein yield: 4 mg/g DW; Lutein productivity: 7.2 mg/L/day. Other major compounds: asthaxanthin 1.5 mg/g DW	Del Campo et al. (2004)
C. protothecoides, wet biomass	Cell disruption and extraction method 1: biomass treated with methanol + dichloromethane (3:1) at dry biomass solvent ratio of 1:100 and ground at 24000 rpm using dispersing homogenizer. Procedure repeated twice Cell disruption and extraction method 2: 0.08 g biomass (DW) treated with 10 N KOH + 2.5 % ascorbic acid, 60 °C, 10 mins followed by addition of 9 ml mixture of methanol + dichloromethane (3:1)	Method 1 removes 98 % lutein compared to method 2. Method 1 extract has other pigments like chlorophyll a & b, β-carotene, while method 2 doesn't have significant chlorophyll	Shi and Chen (1999)

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Table 1.1 Continued.

Microalgae, biomass pretreatment	Cell disruption, extraction and purification	Main compound yield and productivity	Reference
C. vulgaris, , wet biomass	Cell disruption: microalgal suspension (10^9 cells/ml) for pulsed electric treatment (PEF) at 20 kV/cm, for 150 µs in citrate-phosphate McIlvaine buffer (1 mS/cm; pH 7) Extraction: 1ml of 96 % ethanol to 100 µl of PEF treated cells	Carotenoids: 1.1 mg/g DW and co-extraction of 2.8 mg/g chlorophyll	Luengo et al. (2014)
C. vulgaris, freeze drying after mechanical disruption	Cell disruption: bead milling with 0.3–0.5 mm Y_2O_3-stabilized ZrO2 grinding beads, with 1:13 solid water ratio (w/v). Extraction: supercritical CO_2 extraction with flor rate of 30 g/min, pressure of 350 bar, for 3 h at 60° C	Carotenoids yield: 2 mg/g DW and chlorophyll yield of 4 mg/g DW	Safi et al. (2014a)

1.1.5.2 Extraction and processing of proteins from Chlorella

Protein extraction from Chlorella has been carried out using various methods such as alkali treatment, enzyme hydrolysis and mechanical methods such as high pressure homogenization, sonication and bead-milling. There extraction conditions along with protein yields and the properties of protein products have been summarized in Table 1.2. Protein concentrates and hydrolysates from algae or plants despite having good nutritional values, have a number of drawbacks, such as having dark green color and strong grassy flavor, which limit their acceptability. The color is mainly due to plant pigments (chlorophyll and carotenoids). The origin of the grassy taste is linked to specific lipid compounds, usually those formed by oxidative degradation of unsaturated fatty acids. One important finding regarding removal of the green color is that it is difficult to obtain a good degree of discoloration using dried plant or algal protein concentrates, hence extraction has to be carried out on wet protein cake (Fiorentini & Galoppini, 1983). Removal of pigments can increase efficiency of processing of algal protein products. For example, depigmentation of *Scenedesmus* biomass with ethanol resulted in five fold enhancement in the yield of protein hydrolysates (Tchorbanov & Bozhkova, 1988). Therefore, for their acceptability and ease of processing, it is important that removal of pigments from algal proteins is accomplished.

Table 1.2 Current protein extraction methods from Chlorella.

Microalgae, biomass pretreatment	Cell disruption, extraction and purification	Protein yield and properties	Reference
*C. vulgaris, l*yophilization	Cell disruption & extraction: 0.5 dry biomass mixed with 25 ml DI water and disruption carried out using (i) high-pressure homogenizer (HPH) 2700 bar 2 passes, (ii) ultrasonication for 30 mins and (iii) chemical treatment with RO water at pH 12 for 2 h at 40 °C	Protein release of 0.25 g/g DW with HPH, 0.15 g/g DW with chemical treatment and 0.09g/g DW with sonication. Total protein 0.49 g/g DW* Total protein content: 51–58 g/d DW*[a] . Protein productivities 1.97-3.24 ton /hectare/month*[b]; 0.75 g/L/day*[c]	Safi et al. (2014b)
C. vulgaris, wet biomass stored at 4°C for 1 day	Pulsed electric field at specific energy input of 0.55-1.11 kWh kg_{DW}^{-1}, temperature between 25-65 °C	A maximum of 5% protein release achieved. Other biomolecules release were carbohydrates (39%) and small ionic solutes (75%)	Postma et al. (2016)

Table 1.2 Continued.

Microalgae, biomass pretreatment	Cell disruption, extraction and purification	Protein yield and properties	Reference
Chlorella pyrenoidosa, oven dried	i. Cell disruption by ultrasonication, separated protein from lipids and carbohydrates using a three-phase partitioning system formed by mixing biomass with t-butanol and ammonium sulfate. Carezyme™ and Stargen™ which hydrolyze starches to glucose for the increase protein concentration in the middle phase. ii. Middle phase containing proteins was diafiltered to remove salt and precipitated protein by changing pH to 5. iii. Best conditions were with the combination of both commercial enzyme preparations (Stargen™ and Carezyme™) as observed when all of the parameters of TPP were maintained constant such as solvent (t-butanol), ammonium sulphate concentration (40 % w/v), solid load (0.75 g/20 mL), pH (6), incubation time (20 min) and slurry to butanol ratio (1:1.5).	Optimum protein concentration in middle protein concentrate phase of 78.1 % w/w The protein concentrate displayed excellent foaming capacity and, high essential amino acid index compared to soy protein but a slightly lower thermal denaturation temperature	Waghmare et al. (2016)

Table 1.2 Continued.

Microalgae, biomass pretreatment	Cell disruption, extraction and purification	Protein yield and properties	Reference
C. vulgaris frozen paste	i. Cell disruption by high-pressure homogenization (2.7 kbar for 2 passes) at pH 12. ii. Protein concentration by isoelectric precipitation and membrane filtration (300 kDa)	Protein release of 0.5 g/g DW achieved. Concentrated protein fractions displayed good amino acid profile and emulsification properties comparable to commercial ingredients,	Ursu et al. (2014)
C. vulgaris, spray dried	i. Algal slurry (10% dry matter) was carried out using DynoMill apparatus for 3 min followed by spray-drying of algal slurry. ii. The dried algal powder was further treated with ethanol (2.5% w/v) at 45°C for 3h via gentle agitation to remove pigments. Various enzymes were used to create protein hydrolystaes, they were – pancreatin, pepsin and papain, trypsin and beomelain. iii. The hydrolysis was carried out for 4 h at 20 U/g, algae concentration of 10%, temperature of 37°C and the pH considered as optimum for enzymes.	Pancreatin and papain were the most effective with a 52% protein hydrolysates yield at an algal slurry concentration of 15% (w/v).	(Morris et al., 2008)

1.1.6 Pulsed electric field treatment

Pulsed electric field (PEF) technology consist of application of electric treatment for a short time (nanoseconds to microseconds) with an electric field strength ranging from 0.3-80 kV/cm. It is used in the food industry as an alternative to traditional thermal processing methods to deactivate pathogenic microorganisms and quality-associated enzymes, with the benefit of minimal modification of sensorial and nutritional attributes of liquid food products (Barba et al., 2015). PEF has also been used for cell hybridization and electrofusion in the field of genetic engineering and biotechnology (Chang, 1991). The theory of PEF and its application in extraction of intracellular products from algae is briefed in the following sections.

1.1.6.1 Theory

Pulsed electric filed (PEF) or electroporation is a phenomenon that causes temporary or permanent pores in the cell membrane of biological cells due to the application of a high-intensity electric field (kV/cm) in the form of very short pulses (µs or ms). When electric fields are applied to biological cells, it causes build-up of electrical charges at the cell membrane (Schoenbach et al., 1997). Membrane disruption occurs when the induced membrane potential exceeds a critical value of 1 V in various cellular systems, for example around 10 kV/cm for *E. coli* (Castro et al., 1993) and a range of 1-2 kV/cm for plant cells (Martín-Belloso & Soliva-Fortuny, 2011). One of the most studied theory to explain the effect of PEF on cells is known at the electrical breakdown (Zimmermann et al., 1974). It considers the cell membrane as a capacitor occupied by dielectric

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material. A build-up of naturally occurring transmembrane potential occurs due to the accumulation of charges with opposite polarity on either side of the membrane (Zimmermann, 1986). Once an external electric field is applied, stronger polarization of membrane is induced, which results in an increase in the transmembrane potential which then leads to reduction in cell membrane thickness. When a critical value is exceeded, membrane breakdown occurs. An assumption is made that this breakdown causes the development of transmembrane pores which are filled with conductive solution, thus enabling the electrical discharge at the membrane and speeding up membrane decomposition. Irreversible membrane breakdown occurs when the amount and size of the pores is big enough with respect to total membrane surface. The mechanism that elucidates the formation of pores and their stabilization to permit transport phenomena between extra- and intracellular media is not well defined. Kinosita Jr and Tsong (1977) suggested a two-step mechanism for pore formation in which the initial perforation occurs because of an electrical suprathreshold potential which is followed by a time-dependent expansion of pore size. According to this theory, larger pores can be obtained by increasing the electric field intensity and also the duration of pulses or by reducing the ionic strength of the medium. Once a voltage exceeds a threshold value, the diameter of the pores can increase to a point where normal attractive forces cannot reseal the pore even after the removal of the electric field. In biological cells, application of pulsed electric field can cause re-arrangement of the lipid bilayer causing the formation of either temporary or permanent pores which can aid in transport of molecules in and out of cell (Pavlin et al., 2008; Pliquett et al., 2007).

1.1.6.2 Application of PEF for permeabilization on microalgal cells

PEF has been used to facilitate the extraction of various products from microalgae. A PEF treatment of 35 kV/cm (0.5 kWh/kg) and a pulse duration of 1 µs carried out on *Auxenochlorella protothecoides* before freeze-drying of cells caused irreversible permeabilization and could increase the lipid extractability by 70% (Eing et al., 2013). In a study undertaken by Coustets et al. (2013) a continuous process for application of PEF on Chlorella and Nannochloropsis cells was assessed for its ability to impart permeability and ease extraction of cytosolic proteins from microalgal cells. The electric field strength was 6 kV/cm for a duration of 2 ms. The number of bipolar pulses delivered to each cell while travelling in the treatment chamber were 15. For both Nannochloropsis and Chlorella, significantly more release of proteins was observed after PEF than in the control. Postma et al. (2016) evaluated the use of bench-scale continuous flow PEF to selectively release carbohydrates and proteins from *C. vulgaris* at a flow rate of 33 mL/min, at temperatures ranging from 25-65 °C. The applied voltage was 20 kV/cm for a total of 5 µs which corresponded to an energy consumption of 0.55-1.1 kWh/kg of biomass. After PEF treatment, algal cells were mixed mildly at room temperature for 1 hour to allow the diffusion of intracellular compounds. The maximum carbohydrates yield was 4 % and that for proteins was just 4% by the synergetic effect of PEF and high temperature (55-65 °C). Bead-beating was required for additional protein and carbohydrates yield (45 and 58%, respectively). The native-PAGE profile of proteins released after PEF treatment showed that most of them were small molecular weight proteins (< 5kDa). Luengo et al. (2014) studied the effectiveness of PEF on

pigment extraction carotenoids and chlorophyll from wet *C. vulgaris* biomass. They also

checked the effect of PEF at varying electric field strengths on irreversible permeability

of cells by uptake of Propidium Iodide (PI) dye. A minimum electric field strength of 20

kV/cm and a total treatment time of 6 µs was required to cause irreversible permeation

in *C. vulgaris* cells, and application of PEF below this field strength caused reversible

permeation. After PEF treatment at 20 kV/cm for 75 µs, extraction yield for carotenoids,

and chlorophylls a and b increased 1.2, 1.6, and 2.1 times, respectively. A high

correlation was observed between irreversible electroporation and percentage of pigment

yield increase when the extraction was conducted after 1h of the application of PEF

treatment (R: 0.93).

1.1.7 Concept of algal biorefinery

The algal biorefinery concept is based on the ability of different technologies to convert

algal biomass to value added products, biofuels and chemicals. This concept is derived

from the petroleum refinery where multiple products and fuels with applications in

various industries are produced from petroleum. This maximizes the value derived from

the feedstock. Many research groups have discussed the concept, need, economics and

benefits of algal biorefinery (Cheali et al., 2015; Dong et al., 2016; Gebreslassie et al.,

2013; Subhadra, 2011; Uggetti et al., 2014).

However, there are only a few publications that have experimentally attempted the

selective or separate extraction of multiple products from algae. Kumar et al. (2013)

were able to extract agar from alga *Graciliaria verrucosa*, and produce bioethanol from

residue after agar extraction. The mass balance evaluation of the complete process demonstrated that developing a biorefinery approach for using microalgae could be commercially viable. Selective extraction of intracellular components by PEF treatment on *C. vulgaris* cells was attempted by Postma et al. (2016). With the synergistic effect of PEF treatment and heat (55 °C), small intracellular molecules such as carbohydrates could be selectively released (39%), with larger molecules such as proteins staying inside the cell (> 95 %), available to be released separately by complete disruption methods such as bead-milling. An integrated algal biorefinery process was successfully demonstrated by Dong et al. (2016). Their combined algal processing (CAP) used algal slurry after acid pretreatment to produce ethanol. Almost all fermentable sugars were utilized for ethanol production. After ethanol removal, effective lipid extraction (87% FAME) was achieved from algal stillage by application of hexane and distillation of free fatty acids. After a techno-economic analysis, they concluded that CAP could reduce microalgal biofuel cost by 9%. Sari et al. (2016) were able to successfully use de-oiled *Chlorella fusca* biomass to achieve up to 75% protein extraction yield with the help of 1-5% protease mix and 30% protein yield with alkaline extraction. Because of the low initial cost (18 fold lesser) of de-oiled microalgae compared to fresh biomass, working with de-oiled *C. fusca* proved to be much more economical (9-14 fold lower) for protein products.

The commercial potential of algae is tremendous. It encompasses a diverse spectrum of products such as lipids, proteins, carbohydrates and carotenoids. However, various lifecycle and techno-economic analyses have determined that for the sustainability and

marketability of algal production platform, it is vital that multiple high-value products be selectively extracted (Guedes et al., 2011; Perez-Garcia & Bashan, 2015; Safi et al., 2014c)

1.2 Recombinant protein production from algae

Protein therapeutics which encompass vaccines, single chain antibody fragments, immunotoxins and cytokines are currently being produced either by mammalian cells, which is expensive because of intricate growth and nutritional requirements and specialized bioreactor design or in *E.coli* which is inefficient in post-translational modification of proteins. Other systems like plants, yeast and insect cells also have their share of limitations, which hinder their commercial use (Dove, 2002; Mayfield et al., 2007). Eukaryotic microalgae, *Chlamydomonas reinhardtii* which has simple growth requirements, scalable production and rapid growth offers an attractive alternative for production of therapeutic recombinant proteins. Successful expression and production of complex large dimeric functional immunotoxin , single chain antibody fragment, and malaria vaccine have been accomplished in *C. reinhardtii* (Mayfield et al., 2007; Tran et al., 2013). However, the commercialization of proteins expressed in microalgae can only be possible if they can be purified with scalable and inexpensive methods

1.2.1 Utilization of microalgae as a production platform for recombinant therapeutic proteins

The US Food and Drug Administration has approved more than 130 therapeutic proteins which include enzymes, cytokines, vaccines and antibodies in the span of 25 years,

which has led to successful treatment of diseases such as hepatitis, diabetes etc. These proteins are produced in various heterologous expression systems which have their unique advantages and disadvantages in the aspects of protein expression , ease of manipulation and operation yield (Dove, 2002). Currently the most popular expression systems for monoclonal antibodies and complex proteins are transgenic mammalian cells. Low expression titers (except for monoclonal antibodies), expensive operation, need for specialized bioreactors, along with complex nutritional growth requirements are a few of the limitation for recombinant protein production from mammalian cells (Adamson, 1998). Bacterial systems like *E. coli* although efficient for economic production of recombinant proteins, can't be used to produce properly folded proteins, such as soluble proteins with correctly formed disulfide bonds (Baneyx & Mujacic, 2004). Transgenic plants such as tobacco, which are free from human pathogens and capable of post-translational modifications are also being currently evaluated for the production of recombinant proteins. However, factors such as regulatory uncertainty and longer development times are few of the drawbacks for this system (Twyman, 2005). Recent studies have demonstrated that microalgae can be used as a factory of recombinant protein production (Mayfield et al., 2007). Eukaryotic algae are Generally Recognized as Safe (GRAS) and possess several advantages over other systems: (i) cost reduction (ii) faster doubling times translating to quicker growth (iii) not susceptible to viral contamination that can prove harmful to humans (iv) can fold complex human proteins as they have chaperons and cellular machinery (v) *C. reinhardtii* can be grown photo-synthetically or as acetate as the carbon source (vi) all three genomes in *C.*

reinhardtii (nuclear, mitochondrial and chloroplast) can be transformed. Over 40 proteins have been successfully expressed in the chloroplast of *C. reinhardtii* by the Mayfield lab. A few of the reasons of using the chloroplast for expression of proteins are (i) it can be transformed with multiple genes in a single event, due to the availability of multiple insertion sites and also because of the ability to process polycistronic transcript, which allows the entire gene cassette to be regulated by a single promoter (ii) high levels of recombinant proteins can be accumulated in the chloroplast as it possesses minimum gene silencing mechanisms in the plastids and (iii) tools that allow genetic manipulation of chloroplast and over-express proteins already exists.

1.2.2 Single chain antibody fragment

Single chain antibody fragments (scFv) play a major role in the development of therapeutics and diagnostics and account for 35% of antibody fragments in clinical trials (Nelson & Reichert, 2009). A scFV fragment is made up of the smallest functional antigen-binding domain of an antibody (Figure 1.4) and a flexible peptide linker joins the variable heavy (VH) and variable light (VL) chains (Maynard & Georgiou, 2000). The linker (3.5 nm) will usually have hydrophilic residues with stretches of Serine and Glycine for flexibility (Alfthan et al., 1995). scFv fragments have several advantages over full length monoclonal antibodies (mAb) which are (i) enhanced pharmacokinetic properties due to improved tissue penetration and rapid blood clearance and (ii) low immunogenicity because of the absence of Fc region. There are also some disadvantages in using scFvs as therapeutics. Instability due to its small size which

thwarts its accumulation at the target site. Another disadvantage arises due to the lack of

Fc-mediated cytotoxicity which puts a limit on the therapeutic potential of scFvs.

Usually conjugation of scFvs to drugs and toxin is necessary for their effectiveness in

therapies (Monnier et al., 2013). One of the many application of scFvs is to construct

specific immunotoxin molecules (Liu et al., 2012). The Mayfield group at UC San Diego

has successfully demonstrated that *C. reinhartdii* chloroplast can produce and

accumulate full length immunotoxin as cancer therapeutics. One of the immunotoxin

was produced by genetically fusing scFv that recognizes the CD22 antigen on B-cells

leukemia and lymphomas, with a eukaryotic toxin (a truncated variant of *Pseudomonas

aeruginosa* exotoxin A ETA). They were successful in demonstrating the production of

scFv, monomeric and dimeric active immunotoxins from *C. reinhartdii* which bound

specifically to B-cells showing the CD22 molecule and causing them to undergo

apoptosis (Tran et al., 2013).
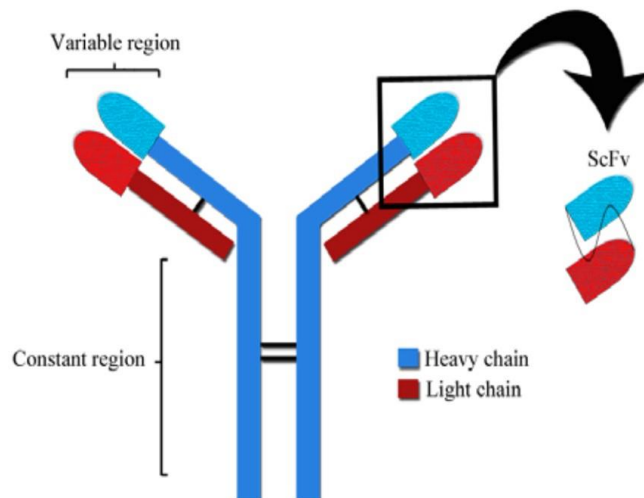
Figure 1.4 Diagrammatic representation of full-length monoclonal antibody (mAb) and single chain variable domain fragment (scFv).

1.2.3 Primary recovery methods

For intracellular protein extraction, cell lysis is usually followed by removal of cell debris by centrifugation or filtration. This cell-free extract contains several impurities such as host cell proteins, DNA and chlorophyll. It is desirable to remove or reduce these impurities before moving on to the use of expensive chromatographic resin. Reduction of impurities can lead to increase in longevity of the chromatographic column (less column fouling). Various precipitation methods have been used for pre-treatment of extracts.

