Biorefining microalgae and plant hosts with extraction, recovery, and purification of multiple biomolecules

by

Chelsea Keiana Dixon

B.S., Texas A&M University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Biological and Agricultural Engineering
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Abstract

Microalgae are a potential feedstock for renewable and sustainable bioproducts and energy but there are significant scientific and engineering challenges to address before widespread acceptance of this platform. In particular, biorefining microalgae serves to maximize biomass valorization and minimize waste to improve process economics. The overall goal of this dissertation was the development of a biological-based microalgae biorefinery to enhance the economic feasibility of *Chlamydomonas reinhardtii* as a source of multiple products including native proteins and lipids. Specific objectives included accumulating biomass enriched in target biomolecules and determining processing strategies that eliminated the need to dry biomass, employed mild conditions to maintain extractability and quality, and minimized application of petroleum-derived and toxic solvents during extraction. The microalgae biorefinery developed included biomolecule accumulation, biomass harvesting, and targeted enzymatic degradation of the cell wall and organelles for release of native proteins and lipids.

Biomass was cultivated, and kinetic studies indicated that 48 h nitrogen deprivation was adequate for protein and lipid accumulation. Four lytic enzymes were screened for their ability to permeate the *C. reinhardtii* cell wall and the *C. reinhardtii*-produced enzyme, autolysin, led to >85% cell disruption. TEM imaging confirmed cell disruption and retention of lipid droplets in organelle remnants indicating that protein, lipids, and starch could be distinctly partitioned and recovered. A design of experiments optimization study determined that incubation of disrupted biomass at pH 12 for 4 h at 45°C resulted in up to 65% of total protein released from disrupted biomass followed by 40-50% protein recovery with isoelectric precipitation. The cell disruption and protein extraction steps were subsequently integrated to minimize unit operations, processing time, and energy inputs. Secondary application of trypsin led to release of ~73% of total lipids

(enriched in triacylglycerols) from the disrupted biomass. Characterization by thin layer chromatography and GC-FID of released lipids revealed similar profiles of enzymatically released lipids as compared to those released by conventional extraction procedures. Finally, the composition of released lipids indicated favorable combustion behavior, high oxidation stability, and suitability as biodiesel. The developed biological-based biorefinery is a promising step towards adoption of microalgae as a source of bioproducts to provide energy and food to meet the needs of a growing population.

The second focus of the work was mitigation strategies for isolation of critical impurities (or potential co-products) while processing microalgae and plant hosts. Specific emphasis was placed on evaluating the impact of proteases, polysaccharides, phenolic compounds and pigments, phytic acid, and host cell proteins on the processing of microalgae and other plant hosts for extraction, recovery, and purification of therapeutic proteins. This review served as evaluation of the broader implications of application of the biorefinery to transgenic microalgae and other plants.

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Table of Contents

List of Figures xvii

List of Tables xxi

Nomenclature xxiii

Acknowledgements xxiv

Dedication xxvi

Dissertation Organization xxvii

Chapter 1 - Green microalgae biomolecule separations and recovery: A review 1

Abstract 1

Introduction 2

Products and downstream processing 3

Commercial products 3

β-Carotene from *Dunaliella* 4

Astaxanthin from *Haematococcus* pluvialis 6

Biofuels 7

Eicosapentaenoic acid (EPA) from *Nannochloropsis* 8

Animal feed formulation with *Spirulina* 8

Human health products from *Chlorella* 9

Cosmetics 10

Emerging products 10

Recombinant proteins 11
Bioplastics/biopolymers 13
Future trends for microalgae products 14
Processing operations for microalgae products 15
Unit operations 15
Economic considerations 18
Extracellular matrices and pretreatment strategies for disruption 21
The diversity of extracellular matrices 21
Traditional ECM disruption techniques 24
Mechanical methods 24
Bead beating 24
High pressure homogenization 26
High shear homogenization 26
Ultrasonication 27
Pulsed electric field 28
Non-mechanical methods 29
Microwave treatment 29
Freezing/unfreezing 29
Chemical application 30
Osmotic shock 31
Algicidal microorganisms 31
Enzymatic hydrolysis for ECM disruption 32
Proteases 33

Carbohydrases 34

Lipases/phospholipases 35

Current applications of aqueous enzymatic processing 35

Product-containing organelles and disruption strategies 36

Chloroplast structure and disruption strategies 36

Lipid droplet structure and disruption strategies 39

Future directions of aqueous enzymatic processing 41

Conclusions 46

Chapter 2 - Fundamental studies on the applicability of aqueous enzymatic processing as part of a microalgae biorefinery 47

Abstract 47

Introduction 48

Biorefinery concept for microalgae biomass: Success and limitations 49

Alternative approaches to cell disruption and extraction for microalgae biorefinery realization 50

Cell disruption via enzymatic hydrolysis as part of aqueous enzymatic processing (AEP) 51

Chlamydomonas reinhardtii 53

Study objectives 54

Materials and Methods 55

Microalgae source 55

Production stain 55

Mating strains 55

Cultivation procedures 55
Characterization of algal biomass 56
Dry weight determination 56
Total protein quantification........ 56
Total lipid quantification........ 57
Protein profile analysis 58
Primary Enzymatic Treatment (PET) of microalgae biomass 58
Candidate enzymes 58
Production of gamete autolysin 59
Enzyme incubation and cell permeability assessment 60
Quantitative estimation of cell permeability 60
Qualitative visualization of cell permeability 60
TEM imaging 60
Evaluation of protein solubilization following PET alone or with combined chemical/mechanical disruption........ 61
Evaluation of extractable lipid content after PET alone or with combined mechanical disruption 61
Modified Bligh and Dyer extraction 62
Hexane extraction 63
Statistical Analysis........ 63
Secondary Enzymatic Treatment (SET) of microalgae biomass 63
Quantification of lipid release following secondary enzymatic treatment 63
Results and Discussion 64