Isoelectric point for a protein (pI) is the pH of the solution where the net charge on the protein reaches zero. When the overall charge nears zero, electrostatic repulsion is minimized, resulting in aggregation primarily due hydrophobic interactions This is

called as isoelectric precipitation (Figure 1.5) and for this particular dissertation acid

precipitation (most algal proteins are acidic in nature). Selective precipitation can be

achieved when separating proteins with different pI. Acid precipitation has been used for

the removal of pigments and phenolics from *Lemna* extracts containing IgG (Barros et

al., 2011; Woodard et al., 2009), reduction of phytic acid concentration in rice seed

extracts containing human lysozyme (Wilken & Nikolov, 2010), and decreasing the

amount of RuBisCo and green pigments of tobacco leaves extracts containing IgG

(Vézina et al., 2009).
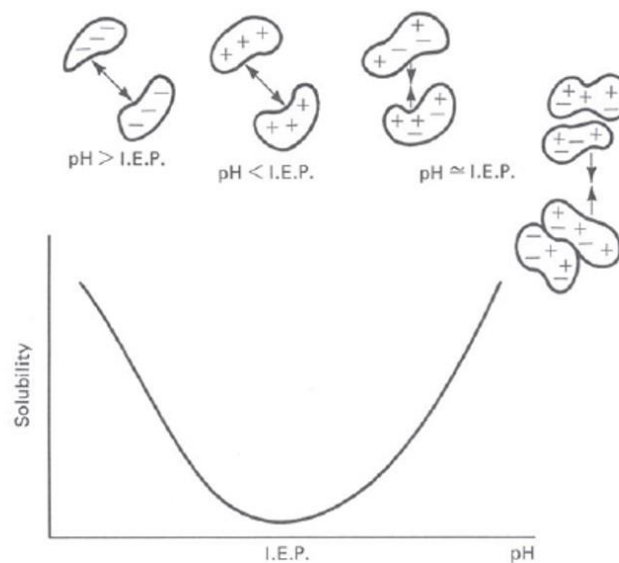
Figure 1.5 Mechanism of isoelectric precipitation shown by the effect on solubility of
proteins by changing pH. Reprinted from Scopes, 2013

A polymer which is usually a natural polyelectrolyte which becomes charged when dissociated in an aqueous solution. The charged polymer is electrostatically attached to the oppositely charged surface of the protein creating a bridging effect (Figure 1.6). Factors such as the charge, molecular weight, concentration, ionic strength have an effect on polymer precipitation of proteins (Scopes, 2013). Cationic polymers are more efficient at the flocculation of cells but neutral and anionic polymers are often not as effective (Kumar et al., 2003). Polyethyleneimine (PEI) was effective in the removal of large quantities of native tobacco impurities and at the same time high recovery of the recombinant acidic protein was achieved from tobacco (Holler et al., 2007). Another popular flocculant used in clarification of extracts is chitosan which is a cationic linear polymer of β-(1–4) linked D-glucosamine monomers generated by the chemical deacetylation of chitin. It is inexpensive, produced from non-mammalian sources and available in purified form that is low in microbial materials, volatile organics and heavy metals. Chitosan has been used in defatting of protein hydrolysates (Novikov & Mukhin, 2001), removal of nucleic acids (Hashimoto et al., 1989), and for the flocculation of algae (Garzon-Sanabria et al., 2012), yeast (Weir et al., 1993) and bacteria (Hughes et al., 1990).
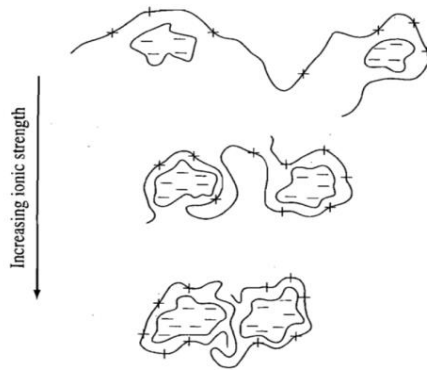
Figure 1.6 Mechanism of polymer precipitation. Reprinted from Scopes, 2013

1.**3 Objectives**

The overall objective of the dissertation was to develop bioprocesses designed to obtain high-value products from microalgae. Two major topics covered in this dissertation are "selective extraction of pigments and proteins from *Chlorella vulgaris*" and "evaluation of pre-treatment methods for primary recovery and capture of an antibody fragment from *C. reinhartdii*"

1.3.1 Selective extraction of pigments and proteins from Chlorella vulgaris

Microalgae are photosynthetic microorganisms and are rich sources of lipids, proteins, carbohydrates, and high-value compounds such as pigments, anti-oxidants and vitamins (Pulz & Gross, 2004). Previous research in the field of microalgae has been focused on extraction of just a single product (Bleakley & Hayes, 2017; Chen et al., 2013; Poojary et al., 2016; Ranjith Kumar et al., 2015). However, various techno-economic analyses

conducted in the past 5 years have determined that unless multiple products can be extracted from biomass, the algal production platform cannot be sustainable (Perez-Garcia & Bashan, 2015; Ruiz et al., 2016; Sari et al., 2016). Therefore, there's a need for focusing on developing or optimizing process that can selectively extract co-products from algal biomass. Since the focus of this segment of research was to develop products which have a potential in the food and nutraceutical market, the overall goal was to develop a process for selective extraction pigments and functional proteins from *C. vulgaris.*

a. Compare high-pressure homogenization, bead-milling and sonication for their effectiveness in protein extraction from wet, frozen *C. vulgaris* and determine the best method amongst the three.

Hypothesis: High-pressure homogenization will be the best method for protein extraction because of higher biomass processing rates.

b. Evaluate ethanol as a solvent for its effectiveness to extract pigments from wet, frozen *C. vulgaris* biomass and optimize solvent ratio and number of stages required.

Hypothesis: Since ethanol is a polar, water miscible solvent, it will be effective in extraction of pigments from wet biomass.

c. Integrate pigment and protein extraction process and determine the impact of pigment removal on extractability, membrane processing, nutritive value and functionality of protein fractions.

Hypothesis: Pigment extraction by ethanol will lead to denaturation or precipitation of proteins which will require alkaline conditions for complete protein solubility.

d. Evaluate the effectiveness of PEF for lutein, chlorophylls and protein extraction from fresh *C. vulgaris*.

Hypothesis: PEF will be effective in creating pores in the cell membrane and thus enhance permeability as well as lutein, chlorophylls and protein release from fresh *C. vulgaris.*

e. Evaluate the impact of handling of cells (storage at 4°C and-80°C or freeze-drying) on extraction of pigments and proteins.

Hypothesis: The process of freeze-drying will impart permeability and thus enhance pigment and protein release.

1.3.2 Evaluation of pre-treatment methods for primary recovery and capture of an antibody fragment

Using the lessons learned from transgenic plants as bioreactors for recombinant protein production, there have been improvements in microalgae biotechnology in the past 15 years (Wilken & Nikolov, 2012). Extraction of intracellular recombinant proteins from microalgae is usually accompanied by release of impurities such cell debris, nucleic acids, chlorophyll and host cell proteins. One of the major roadblocks in the purification of recombinant proteins is removal of impurities before loading the extract onto the first chromatography column. Therefore, there's a need to evaluate pre-treatment methods

that are effective in removal of host cell impurities from algal extracts. The specific objectives for this segment of research were as follows:

a. Evaluate chitosan precipitation as a pretreatment method to reduce impurities like host cell proteins, nucleic acids and chlorophyll.

   Hypothesis: Due to the cationic nature of chitosan, it will be effective in removal of acidic host cell proteins, chlorophyll (which is associated with proteins) and negatively charged DNA.

b. Compare chitosan to acid precipitation in terms of impurity removal.

   Hypothesis: Since acid precipitation will be undertaken at an isoelectric point of majority of algal proteins, it will be effectively in reduction of host cell proteins and associated chlorophyll, although it won't be as efficient as chitosan for DNA removal.

c. Determine if the chromatography capture step improves purity of target protein.

   Hypothesis: Due the charge of target protein, the chromatography (anion exchange) capture step will be effective in separating the single chain antibody fragment from host cell proteins.

CHAPTER 2

PROCESS FOR SELECTIVE EXTRACTION OF PIGMENTS AND FUNCTIONAL PROTEINS FROM *CHLORELLA VULGARIS*

2.1 Overview

Extraction of multiple high-value products is recommended for sustainability of the microalgal production platform. This study proposes a process for selective extraction of carotenoids and chlorophylls with ethanol, followed by alkaline pH extraction of proteins from wet *Chlorella vulgaris* biomass. A biomass-to-solvent ratio of 1:5 and 3 extraction stages were required to achieve maximum extraction yield of chlorophylls and carotenoids. The main compounds in the ethanol extract were identified as lutein, chlorophyll a and chlorophyll b. The lutein and total chlorophyll yield in the extracts were 5.4 mg/g and 15.4 mg/g dry biomass, respectively. Effective protein release from wet biomass was contact-time dependent and more than 76% of total protein could be extracted in 15 minutes via bead-milling, and in 6 minutes by high-pressure homogenization at 15000 psi. Ethanol extraction of pigments affected protein solubility, and an alkaline pH was required to release the same total protein content. Concentration and fractionation of proteins was carried out using a two-stage membrane filtration process and 78-80 % of proteins remained in the 300 kDa retentate. Ethanol treatment and higher pH conditions did not impact membrane filtration, nutritive value, nor the emulsification properties of protein concentrates.

2.2 Introduction

Microalgae are photosynthetic microorganisms and are rich sources of lipids, proteins, carbohydrates, and high-value compounds such as pigments, anti-oxidants and vitamins (Pulz & Gross, 2004). The various uses of microalgae for food, feed, and energy were identified decades ago (de la Noue & de Pauw, 1988). Numerous studies have been conducted focusing on enhancing biomass growth (Tandon & Jin, 2017), and optimizing processes to obtain a single product from microalgae like lipids or carbohydrates for biofuels production (Chen et al., 2013; Ranjith Kumar et al., 2015), proteins for food and feed (Bleakley & Hayes, 2017), and pigments for nutraceutical applications (Poojary et al., 2016). However, life-cycle and techno-economic analyses published in the past ten years indicate that the algal platform would not be sustainable unless multiple high-value products can be generated from algal biomass (Perez-Garcia & Bashan, 2015; Ruiz et al., 2016; Sari et al., 2016). Therefore, it is important to develop processes aimed at optimizing multiple product extraction which can maximize value of algal feedstock. Based on cell accumulation levels and current market value, proteins, chlorophylls, and carotenoids from microalgae hold promise as potential co-product candidates, especially if they could be selectively and inexpensively extracted.

The microalga *Chlorella vulgaris* is established as a good source of protein and carotenoids (Safi et al., 2014c). The protein content of *C. vulgaris* can amount to 58% of the cell dry weight (DW) (Safi et al., 2014c), and essential amino acid profile of extracted proteins compares well to the standards recommended by WHO/FAO (Becker,

2004; FAO & WHO, 1973). *C. vulgaris* protein fractions obtained after high-pressure homogenization and membrane filtration displayed emulsification properties comparable to soy protein isolate and sodium caseinate (Ursu et al., 2014). The lutein (carotenoid) content in Chlorella can reach concentrations as high as 7 mg per gram cell dry weight (Safafar et al., 2016). Dietary intake of lutein helps with preventing early atherosclerosis, decreasing the rate of age-related macular degeneration (Dwyer et al., 2001), and ameliorating the onset or progression of cataracts (Shao et al., 2008). Chlorophyll, a major pigment in plants and phototrophic algae like *Chlorella* (Safi et al., 2014c), when isolated as a co-product, could also provide a revenue stream. Cited chlorophyll health-benefits include immune system stimulation, blood and liver detoxification, and relief from sinusitis, fluid buildup, and skin rashes (Ferruzzi & Blakeslee, 2007).

To capture the potential health benefits and value of algal co-products, one has to evaluate the suitability of extraction methods related to their impact on product yield, quality, and overall production cost. Various cell disruption techniques such as sonication, high-pressure homogenization and bead-milling have been tested for protein extraction from *C. vulgaris* biomass (Safi et al., 2015; Safi et al., 2014b). Although the initial condition (dried, frozen, or wet) of tested algal biomass was not always clearly stated, bead-milling and high-pressure homogenization were more effective than sonication in releasing intracellular proteins by disrupting the rigid cell wall (Blumreisinger et al., 1983) of *Chlorella sp.*. The state of the harvested biomass (wet or frozen) and the subsequent drying method are also important variables to consider when comparing the effectiveness of cell disruption and protein extraction. First, freezing

and/or drying processes changed cell wall and membrane properties to a different degree compared to wet-harvested cells (Lin, 1985). Second, the extractability (solubility) of algal proteins could be substantially affected by heat-drying. And third, freezing or freeze-drying of algal biomass are not scalable methods, while heat-drying is an energy intensive process that would increase processing costs (Cha et al., 2009; Damergi et al., 2017; Luengo et al., 2014; Weschler et al., 2014).

Carotenoids and chlorophylls have previously been extracted from *Chorella sp.* biomass using organic solvents such as ethanol. Most of the reported processes utilized dried algal biomass as a source material (Cha et al., 2009; Damergi et al., 2017; Safi et al., 2014a) and, in some cases, high temperatures for pigment extraction (Cha et al., 2009; Damergi et al., 2017), which are not optimal conditions if recovery of protein co-products are desired. Thus, developing processing conditions that would allow the selective extraction of lutein and chlorophylls followed by functional and nutritive protein fraction would advance our understanding and assessment of a multiproduct *Chlorella vulgaris* platform. Although processing parameters for extracting either pigments or proteins have been assessed, an integrated process for selective extraction of these biomolecules has to be evaluated. Hence, the overall goal of this study was to develop optimal processing methods and conditions to permit selective co-extraction of pigments (lutein and chlorophylls) and quality protein from wet *C. vulgaris* biomass. The ancillary objectives of the experimental work were to determine (i) the best cell lysis method(s) for complete disruption and protein release from wet *C. vulgaris* biomass, (ii) the efficiency of solvent extraction to obtain a high-value pigment fraction

from *C. vulgaris* and (iii) the impact of pigment removal on extractability and functionality of protein fractions.

2.3 Methodology

2.3.1 Cell disruption methods for protein extraction

Frozen *C. vulgaris* biomass with 24% (w/w) solids content was supplied by Global Algae Innovations (HI, USA). Biomass was stored at -80 °C and thawed at room temperature for use. This freeze-thawed biomass will be referred to as wet biomass. Three cell lysis methods were investigated and compared: high pressure homogenization (Emulsiflex C3, Avestin), ultra-sonication (CL-188, Qsonica Sonicator), and bead milling (GenoGrinder 2000, SpexSamplePrep). High-pressure homogenization was performed at 15000 psi, bead milling was done using 0.5 mm glass beads at 1500 strokes/min, and sonication was performed using 30 s on/off intervals at 50% amplitude. Biomass was dispersed in RO (reverse osmosis) water at pH 7 with a wet biomass-to-water (w/v) ratio of 1:10. The varying volumetric capacity of cell lysis equipment determined the amount of processed cell suspension; 10 mL of suspension was used for ultra-sonication, 1 mL for bead beating and 100 mL for high-pressure homogenization. For cell lysis experiments at pH 12, cell suspensions were adjusted to pH 12 with 2 M NaOH. The temperature of cell lysates was maintained below 25 °C. Cell lysates were clarified by centrifugation for 10 minutes at 15000 x g, and supernatants analyzed for total soluble protein. After high-pressure homogenization at pH 7 and 12, the remaining

intact cells were counted using a hemocytometer (Bright Line, Hausser Scientific) and turbidity measurements were carried out at OD (optical density) of 750 nm.

2.3.2 Ethanol extraction of carotenoids and chlorophylls

Frozen *C. vulgaris* biomass was mixed with 95% ethanol at wet biomass-to-solvent (w/v) ratio of 1:3, 1:5 and 1:10, for 30 minutes at room temperature (22-25 °C). Ethanol was recovered by centrifugation at 7500 x g at 4 °C for 10 minutes and supernatant absorbance at 470 nm, 649 nm, and 664 nm were recorded. Ethanol extraction of the same biomass sample was repeated two more times - a total of 3 extraction stages. Chlorophyll and carotenoid concentrations in the supernatants were estimated using the following equations (Lichtenthaler, 1987):

Chlorophyll **a** (µg/mL) = $(13.36 \times A_{664}) - (5.19 \times A_{649})$ (1)

Chlorophyll **b** (µg/mL): $(27.43 \times A_{649}) - (8.12 \times A_{664})$ (2)

Total carotenoids (µg/mL): $(1{,}000 \times A_{470} - 2.13 \times \text{Chlorophyll } \mathbf{a} - 97.64 \times \text{Chlorophyll } \mathbf{b})$ /209   (3)

After the ethanol treatment, cells were lysed using high-pressure homogenization either at pH 7 or pH 12. The cell debris was removed by centrifugation at 15000 x g, 4 °C for 15 minutes and the supernatant analyzed for total protein content.

2.3.3 RP-HPLC analysis of ethanol extracts

Lutein, chlorophyll a and chlorophyll b standards were purchased from Sigma Aldrich. A Dionex HPLC (Thermo Fisher) system equipped with an ASI-100 automated sample

injector, PPA-100 photodiode array detector, and P680 HPLC pump was used for analysis of ethanol extracts. 20 µL aliquots were injected onto a RP-C-18 column (Thermo Scientific Acclaim TM 4.6 x 250 mm, 5 µm). A gradient elution of 0-40% (v/v) dichloromethane in methanol was started immediately after injection and was run for a total of 30 minutes. Absorbance was measured at 450 nm and 652 nm.

2.3.4 Concentration of protein extracts by tangential flow ultrafiltration

Clarified algal extracts were fractionated and concentrated using a two-stage tangential flow ultrafiltration. A 300 kDa molecular weight cut off (MWCO) hollow fiber module (Spectrum Laboratories) was used in the first stage. The 300 kDa permeate was concentrated by 3 kDa MWCO hollow fiber module in the second stage. Both modules were made of hydrophilic modified polyethersulfone (mPES) membrane that provides higher flux rates and low protein binding for better product yields. Protein retentates (300 kDa and 3 kDa) were first concentrated four times and then diafiltered with three volumes of RO water adjusted to either pH 7 or 12. Membrane filtration was conducted at a constant transmembrane pressure (TMP) of 7 psi. The permeate flux (measured as $L/m^2/h$ or LMH), normalized average flux (permeate flux/TMP) and protein concentrations in both membrane retentates and permeates were recorded for all samples. Membrane filtration was performed at the recommended Spectrum Labs shear rate of 2000 s^{-1}. Control protein concentrate samples (from algal biomass not treated with ethanol) were prepared by cells homogenization at pH 7 and pH 12 followed by a two-stage membrane processing as previously described. Protein concentrates and

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controls generated at each pH were used for emulsification studies. Representative samples of each protein concentrate were freeze-dried and submitted for amino acid analysis.

2.3.5 Protein, amino acid, lipids, and carbohydrates analyses

Total protein content in biomass was determined by a modification of the method proposed by Rausch (1981). Wet biomass aliquots (0.3 g) were mixed with 1 mL of 0.5 M NaOH and heated at 80 °C for 10 minutes. The suspension was then cooled to room temperature and centrifuged for 10 minutes at 15000 x g. The pellet was re-extracted under the same conditions as the first step. The pellet from the second step was mixed with 0.5 M NaOH, heated for 10 minutes at 100 °C, suspension cooled to room temperature, sonicated for 1 minute at 50% amplitude (CL-188, Qsonica Sonicator) and centrifuged at 15000 x g for 10 minutes. Supernatants from all three extraction steps were combined and total soluble protein determined using the Bradford assay (Bradford, 1976a). Bradford method for total soluble protein determination in extracts and processed fractions was conducted using a microplate protocol with Bovine serum albumin (BSA) as a standard (working range from 1 to 25 μg/mL and 25 to 1500 μg/mL) and Coomassie plus assay kit (Thermo Scientific). Absorption at 595 nm was measured using the VERSA max microplate reader (Molecular Devices, CA). The Protein Chemistry Lab at Texas A&M University performed the amino acid analysis of 300 kDa and 3 kDa protein concentrates. Freeze-dried protein concentrate samples were hydrolyzed at 110 °C for 20 hours along with the internal standards, Norvaline and

Sarcosine (Sigma Aldrich). Amino acids were derivatized and separated on a reversed-phase HPLC column (2.1 x 200 mm, Hypersil AA-ODS) from Thermo Fisher. Total lipids and carbohydrates in the protein concentrates were determined by the Bligh and Dyer (1959) and phenol-sulfuric acid (DuBois et al., 1956) methods, respectively.