Biomolecule accumulation kinetics 64
Primary enzymatic treatment of microalgae biomass 68
Enzyme incubation and cell permeability assessment 68
Autolysin as a pretreatment technique 69
Effect of incubation time on cell disruption 69
Effect of incubation temperature on cell disruption 71
Evaluating the effect of autolysin on enhancing lipid and protein extractability 73
Biomolecule release following Primary Enzymatic Treatment (PET) 76
Effect of increased temperature and incubation time on protein solubility 76
Lipid release due to increased temperature and extend incubation 78
Secondary Enzymatic Treatment (SET) of Microalgae Biomass 79
Summary 82
Chapter 3 - Enhancing protein extraction from *Chlamydomonas reinhardtii* as part of a biological-based biorefinery model 84
Abstract 84
Introduction 85
Materials and Methods 89
Laboratory equipment and reagents 89
Microalgae source 89
Production strain 89
Mating strains 89
Cultivation procedures 89

Autolysin production 90
Biological-based biorefinery approach 90
Dry weight determination 92
Primary enzymatic treatment (PET) 92
Enhanced protein (EP) extraction after PET of microalgae 93
Protein content quantification 94
Experimental Design 94
Two level full factorial design 94
Design augmentation to face centered central composite design 94
Optimization of protein extracted (%) and model validation 95
Integration of enhanced protein extraction step into overall bioprocessing procedures 96
Protein recovery via isoelectric precipitation 96
Protein profile analysis 97
Statistical analysis 97
Results and Discussion 97
Statistical analysis and model fitting 97
Effect of independent variables 100
Effect of pH 100
Effect of time 102
Effect of temperature 103
Optimization of protein solubilized (%) and model validation 103
Integration of the EP extraction step into biorefinery process 105

Protein recovery via isoelectric precipitation 106
Conclusions 112
Chapter 4 - Evaluating the effects of a biological-based biorefinery model on lipid release from *Chlamydomonas reinhardtii* 113
Abstract 113
Introduction 114
Materials and Methods 116
Microalgae Source 116
Production stain 116
Mating strains 116
Cultivation procedures 116
Dry weight determination 117
Enzyme source 117
Autolysin production 117
Biological based biorefinery procedures 118
Primary Enzymatic Treatment (PET) 118
Enhanced Protein (EP) extraction 119
Combined PET-EP 119
Lipid Extraction 119
Modified Bligh and Dyer extraction 119
Hexane extraction 120
Lipid Profile Analysis 120
Thin layer chromatography (TLC) 120

Preparation of fatty acid methyl esters (FAMEs) 120

GC-FID analysis 121

Fatty acid quantification 121

Biodiesel potential analysis.. 121

Results and Discussion 122

Evaluating lipid composition following nitrogen deprivation of *C. reinhardtii*..... 122

Characterizing lipid release due to biorefinery procedures 125

Effect of biomass state on lipid release after PET or EP step............................ 125

Effect of cell pretreatment on lipid release 129

Evaluating biodiesel potential of lipids released during biorefinery procedures 135

Conclusions and recommendations 137

Chapter 5 - The impact of six critical impurities on recombinant protein recovery and purification from plant hosts.. 140

Abstract 140

Introduction... 141

Evolution of molecular pharming 141

Recent successes in plant made recombinant proteins 142

Trends in recombinant protein products 143

Plant expression platforms and implications on downstream processing.................. 148

The plant platform for recombinant protein products.. 148

Leaf-based systems 149

Seed-based systems.. 151

Bioreactor-based systems... 152

The downstream process ... 154

Recurring challenges in downstream processing ... 157

The 6Ps ... 158

Proteases ... 158

Proteins derived from the host ... 165

Phytic acid (salts) ... 174

Polysaccharides ... 181

Phenolics and pigments ... 184

Summary and conclusion ... 193

Chapter 6 - Conclusions and recommended future work ... 195

Conclusions ... 195

Future work ... 197

References ... 199

Appendix A - Copyright permissions ... 225

List of Figures

Figure 1.1 Downstream processing steps for the recovery of proteins, carbohydrates, lipids, and pigments 19

Figure 1.2 Cell disruption alternatives 25

Figure 1.3 Aqueous enzymatic processing scheme workflow and design considerations 43

Figure 1.4 An alternative and holistic approach to aqueous enzymatic processing of microalgae for biorefining 45

Figure 2.1 Major steps of proposed biorefining procedures 54

Figure 2.2 (a) Lipid content and (b) protein content (g / g dry weight) for cells grown in nitrogen replete (TAP) media or nitrogen deplete (TAP-N) media.. 65

Figure 2.3 Coomassie-stained SDS-PAGE gel of molecular weight protein profiles for TAP and TAP-N *C. reinhardtii* cultures over time in hours.. 67

Figure 2.4 Cell permeability imaging assessment (a) using NP-40 to evaluate biomass treated with different enzymes for 2 h and (b) using Sytox® green under fluorescence microscope for negative control samples (no autolysin) and autolysin-treated biomass. 69

Figure 2.5 TEM images of *C. reinhardtii* cells incubated with TAP-N (negative control) or autolysin for 2 h and 24 h at 890x (a, c) and 2900x (b, d) magnification. Letters indicate cellular components: (S) starch granules, (LD) lipid droplets, and (CW) cell wall. 71

Figure 2.6 Cell lysis (%) after PET with autolysin over time at different temperatures. 72

Figure 2.7 Extractable lipids (% of total) via (a) Bligh and Dyer hexane extraction for different biomass pretreatments and (b) solubilized protein (% of total) following PET and negative control with or without secondary disruption. Soluble protein (%) is based on a total protein reference and extractable lipid (%) is based on a total lipid reference.. 75

Figure 2.8 (a) Impact of temperature on solubilized protein (% of total) released after PET or negative control treatment with 20 h incubation and(b) impact of time on solubilized protein (% of total) at 37˚C post PET. 77

Figure 2.9 (a) TEM image of solid residue after PET and (b) Lipid release (% of total) into aqueous phase (supernatants) of biomass treated with (-) control or PET........................... 78

Figure 2.10 (a) Lipid release (% of total) into the aqueous phase (supernatants) after PET and PET+SET and(b) fluorescence microscopy imaging of lipid droplets (yellow fluorescence) stained with Nile Red.. 80

Figure 2.11 TEM images of *C. reinhardtii* biomass subjected to (a) PET only or (b)PET and SET at 6400x (1,2,3), and 2900x (4) magnification. Letters indicate cellular components: (S) starch bodies, (LD) lipid droplets, and (T) thylakoids. 81

Figure 3.1 Process flow diagram of cell disruption and enhanced protein extraction procedures on (a) PET residue or (b) crude PET lysate. PET, primary enzymatic treatment, of *C. reinhardtii* biomass; EP, enhanced protein extraction, with combination of pH, time, and temperature; PET-EP, primary enzymatic treatment with EP extraction of unclarified PET lysate.. 91

Figure 3.2 (a-c): Contour plots for protein extracted (%) as a function of a) time and pH, b) temperature and pH, and c) temperature and time. (d-f) 3D Response surface plots for protein solubilized (%) as a function of d) time and pH, e) temperature and pH, and f) temperature and time.. 101

Figure 3.3 Distribution of total protein in extracts and residues resulting from separated PET and EP extraction procedures (Case 1) and integrated PET-EP extraction procedures (Case 2).. 106

Figure 3.4 Protein recovery from UTS (ultrasonication), PET (primary enzymatic treatment), and PET-EP (PET plus enhanced protein). (a)Precipitation efficiencies after isoelectric precipitation at pH 4.5. (b) Qualitative comparison of supernatant and pellet of UTS extract (left) and PET or PET-EP extract (right). (c)Protein profiling with gel electrophoresis. Lane 1 (MW marker), lane 2 (total protein), lane 3 (UTS extract), lane 4 (PET extract), lane 5 (EP- extract, pH 12, 4h, 45°C), lane 6 (PET-EP (I) extract, pH 12, 4h, 45°C); lane 7 (PET-EP (II) extract, pH 7, 4h, 45°C); lane 8 (PET-EP (III) extract, conditions: pH 7, 4h, 23°C); lane 9 (SR(I)-secondary residue after PET-EP(1)); lane 10 (SR(II)-secondary residue after PET-EP(II)); and lane 11 (autolysin solution) 109

Figure 4.1 TLC separation of lipids from nitrogen-replete (TAP) and nitrogen-deprived (TAP-N) cultures. 122

Figure 4.2 (a) Comparison of lipid classes in TAP and TAP-N cultures and (b) TAG accumulation in TAP and TAP-N cultures. 123

Figure 4.3 Comparison of FAME composition in (a) TAG and (b) polar lipids of TAP and TAP-N cultures. 124

Figure 4.4 TLC separation of lipids in aqueous extracts after PET (- control), PET, and EP of fresh biomass and lipids in autolysin solution. 127

Figure 4.5 TLC separation of lipids in aqueous extracts after PET (- control), PET, and EP of frozen biomass and lipids in autolysin solution. 128

Figure 4.6 Process flow and TLC separation of lipids in aqueous extracts after various cellular pretreatments 131

Figure 4.7 Comparison of fatty acid composition in (a) TAG, (b) FFA, (c) DAG, and (d) polar lipids released into aqueous extracts after various cell pretreatments 133

Figure 5.1 Downstream processing diagram with unit operations for recombinant protein purification from leaf-based, seed-based, and bioreactor-based systems 155

Figure 5.2 Sugarcane juice (left) and sugarcane extract (right) 193

List of Tables

Table 1.1 Summary of recent studies employing enzymes for cell wall pretreatment 37

Table 2.1 Mild cell disruption technique comparison (adapted from Phong et al. 2018 and Vanthoor-Koopmans et al. 2013) 51

Table 2.2 Summary of recent studies using enzymes for cell wall pretreatment (adapted from (Dixon and Wilken, 2018)). 53

Table 2.3 Enzymes used for cell permeabilization, cell wall target, compatible conditions and dosage guidelines for incubation. Adapted from [1]Wilken and Nikolov (2011), Nakimbugwe et al., (2006), [2] Yoshida and Noda (1965), [3] Bergmann et al., (1939) and [4] Jaenicke et al., (1987). 59

Table 3.1 Variables and levels considered for protein extracted (%) 93

Table 3.2 Experimental matrix for 2 level full factorial design with center runs and experimental and predicted responses 98

Table 3.3 Augmented FCCD with axial runs and additional center runs and associated experimental and predicted responses 98

Table 3.4 Analysis of variance (ANOVA), test of significance for protein extracted (%) for reduced quadratic model 99

Table 3.5 Validation points for protein extracted (%) at optimized and industrially relevant conditions 105

Table 3.6 Comparison of protein precipitation from UTS and PET extracts with comparable protein concentrations and/or buffer composition after pH adjustment to 4.5 107

Table 4.1 TAG fatty acid composition in PET extracts and autolysin solution 129

Table 4.2 Lipid class mass measurements in aqueous extraction after various cellular pretreatments ... 132

Table 4.3 Biodiesel properties calculated from the fatty acid profile of TLC-separated TAGs in UTS, PET and PET-EP Extracts ... 136