2.3.6 Emulsification studies

Emulsifying activity and stability of samples were determined by the method of Yasumatsu et al. (1972). Emulsification studies of algal concentrates prepared at pH 7 were carried out without pH adjustments. Algal samples prepared at pH 12 were adjusted to pH 7 prior to emulsification experiments. Soy protein isolate (SPI, Now Sports) powder was dissolved in RO water at pH 12 and adjusted to pH 7 before emulsification experiments. Emulsification activity (EA) was determined by homogenizing pH 7.0 - adjusted protein samples with peanut oil. Protein samples (30 mL) at a concentration of 0.1% (w/v) were mixed with 30 mL of peanut oil in 150 mL plastic cylinders, homogenized at 9800 rpm for 2 minutes using a high shear mixer (Silverson L4RT), transferred to 15 mL plastic tubes and centrifuged at 3500 x g for 5 minutes (Fisher Scientific, Model 225). Emulsifying activity (EA) was calculated by equation 4 given below. Emulsion stability (ES) was determined by heating freshly-prepared emulsions for 30 minutes at 80 °C. The ES value was calculated by equation 5. To calculate protein accumulated in the emulsified layer, samples drawn from the bottom (aqueous) layer were centrifuged at 8000 x g for 5 minutes to remove any oil

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droplets remaining in the aqueous layer. Total protein (TP) measurements were carried out on the oil-free aqueous layer.

$$EA \ (\%) = \frac{height \ of \ the \ emulsifed \ layer}{total \ height \ of \ mixture} \ x \ 100 \qquad\qquad (4)$$

$$ES \ (\%) = \frac{height \ of \ the \ remaining \ layer}{total \ height \ of \ mixture} \ x \ 100 \qquad\qquad (5)$$

2.3.7 Statistical Analysis

One way analysis of variance (ANOVA) was conducted for statistical analysis of the experimental data using JMP Pro 12 (SAS) software. Either one or two-factor design were performed to optimize process parameters. All experiments were done in triplicates. To compare significant differences between treatments, a Tukey adjustment was made for a family wise error rate of 0.05.

2.4 Results and discussions

2.4.1 Effect of cell lysis method and pH on protein extraction

One of the objectives of this study was to determine the effect of cell disruption method and pH on protein extraction yield. Three methods of cell disruption were first compared for their effectiveness in releasing of soluble *C. vulgaris* protein at pH 7 and wet biomass-to-water (w/v) ratio of 1:10. (Figure 2.1). The results of high-pressure homogenization shown in Figure 2.1a indicate that maximum extraction yield of *C. vulgaris* protein expressed as percent of total protein in the extract (% TP) can be achieved with 3 passes as the 4th and 5th pass do not significantly increase the extraction

yield. The maximum amount of protein extracted by homogenization was 76% of the total protein content of *C. vulgaris* biomass. The maximum amount of protein released by high-pressure homogenization (76%) was used as benchmark for comparison of effectiveness of the other two methods. Bead-milling experiments were carried out by varying bead concentration and contact time (Figure 2.1b). The data in Figure 2.1b show that bead concentration had the greatest impact on protein release. A concentration of 0.8 g beads per mL of biomass suspension and 15 min of contact time released the same amount of protein (76%) as the high-pressure homogenization method. In the case of ultrasonication, TP increased steadily and reached a maximum value of 76% at 40 min contact time (Figure 2.1c). No statistical difference was found in protein release after 30 min of contact time. However, a large variation is observed at 30 min, so at least 35 min of sonication is recommended to ensure maximum protein release. The increasing trend in protein release with time (Figure 2.1b), indicates that bead milling contact time of more than 15 min would have resulted in a higher than 76% protein yield as reported by Safi et al. (2015); they measured about 80% protein release from *C. vulgaris* after 15 min and 96% after 40 min contact time. The homogenization data from the Safi group were similar to ours (66% protein extraction in 5 min or 2 passes) whereas the sonication protein yield (16% TP in 30 min) was distinctly lower than that achieved in this work (76%). A possible explanation for lower of sonication efficiency in the Safi study (Safi et al., 2015) is the difference in biomass properties as they used freeze-dried versus our use of wet biomass. Freeze- and spray-drying of algal cells typically results in the formation of cell clumps (aggregated algal cells) which, if not properly dispersed in

water before sonication, could affect protein extraction kinetics. Safi et al. (2015) also

noted that the mass transfer kinetics of algal protein into the aqueous phase was

significantly faster during homogenization than bead milling. In conclusion, in terms of

process scalability and productivity (amount of protein released per unit contact time),

ultrasonication (30 min) and bead milling (15 min) are clearly less effective methods

than homogenization (6 min contact time over 3 passes). Since the exact reason for

partial protein extractability (76%) for cells disrupted by homogenization is not quite

clear, we also tested the effect of alkaline pH on protein yield. Alkaline pH (pH >10) is

typically used for production of protein concentrates and isolates from oilseeds (Moure

et al., 2006), defatted and non-defatted *Nannochloropsis* sp. (Gerde et al., 2013), and has

also been applied to increase protein extractability from *C. vulgaris* (Ursu et al., 2014).
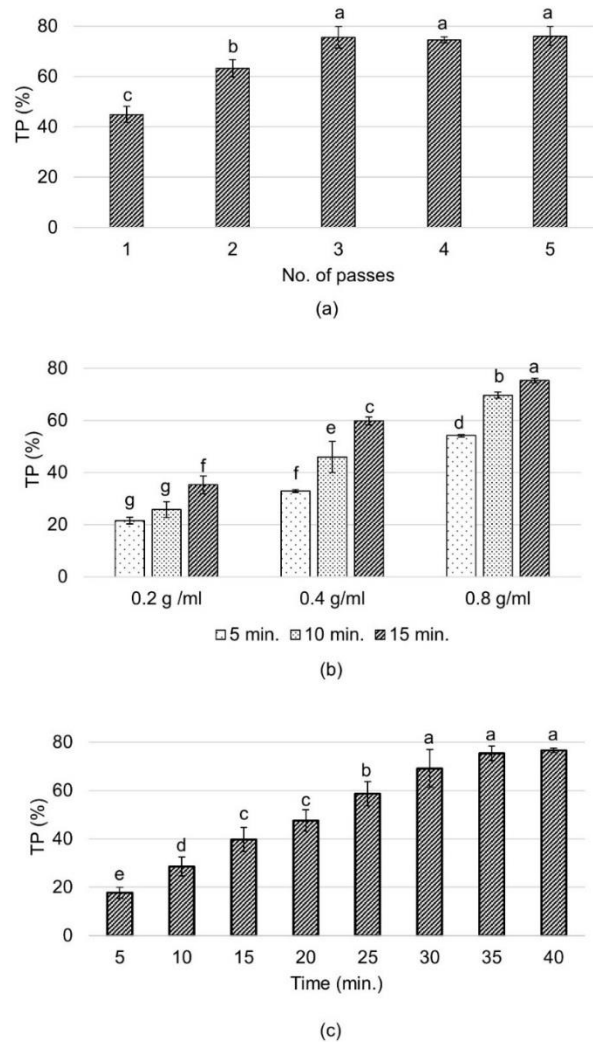
Figure 2.1 (a) Protein release using high-pressure homogenization at 15000 psi (b) bead-milling at various bead concentrations and time of treatment (c) ultrasonication at 50% output cycle with 30 s on/off cycles. 100% TP corresponds to 195 mg/g dry biomass. Within each treatment, levels (TP) not sharing the same letter are significantly different ($\alpha = 0.05$).

The effect of homogenization pH on protein release is compared in Figure 2.2a. The

main difference between the two conditions is a slightly faster release of algal protein at

pH 12 than pH 7. The maximal extractable protein value (% TP) at pH 12 is reached

after two passes compared to 3 passes at pH 7. The TP values at pH 12 and pH 7 after two and three passes, respectively, were not significantly different than to those reached after 5 passes (Figure 2.2a). Cell disruption (% cell count) data in Figure 2b show similar trend i.e. the number of cell that could be viewed and counted were consistently lower in pH 12 than pH 7 lysates (Figure 2.2b). Direct correlation between cell disruption and protein release is not possible because cells that are not fully lysed to cell debris level are sufficiently permeabilized to release the soluble protein. For example, unlysed cell count after 5 passes was about 10% at both pHs, 20% after 3 passes at pH 7, and 30% after 2 passes at pH 12, yet similar TP values were measured in the clarified lysates. Nevertheless, cell count was a useful method to monitor and assure batch-to-batch disruption efficiency and consistency. Turbidity is known to align closely with cell counting for *C. vulgaris* (Spiden et al., 2013). A linear relationship could be established between turbidity and cell count at pH 7 and 12 (Figure 2.2d) which was statistically significant ($p < 0.0001$). At 95 % confidence level, it was found that at pH 7, turbidity was between 6.8-12.7 % higher than the cell count. For cell lysis at pH 12, turbidity was between 4.7-9.9 % higher than cell count. Thus, turbidity can be used as a quick predictor of the extent of cell lysis.
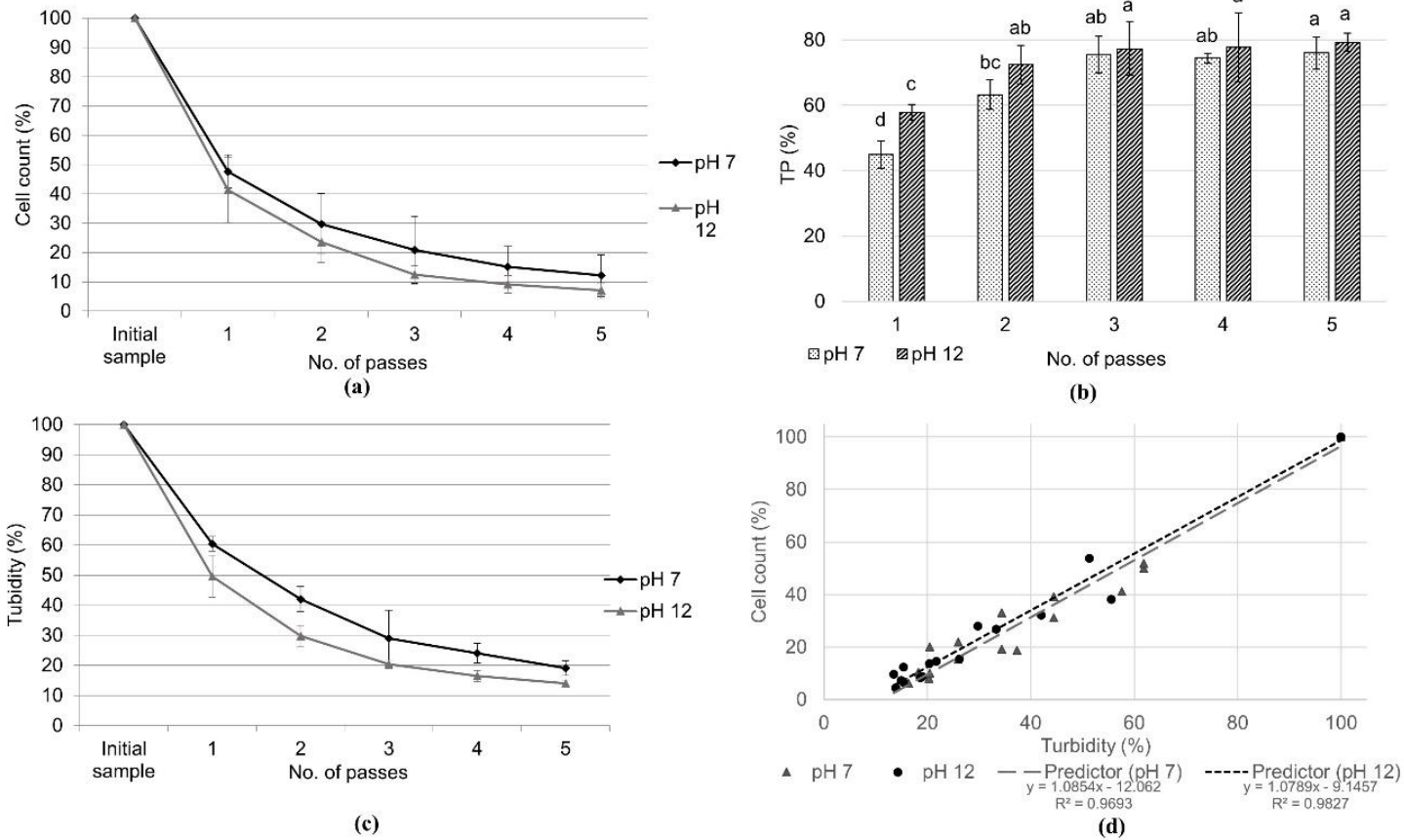
Figure 2.2 (a) Protein release at pH 7 and pH 12 during high-pressure homogenization; TP values not sharing the same letter are significantly different ($\alpha = 0.05$).(b) Effect of the number of passes through the homogenizer on the cell count. (c) Effect of number of passes in the high-pressure homogenizer on turbidity. (d) Linear relationship between the cell count and turbidity during high-pressure homogenization.

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Energy calculations even though not used directly in this study, can be extremely helpful for checking the feasibility of commercialization of algal derived products (Lee et al., 2012; Yap et al., 2015). Based on data acquired from high-pressure homogenization, an energy consumption of 87 MJ/kg of dry biomass would be required for release of TSP from *C. vulgaris*. Lee et al. (2012) calculated the energy requirement of high-pressure homogenization assisted cell disruption of *Chlorococcum sp*. undertaken by Halim et al. (2012) to be 529 MJ/kg of dry biomass. The energy estimate of 87 MJ/kg of dry biomass is significantly lower than that estimated by Lee et al. (2012) primarily because the starting density of the algal suspension used by us was 1,501,771 $\pm$ 216,705 cells/ mm^3 compared to that of 65,476 $\pm$ 24,227 cells/mm^3 used by Lee et al. (2012). In a separate experiment (data not shown) it was observed that the biomass: buffer dilution ratio could be decreased from 1:10 to 1:5 for high-pressure homogenizer to achieve similar protein release (3 passes at 15000 psi for complete TSP recovery) from the cells. This could reduce the energy requirement for release of complete TSP fraction to 43.5 MJ/kg of dry biomass. However, increasing the biomass density sometimes resulted in blockages/clogging during homogenization. If homogenization can be carried out at high-biomass densities, without any operational difficulties, energy consumption can be reduced significantly. For example, Yap et al. (2015) found indistinguishable differences in cell rupture between *Nannochloropsis sp*. slurry concentrations of 0.25% w/w, 2.5% w/w and 25% w/w solids. Their energy requirements calculated using a three-phase power meter at a homogenization pressure of 21,755 psi ranged from 160-1.6 MJ/kg dry algae for slurries containing 0.25-25 % w/w solids.

2.4.2 Carotenoid and chlorophyll extraction from Chlorella biomass dispersed in ethanol

The main reason for investigating ethanol extraction of *C. vulgaris* biomass was to establish the feasibility of extracting high-value co-products such as carotenoids and chlorophylls from algal biomass before lysing cells completely for protein release. Specific tasks were 1) to determine if thawed cell biomass with 24% (w/w) solids content would allow ethanol diffusion into and extract pigments (carotenoids and chlorophylls) from the chloroplast and 2) to identify and quantify the yield of extracted pigments (carotenoids and chlorophylls), and 3) to determine the impact of ethanol on subsequent protein extraction yield. The choice of ethanol as solvent was guided/driven by potential applications of extracted co-products in nutraceutical and food industries i.e. solvent GRAS status (Joana Gil-Chávez et al., 2013) and solvent compatibility (miscibility with water) with planned extraction of wet biomass. Ethanol extraction of wet *C. vulgaris* biomass was investigated using three biomass-to-ethanol ratios and three biomass extractions stages. The presence of chlorophylls and carotenoids in the aqueous ethanol phase (Figure 2.3) demonstrates that ethanol was able to reach the chloroplast and extract chloroplast pigments. Microscopic examination of the culture before and after ethanol extraction did not reveal any cell lysis that would had contributed to pigment release in the extract. Ethanol extracts also did not show the presence of

carryover protein by Bradford assay.
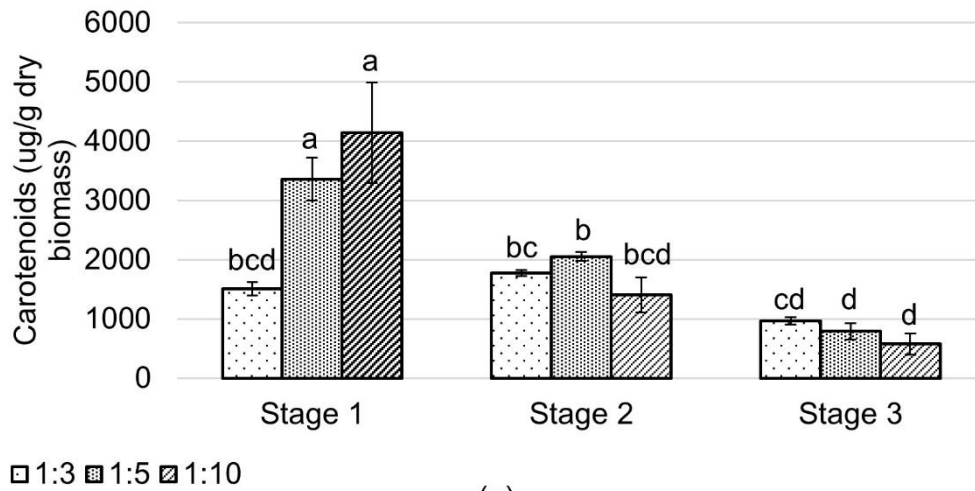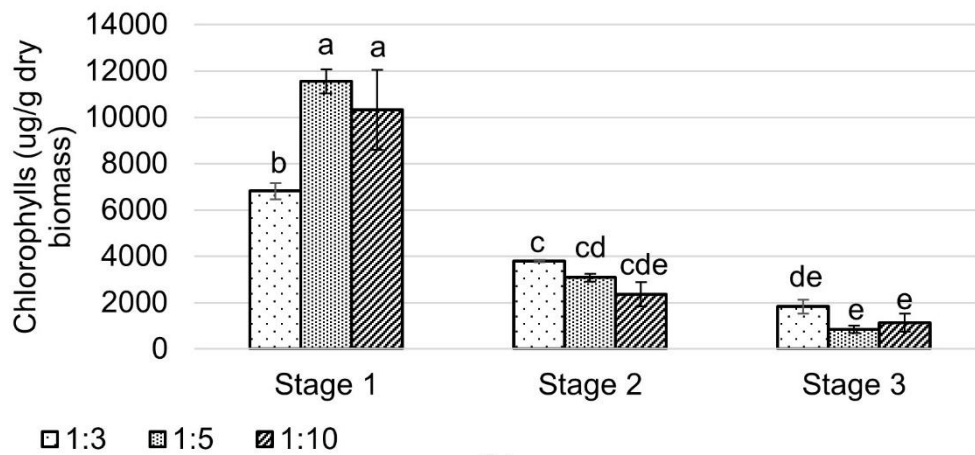
Figure 2.3 (a) Carotenoids extracted at different solvent to biomass ratios (b) Chlorophylls extracted at different solvent-to-biomass ratios. For each graph, carotenoid or chlorophyll values not sharing the same letter are significantly different ($\alpha= 0.05$).

The data in Figure 2.3a and 2.3b indicate that the ethanol phase at the 1:3 water:ethanol

ratio did not have a sufficient solubilization capacity for hydrophobic carotenoids and

chlorophylls in the first extraction stage compared with the 1:5 and 1:10 ratios. The higher water concentration in the ethanol phase at 1:3 ratio (than at 1:5 or 1:10) increases the solvent polarity resulting in lower solubility of both carotenoids and chlorophylls. Therefore, in contrast to the other two solvent-to-biomass ratios, the second extraction stage with 1:3 ratio contained significant amounts (41%) of extractable carotenoids. The third extraction stage of 1:5 and 1:10 extractions contribute less than 15% of total extractable carotenoids and chlorophylls. Therefore, to reduce the amount of solvent usage and biomass separations cost at each stage, we determined that 2 extraction stages at 1:5 solvent-to-biomass ratio is optimal.