Nomenclature

AEP aqueous enzymatic processing
ATPS aqueous two-phase system
BCA bicinchonicic acid assay
BPTI bovine pancreatic trypsin inhibitor
BvLz bovine lysozyme
CAP combined algal processing
CBH-I cellobiohydrolase I
CFPP cold filter plugging point
CN cetane number
CO_2 carbon dioxide
CSFV classical swine fever virus
DAG diacyylcylercol
DARPA Defenese Advanced Research Projects Agency
DBCs dynamic binding capacities
DMSO dimethyl sulfoxide
DoE design of experiments
DSP downstream processing
DW dry weight
EBA expanded bed adsorption
ECM extracellular matrix
EDTA ethylene diamine-tetra-acetic acid
EP enhanced protein
EPA eicosapentaenoic acid
EPO erythropoietin
ERT enzyme replacement therapy
FAME fatty acid methyl ester
FFA free fatty acid
FGF-2 alpha-antitrypsin fibroblast growth factor
GC-FID gas chromatography-flame ionization detection
HA hemagglutinin
HBcAg Hepatitis B virus capsid antigen
HBsAg hepatitis B virus surface antigen
HCl hydrochloric acid
HCP host cell proteins
hEC-SOD human extracellular superoxide dismutase
hFIX human coagulation factor IX
hGH human growth hormone
HIS histidine
HOGP *Haematococcus* oil globule protein
HPH high pressure homogenization
HSH high shear homogenization
IFN-γ interferon gamma
IgG immunoglobulin G
IV iodine value
KCl potassium chloride
LD lipid droplet
LDSP lipid droplet surface protein
LED light-emitting diode
LHC light harvesting complex
LTB heat labile enterotoxin B
mAbs monoclonal antibodies
MAE microwave-assisted extraction
MLDP major lipid droplet protein
NaOH sodium hydroxide
NF nanofiltration
NP-1 neutrophil peptide 1
O&M operational and maintenance
OS oxidative stability
PAP parallel algal processing
PBR photobioreactor
PEF pulsed electric field
PEG polyethylene glycol
PEI polyethylenimine
PES polyethersulfone
PET primary enzymatic treatment
PMP plant-made pharmaceutical
PMSF phenylmethylsulfonyl fluoride
PS protamine sulfate
PVPP prehydrated polyvinylpirrolidone
rHSA recombinant human serum albumin
RP recombinant protein
RSM response surface methodology
RuBisCO Ribulose-1,5-bisphosphate carboxylase/oxygenase
SET secondary enzymatic treatment
siRNA small interfering RNA
TAG triacylglycerol
TAP TRIS acetate phosphate
TEM transmission electron microscopy
TLC thin layer chromatography
TMP transmembrane pressure
TRIS trishydroxymethyl aminomethane
TSP total soluble protein
UF ultrafiltration
UTS ultrasonication
VP validation point

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Dedication

This dissertation is dedicated to my fiancé Jervon, for all the days, weeks, and years we were apart so that I could pursue this goal. Thank you for all the miles you drove down I-70, for all the video chats, and for all the late-night phone calls. Most importantly thank you for believing in me even when I didn't and for never doubting my ability to finish.

The dissertation is also dedicated to my family, in particularly my mom for pushing me to achieve greatness. I remember coming home in grade school and being forced to practice writing. It was all worth it in the end because I've written a whole dissertation and none of it would be possible if you hadn't expected greatness. For that, I'm forever grateful.

Dissertation Organization

This dissertation is composed of six chapters covering a processing project and its implications on using wild type/transgenic microalgae and other plants hosts as platforms for extraction, recovery, and purification of multiple biomolecules. Chapter 1 is an extensive review of green microalgae, their extracellular matrices and organelle composition with specific emphasis of implications to separations and purifications technologies.

Chapters 2-4 are technical studies reporting the experimental work of the development, optimization, and evaluation of enzyme-based biorefinery strategies for extraction and recovery of native proteins and lipids from *Chlamydomonas reinhardtii.* Chapter 2 describes fundamental efforts to use aqueous enzymatic processing for biorefining microalgae. Harvesting and biomolecule accumulation kinetics are investigated and enzymes to mediate cell wall and organelle disruption are determined. Chapter 3 details a design of experiments study for enhancing native protein extraction and recovery. pH, time, and temperature conditions facilitating protein release and recovery by isoelectric precipitation are discussed. Chapter 4 describes characterization of lipids released during the cell disruption and protein extraction procedures. Fatty acid profiles of lipids released during the enzyme-based biorefinery were compared to those resulting from traditional extraction methods, and biodiesel properties were predicted.

Chapter 5 is a review of mitigation or isolation strategies for impurities (potential co-products) encountered during processing microalgae and plants hosts. The review is an examination of the potential implications of the developed biorefinery procedures in relation to using transgenic microalgae and other plant hosts as a source of recombinant proteins. Finally,

chapter 6 presents general conclusions of the project and recommendations for future research efforts.

Chapter 1 - Green microalgae biomolecule separations and recovery: A review[1]

Abstract

Microalgae biomass has garnered significant attention as a renewable energy feedstock and alternative to petroleum-based fuels. The diverse metabolism of green microalgae species additionally provides opportunities for recovery of products for feed, food, nutraceutical, cosmetic, and biopharmaceutical industries. Recently, the concept of using microalgae as part of a biorefinery model has been adopted in place of refinery methods focused on recovering one target product. This has led to producers exploring co-production of high value and high-volume products in an effort to improve process economics. With numerous potential products and applications, the biomass source or specific strain must be carefully selected to accumulate extractable levels of the target molecule(s). It is additionally imperative to understand the morphology and metabolism of the selected strain to cost-effectively manage all stages of commercial production. This review will focus specifically on microalgae in the division of Chlorophyta, or green algae and their extracellular matrices (ECM), potential for commercial products, and finally describe a holistic approach for biomolecule extraction and recovery. Additionally, cell disruption and fractionation methods for recovery of biomolecules for commercial products are highlighted along with an alternative method, aqueous enzymatic processing for multiple biomolecule extraction and recovery from green microalgae. An

[1] This chapter has been published as a peer-reviewed journal article. Dixon C, Wilken LR (2018) Green microalgae biomolecule separations and recovery. Bioresour Bioprocess 5:14. https://doi.org/10.1186/s40643-018-0199-3. This is an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

emphasis is placed on connecting the morphological characteristics of microalgae ECM or organelle membranes to implications on separation and purification technologies.