The identity and composition of ethanol extracts was confirmed by RP-HPLC (Figure 2.4). Three primary compounds present in the ethanol extracts were identified as lutein (1), chlorophyll a (2), and chlorophyll b (3) (Figure 2.4). Peaks marked (a) on the chromatogram could not be identified. Based on the elution times (relative hydrophobicity), peak (b) and two (c) peaks are most likely other xanthophylls (Gouveia et al., 1996; Orosa et al., 2000). The presence of lutein as the most abundant carotenoid (88%) in *C. vulgaris* extracts is not surprising since several Chlorophycea species are known to contain higher lutein levels than other carotenoids (Cordero et al., 2011). Lutein is less hydrophobic that other carotenoids such as β-carotene (Braumann & Grimme, 1981), so it is expected that a polar solvent like ethanol would solubilize and extract lutein more effectively than other carotenoids. The lutein yield obtained in this study (5.4 mg/g dry biomass) is comparable to lutein yields (4.7-7.14 mg/g dry biomass) from *C. vulgaris* reported in the literature (Safafar et al., 2016; Shi et al., 1997). The

chlorophyll yield obtained in this study (15.4 mg/g dry biomass) also falls in range of previously reported values (10-30 mg/g dry weight) for *C. vulgaris* (Safafar et al., 2016; Safi et al., 2014c).
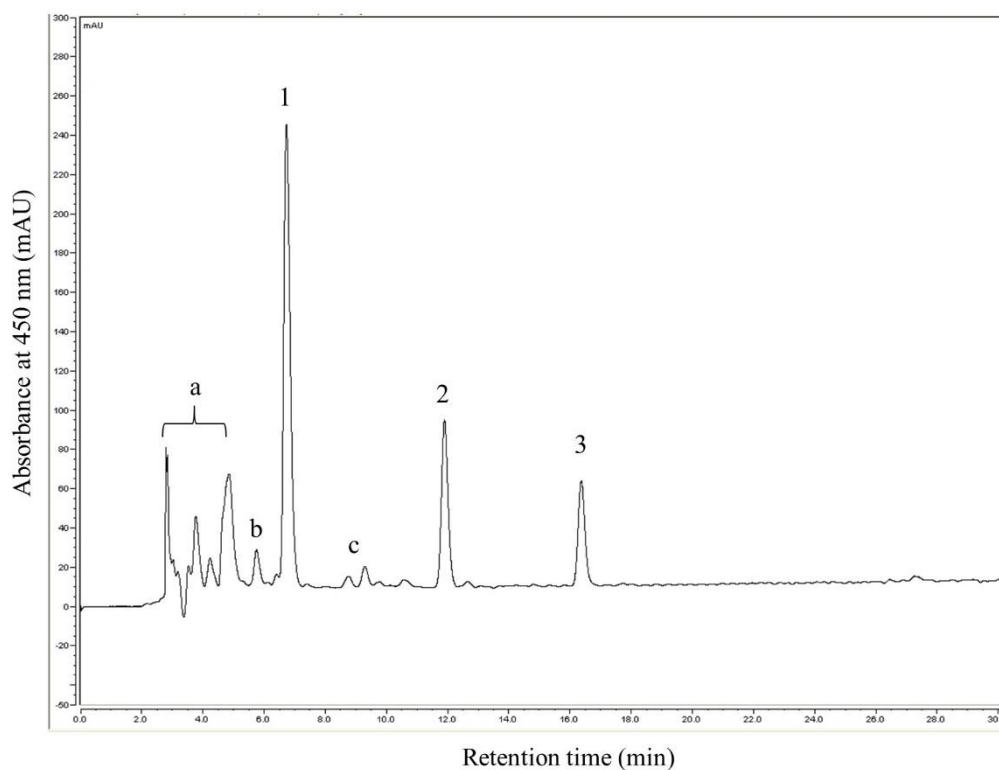
Figure 2.4 HPLC chromatogram of ethanol-extracted compounds from *C. vulgaris*: 1 – lutein; 2- chlorophyll b; 3- chlorophyll a; a- unidentified; b and c- other carotenoids.

To determine to what extent the freeze-thaw process contributed to cell permeabilization that allowed ethanol diffusion into the chloroplast, we compared ethanol extraction of pigments from freshly harvested cells to those that were frozen overnight at -80 °C and thawed before solvent extraction. After mixing with ethanol, freeze-thawed cells

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released 2.3-fold more lutein and 15-fold more total chlorophyll than fresh cells. These experiments established that when using freshly-harvested cells, they would have to be permeabilized for enhanced extraction of pigments.

2.4.3 Production of protein concentrates from ethanol-treated Chlorella biomass

Before proceeding to prepare protein concentrates by microfiltration, we determined the extent of ethanol's effect on protein solubility by comparing protein extraction yields of treated and non- ethanol treated *Chlorella* biomass at pH 7. The protein extraction yield of ethanol-treated biomass by high-pressure homogenization at pH 7 revealed that ethanol had reduced protein extractability from 76% to 53% of TP (Figure 2.5). Because in this work we used wet (24% w/w) *Chlorella* biomass, ethanol would be expected to affect protein solubility due to reversible protein denaturation and aggregation (Bull & Breese, 1978; Herskovits et al., 1970). Because aqueous alkali (pH 11-12) extraction is typically used to solubilize protein complexes and aggregates in protein bodies of oil and grain seeds (Wang et al., 2005; Zhang et al., 2009a), we tested protein extraction of ethanol-treated biomass at pH 12. As data in Figure 2.5 indicate, pH 12 extraction was effective in solubilizing algal proteins which were affected by ethanol, resulting in the same protein recovery yield as the control (Figure 2.5).
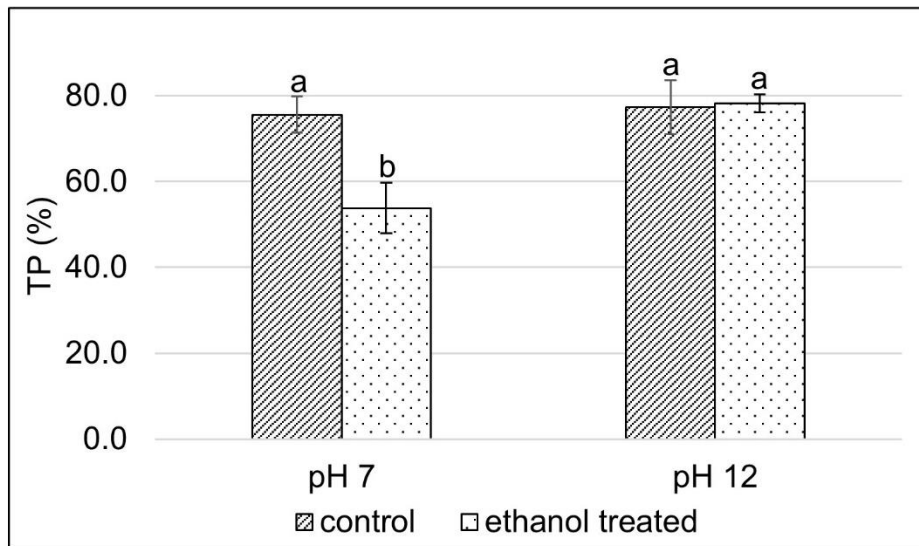
Figure 2.5 Effect of ethanol extraction on protein release at pH 7 and pH 12. 100% TP corresponds to 195 mg/g dry biomass. TP values not sharing the same letter are significantly different (α= 0.05).

Protein concentrates from ethanol-treated biomass were produced by a two-stage membrane filtration process as illustrated in Figure 2.6. Briefly, ethanol-extracted biomass was homogenized with 3 passes at pH 12 to maximize protein release. Control extracts at pH 7 and pH 12 were similarly prepared by omitting the ethanol extraction step. Homogenized extracts were clarified by centrifugation and then processed by a two-stage membrane filtration. The first stage, which utilizes a 300 kDa hollow fiber membrane, was chosen to retain large proteins with molecular weights above 500 kDa such as RuBisCo and the macromolecular complexes of photosystem I and II (Barbeau & Kinsella, 1988; Tanaka et al., 2017; Ursu et al., 2014). The 300 kDa protein concentrate was washed by diafiltration to remove smaller proteins and non-protein

molecules (sugars, ash, etc.). Proteins in the 300 kDa permeate were concentrated and

washed by diafiltration in the second stage (3 kDa membrane).
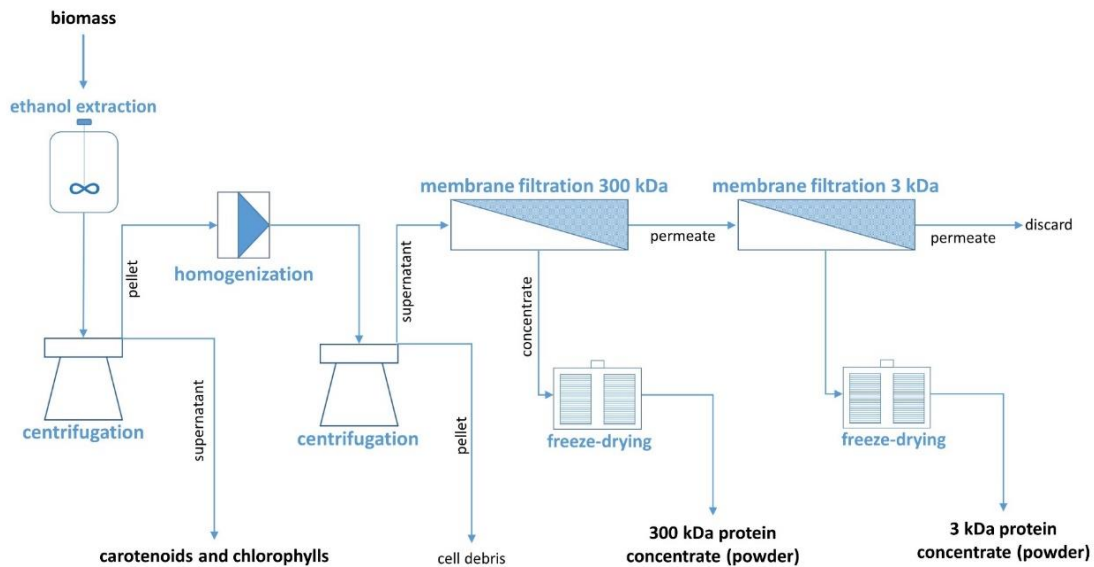
Figure 2.6 Process for selective extraction of pigments and subsequent protein fractionation and concentration by tangential flow filtration. Pigments are first extracted with ethanol from the wet, freeze-thawed biomass after which the cells are subjected to complete lysis using high-pressure homogenization for release of proteins. Protein concentrates are then obtained using membrane filtration.

A membrane processing concern we had regarding the protein extraction at pH 12 was

potential dissociation of macromolecule complexes under alkaline conditions to lower

MW subunits (15-50 kDa) (Aro et al., 2004; Barbeau & Kinsella, 1988). The increase of

small MW protein fraction in pH 12 extracts compared to pH 7, could reduce protein

concentrate yield on the 300-kDa membrane and/or cause extensive pore fouling leading

to flux reduction and longer processing times. The membrane filtration performance of the three extracts, pH 7 and 12 control, and pH 12 ethanol-treated biomass was compared by monitoring respective permeate fluxes at constant TMP (Table 2.1). The normalized average flux on 300 kDa membrane remained between 3.4 and 3.5 LMH/psi for all protein extracts regardless the extraction pH and ethanol pretreatment. Similarly, the permeate flux on the 3 kDa membrane remained within 4.4-4.7 LMH/psi range throughout the entire process, concentration and diafiltration. The higher normalized flux measure on the 3 kDa than 300 kDa membrane was due to lower protein concentration (0.5 g/L) in the 3 kDa feed compared to 300 kDa (3.5 g/L). The permeate flux on the 3 kDa membrane was only 2.5-fold lower than the normalized water flux of cleaned membrane. On the other hand, the normalized flux on 300 kDa membrane was 29-fold lower than the normalized water flux on the same membrane, suggesting stronger pore restriction and general membrane fouling. Protein distribution between the two concentrates (the amount of protein retained on each membrane) seemed unaffected by extraction pH and ethanol pretreatment i.e. 78-80% of the protein has been retained by the 300 kDa membrane. In conclusion, alkaline pH and/or ethanol treatment did not seem to adversely affect membrane performance and protein distribution in 3 and 300 kDa membrane concentrates.

Table 2.1 Normalized permeate flux on 300 kDa and 3 kDa MWCO membranes and protein distribution in membrane concentrates. Values reported are from a single selected run with the same starting protein concentration and run on membranes which had at least 80% water flux recovery.

Biomass processing conditions	Normalized average flux (LMH/psi)		Distribution of total protein (%)	
	300 kDA	3 kDa	300 kDA	3 kDa
pH 12 ethanol-treated	3.4	4.7	78	20
pH 12 control	3.5	4.4	79	15
pH 7 control	3.5	4.4	80	13

In order to check whether ethanol treatment or high pH impacted the nutritive value of protein concentrates, amino acid analysis was conducted on freeze-dried powders of concentrates (Table 2.2). All protein fractions obtained from the membrane filtration process have a comparable amino acid profile and distribution of essential amino acids, with the exception of 3 kDa concentrate of pH 7 control, which has slightly lower amounts of histidine, lysine, and phenylalanine. Extraction of proteins at pH 12 for both control and ethanol-treated biomass did not adversely affect the nutritive value of the protein concentrates. The 300 kDa concentrate that was generated by pH 12 extraction of ethanol-treated biomass contained 57% (w/w) protein, 25% (w/w) lipids, and 14% (w/w) carbohydrates, and had essential amino acid score well above the recommended FAO/WHO standards (Table 2.3). While in comparison to whole egg, this algal protein fraction falls short in some essential amino acids such as histidine, methionine (75-80%)

and lysine, valine, isoleucine (85-88%), it fares exceptionally well with respect to plant

protein alternatives such as defatted soy flour.

Table 2.2 Amino acid compositions (g/100 g protein) of 300 kDa and 3 kDa
concentrates of protein extracts from biomass under various processing conditions
(duplicate values).

Amino Acid	pH 7 control		pH 12 control		pH 12 ethanol -treated	
	300 kDa	3 kDa	300 kDa	3 kDa	300 kDa	3 kDa
Asparagine/Aspartic acid	9.5	9.3	10.9	12.3	10.9	12.6
Glutamine/Glutamic acid	10.5	15.2	11.2	13.5	10.9	14.1
Serine	5.9	5.8	5.2	5.0	5.1	4.9
Histidine*	1.5	0.9	1.7	1.5	2.0	1.4
Glycine	10.1	10.4	5.8	5.4	5.7	5.5
Threonine*	5.8	5.8	5.8	5.7	5.7	5.8
Alanine	10.9	13.8	7.9	7.7	7.8	7.7
Arginine	4.9	5.7	7.4	6.0	7.4	5.6
Tyrosine	2.8	1.3	3.2	2.6	3.5	2.5
Valine*	7.0	6.2	6.2	6.5	6.1	6.5
Methionine*	2.1	1.8	2.4	2.5	2.3	2.4
Phenylalanine*	4.5	2.7	6.7	5.5	6.3	5.2
Isoleucine*	4.9	4.0	5.1	5.1	5.0	5.1
Leucine*	9.1	6.3	10.2	9.3	9.9	9.0
Lysine*	5.5	3.5	5.3	6.4	5.9	6.5
Proline	4.9	7.3	5.2	5.1	5.3	5.2

*Essential amino acids

Table 2.3 Essential amino acid composition (g/100 g protein) and scoring for 300 kDa concentrate of protein extracted at pH 12 after ethanol-treatment.

Amino acid	pH 12 300 kDa	FAO/ WHO[a]	Whole egg[b]	Soy flour[c]	Scoring* (%)		
					d	e	f
Histidine	2.0	1.5	2.6	2.5	> 100	78	82
Threonine	5.7	2.3	5.3	3.6	> 100	> 100	> 100
Valine	6.1	3.9	7.2	1.2	> 100	85	> 100
Phenylalanine + Tyrosine	9.8	3.8	10.6	8.4	> 100	92	> 100
Isoleucine	5.0	3.0	5.8	4.7	> 100	86	> 100
Leucine	9.9	5.9	9.0	7.7	> 100	> 100	> 100
Lysine	5.9	4.5	6.7	5.8	> 100	88	> 100
Methionine	2.3	1.6	3.0	1.1	> 100	78	> 100

[a] WHO/FAO/UNU (2007)
[b] Fabregas and Herrero (1985) and Geigy (1975)
[c] Friedman (1996)
[d] Scoring with respect to FAO/WHO standards
[e] Scoring with respect to whole egg protein
[f] Scoring with respect to soy flour
* Scoring calculated as $= \dfrac{\text{amino acid content in algal concentrate}}{\text{amino acid content in reference protein}} *100$

2.4.4 Emulsification properties

Emulsification studies were undertaken to determine if alkaline pH or ethanol extraction impacted functional properties of algal protein concentrates. The results in Table 2.4 indicate there was no significant impact of pH and ethanol treatment on emulsifying activities (EA) of algal protein concentrates. All algal concentrates had emulsifying activities comparable to soy protein isolate (SPI), with the exception of the 3 kDa concentrate of the pH 7 control sample. Emulsion stability (ES) also was not affected by ethanol extraction or pH, and ES values for ethanol-treated algal concentrates were

significantly lower than for SPI, although the values didn't differ by much . From a

nutritive standpoint, maximizing protein inclusion in the emulsion (% emulsified

protein) is a desirable outcome. Thus, pH 12 concentrates adjusted to pH 7 performed

better than the pH 7 extracts because smaller amount of protein from algal extracts at pH

7 end up in the emulsified layer than their pH 12 counterparts. This observation could be

explained by partial unfolding of proteins extracted at pH 12, which resulted in an

increase of protein surface hydrophobicity (Jiang et al., 2009).

Table 2.4 Emulsifying ability (EA), emulsion stability (ES) and amount of protein emulsified for different processing conditions. For each observation, means which do not share a common superscript letter are significantly different (p< 0.05).

Protein Type	Processing conditions	EA (%)	ES (%)	Protein emulsified (%)
300 kDa concentrate	pH 7 control	$57^{ab} \pm 1.2$	$55^b \pm 1.7$	$74^c \pm 2.6$
	pH 12 control, adjusted to pH 7	$57^{ab} \pm 1.3$	$55^b \pm 0.4$	$83^b \pm 0.8$
	pH 12 ethanol-treated, adjusted to pH 7	$56^{ab} \pm 1.2$	$56^b \pm 1.3$	$83^b \pm 2.0$
3 kDa concentrate	pH 7 control	$54^b \pm 0.2$	$52^b \pm 1.2$	$68^d \pm 1.9$
	pH 12 control, adjusted to pH 7	$57^{ab} \pm 1.3$	$54^b \pm 0.6$	$78^c \pm 1.2$
	pH 12 ethanol-treated, adjusted to pH 7	$56^{ab} \pm 0.6$	$56^b \pm 1.4$	$75^c \pm 3.6$
Soy Protein Isolate	Solubilized at pH 12, adjusted to pH 7	$60^a \pm 2.4$	$59^a \pm 1.9$	$90^a \pm 0.2$

2.5 Conclusions

An optimized process for performing selective extraction of multiple products such as carotenoids, chlorophylls and functional proteins has been developed. High-pressure homogenization and bead-milling were more effective in releasing protein from *C. vulgaris* biomass than sonication. For maximum removal of carotenoids and chlorophylls before protein extraction, an optimized biomass to ethanol ratio of 1:5 and 2 stages of extraction were used. Development of an effective permeabilization methods to replace the unscalable freeze-thaw process would be required to commercialize co-production of lutein, chlorophyll and protein. After ethanol treatment of biomass to remove pigments, protein extraction at pH 12 was necessary to maximize protein release. Membrane processing and concentration of extracted protein from ethanol-treated biomass can be performed with the same adeptness as with the control samples. The high-molecular weight protein fraction (300 kDa MWCO protein concentrate) after extraction of carotenoids and chlorophyll has an essential amino acid profile that surpasses FAO/WHO recommendations and exhibits emulsification properties comparable to commercial plant-based protein emulsifiers such as SPI. The potential applications of algal concentrates in the food industry will ultimately depend on their production cost and pricing compared to plant protein concentrates. Optimizing processing parameters to obtain multiple revenue streams from different components, as this study presents, can improve the overall economics.

CHAPTER 3

EFFECT OF PULSED ELECTRIC FIELD ON PERMEABILIZATION AND SUBSEQUENT LUTEIN, CHLOROPHYLLS AND PROTEIN EXTRACTION FROM *CHLORELLA VULGARIS*

3.1 Overview

The effect of pulsed electric field (PEF) on imparting permeability and enhancing pigment extraction from *Chlorella vulgaris* has been evaluated. PEF treatment of fresh cells enhanced the lutein (2.2 ± 0.1 fold) and total chlorophyll yield (5.2 ± 3.4 fold). Biomass concentrations as high as 56 g/L did not affect PEF efficiency and pigment extraction yields. Multi-stage extraction did not result in release of higher lutein amounts than one-stage extraction for 45 min after PEF treatment. Application of an electric field intensity of 6250 V/cm and pulse duration of 620 µs at a biomass concentration of 56 g/L was optimal for achieving maximum lutein yield by a single-stage ethanol extraction for 45 min. The storage of cells at 4°C or -80°C did not have a major impact on the permeability or pigment extraction behavior and if needed can be used instead of fresh cells. The process of freeze-drying itself permeabilized the cells and improved pigment extraction. Minimal protein release could be achieved by PEF treatment (8-14%) and freeze-drying (10%).

3.2 Introduction

Microalgae are photosynthetic microorganisms that are a rich source of lipids, proteins, carbohydrates, and high-value compounds such as pigments, anti-oxidants and vitamins (Pulz & Gross, 2004). Several techno-economic analyses published in the past five years have emphasized the need for extraction of multiple products from microalgae for the commercial sustainability of the algal platform (Perez-Garcia & Bashan, 2015; Ruiz et al., 2016; Sari et al., 2016). Hence it is critical to focus on development of processes aimed at optimizing multiple product extraction to maximize the value of algal feedstock. Based on cell accumulation levels and current market value, proteins, chlorophylls, and carotenoids from microalgae hold promise as potential co-product candidates, especially if they could be selectively and inexpensively extracted.

Chlorella vulgaris is one the several microalgae species that are known to be a good source of proteins and pigments such as lutein and chlorophylls (Safi et al., 2014c) which offer several health and nutraceutical benefits. Lutein content in *Chlorella sp.* 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 (Safafar et al., 2016; Safi et al., 2014c). Dietary intake of lutein prevents early atherosclerosis, decreases the rate of age-related macular degeneration (Dwyer et al., 2001), and ameliorates the onset or progression of cataracts (Shao et al., 2008). Chlorophyll, when isolated and purified, could also provide a revenue stream. Cited chlorophyll health-benefits include immune system stimulation, blood and liver detoxification, and relief from sinusitis, fluid

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buildup, and skin rashes (Ferruzzi & Blakeslee, 2007). The protein content of *C. vulgaris* can reach up to 58% of the cell dry weight (DW) (Safi et al., 2014c), and essential amino acid profile of extracted proteins exceeds the standards recommended by WHO/FAO (Becker, 2004; FAO & WHO, 1973).

A typical downstream processing of microalgae starts with complete cell lysis with high-pressure homogenization or bead-milling that results in extraction of a mixture of intracellular proteins and lutein, chlorophylls and other pigments (Safi et al., 2015; Spiden et al., 2013). The fractionation of these potential products from the complex algal cell lysates is difficult and prohibitively expensive because it involves multiple separations steps. A possible alternative to complete cell lysis is controlled permeabilization of cell biomass to allow the development of sequential and/or selective extraction to generate multiple revenue streams. Pulsed electric field (PEF) or electroporation is a phenomenon that causes temporary or permanent pores in the cell membrane of biological cells due to the application of a high-intensity electric field (kV/cm) in the form of very short pulses (μs or ms). If the electric field is applied with sufficient intensity, it causes increased permeability in the cells due to formation of pores or local defects in the cytoplasmic membrane (Castro et al., 1993; Ivorra, 2010). PEF has been successfully applied for the enhance extraction of pigments from *C. vulgaris* (Luengo et al., 2014; Luengo et al., 2015). However, PEF was not effective for release of proteins (Postma et al., 2017; Postma et al., 2016), since it results in formation of small pores that did not allow the release of larger molecules like proteins. Previous PEF studies conducted on *C. vulgaris* have been focused on the

application of this method for extraction of either pigments (Luengo et al., 2014) or proteins (Postma et al., 2017). Although the information obtained from these investigations is useful, it is limited when the goal is to design a process for selective extraction of multiple products from microalgae. The design of selective extraction of fractionation methods requires data on all products of interest as well as on undesirable impurities. Hence, the objectives of this study were to determine 1) the effectiveness of PEF on cell permeabilization and subsequent release of lutein, chlorophylls and protein from freshly harvested *C. vulgaris* and 2) the impact of storage conditions, if required on co-product extractability and yield. The reason for the latter is that the location of the biomass cultivation facility may not be close to the processing facility and would then require refrigeration (at 4°C), freezing (at -20 to -80°C) or drying (spray- or freeze-drying) of harvested algal biomass.

3.3 Materials and methods

3.3.1 Cultivation of C. vulgaris

Chlorella vulgaris (UTEX 26, Austin, TX) was grown in Bold's Basal Media (BBM) with nutrients as follows: K_2HPO_4 (75 mg/L), KH_2PO_4 (175 mg/L), $MgSO_4.7H_2O$ (75 mg/L), $NaNO_3$ (250 mg/L), $CaCl.2H_2O$ (25 mg/L), NaCl (25 mg/L), $EDTA-Na_4$ (50 mg/L), KOH (31 mg/L), $FeSO_4.7H_2O$ (4.98 mg/L), H_2SO_4 (1.84 mg/L), H_3BO_3 (11.42 mg/L), $ZnSO4.7H2O$ (1.42 mg/L), $MnCl_2.4H_2O$ (0.23 mg/L), $CuSO_4.5H_2O$ (0.25 mg/L), $Co(NO_3)_2.6H2O$ (0.08 mg/L), $Na_2MoO_4.2H_2O$ (0.192 mg/L). Glucose was added at a concentration of 10 g/L. 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 1500 lux. 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

3.3.2 Pulsed electric field optimization for lutein and chlorophylls extraction from fresh C. vulgaris

Freshly harvested cells were re-suspended in 0.04 % (w/v) NaCl (1.1 mS/cm) solution. Pulsed electric field (PEF) was carried out using Gene Pulser Xcell ^{TM} (Bio-rad) electroporation system and 4 mm electroporation cuvettes (Bio-rad). Control (non-PEF) and PEF-treated cells were centrifuged at 7500 x g, 10 min at 4°C to remove electroporation buffer within 30 min of PEF treatment. Electroporation buffer removed after PEF was used for protein analysis. Cell samples were collected for assessment of permeability using SYTOX dye (Thermo Fisher). 95% ethanol was added to the cells at solvent concentration of 50 ml/g of dry biomass. Cells were removed by centrifugation and supernatants were analyzed for lutein and total chlorophyll content.

3.3.2.1 Kinetics of lutein and chlorophyll release

Biomass suspension at 14 g/L ($OD_{750} = 20$) was subjected to PEF treatment at an electric field strength of 6250 V/cm for a duration of 1 ms followed by pigment extraction with

ethanol. Samples were collected for analysis at time intervals ranging from 2.5 to 120 min and one after 24 hours of mixing.

3.3.2.2 One stage vs multi-stage extraction of lutein and chlorophylls for PEF at different biomass densities

Cell suspensions at 3 biomass densities- 14 g/L ($OD_{750} = 20$), 28 g/L ($OD_{750} = 40$) and 56 g/L ($OD_{750} = 80$) were subjected to PEF treatment at an electric field strength of 6250 V/cm for a duration of 1 ms. For multi-stage pigment extraction, biomass 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.

3.3.2.3 Determination of minimum energy input for lutein release

Cell suspension at 56 g/L ($OD_{750} = 80$) was subjected to PEF treatment at an electric field strength of 6250 v/cm 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. The volumetric (W_v) specific energy input was calculated as previously described in the literature (Postma et al., 2017; Salerno et al., 2009; Sheng et al., 2012):

$$W_v(kWh/m^3) = \frac{E^3.t.N.\sigma}{3600000} \tag{6}$$

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

$$W_m \left(\frac{kWh}{kg} \right) = \frac{W_v}{C} \qquad (7)$$

where C is the concentration of (dry) biomass in kg/m^3.

3.3.3 Effect of cell handling on permeability and lutein, chlorophylls and protein release

Fresh cells were subjected to PEF immediately after harvesting. Biomass from the same batch was stored at 4°C for 4 days, or at - 80°C for a week. Biomass was also freeze-dried (Lanconco) at -50°C and 0.12 mPa for 24 h. PEF was carried out at 6250 kV/cm and a pulse duration of 620 µs. Ethanol extraction was undertaken for 45 min.

3.3.4 Analysis and quantification of lutein and chlorophylls using HPLC

Lutein, chlorophyll a and chlorophyll b standards were purchased from Sigma Aldrich. A Dionex Ultimate -3000 HPLC (Thermo Fisher) system equipped with an automated sample injector, diode array detector and pump, was used for analysis of ethanol extracts. 20 µL aliquots were injected onto a RP-C-18 column (Thermo Scientific Acclaim TM 4.6 x 250 mm, 5 µm). A gradient elution of 0-40% dichloromethane in methanol was started immediately after injection and was run for a total of 30 minutes. Absorbance was measured at 450 nm and 652 nm.

3.3.5 SYTOX green nucleic acid stain for assessment of permeability

Cells suspension was diluted to OD$_{750}$= 0.25. 0.5 µL of SYTOX green nucleic acid stain (5 mM solution, Thermo Fisher) was added to 1 ml of the diluted cell suspension,

vortexed for 30 seconds and incubated for 30 min. Cells were analyzed for absorption of

SYTOX using FACSCalibur™ Analyzer (Becton Dickinson, located at Flow Cytometry

Core Laboratory, Department of Veterinary Pathobiology at Texas A&M University).

Cells were excited using a 488 nm argon-ion laser and the emission was read at 530 nm.

3.3.6 Protein analysis

Total protein content in biomass was determined by a modification of the method

proposed by Rausch (1981). 1 ml of biomass at $OD_{750}= 20$, was centrifuged at 7500 x g

for 10 min to remove supernatant, and then mixed with 1 mL of 0.5 M NaOH and heated

at 80 °C for 10 minutes. The suspension was then cooled to room temperature and

centrifuged for 10 minutes at 15000 x g. The pellet was re-extracted under the same

conditions as the first step. The pellet from the second step was mixed with 0.5 M

NaOH, heated for 10 minutes at 100 °C, suspension cooled to room temperature,

sonicated for 1 minute at 50% output capacity (55 W, CL-188, Qsonica Sonicator) and

centrifuged at 15000 x g, for 10 minutes. Supernatants from all three extraction steps

were combined and total soluble protein 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) and absorption at 562 nm was measured using the VERSA

max microplate reader (Molecular Devices, CA). NuPAGE Novex Bis-Tris pre-cast

gradient gels (4-12%, Thermo Fisher) were used for SDS-PAGE electrophoresis.

Reducing buffer was prepared using LDS sample buffer (4X, Thermo Fisher) containing

10% of reducing agent (Thermo Fisher). Reduced samples were prepared using a 1:4

ratio reducing-buffer: sample and heated at 70 ºC for10 min. MES SDS Running Buffer

(20X, Thermo Fisher) stock solution was used to prepare 1X running buffer in RO

water. Antioxidant (Thermo Fisher) was added to ensure reduced samples during

electrophoresis. Gels were run for 35 min at constant voltage (200 V). For SDS

analysis, the gels were stained in Coomassie[TM] G-250 stain (Thermo Fisher) for 3 hours

followed by destaining in RO water.

3.3.7 Statistical Analysis

One way analysis of variance (ANOVA) was conducted for statistical analysis of the

experimental data using JMP Pro 12 (SAS) software. Either one or two-factor design

were performed to optimize process parameters. All experiments were done in

triplicates. To compare significant differences between treatments, a Tukey adjustment

was made for a family wise error rate of 0.05.

3.4 Results and discussions

3.4.1 Identification of compounds in ethanol extracts

Carotenoids and chlorophylls are part of the light harvesting complex (LHC) situated in

the thylakoid membrane inside the chloroplast (Ballottari et al., 2013). These pigments

are usually co-extracted with the solvent application on algal biomass (Luengo et al.,

2014; Pasquet et al., 2011). As seen in the chromatogram in Figure 3.1, the main

carotenoid present in the ethanol extracts from *C. vulgaris* is lutein (peak 1). Other

carotenoids, though in small amounts were also found in the ethanol extracts. Based on

their elution behavior (relative hydrophobicity) (Gouveia et al., 1996; Orosa et al., 2000), compounds eluting before lutein may include neoxanthin, violaxanthin or astaxanthin (region a), and those eluting just after lutein (region b), could be canthaxanthin and other xanthophylls. α- and β-Carotene (region c) are expected to elute at the very end. The presence of lutein as the most abundant carotenoid in *C. vulgaris* extracts is not surprising since several Chlorophycea species are known to contain higher lutein levels than other carotenoids (Cordero et al., 2011). Also, because of the higher polarity of ethanol (compared to other organic solvents), more effective solubilization of lutein than comparatively hydrophobic carotenoids such as β-carotene (Braumann & Grimme, 1981) is expected and explains the greater amount lutein determined in the ethanol extracts. Other major pigments that were co-extracted were chlorophyll b (peak 2) and chlorophyll a (peak 3) which will be quantified as total chlorophyll along with the lutein.
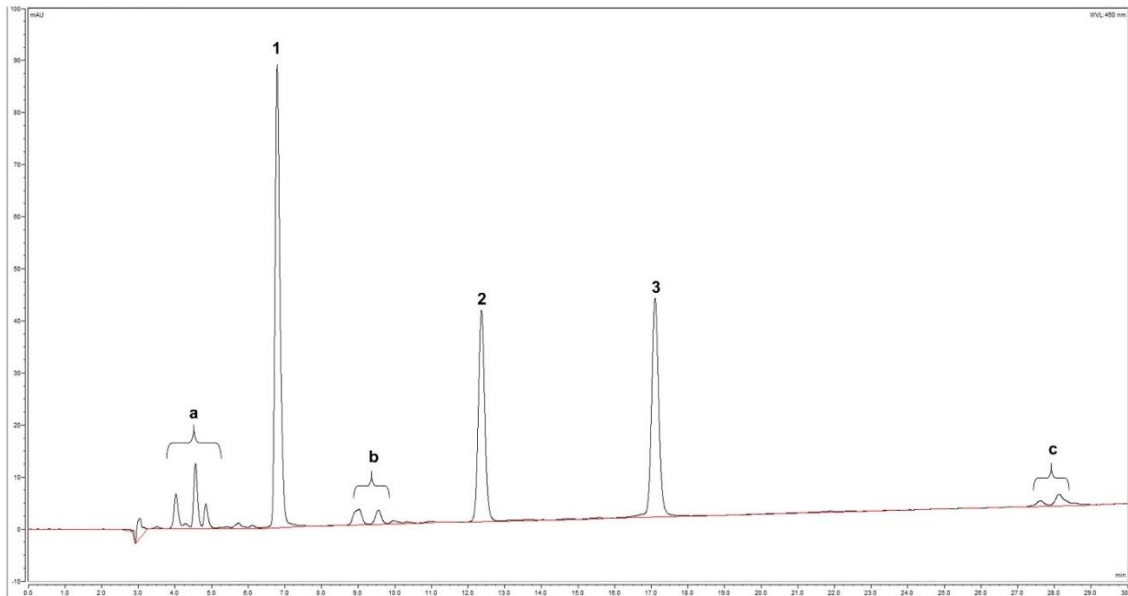
Figure 3.1 Chromatogram for pigments released in ethanol extract. Major compounds: 1-lutein, 2 – chlorophyll b and 3 – chlorophyll a. Other compounds: regions a and b – other xanthophylls like neoxanthin, violaxanthin, astaxanthin and canthaxanthin; region c - α- and β-carotene.

3.4.2 Optimization of lutein and chlorophylls release from fresh C. vulgaris by PEF treatment

3.4.2.1 Kinetics of lutein and chlorophylls extraction

In order to determine the kinetics of lutein and chlorophylls release, the pigments' concentration in ethanol extracts from non-treated (control) and PEF-treated fresh *C. vulgaris* biomass was measured at various time points (Figure 3.2). A logarithmic trend ($y = 614.33\ln(x) + 2104$, $R^2 = 0.92$) is observed for lutein release from PEF-treated cells (Figure 3.2a) over the time period of 120 min. An initial fast extraction rate ($y = 197.23x + 2047$, $R^2 = 0.95$) during first 7.5 min is was followed by leveling off to a maximum

lutein concentration of 5 mg/g. No significant difference ($p < 0.05$) was found after 45 min mixing with ethanol. In fact, even after 24 hours of mixing (time point not shown), the lutein yield did not exceed 5.2 mg/g. The lutein yield obtained in this study is comparable to lutein yields (4.7-7.14 mg/g dry biomass) from *C. vulgaris* reported in the literature (Safafar et al., 2016; Shi et al., 1997). The amount of lutein released from control samples (Figure 3.2 a) in this batch of biomass was considerably lower (2.4 fold) even after 120 min of mixing. Lutein yield could reach the maximum for 5 mg/g in the 24 hour sample for the control biomass (time point not shown) indicating a much slower rate of diffusion of lutein from cells not treated with PEF.

Since chlorophylls (a and b) are the other major pigments in the ethanol extracts, their kinetics are also reported (as total chlorophyll) in this study (Figure 3.2b). Similar to lutein, a logarithmic trend ($y = 1377\ln(x) - 65.15$, $R^2 = 0.97$) is observed for total chlorophyll yield for PEF-treated samples over the period of 120 min with a linear region of faster rate of extraction of chlorophylls till 10 min ($y = 164.13x + 1334$, $R^2 = 0.98$). The total chlorophyll yield reached to 6.8 mg/g after 2 hours of mixing with no significant difference after 60 min. However, after 24 hours of mixing (time point not shown), the chlorophyll yield reached a maximum value of 13 mg/g indicating that more than 2 hours of extraction will be required if chlorophylls were the desired product. PEF was effective in releasing more chlorophylls (9.7 fold) than the control sample after 120 min of mixing. Even after 24 hours (data point not shown), the total chlorophyll release from control sample was still 5.4 fold lower than the yield obtained with PEF-treated cells. The significant difference between control and PEF-treated cells can be explained

by the formation of pores in cell membrane by application of high-intensity electric fields which can allow enhanced transport of molecules in and out the cell (Pavlin et al., 2008; Pliquett et al., 2007).
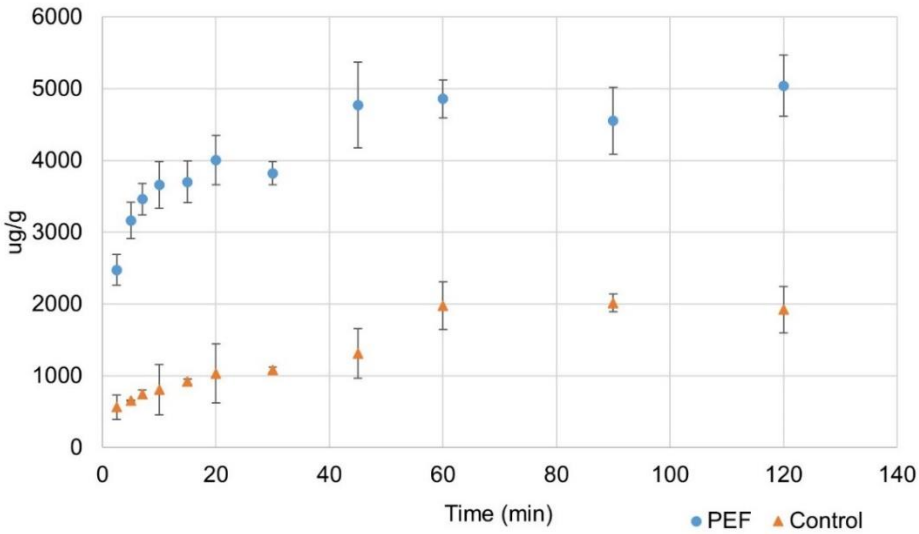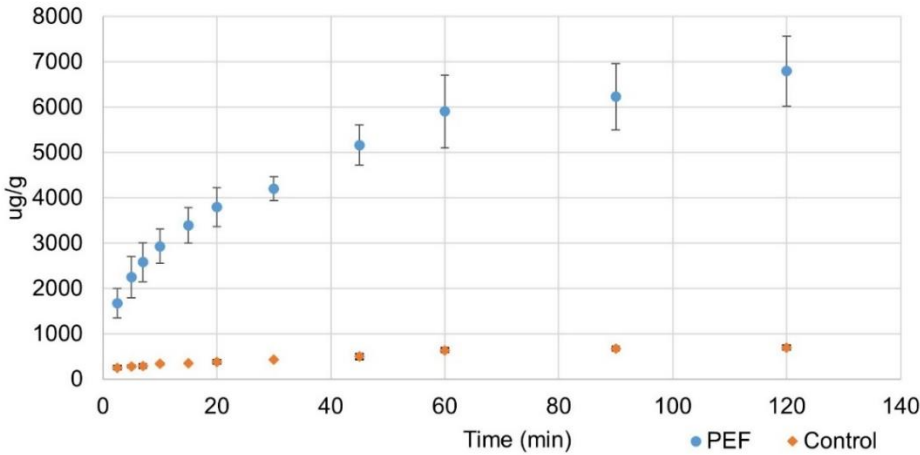
Figure 3.2 Kinetics of lutein (a) and total chlorophyll (b) release from control and PEF treated fresh *C. vulgaris* biomass. Values reported are averages of triplicates.

3.4.2.2 One stage vs multi-stage extraction of lutein and chlorophylls for PEF at different biomass densities

As established from the kinetics of pigment extraction, PEF treatment had faster initial rate of release of lutein. A multi-stage (3 stages) extraction on control and biomass treated with PEF at three different biomass densities was undertaken. PEF-treated cells at different biomass densities (14 g/L, 28 g/L and 56 g/L) were subjected to the same electric field strength (6250 kV/cm, 1 mS) and their ethanol extracts were compared to determine the maximum concentration of cells at which effective pigment extraction can be carried out.

 Biomass densities didn't not impact lutein yield for multi-stage extraction from PEF treated cells (Figure 3.3a), with 83-88% being extracted in this first stage, 13-16% extracted in the second stage and only 1-4% extracted in the second stage. For control samples, the first stage attained 36% lutein yield, the second stage could achieve 40% yield, and even the third stage has considerable amount of lutein (17%). Similar trend is observed for chlorophylls release for multi-stage extraction (figure 3.3b), with the first stage comprising of 27-29% of total chlorophyll. The second stage for PEF carried out 14 g/L biomass concentration could extract slightly more total chlorophyll (25%) compared to the second stage of the other two biomass concentrations (27-29%). The third stage of extraction for all PEF-treated samples had the least amount of chlorophylls (9-12%). For the control sample (Figure 3.3b), the first stage had the least amount of chlorophylls (3%), with more chlorophyll in the second (9%) and third (7%) stages.