Introduction

Microalgae, a large and diverse group of unicellular photo- and heterotrophic organisms, have significant potential for production of a vast array of valuable products for diverse industries. Microalgae use solar energy, nutrients, and carbon dioxide (CO_2) to produce proteins, starch, lipids, and other biomolecules. Much research has been conducted in regard to using microalgae biomass as an alternative fuel source, but other valuable products can be sourced from microalgae including bioactive compounds for human health and nutrition (omega-3 fatty acids), biopharmaceutical, cosmetic, and feed industries (Mercer and Armenta, 2011; Skjånes et al., 2013). Value-added products include carotenoids; phycobiliprotein pigments; vitamins C, E, and biotin; fatty acids (linolenic, arachidonic, etc.); and recombinant proteins (Converti et al., 2009).

While microalgae has demonstrated potential as an alternative and sustainable biomass source for biofuels and bioproducts, techno-economic assessments have repeatedly concluded that microalgae-derived fuels, animal feed inputs, and bulk chemicals cannot currently compete with market prices (Benemann et al., 2013; Chauton et al., 2015). Central to this barrier to commercialization of microalgal products are the processing techniques used to extract and recover biomolecules. The most critical step to access internally stored biomolecules is cell disruption. Current processes are energy-intensive, expensive, and/or utilize organic solvents, which has significant environmental implications. To improve process economics and promote product commercialization, processes should be robust, energy-efficient, minimize the environmental impact, and maintain product quality. Thus, alternative processing techniques

such as enzymatic hydrolysis are being explored for cell disruption. Enzymatic hydrolysis, in addition to serving as pretreatment for cell disruption, can be selectively used for extraction of valuable biomolecules produced in organelles. By employing a strategy whereby algal species are classified based on ECM characteristics, enzymes specifically targeting components found in the ECM can be used to permeabilize and/or lyse the matrix prior to additional processing. Additionally, identifying structural components of organelle membranes can similarly allow for targeted enzymatic hydrolysis of organelles and facilitate extraction of biomolecules. This paper discusses traditional and emerging products and general downstream processing (DSP) of green microalgae and reviews the cell morphology of several microalgae species including *Dunaliella*, *Haematococcus*, *Nannochloropsis*, *Spirulina*, *Chlorella*, and *Chlamydomonas* through classification of their ECM. Additionally, cell disruption and fractionation methods for recovery of biomolecules are discussed along with an alternative processing strategy for multiple biomolecule extraction and recovery from microalgae.

Products and downstream processing

Commercial products

The first unialgal cultures (*Chlorella vulgaris*) were produced in 1890. After 1948, concurrent research efforts into new applications of algal cultures occurred in the United States, Japan, and Germany with a primary focus on algae use for food production (Burlew, 1953). This research ultimately spurred the first industrial-scale production of *Chlorella* biomass in Japan in the 1960s. Dried whole cells were supplied to research institutes to develop nutritional and medical applications and mass cultivation techniques, which led to the *Chlorella* health food industry known today (Borowitzka, 2013). This was followed by *Spirulina* production in Mexico in the 1970s and *Dunaliella salina* production in Australia in the mid-1980s (Milledge, 2011). In

the 1980s and 1990s, the US Department of Energy funded the Aquatic Species Program with a goal of producing oil and ultimately biofuels from microalgae. The program funding was ultimately cut due to budgetary pressures, but the foundation for generating cost-effective and scalable processing of microalgae into biofuels was set. Although microalgae from biofuels continues to be a primary goal for the research community, other microalgae-derived bioproducts have been commercially developed. Currently, the microalgae product market includes bioactive compounds from a variety of different green microalgae. Genera most commonly used for commercial production include *Dunaliella*, *Haematococcus*, *Nannochloropsis*, *Chlorella*, and *Chlamydomonas*. Additionally, the cyanobacterium or blue-green algae *Spirulina* is used for commercial products. The unique metabolisms of selected green microalgae species have been exploited for the production of β-carotene using *Dunaliella*, astaxanthin using *Haematococcus* pluvialis, Eicosapentaenoic acid (EPA) from *Nannochloropsis sp*ecies, components of animal feed formulation from *Spirulina*, human health products from *Chlorella* species, and cosmetics and biofuel feedstock sourced from multiple green microalgae species. In many instances, the aforementioned microalgae species are manipulated during the cultivation stage to redirect their metabolisms toward production or accumulation of valuable products at the expense of diminished cell growth.

β-Carotene from *Dunaliella*

β-Carotene is a provitamin A carotenoid with success as a natural food pigment, coloring agent, or health food (Markou and Nerantzis, 2013) and has additionally been recognized for its antioxidant properties and role as an essential nutrient (Singh et al., 2016). The carotenoid is non-polar; lipophilic; insoluble in water, acids, and alkalis; but soluble in benzene, chloroform,

and carbon disulfide. The halotolerant microalgae, *Dunaliella salina*, is used for the commercial production of 8.5–30% of the global β-carotene supply per year (Ericksen, 2016).

By the year 2018, the global market for β-carotene is expected to be US$334 million (BCC Research, 2011). Global producers include companies in Australia, Israel, India, and China. β-Carotene can comprise up to 14% dry weight of *D*. *salina* and accumulates within lipid globules in the chloroplast interthylakoid space (Markou and Nerantzis, 2013; Shariati and Reza, 2011) under stress parameters including high salinity, high temperature, high light, and nutrient limitation (Haghjou and Shariati, 2007; Nguyen et al., 2016). Since β-carotene accumulates under suboptimal growth conditions, manufacturers must establish a balance between biomass production and product accumulation. Biomass production can be "intensive" in which all cultivation factors are controlled to affect cell growth and chemistry or "extensive" in which growth is slowed down via cultivation in a brine solution to promote β-carotene accumulation (Raja et al., 2007). Extensive production occurs in large unstirred outdoor ponds while intensive production occurs in paddle wheel stirred raceway ponds (Borowitzka, 1990). Harvesting and dewatering strategies for *D*. *salina* are complicated by the lack of a protective cell wall and natural buoyancy. Successful lab- and pilot-scale strategies for harvesting include high pH-induced flocculation–flotation and membrane filtration (Besson and Guiraud, 2013; Monte et al., 2018). Extraction of β-carotene commercially can occur via traditional solvent extraction, but concerns about toxicity and residuals limit market acceptance. Patented, commercial extraction methods include supercritical CO_2, biomass saponification followed by solvent extraction, or hot oil extraction (Borowitzka and Borowitzka, 1990). Other extraction methods include edible oil (vegetable oil) extraction, microwave-assisted extraction, ultrasound-assisted extraction

(Kyriakopoulou et al., 2015), and "milking" D. *salina* in closed photobioreactors (PBR) with the addition of an organic phase (dodecane) (Kleinegris et al., 2010).

Astaxanthin from *Haematococcus pluvialis*

Astaxanthin is a carotenoid used as a natural pigment source for aquaculture with antioxidant activities and human health implications in skin cancer pathogenesis, coronary heart disease, and infection resistance (Yaakob et al., 2014). The global astaxanthin market is predicted to be US$1.1 billion by 2020 (Industry Experts, 2015). *Haematococcus pluvialis* is used to produce 280 metric tons of astaxanthin annually which accounts for < 1% of the global market with the remaining astaxanthin sourced from other marine life, *Phaffia* yeast, Paracoccus bacteria, or chemical synthesis (Panis and Carreon, 2016). Astaxanthin is polar, lipophilic, and soluble in acetone, acetic acid, chloroform, pyridine, and dimethyl sulfoxide (DMSO) (with heat application) (Kim et al., 2008).

Commercially, *Haematococcus pluvialis* is grown in indoor and outdoor PBR or hybrid pond–PBR systems to control contamination. Typically, a two-step cultivation process is used including accumulation of vegetative cells in optimal growth conditions followed by exposure to suboptimal environmental or nutrient stresses (Sarada et al., 2002). During stress conditions, vegetative cells become hematocysts and accumulate astaxanthin to 1.5–3.0% dry (Shah et al., 2016). The cells are then harvested via settling and centrifugation, disrupted rapidly with homogenization or ultrasonication to retain astaxanthin integrity, and cracked or pulverized to enhance extraction and recovery.

Biofuels

The high lipid and carbohydrate content of green microalgae confers the possibility of conversion to biofuels (Sahay and Braganza, 2016) including biodiesel, bioethanol, biomethane, and bio-oil (Lam and Lee, 2012). *Chlorella vulgaris* and *Nannochloropsis oculata* have been successfully used for the production of biodiesel while *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, and *Spirulina sp.* are all considered good candidates for bioethanol production. Additionally, *Chlorella* and *Chlamydomonas* sp. can be used for biogas production. Production begins with accumulation of lipid-rich algal biomass and is followed by harvesting, dewatering, lipids/sugar extraction and conversion, and additional processing of biomass for valuable co-products (Sing et al., 2013). Flocculation and subsequent flotation are commonly used for harvesting microalgae for biofuels because this technique can handle the diversity in shape, size, specific weight, and surface charge of various microalgae cells. Following harvesting and dewatering, microalgae are subject to cell disruption or pretreatment strategies. Cells can be mechanically pressed for access to lipid precursors of biodiesel or bio-oil or enzymatically hydrolyzed for access to fermentable sugars for bioethanol. Lipids are extracted with conventional solvents, green solvents, subcritical water, supercritical CO_2, or co-solvent mixtures (ionic liquids/polar covalent molecules) (Sing et al., 2013). Lipids are converted to biodiesel via transesterification, pyrolysis, or hydrogenation. Enzymatic saccharification hydrolyzes carbohydrates into simple sugars that are then fermented to bioethanol (Hernández et al., 2015). Hydrothermal liquefaction is used to produce bio-oil from microalgae in an aqueous conversion environment with no prior drying (Guo et al., 2015). Microalgae biofuels are advantageous due to the high oil content of various species and the cultivation time. Additionally, one species can be used for multiple fuels (i.e., biodiesel produced from lipids and bioethanol production from

lipid-depleted residual biomass) (Gutiérrez-Arriaga et al., 2014). Challenges for widespread commercialization of microalgae biofuels include the diversity in size and morphology of lipid-producing algae strains, harvest of dilute algae suspensions, and translation of laboratory- and pilot-scale techniques for commercial operations. Additionally, harvesting and dewatering operations greatly impact economically feasibility (Wu et al., 2012).

Eicosapentaenoic acid (EPA) from *Nannochloropsis*

Eicosapentaenoic acid (EPA) is a marine-derived omega-3 fatty acid and essential fat with various human health applications (Swanson et al., 2012). The 2016 global market for omega-3 products is US$34.7 billion (Pakaged Facts, 2012). The current source of EPA is mainly fish oil, but *Nannochloropsis sp*ecies are promising alternative producers (Chauton et al., 2015) as they can produce EPA to levels of 1.1–12% dry weight depending on culturing conditions (Camacho-Rodríguez et al., 2013; Chen et al., 2013b, 2013a; Chen and Davis, 2016; Ma et al., 2016). On a commercial scale, *Nannochloropsis sp*. are cultivated photoautotrophically using natural light and carbon dioxide from power plants or flue gas in raceway ponds. The microalgae can additionally be grown heterotrophically and mixotrophically, but on a smaller bench- or pilot-scale. Following culturing, algae cells are separated from media via filtration, flocculation, or centrifugation and then dried. EPA is extracted via solvent (hexane), refined by degumming, bleaching, or deodorization, and encapsulated (Adarme-Vega et al., 2012).