A single stage extraction comprising of 45 min of mixing was also carried out on the same batch of biomass to compare the lutein and total chlorophyll yield with multi-stage extraction (Figure 3.4). The total yield of lutein from multiple stages for the control is not significantly different than that from PEF-treated cells (Figure 3.4a), and is higher than that attained with a the 45 min, single stage extraction, as the addition of fresh solvent in each stage expedited the transfer of lutein from cells to ethanol. However, as explained earlier, control samples needed at least two stages of extraction (76%) to get close to lutein yield obtained by PEF treatment in one stage (83-88%). Multi-stage extraction of lutein led to slightly higher yields (though not significant, except for PEF at 28g/L) for PEF-treated samples than single-stage extraction for 45 min. Multi-stage extraction results in higher yield (56-64%) of chlorophylls (Figure 3.4b) for all PEF-treated samples than their single stage counterparts (40-46%). Within the control samples too, higher amounts of chlorophylls are extracted (18%) with multiples stages than a single stage of 45 min (6%), although these amounts are considerably lower than all PEF-treated samples.

One thing to note from this set of results is that the introduction of fresh solvent during multi-stage extraction closes the gap in the amount of lutein released from control and PEF-treated samples but does not for chlorophylls. This is because for the control samples, initial rate of extraction of lutein is 2.4 fold higher than that for chlorophyll (Figure 3.2). With three extraction stages, lutein yield equivalent to the total amount of lutein present in the cells which ranges from 4.7-7.14 mg/g dry biomass (Safafar et al., 2016; Shi et al., 1997), can be achieved. However, the slower extraction rate of

chlorophylls from non-PEF treated cells, coupled with the fact that compared to lutein, higher amount of total chlorophyll (10-30 mg/g dry biomass) is synthesized in *C. vulgaris* (Safafar et al., 2016; Safi et al., 2014c), makes it difficult to achieve chlorophylls yield comparable to PEF-treated samples. Multi-stage extraction in this case, would require not only thrice the amount of solvent but also two more centrifugation steps adding energy and capital investment cost on a large scale. Since no significant difference was observed in the amount of lutein (our primary product of interest) extraction for PEF-treated samples, a one-stage extraction for 45 min was deemed sufficient for maximum lutein yield. Another important finding from this set of studies is that PEF undertaken on different biomass concentrations didn't have an impact on pigment extraction. Faster processing rates can be achieved when higher concentrations of biomass can be treated with PEF with the same effectiveness. In fact, successful operation of PEF has been conducted at a biomass density of 100 g/L (Eing et al., 2013), so there is potential to increase the biomass concentration even beyond 56 g/L at bench scale.
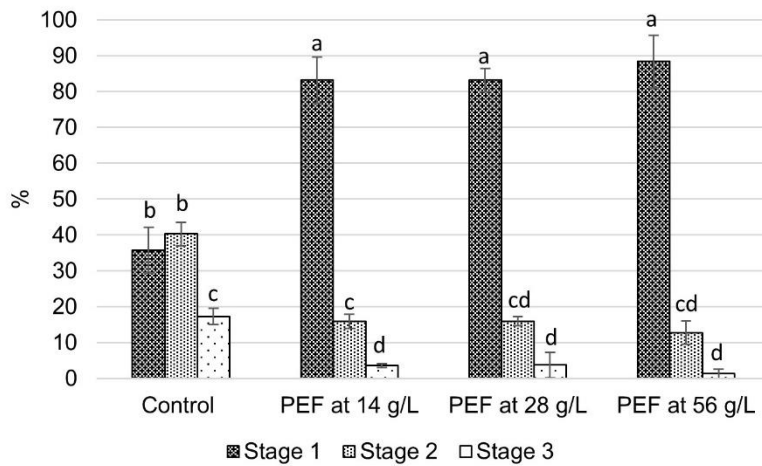
(a)
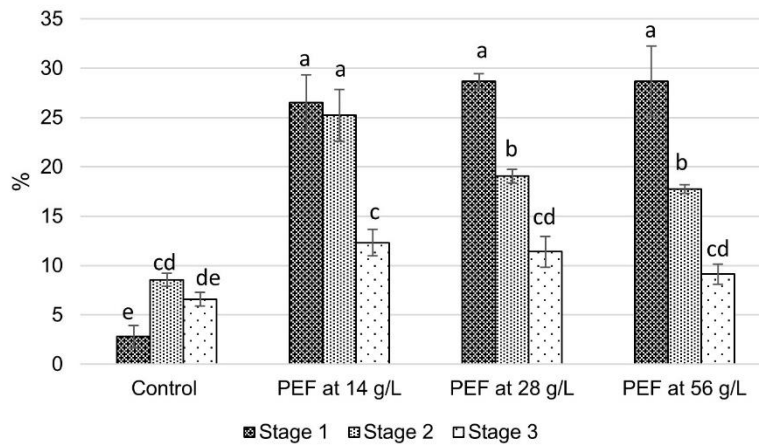
(b)

Figure 3.3 (a) Lutein release using a multi-stage extraction model for PEF undertaken at different biomass concentrations (b) Total chlorophyll release using a multi-stage extraction model for PEF undertaken at different biomass concentrations. 100 % lutein or total chlorophyll value was assigned to the sample with the highest pigment yield. For each graph, values not sharing the same letter are significantly different (α= 0.05).

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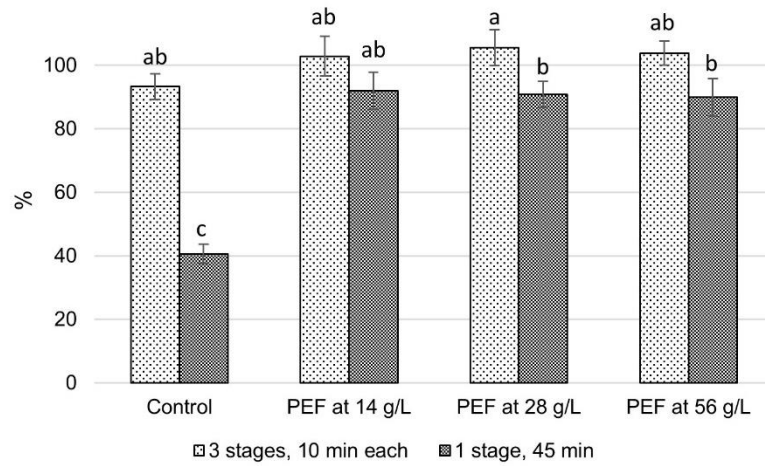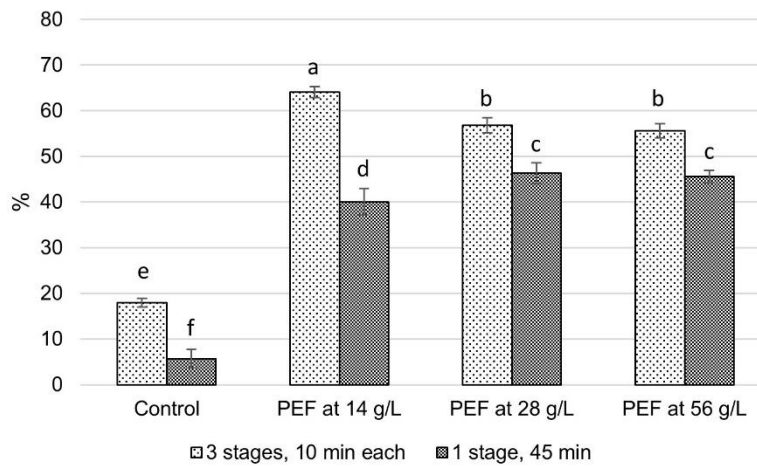
Figure 3.4 (a) Comparison of lutein yield from one-stage vs multi-stage for PEF undertaken at different biomass concentrations (b) Comparison of total chlorophyll yield from one-stage vs multi-stage for PEF undertaken at different biomass concentrations. 100 % lutein or total chlorophyll value was assigned to the sample with the highest pigment yield. For each graph, values not sharing the same letter are significantly different (α= 0.05).

Permeabilization of cells was evaluated by SYTOX green nucleic acid stain. PEF

treatment could permeabilize cells with the same efficiency irrespective of the biomass

concentration (Figure 3.5) which could explain why no difference in pigment extraction behavior was observed. SYTOX Green nucleic acid stain is green-fluorescent dye which does not cross intact cell membranes but can easily penetrate compromised membranes and exhibits >500 fold fluorescence enhancement upon binding with nucleic acids. Control or non-PEF treated cells have a large population of cells (98%) which are intact (Figure 3.5), whereas PEF treatment allowed the absorption of SYTOX, with 87-88% of the cells having a permeable cell membrane.

(a)   (b)
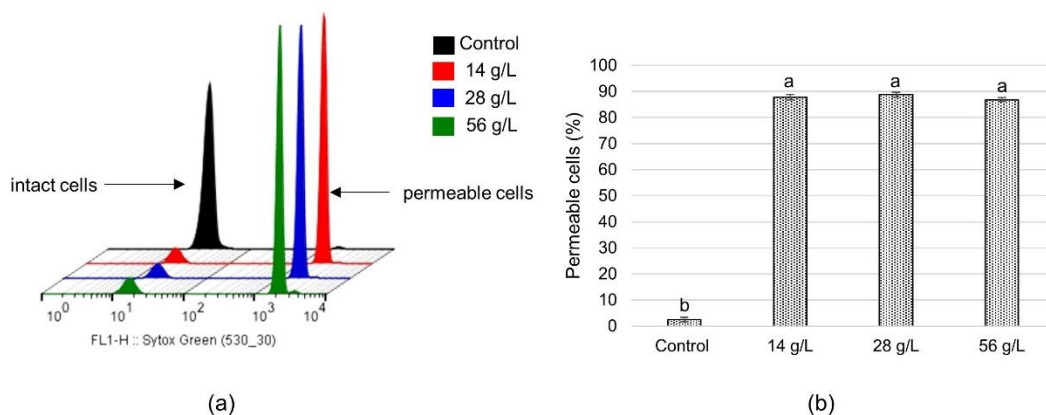
Figure 3.5 (a) Histogram for absorption of SYTOX and (b) percentage of permeable cells which absorbed SYTOX for control and PEF-treated samples at different biomass densities. Values not sharing the same letter within each treatment are significantly different (α= 0.05).

3.4.2.3 Determination of minimum energy input for PEF

The objective of this set of experiments was to minimize the PEF energy input without affecting the lutein yield. This was established by subjecting freshly harvested *C. vulgaris* cells at a biomass concentration of 56 g/L to a constant electric field of 6250

V/cm but varying the duration of pulses (1ms, 620µs and 240µs). The amount of lutein

extracted (Figure 3.6a) was not significantly different between pulse duration of 1 ms

and 620 µs, and decreased (70%) when the pulse duration was 240 µs. Similar trend is

seen in the total chlorophyll yield (Figure 3.6b) with the pulse duration of 240 µs

releasing only 42% of the pigment compared to PEF samples at 1 ms and 620 µs.

Highest amount of cells (87%) were permeable when the pulse duration was 1 ms and

gradually decreased with shorter pulse durations of 620 µs (70%) and 240 µs (43%)

(Figure 3.6c).

While we are not providing a prediction model for pigment release based on

permeability (SYTOX), the data suggested strong correlation between extent of

permeabilization and pigment extraction. Through our optimization studies, it was

established that a biomass density of 56 g/L, electric field strength of 6250 V/cm and a

pulsed duration of 620 µs were optimum for the maximum yield of lutein from fresh *C.*

vulgaris. The mass specific energy input for these conditions comes up to be 0.13

kWh/kg of dry biomass, which falls in range (0.01-2 kWh/kg) of energy inputs used in

the literature for continuous flow PEF-treatment for the formation of small pores on the

cell membrane of *C. vulgaris* to enhance release of intracellular compounds (Postma et

al., 2017).
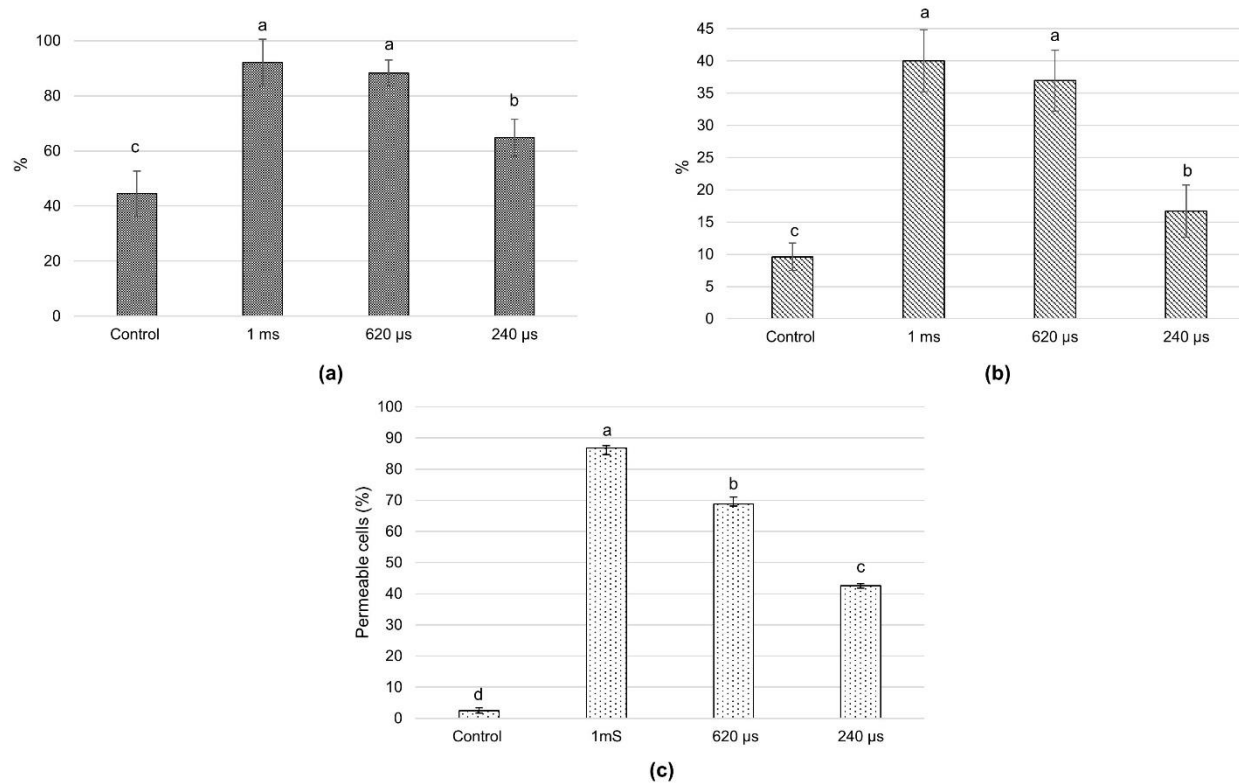
Figure 3.6 (a) Lutein yield, (b) total chlorophyll yield and (c) percentage of permeable cells for control and PEF samples treated with varying pulse durations. 100 % lutein or total chlorophyll value was assigned to the sample with the highest pigment yield. For each graph, values not sharing the same letter are significantly different ($\alpha = 0.05$).

3.4.2.4 Protein release after PEF treatment from fresh C. vulgaris

Though the primary purpose of treating biomass with PEF was to enhance the release of pigments such as lutein, protein release due to the application of PEF was also monitored. Ideally, we would like the protein fraction to stay inside the cells, available for selective extraction later in the process. Protein in the buffer for biomass treated with varying PEF parameters such as biomass concentrations (14 g/L, 28 g/L and 56 g/L at 6250 V/cm for 1 ms) and pulse durations (1 ms, 620 µs and 240 µs at biomass concentration of 56 g/L and 6250 V/cm) was measured (Figure 3.6). Varying biomass concentration during PEF did not have an impact on the protein release, as a small fraction (12-14%) of the total protein (TP) is released. This trend is similar to that of pigment extraction (Figure 3.4) and level of permeability (Figure 3.5). When the specific energy input was changed by varying the pulse lengths, slightly lower amount of protein is released (9-10%). Other authors (Postma et al., 2017; Postma et al., 2016) also observed that PEF was not able to generate sufficiently large openings in the cell membrane to release proteins. Application of PEF with specific energy inputs as high as 100 kWh/kg was able to release only 5% protein from *C. vulgaris* and 13% protein from *Neochloris oleoabundans* (Postma et al., 2017). Even aqueous extraction of protein under alkaline conditions (pH 11) after PEF, could yield only 10% protein from *Nannochloropsis* (Parniakov et al., 2015).
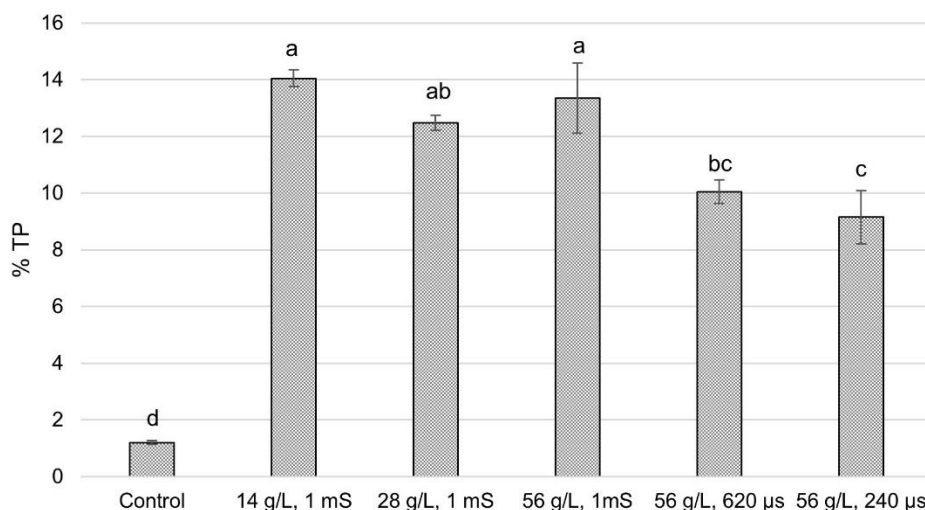
Figure 3.7 Protein release after PEF treatment of samples at different biomass concentrations and pulse durations. Values expressed as percent of total protein (TP). Values not sharing the same letter are significantly different (α= 0.05).

3.4.3 Effect of cell storage on permeability, lutein, chlorophylls and protein release

3.4.3.1 Effect of cell storage on permeability, lutein and chlorophylls release

All our previous experiments were undertaken on freshly harvested *C. vulgaris* biomass grown in our lab. However, in reality it not always possible to grow large quantity of biomass on site or work with harvested biomass immediately. This would require either the storage or transport of biomass during which cells are subjected to refrigeration (at 4°C), freezing (at -20 to -80°C) or drying (spray- or freeze-drying). The main objective of this section was to check the impact of handling of biomass on permeability and pigment release. Also, PEF was undertaken on different types of cells to check if it enhances permeability and pigment extraction. Amongst the control (non-PEF) samples of cells undergoing different handling conditions, the entire cell population (99.7%) was

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completely permeable for freeze-dried cells (Figure 3.8), indicating that the process of drying made the cell membrane completely porous. The 4°C control sample had permeability similar to fresh cells (1-2%) and the frozen cells had a slightly higher extent of permeability (7%). In terms of lutein extractability (Figure 3.8b) among control samples, freeze-dried cells performed the best, extracting 1.5 fold more (83%) lutein than the other three samples (54-58%). Higher (3.3 fold) total chlorophyll yield (Figure 3.8c) was obtained for freeze-dried control sample (27%) compared to the other three control samples (6-9%). With the application of PEF (Figure 3.8), similar to the fresh cells, biomass stored at 4°C and -80°C, displayed an increase in permeability (70%), and increase in lutein (2 fold) and chlorophylls extraction (3.5-5.8 fold). The PEF-treated freeze-dried sample, also showed enhanced release of lutein (1.3 fold) and chlorophylls (1.9 fold) compared to its control sample. Fresh, 4°C and frozen cells showed similar trends in terms of permeability and pigment release, requiring the application of PEF for enhancement of both parameters. The process of freeze-drying itself (without PEF) imparted permeability and aided in extraction of pigments, which works well for the bench-scale studies. However, the high-energy consumption during lyophilization can be a deterrent to its scalability.
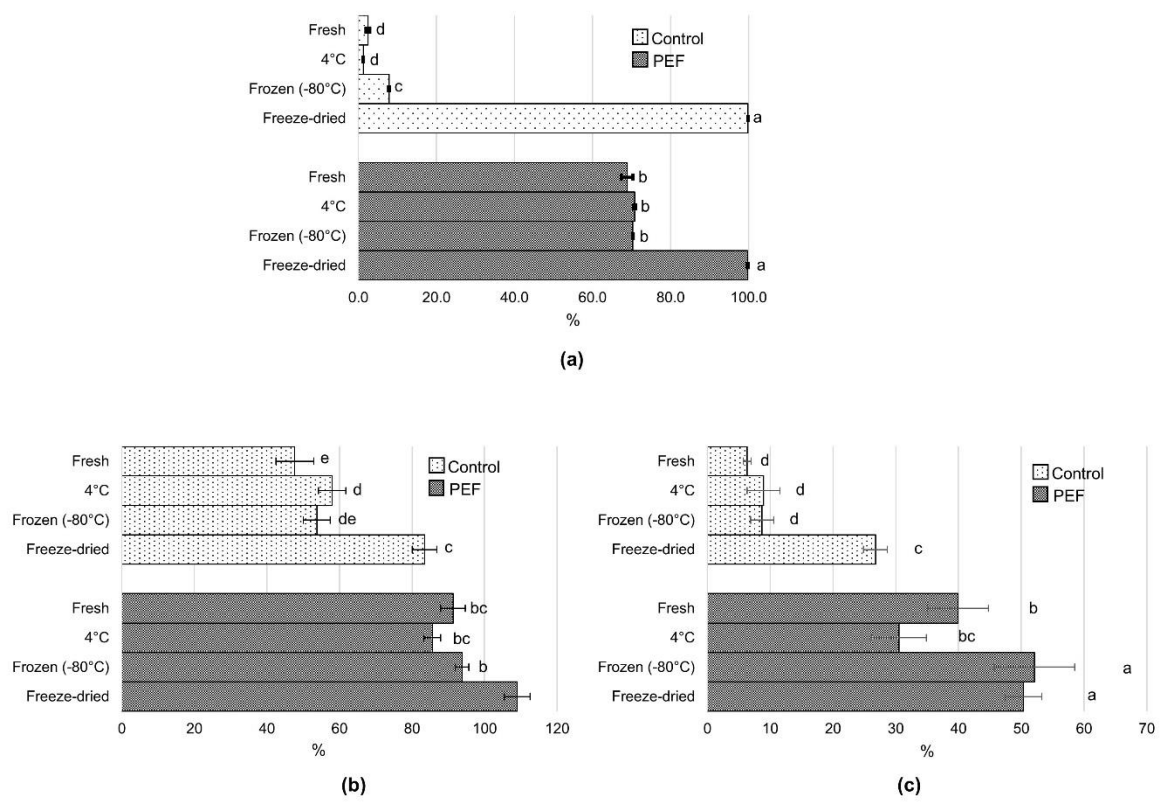
Figure 3.8 Effect of cell storage and PEF treatment on (a) lutein, (b) total chlorophyll yield and (c) extent of permeability. 100 % lutein or total chlorophyll value was assigned to the sample with the highest pigment yield. For each graph, values not sharing the same letter are significantly different ($\alpha= 0.05$).