Animal feed formulation with *Spirulina*

As an edible microalgae, *Spirulina* is not only a human food supplement, but also valuable as an animal feed formulation component due to its high protein (60–70% dry weight), vitamin, and mineral contents (Harun et al., 2010). Global production as of 2010 was 5000 metric ton/year (Norsker et al., 2011) with producers in the United States, China, India, and

Chile. Commercial production occurs in shallow raceway ponds mixed by a paddle wheel (Belay, 2013) in high saline and alkaline conditions and the biomass is then harvested and processed for inclusion in animal feed formulations (Yaakob et al., 2014). Harvesting and processing include filtration, concentration, neutralization, grinding/homogenization, and dehydration. Drying methods include drum drying, spray drying, sun drying, solar drying, cross-flow air drying, vacuum-shelf drying, and freeze drying (Belay, 2013). Typically, *Spirulina* is neutralized with an acidic solution and dried or dehydrated as this allows for easy integration into animal feed formulations (Ahsan et al., 2008).

Human health products from *Chlorella*

The high content of bioactive compounds in *Chlorella* makes it an attractive source as a nutritional food and human health product with global production in excess of 2000 metric tons/year (Ramaraj et al., 2016). In particular, the carbohydrate and protein contents have led to the production of nutraceutical tablets, teas, and noodles among other products (Liu and Chen, 2014). The majority of commercial *Chlorella* production is done using mixotrophic mass cultivation (Hudek et al., 2014) in PBR with various geometries. A small percentage of industrial *Chlorella* cultivation occurs heterotrophically in fermenters but high operating costs limit widespread commercial use. Various harvesting strategies such as flocculation, flotation, filtration, gravity sedimentation, and centrifugation (Liu and Chen, 2014) are employed on a commercial scale. To access carbohydrates, mostly contained within the cell wall, a disruption technique must be employed. Commercially, this can include high pressure homogenization, enzymatic lysis, bead milling, or grinding (Huang et al., 2016). When sold as a health food, dried *Chlorella* biomass is most commonly pulverized or spray dried and supplied in tablet or capsule form for consumption.

Cosmetics

Several species of microalgae have been used for skin and hair care products including *Spirulina*, *Chlorella*, *Dunaliella*, and *Nannochloropsis* extracts. More specifically, carotenoids such as astaxanthin, β-carotene, and lutein can be included as part of topical cosmetic products for protection against hyper-pigmentation or UV-induced damage (Mourelle et al., 2017; Wang et al., 2015). Similarly, polysaccharides from various green microalgae species can be included in cosmetic products for the purposes of antioxidant activity, gelling, or thickening (Mourelle et al., 2017). Due to the application of these products on the skin of consumers, consistent quality and controlled growth environments are necessary for the microalgae bioproduct precursors. The microalgae are grown in PBR with optimal light and nutrients which leads to consistent accumulation of the bioactive substances for skin care products. For whole cell products, biomass is centrifuged or filtered to separate cells from growth media. The biomass is then lyophilized, spray dried, of flash dried to produce cake, flakes, powder, or flour (Brooks and Franklin, 2009). When intracellular products are of interest, whole cells are disrupted via high pressure homogenization or ultrasonication (Coragliotti et al., 2010). The lysate is then centrifuged or filtered prior to lyophilization or drying with heat. Polysaccharides and proteins can be recovered with precipitation or tangential flow filtration (Coragliotti et al., 2010) while oil is typically recovered with solvents or supercritical CO_2 (Brooks and Franklin, 2009). The final products or extracts are often decolorized by bleach, solvents, activated carbon, high salt solutions, or enzymes and then incorporated into cosmetic products (Coragliotti et al., 2010).

Emerging products

While the aforementioned products have well established pilot and/or commercial production streams, there are emerging opportunities to additionally capitalize on green

microalgae metabolic diversity. Research is currently being conducted in using the microalgal platform for recombinant protein expression and accumulation (Rasala and Mayfield, 2015), bioplastics/biopolymers (Wang, 2014), and bioremediation (Ummalyma et al., 2018). Research advancements have been made but a better understanding of the cellular processes and their response to environmental stimuli are critical for commercialization.

Recombinant proteins

Recombinant proteins (RP) such as antibodies, immunotoxins, subunit vaccines, and industrial enzymes have been expressed in microalgae (Hempel and Maier, 2016; Rasala and Mayfield, 2015; Scranton et al., 2015; Yusibov et al., 2016). *Chlamydomonas*, *Chlorella*, and *Dunaliella sp.* are generally regarded as safe organisms and can be potentially used for RP production, but most studies have focused on *Chlamydomonas reinhardtii*.

Completed sequences for the nuclear, chloroplast, and mitochondrial genomes have allowed for the establishment of transformation methods and the introduction of recombinant molecules into *C. reinhardtii*. The expression level of most RP in *C. reinhardtii* ranges from 0.1 to 5% of total soluble protein (TSP) but can be as high as 20% TSP. Expression in the nucleus or chloroplast is most common with the chloroplast having distinct advantages including the possibility of targeted DNA integration, disulfide bond formation, the absence of gene silencing mechanisms, and high level of expression (Guzmán-Zapata et al., 2016; Rasala and Mayfield, 2011; Scaife et al., 2015). Nuclear expression, while allowing RP secretion and glycosylation, suffers from lower accumulation levels, transgene silencing, and positional effects (Rasala and Mayfield, 2015; Scranton et al., 2015).