While permeability assessment with SYTOX is a good predictor of intracellular pigment release, it has limitations. SYTOX is small (~600 Da) in size (Johnson & Criss, 2013), so its absorption behavior wouldn't change with the increase of pore size or disruption extent of cell membranes. For example, if freeze-thawing or freeze-drying weakens the cell membrane due to ice-crystal formation (Steponkus, 1984) , the subsequent PEF treatment, because of the already destabilized cell membrane, would cause a greater

extent of disruption (e.g. bigger pores) than it would in PEF-treated freshly harvested cells. This phenomena of greater extent of cell membrane disintegration cannot be differentiated by SYTOX assay. Therefore, the most appropriate method to check the effectiveness of cell disintegration method (like PEF), is the direct measurement of the biomolecules of interest.

3.4.3.2 Effect of cell storage on protein release

Protein release for control and PEF-treated cells was recorded and analyzed by SDS-PAGE (Figure 3.9). Fresh, 4°C, and frozen controls released trace amounts of protein (1.2-1.6%), but the freeze-dried control released significantly more protein (11.9%). The induction of permeability (Figure 3.8a) due to the process of freeze-drying, must have allowed higher amounts of protein release compared to other samples. PEF treatment on fresh, 4°C, and frozen cells lead to increase (7 fold) in protein release compared to their respective controls with the exception of PEF-treated freeze-dried samples. SDS-PAGE analysis (Figure 3.9b) of protein samples revealed a protein band at 106 kDa for control as well as PEF samples for freeze-dried and frozen cells. While the origin of these band is not clear, it could be glycoprotein (Blumreisinger et al., 1983; Loos & Meindl, 1982; Voigt et al., 2014) releasing from a disintegrated cell wall because of freezing. Another distinct band is seen at 5.3 kDa for both control and PEF samples of freeze-dried cells and the PEF sample of frozen cells. An extremely faint (which appears with staining for a long time, but disappears after short destaining) band is seen at 7.8 kDa for fresh and 4°C, PEF-treated samples. The smaller molecular weight bands could be cytoplasmic

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proteins diffusing due to the induction of permeability of the cells either because of PEF or freeze-drying. The intensity and number of visible protein bands doesn't necessarily match the protein amounts measured by the BCA assay, because SDS-PAGE was developed by Coomassie^TM , which is unable to stain low molecular weight proteins (<3kDa) and peptides. However, these smaller proteins and peptides releasing due to enhanced permeability caused by PEF-treatment or freeze-drying, can be measured by the BCA (Chemistry of Protein Assays, Thermo Fisher Scientific).
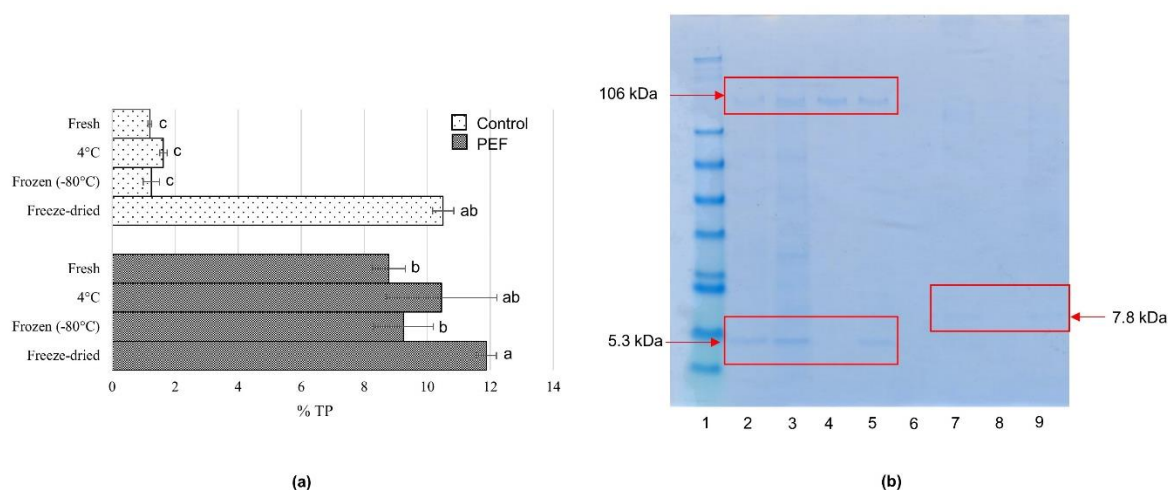
Figure 3.9 (a) Effect of cell handling on protein release with or without PEF treatment (b) SDS-PAGE gel for protein release; lane 1: marker; lane 2: control, freeze-dried; lane 3: PEF, freeze-dried; lane 4: control, frozen; lane 5: PEF, frozen; lane 6: control, 4°C; lane 7: PEF, 4°C; lane 8: control, fresh; lane 9: PEF, fresh.

3.5 Conclusions

PEF was successful in imparting permeability and enhancing extraction of pigments from *C. vulgaris*. PEF parameters were optimized based on the extraction of lutein, which was our primary pigment of interest. Application of an electric field intensity of 6250 V/cm and pulse duration of 620 µs at a biomass concentration of 56 g/L, followed by a single-stage ethanol extraction for 45 min was optimum for the attainment of maximum lutein yield. Cells stored at 4°C or -80°C didn't have a major impact on the permeability or pigment extraction behavior and if needed can be used instead of fresh cells. The process of freeze-drying itself permeabilized the cells and improved pigment extraction. Majority of the proteins (>85%) remained inside the cells after PEF or freeze-drying, which can be extracted later by complete cell lysis methods like high-pressure homogenization or bead-milling. To determine the commercial viability of selective extraction of pigments and proteins from *C. vulgaris,* a techno-economic analysis of processing parameters optimized on the bench-scale has to be undertaken.

CHAPTER 4

EVALUATION OF CHITOSAN AND ACID PRECIPITATION AS PRE-TREATMENT METHODS FOR PRIMARY RECOVERY AND CAPTURE OF ANTIBODY FRAGMENT FOR *C. REINHARTDII**

4.1 Overview

The effectiveness of mechanistically different precipitation methods (polymer and isoelectric) for reduction of DNA, chlorophyll, and host cell protein in cell-free extracts was compared. Acid precipitation was effective in reducing host cell protein (69%) and chlorophyll (72%) but not DNA (9%). Chitosan precipitation performed better than acid precipitation for removal of impurities, reducing DNA by 91%, chlorophyll by 98% and host cell protein by 81%. After pretreatment, the clarified cell extracts were loaded onto an anion-exchange Capto-Q column to improve purity of the target protein (αCD22scFv). The target protein completely adsorbed onto Capto Q resin at pH 8 from acid- and chitosan-pretreated extract after reducing the extract ionic strength to below 4 mS. Capture chromatography of αCD22scFv from chitosan- and acid-pretreated extracts resulted in 20 and 13 purification fold, respectively.

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4.2 Introduction

Using the lessons learned from transgenic plants as bioreactors for recombinant protein production, there have been improvements in microalgae biotechnology in the past 15 years (Wilken & Nikolov, 2012). Specht et al. (2010) have reviewed the benefits of using microalgae over traditionally used bacterial or mammalian cells for the production of recombinant proteins. The chloroplast of *Chlamydomonas reinhardtii* alone has been used for the expression of more than twenty recombinant proteins (Rasala et al., 2010; Surzycki et al., 2009; Tran et al., 2013).

Extraction of intracellular recombinant proteins from microalgae is usually accompanied by release of impurities such cell debris, nucleic acids, chlorophyll and host cell proteins.   One of the major roadblocks in the purification of recombinant proteins is removal of impurities before loading the extract onto the first chromatography column. Centrifugation and microfiltration alone are not efficient in clarification of the turbid extracts (Thömmes & Etzel, 2007).

In order to increase process robustness and clarification efficiencies, pre-treatments have be carried out by isoelectric precipitation (Barros et al., 2011; Woodard et al., 2009), ammonium sulfate precipitation (Lai et al., 2010), and polymer-mediated flocculation or precipitation (Hashimoto et al., 1989; Holler et al., 2007). For example, low saturation ammonium sulfate solutions (25–30%) have been successful in reducing concentrations of native plant proteins, protein aggregates, and cell debris (Garger et al., 2000; Lai et al., 2010). Acidification of leafy extracts and cell homogenates to pH <5.0 led to

precipitation of the most abundant plant protein (RuBisCo), cell debris, as well as chlorophyll pigments attached to the protein and debris (Garger et al., 2000; Woodard et al., 2009). Cationic polymers, such as polyethylenimine (PEI) and chitosan, have been used to flocculate whole cells and cell debris (Garzon-Sanabria et al., 2012; Holler et al., 2007; Persson & Lindman, 1987), remove nucleic acids (Hashimoto et al., 1989), or to precipitate recombinant β-glucuronidase (pI 5.5) from low ionic strength tobacco extracts (Holler et al., 2007).

One of the major challenges in clarification of algal extracts for purification of acidic recombinant proteins is the abundant presence of other host cell acidic proteins such as RuBisCo. Conditions that favor formation of native (rubisco) proteins precipitation may also be favorable for co-precipitation of acidic recombinant proteins. The impact acid precipitation on *C. reinhardtii* extracts for reduction of DNA, host cell protein and chlorophyll has been studied previously by Munjal (2014). This study, an extension to that done by Munjal (2014) checks the impact of chitosan precipitation on removal of host cell impurities. After conditioning of the extracts, further purification is usually carried out by capture chromatography step to concentrate the target recombinant protein. Some of the plant-derived recombinant proteins have been captured by anion-exchange adsorption (Holler & Zhang, 2008), cation exchange adsorption (Zhang et al., 2009b), affinity (Nikolov et al., 2009) and hydrophobic interaction chromatography (Xu et al., 2007). It is important that conditions applied for clarification of extracts is compatible with subsequent chromatography capture step. Therefore, the objectives of this work were to evaluate 1) chitosan precipitation as a pretreatment method to reduce

impurities like host cell proteins, nucleic acids and chlorophyll 2) compare chitosan precipitation to acid precipitation in terms of impurity removal 3) check if the chromatography capture step is compatible with pre-treatments methods and if it improves purity of target protein.

4.3 Materials and methods

4.3.1 Gene constructs for αCD22 scFv

In the construct, the endogenous psbA locus was replaced by αCD22 scFv via direct homologous recombination. Thus, transgene expression in these strains is regulated by the psbA promoter and the 5' and 3' untranslated regions (UTRs) and, therefore, is light inducible. A kanamycin resistance cassette was incorporated for selection. The variable domains of a human antibody against the B-cell surface antigen CD22 were separated by a linker consisting of four glycines and a serine repeated four times (4×G4S) to create an scFv (Tran et al., 2013). The gene cassettes (αCD22 scFv) was ligated with a sequence coding for a 1× Flag peptide (DYKDDDDKS) and separated by a sequence that encodes a Tobacco etch virus (TEV) protease cleavage site (ENLYFQG) (Tran et al., 2013).

4.3.2 Cultivation of recombinant αCD22 scFv C. reinhartdii strains

Algal biomass from a single Tris acetate phosphate agar plate containing 150 µg/ml kanamycin) was transferred to 100 mL of TAP (Tris acetate phosphate) media without kanamycin and grown for 3 days. Subsequent volumetric culture scale up was performed using 10 % inoculum in the exponential phase (100 mL) in 1-L of fresh TAP media

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containing 25µg/mL kanamycin. One liter cultures were grown heterotrophically (in the dark) for 5 days reaching ~ 4 to 5×10^5 cells/mL. At the end of the fifth day, the biomass from 1-L cultures was resuspended in 1-L of fresh TAP media containing 25 µg/mL kanamycin and grown for 1 day reaching about 10^6 cells/mL. The cultures were then exposed to light at 300 µmol m-2s-1 for 24 hours to induce recombinant protein synthesis. Cell growth was monitored daily by counting cells using a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA) and measuring optical density at 750 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer.

4.3.3 Protein extraction

C. reinhardtii cultures producing recombinant proteins were grown in liquid media until they reached the desired cell concentration of ~10^6cells/mL. At the end of the light exposure period, cells were harvested by centrifugation at 10,000 ×g for 15 min at 4 ºC. Pelleted algal biomass was washed with fresh TAP media, weighted, and then resuspended at 1:5 biomass-to-lysis buffer ratio (50 mM Tris-HCl, 400 mM NaCl, and 0.5% Tween 20, pH 8.0). The buffer contained a complete protease inhibitor cocktail (Roche-Mannheim, Germany) dissolved in 200 mL of the buffer. Algal cells were lysed by sonication for 8 min with 30 s on/off intervals at 4 °C using sonicator (150 W, Sonifier 250, Branson, USA) at 30% output control and 30% duty cycle with a micro probe (1/8' microtip A3-561 Branson, USA). Cell lysates were centrifuged (10,000 x g for 10 min) to produce cell-free extracts. Cell-free extracts were centrifuged (10,000 x g for 10 min) and filtered with PES 0.45 µm syringe filter to produce clarified extracts.

4.3.4 Pretreatment of cell-free extracts

Polymer pretreatment was done with 10 mg/mL stock solution of chitosan in 1% acetic acid solution (Sigma Aldrich Company, USA). The stock solution of chitosan was prepared by mixing chitosan in 1% acetic acid solution for 24 h at 60 rpm and room temperature. Thirty milligrams of chitosan (3 ml stock) was added per gram of algae biomass followed by vortexing for 30 sec. The lysate pH was then adjusted to 5.0 with 0.1 N HCl and mixed (end over end) for 30 minutes at room temperature. The clarified cell extract was then readjustment to pH 8.0 and centrifuged at 6000 x g for 10 min for precipitating out chitosan and chitosan bound impurities from the extract. The supernatants were filtered with PES 0.45 µm syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and αCD22 scFv by methods discussed below.

Acidic precipitation was performed by adding 0.5 N HCl (dropwise with gentle shaking) to *C. reinhardtii* cell lysates or cell-free extracts until the pH dropped to 4.5. Agitation was provided by end-over–end gentle mixing for 15 min, followed by centrifugation at 10000 x g for 10 min. The acidic supernatants were adjusted to pH 8 with 1 N NaOH. The supernatants were filtered with PES 0.45 µm syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and αCD22 scFv by methods discussed below.

4.3.5 Protein analysis

Filtered algal crude extract and purified samples were analyzed by SDS-PAGE, Western blot, and total eluted protein determined by Bradford assay (Bradford, 1976b). Host cell protein/Total soluble protein from crude extract and purified samples were quantified using the microplate protocol (working range from 1 to 25 µg/mL and 25 to 1500 µg/mL) Coomassie plus (Bradford, 1976b) assay kit (Thermo Scientific). Absorbance at 595 nm was measured using the VERSA max microplate reader.

NuPAGE Novex Bis-Tris pre-cast gradient gels (4 - 12 %) from Invitrogen^{TM} (1.5 mm x 10 wells), (Cat no. NP0335BOX) were used for SDS-PAGE electrophoresis. Reducing buffer was prepared using LDS sample buffer (4X) (NuPAGE NP0007) containing 10% of reducing agent (Cat no. NP0004). Reduced samples were prepared using a 1:4 ratio reducing-buffer: sample and heated at 70 ºC for10 min. MES SDS Running Buffer (20X) (Cat no. NP0002) stock solution was used to prepare 1X running buffer in RO water. Antioxidant (NuPAGE NP0005) was added to ensure reduced samples during electrophoresis. Gels were run for 35 min at constant voltage (200 V). For SDS analysis, the gels were stained in Coomassie^{TM} G-250 stain (Cat no. LC6065) for 3 hours followed by destaining in RO water. For Western blot analysis, the gel was transferred to nitrocellulose membranes using iBlot® 7-Minute Blotting System, Life Technologies Corporation.

After protein transfer to a nitrocellulose membrane, the membrane (free sites) was blocked with 2.5 % non-fat milk in TBS containing 0.05 % Tween 20 at pH 7.5 buffer

for 1 h to prevent nonspecific binding of the detection antibodies. FLAG-tagged

recombinant proteins (αCD22 scFv and Pfs25) were detected by using anti-FLAG M2-

AP (alkaline phosphatase conjugated) antibody from Sigma Aldrich (Carvalho, A9469)

at a concentration of 1:1,000. After incubation with the antibody for 1 h, the membrane

was washed with TBS containing 0.05 % Tween 20 at pH 7.5, buffer and blots were

visualized (developed) with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-

indolyphosphate p-toluidine salt (BCIP) (Sigma FAST B5655) dissolved in 10 mL of

filtered RO water.

4.3.6 FLAG affinity purification

Samples were filtered using polyethersulfone (PES) 0.45 µm filter and mixed with anti-

FLAG affinity resin (Sigma Aldrich A4596) equilibrated in the same lysis buffer used

for protein extraction. Approximately 1 mL of resin was used per every 4 g of wet algal

biomass. Binding of the recombinant protein to the affinity resin was performed for 2 h

at 4 ºC by continuous end-over-end mixing in a Glass Col rotor at ~ 33 rpm (40 % speed

control). Affinity resin was washed with 10 column volumes (CV) of lysis buffer

followed by 3 CV of lysis buffer without tween. The washed FLAG resin was

transferred into Bio Spin disposable chromatography columns (Bio Rad, Cat # 732-

6008) for protein elution at room temperature. Recombinant protein was eluted at pH

3.5 using 5 CV of 100 mM glycine buffer, pH 3.5 that contained 400 mM NaCl. Eluted

protein fractions were collected in 5 tubes containing a predetermined amount of 1M

Tris-HCl, pH 8.0 to immediately increase the pH of eluted protein to pH 8.0 and avoid

protein denaturation. Typically, three elution fractions (E2 to E4) were used for estimation of purity and yield, although some losses were occurring by not taking into account E1 (Elution fraction 1). By pooling these three fractions, more than 80% of extracted FLAG-tagged proteins were recovered. Extraction buffer and all the materials used including the sonication probe (1/8' microtip A3-561 BRANSON, USA) were cooled in advance.

The FLAG affinity purification method was used as a convenient analytical tool to determine the recombinant extraction yield. The resin was added in sufficient amounts to bind all available FLAG fusion protein present in clarified extracts. Cell debris and supernatants at the end of the batch adsorption period were regularly analyzed by western blotting to assure complete extraction and adsorption, respectively. Although minor recombinant protein losses have occurred during resin washing and pH 3.5 elution from the anti-FLAG resin, this determination of recombinant protein concentration was considered appropriate for estimating recombinant protein in extracts.

4.3.7. DNA determination

The DNA concentration in the algae extract was determined using a Quant-iT™ PicoGreen ® dsDNA Reagent Kit from Molecular Probes (Eugene, OR). The assay was performed using a fluorescence plate reader, Spectra Max Gemini XS, from Molecular Devices following the procedure recommended by the manufacturer. The samples were excited at 480 nm, and the fluorescent emission intensity at 520 nm was measured. The

assay limit is 250 pg/mL. Standard curve (Range 1-1000 ng/ml) was made using lambda DNA standard present in the kit.

4.3.8 Chlorophyll determination

Chlorophyll (a and b) was measured by measuring the absorbance at 652 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer. Blanks were prepared with lysis buffer containing ammonium sulfate or chitosan for respective pretreatments.

4.3.9. Turbidity measurement

It was measured by measuring the optical density at 750 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer. Lysis buffer (with/without chitosan) was used as blank.

4.3.10. Statistical analysis

Design Expert software was used for the experimental design and analysis. The statistical significance of the models was evaluated by the analysis of variance (ANOVA). Significantly different means ($p < 0.05$) were separated by the Tukey's test

4.4 Results

4.4.1 Evaluation of pretreatment methods in primary protein recovery

Data on acid precipitation for its effectiveness on reduction of impurities was previously obtained in our lab (Munjal, 2014). Cationic chitosan polymer was chosen over polyethylene amine (PEI) for this investigation because hydrophilic chitosan polymer

backbone exhibits a greater ionic-strength tolerance than PEI. Polymeric chitosan has a

pKa value of 6.3, and is soluble at acidic pH values and insoluble at pH values above

6.3. The impact of the acid and polymer pretreatment methods on cell free extracts is

summarized in Table 4.1. DNA, chlorophyll pigments, host cell proteins, and

recombinant protein αCD22scFv after each pretreatment were determined and

normalized against the control. Control experiments (no pretreatment) were performed

from the same algae batch to minimize the effect of batch-to-batch variability.

Table 4.1 Effect of pretreatment method stage of implementation on residual DNA, chlorophyll, host cell protein, and αCD22scFv yield in algae extracts. Values given are averages from 3-5 replicates ± standard deviations. [a,b,c] For each observation, means which do not share a common superscript letter are significantly different from the control no pretreatment ($p < 0.05$).

	Residual Host Cell Protein (%)	Residual DNA (%)	Residual Chlorophyll (%)	αCD22scFv yield (%)
Control	$100^a \pm 14.5$	$100^a \pm 9.12$	$100^a \pm 4.8$	$100^{ab} \pm 16.3$
Acid precipitation at pH = 4.5	$31.1^b \pm 9.3$	$91.7^a \pm 7.6$	$28.4^b \pm 14.7$	$118.8^a \pm 21.8$
Chitosan flocculation/ precipitation	$17.9^b \pm 7.1$	$8.9^b \pm 7.9$	$1.37^c \pm 1.1$	$73.58^b \pm 11.8$

In a 2D-gel of algal proteins analyzed by Ursu et al. (2014), it was found that a major portion of algal proteins have a pI between 4.0-5.5. Therefore, it was not surprising that acid precipitation was effective in reduction of host cell proteins by 69% (Table 4.1). There was also a 72% decrease in amount of chlorophyll. Pigments such as chlorophyll are associated with protein complexes of photosystem I and II (Caffarri et al., 2014), therefore, the general trend of chlorophyll reduction seems to follow that of host cell protein removal. However, acid precipitation was able to reduce only 9% of the DNA and this is where positively charged chitosan was more efficient, reducing DNA content by 91% which in turn resulted in a less viscous and easier to filter extracts. Chitosan precipitation could also reduce significantly more HCP (81%) and chlorophyll (98%) than acid precipitation. Figure 4.1 also confirms the significant reduction in HCP. For the same protein loading, much lighter protein bands are visible on the SDS-PAGE gel (Figure 4.1a). The presence of target protein after chitosan precipitation is confirmed by the Western blot (Figure 4.1b). Chitosan was also much more effective in reducing the turbidity of extracts (35 ± 9.7 fold) before filtration than acid precipitation (1.8 ± 0.2 fold). Lower viscosity of chitosan treated extracts along with the significant decrease in turbidity improved 0.45 µm filtration efficiency significantly. Munjal (2014) observed a threefold increase in filtration throughput (processed volume /area) with chitosan pretreatment, compared to 1.5 fold with acid precipitation. One drawback of using chitosan for removal of impurities was the loss of target protein by 26%. This could be improved by optimization of parameters such as chitosan concentration and pH for mixing.

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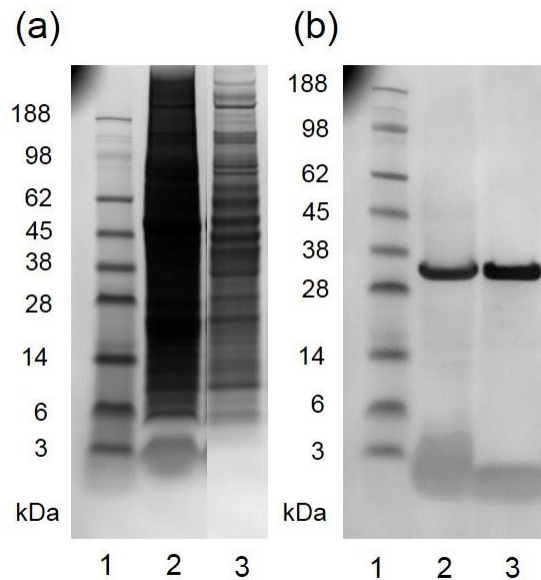
Figure 4.1 Effect of chitosan precipitation on HCP and αCD22scFv a) Coomassie-stained SDS-PAGE; (b) Western blot analysis of αCD22scFv samples using anti-FLAG-AP conjugated antibody. Lane 1: Molecular weight marker (kDa); Lane 2: Control/non-pretreated clarified cell lysate for; Lane 3: Clarified cell lysate after chitosan precipitation.

4.4.2 Capture chromatography of αCD22scFv from acid and chitosan pretreated extracts

Acid and chitosan pretreated samples were analyzed by adsorption to and elution from HiScreen Capto Q resin using linear salt gradient. αCD22scFv eluted at a conductivity range of 11-34 mS/cm (Figure 4.2).
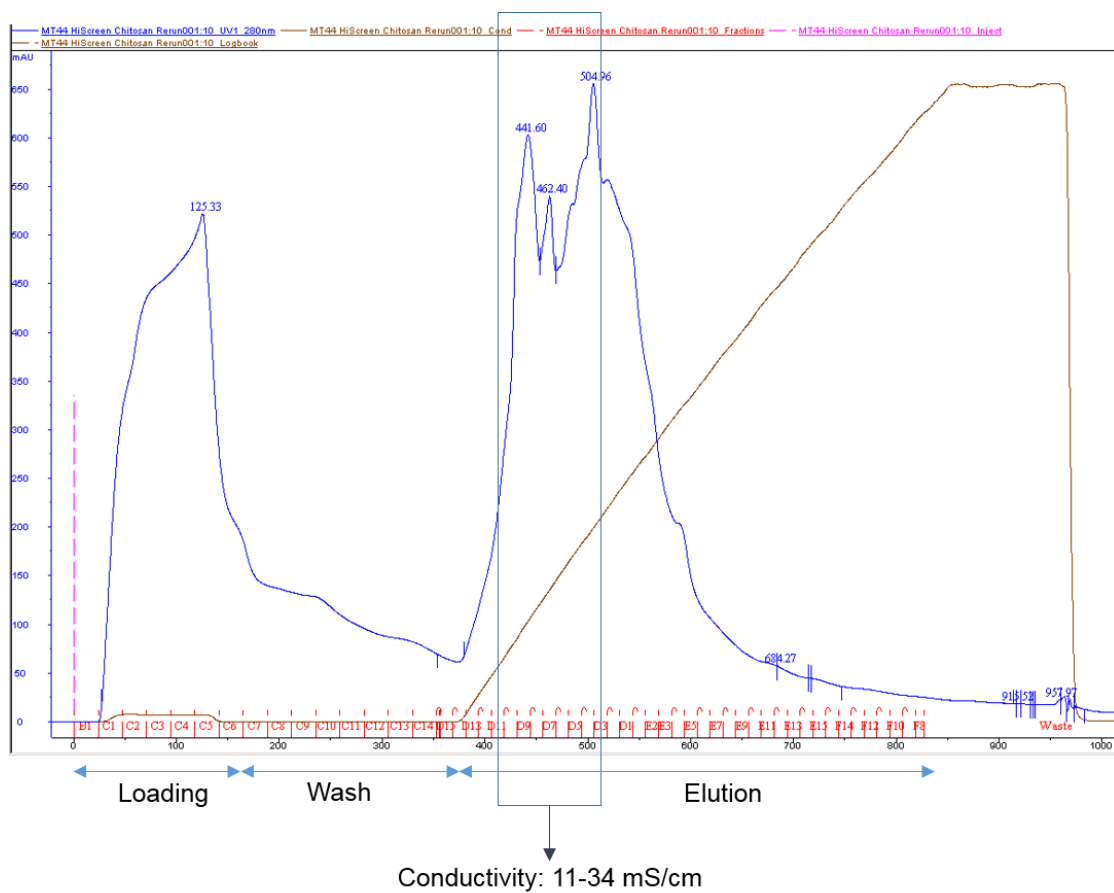
Figure 4.2 Protein elution profile (blue curve) of chitosan pretreated extracts from HiScreen Capto Q. αCD22scFv elutes at conductivity of 11-34 mS/cm.

Western blot and SDS-PAGE were carried out on eluted fractions (Figure 4.3) for confirmation of the presence of αCD22scFV and to determine which fractions containing αCD22scFv should be pooled for quantification by FLAG affinity. αCD22scFv pool was made of four fractions that eluted at conductivity between 13 and 26 mS/cm (Figure 4.3, lane 3-6) About 10% of additional αCD22scFv was detected in fractions that were eluted at 11 mS/cm and between 27-34 mS/cm, but were not included

because of very low αCD22scFv concentrations. The purity, yield and purification fold

at each stage of the process is given in Table 4.2.
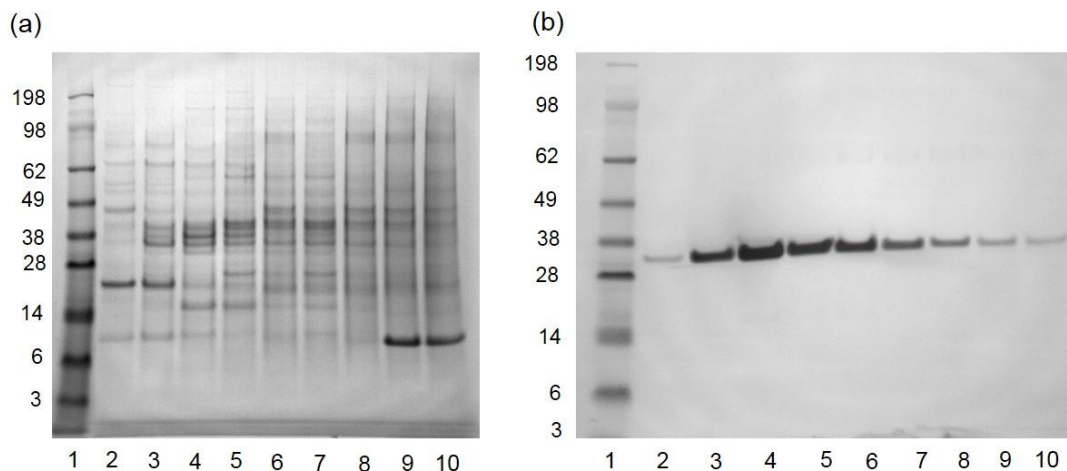
Figure 4.3 Purification of αCD22scFv after chitosan precipitation and adsorption to Capto Q. (a) Coomassie-stained SDS-PAGE; (b) Western blot analysis of αCD22scFv samples using anti-FLAG-AP conjugated antibody. Lane 1: Molecular weight marker (kDa); Lane 2: Elution fraction 1(11.03 mS/cm); Lane 3: Elution fraction 2 (13.67 mS/cm); Lane 4:Elution fraction 3 (17.16 mS/cm); Lane 5: Elution fraction 4 (20.90 mS/cm); Lane 6: Elution fraction 5 (22.7 mS/cm); Lane 7: Elution fraction 6 (25.5 mS/cm); Lane 8: Elution fraction 7 (27.89 mS/cm); Lane 9: Elution fraction 8 (30.71 mS/cm); Lane 10: Elution fraction 9 (33.45 mS/cm).

Table 4.2 Purity, yield and purification fold at each stage of Capto Q purification process for chitosan and acid pretreated extracts

Sample	Chitosan precipitation			Acid precipitation		
	Purity (% TSP)	Yield (%)	Purification fold	Purity (% TSP)	Yield (%)	Purification fold
Clarified cell lysate	0.08	100	1.0	0.12	100	1.0
Pretreated extract	0.25	88	3.0	0.39	85	3.0
αCD22scFv pool	1.7	40	20 (6.8)	1.6	26	12 (4.1)

In case of chitosan precipitation, purity increased 3 fold after the pretreatment and 6.8 fold after the anion exchange chromatography giving an overall purification fold of 20. For acid precipitation, purity increased 3 fold after the pretreatment and 4 fold after the anion exchange step – a total purification fold of 12. Interestingly, chitosan pretreatment yield of αCD22scFv from clarified cell lysate in this experiment was 88% - the high end of chitosan precipitation yields observed in Table 4.1. From this data and we can confirm both pretreatments would be compatible with anion exchange adsorption. We believe that "cleaner" extracts obtained with the chitosan pretreatment are the reason for

greater overall yield i.e. 40% vs 26% for acid treated extract. The notable difference between the two experiments was the chlorophyll-stained resin after the adsorption of the acid-pretreated extract but not with the chitosan one. The low purity of αCD22scFv after the anion exchange capture step is a reflection of the low initial titer of αCD22scFv in cell lysates.

4.5 Discussion

Investigation of different primary recovery methods, viz. acid precipitation and cationic polymer (chitosan) precipitation on cell free extract of *C. renihardtii,* revealed interesting insights. Acid precipitation reduced HCP in cell free extracts by 69%, chlorophyll by 71% and DNA by 9%. Chitosan pretreatment on clarified cell lysate showed even better results in terms of HCP (82%), chlorophyll (98%), and DNA (91%) reduction.

The observed loss of αCD22scFv with cell free extract (26%) during chitosan precipitation at pH 5 was somewhat unexpected. The small size of αCD22scFv (28 kDa) and flocculation pH near the pI of αCD22scFv should not have affected the protein yield that was previously observed with cationic polymer precipitation of large proteins (Agerkvist et al., 1990; Holler et al., 2007). Therefore, possible nonspecific interaction between the recombinant protein and chitosan, beyond the simple enmeshment, might be the reason for lower than expected αCD22scFv yield. The 74% αCD22scFv yield during the chitosan pretreatment is below the desirable 85% primary recovery yield, but

potential benefit of significant DNA, chlorophyll, and HCP reduction for increasing the efficiency of the capture chromatography step could offset lower recovery yield.

Chitosan and acid pretreated extracts followed by anion exchange capture of αCD22scFv by Capto Q resulted in an overall purification fold of 20 and 12, respectively. Thus, anion exchange chromatography supplemented with the pretreatment methods on algae extracts can have a synergistic beneficial effect on impurity reduction and target protein purity. The pretreatment methods tested in this work have pros and cons, which depend on the target protein properties and pretreatment compatibility with potential capture methods. Pretreatment by acid and/or chitosan precipitation significantly reduces extract impurities and would be beneficial to any capture chromatography step as reduction in DNA, HCP, and chlorophyll pigments increases resin lifetime, prevent column compression, and decrease column fouling (Barros et al., 2011). On the other hand, acid and chitosan precipitation require additional pH and ionic strength adjustment of pretreated extracts for efficient adsorption of the target protein on ion exchange resin.

4.6 Conclusions

Chitosan pretreatment was effective in reducing impurities in like HCP (82%), chlorophyll (98%), and DNA (91%) from clarified cell extracts of *C. reinhartdii* and better than acid precipitation in terms of impurity reduction. Overall, both pretreatments coupled with anion exchange capture were successful in increasing the purification fold of the investigated recombinant protein. In summary, the selection of a pretreatment method for conditioning of algae extracts should be chosen on a case-by-case basis. The

comparison of the two precipitation methods and their compatibility with the capture

chromatography step on *C. reinhardtii* extracts lays down a road map for further testing

and process development of the recombinant protein production platform from

microalgae.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Conclusions and recommendations are divided into the two parts: (i) selective extraction of pigments and proteins from *Chlorella vulgaris* and (ii) evaluation of pre-treatment methods for primary recovery and capture of an antibody fragment from *C. reinhartdii.*

5.1 Selective extraction of pigments and proteins from *C. vulgaris*

The commercial sustainability of microalgal production platform is possible only with selective extraction of multiple high-value compounds. It is essential to focus on optimizing processing parameters to obtain multiple revenue streams from different microalgal biomolecules. The first part of this dissertation investigated the development of process that resulted in selective extraction of pigments and functional proteins from microalga, *Chlorella vulgaris.* Work on this topic was initiated by working with frozen, wet biomass.

The major conclusions for this segment of research were as follows:

a) In terms of process scalability and productivity, high-pressure homogenization was the more effective in protein release compared to bead-milling and sonication

b) Ethanol was effective in extracting a pigment stream consisting of carotenoids and chlorophylls, while keeping the protein fraction inside the cells. Wet biomass

to ethanol ratio of 1:5 and 2 extraction stages yielded optimum amount of carotenoids and chlorophylls.

c) Reduction (1.4 fold) in protein extractability was observed after ethanol treatment of biomass, because of reversible protein denaturation and aggregation. Alkaline conditions (pH 12) were required to solubilize protein aggregates. Ethanol treatment of biomass or alkaline pH conditions required for complete release of proteins did not have any negative impacts on membrane processing parameters. Ethanol treatment of pigments also did not affect the functional properties of proteins such as the emulsifying ability or stability. In fact, the main protein concentrate generated out of this process had an essential amino acid profile that surpasses FAO/WHO recommendations and exhibits emulsification properties comparable to commercial plant-based protein emulsifiers such as soy protein isolate.

Since a process to achieve selective extraction of pigments and functional proteins was established, the next set of studies were focused on imparting permeability and enhancing pigment release from *C. vulgaris*. To achieve this, pulsed electric field (PEF) technology was utilized and fresh biomass was used. The effect of handling of cells (storage at 4°C and -80°C) and freeze-drying on permeability and pigment extraction was also evaluated. The major conclusions for this part of the research were as follows:

a) Application of PEF resulted in faster rate of extraction of and higher yields of lutein and chlorophylls. An electric field intensity of 6250 V/cm, pulse duration

of 620 µs, a biomass density of 56 g/L followed by single stage ethanol

extraction for 45 min was optimum for lutein extraction for fresh *C. vulgaris*

biomass. At these conditions, lutein yield was enhanced 2 fold and chlorophyll

yield was enhanced 4 fold. 90% of the protein remained inside the cells available

to be selectively extracted by complete cell lysis.

b) Cells stored at 4°C or -80°C didn't have a major impact on the permeability or

pigment release. The process of freeze-drying itself permeabilized the cells and

improved pigment extraction. However, majority of the proteins (>88%)

remained inside the cells after freeze-drying.

Recommendations for future work include:

a) Techno-economic analysis based on process parameters optimized at bench

scale to determine the scalability and commercial feasibility.

b) Incorporation and analysis of additional extractable algal biomolecules such

as lipids and carbohydrates

c) Further studies on the functionality and nutritional value of protein

concentrates such as foaming, pH stability, gelling, digestibility and bio-

activity

d) Optimization of protein purification process to produce protein isolates,

which have a higher commercial value.

5.2 Evaluation of pre-treatment methods for primary recovery and capture of an antibody fragment from *C. reinhartdii*

This second part of this dissertation was focused on investigation of chitosan precipitation on clarification of impurities in algal extracts such as DNA, chlorophyll and host cells proteins (HCP) for the purification of a single chain antibody fragment (αCD22scFv). Chitosan pretreatment was also compared to acid precipitation. The major conclusions for this segment of research were as follows:

a) Chitosan pretreatment on clarified cell lysate showed excellent results in terms of HCP (82%), chlorophyll (98%), and DNA (91%) reduction.

b) Chitosan precipitation was better than acid precipitation (HCP by 69%, chlorophyll by 71% and DNA by 9%) in terms of impurity reduction.

c) Chitosan and acid pretreated extracts followed by anion exchange capture of αCD22scFv by Capto Q resulted in an overall purification fold of 20 and 12, respectively. Thus, anion exchange chromatography supplemented with the pretreatment methods on algae extracts can have a synergistic beneficial effect on impurity reduction, filtration and target protein purity.

Recommendations for future work include:

a) Increase in the expression of level of αCD22scFv

b) Screening of more resins for capture and intermediate purification chromatography steps to increase purity of target protein.

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