The first antibody expressed was a large single-chain antibody against Herpes simplex virus glycoprotein D in 2003 (Rasala and Mayfield, 2015). Complete human Immunoglobulin G

(IgG) antibody against anthrax has also been expressed and more recently, mono and dimeric single-chain immunotoxins were expressed in the *Chlamydomonas* chloroplast (Tran et al., 2013). Other RP expressed in the *Chlamydomonas* chloroplast include the E2 protein, an antigen for vaccines against classical swine fever virus (CSFV), at levels of 1.5–2% TSP, Viral Protein 1-Cholera toxin B (VP1-CTB) vaccine against foot and mouth disease virus at levels up to 3% TSP (Yan et al., 2016), and oncoproteins and antigens for cancer treatment/prevention (Demurtas et al., 2013).

Various processing steps have been utilized for the extraction and purification of recombinant proteins from *C. reinhardtii*. For therapeutic applications, RP are subject to additional processing and purity requirements. Thus, the effects of all downstream processing strategies must be closely monitored and optimized to retain the RP integrity and activity. Munjal et al. (2015) reported the pretreatment of cells expressing a single-chain antibody fragment (αCD22scFv) in the chloroplast with ultrasonication followed by reduction of chlorophyll and precipitation of host cell proteins from cell lysates with chitosan (Munjal et al., 2015). The αCD22scFv was then purified from clarified extract using capture chromatography. Cells expressing Plasmodium falciparum surface protein 25 (Pfs25TBV/Pfs25), a subunit vaccine candidate for malaria, were likewise pretreated with ultrasonication with purification from clarified extract using affinity chromatography (Munjal et al., 2014).

While RP expression in other green microalgae has not been as thoroughly explored as in *Chlamydomonas reinhardtii*, *Chlorella ellipsoidea* has successfully expressed therapeutic recombinant proteins including mature rabbit neutrophil peptide 1 (NP-1) for innate immune system defense and flounder growth hormone for aquaculture (Rasala and Mayfield, 2015). Similarly, *Dunaliella tertiolecta* has expressed industrially applicable bioactive xylanases, α-

galactosidases, and phytases (Rasala and Mayfield, 2015; Yan et al., 2016). *Dunaliella salina* has similarly been used to express hepatitis B virus surface antigen (HBsAg) to 1.6–3.1 ng/mg of total protein (Yan et al., 2016). Although these examples of successful expression prove microalgae to be a viable option for RP production, improvements in integrated process development providing high yields and protein stability are critical for commercialization.

Bioplastics/biopolymers

Bioplastics are biodegradable plastics from renewable biomass sources with a global market value expected to be US$10 billion by 2020 (Oilgae, 2016). Microalgae-derived bioplastics are a combination of microalgae biomass and polymers/additives that are molded or extruded into their final form (articles, sheets, and films). Bioplastics from microalgae are used in the packaging, catering, gardening, medical, and automotive industries (Rajendran et al., 2012) and can be classified as hybrid-based plastics, cellulose-based plastics, polylactic acid, or biopolyethylene (Beetul et al., 2016). *Spirulina* and *Chlorella* are commonly used for bioplastic production based on their small cell size and protein composition that allows for conversion to bioplastics without prior treatment (Zeller et al., 2013). Microalgae biomass and proteins are converted into bioplastics through a process of denaturation/digestion/fermentation, plasticization, blending, and compatibilization (Wang, 2014). Plasticization improves flexibility and durability through the addition of non-volatile, organic molecules such as glycerol, sorbitol, saccharose, urea, triethylene glycol, or polyethylene glycol. Blending mixes compatible polymers such as polyethylene or poly(vinyl alcohol) to the plasticized biomass and compatibilization stabilizes the blended polymers by modifying their interfacial properties. After compatibilization, the bioplastics are molded or extruded with heat and pressure. Microalgae-derived bioplastics have relatively simple production procedures and unlike soy or other

common feedstocks for bioplastics, use of microalgae has less impact on food supply (Wang, 2014). For commercial viability, the functional properties of microalgae proteins must be improved and methods for removing odor-causing volatiles must be developed (Wang, 2014).

Future trends for microalgae products

For microalgae to extend beyond traditional markets, a concerted effort to diversify products is necessary. Microalgae production companies seek to reach new industries such as green chemicals, polymers/plastics, and therapeutics. A biorefinery model is increasingly employed whereby biomass is converted into products for different industries to maximize biomass utilization and minimize residual waste (Zhu, 2015). In addition, microalgae producers are exploring co-production of high value and high volume products in an effort to improve process economics (Barbosa and Wijffels, 2015) and increase product range.

Life cycle assessment studies have previously concluded that producing algal biomass with the sole intent of using the accumulated lipids (or starch) for biofuel production is not environmentally (Gnansounou and Raman, 2016) or economically (Soratana et al., 2014) advantageous. Thus, production and recovery of higher value products like fine chemicals, carotenoids, and therapeutic recombinant proteins along with lipids can promote commercialization. To improve product (target biomolecule) accumulation, candidate algae strains have been genetically manipulated (Singh et al., 2016). With the accumulation of multiple products, producers will need to incorporate methods for assessing the effects of manipulating microalgae metabolism on cultivation strategies. Thus, cultivation and harvesting methods have been studied and optimized while many techniques for extraction and recovery have been developed at bench- and pilot-scale. Additionally, alternative extraction techniques such as enzymatic hydrolysis are being explored for translation into industrial-scale processing.

Processing operations for microalgae products

Unit operations

Microalgae have been demonstrated as a source of biomolecules for pigments/dyes, feed, biofuels, cosmetics, nutraceuticals, and even therapeutic molecules, but complex biological structure and costly processing requirements limit industrial-scale production and distribution of products. The DSP of microalgae for valuable bioproducts includes generally includes four major stages: (1) cell disruption/pretreatment; (2) extraction; (3) fractionation, purification, and/or biochemical conversion; and (4) final formulation. Figure 1.1 illustrates the potential techniques employed at each DSP stage for recovery of common biomolecules (proteins, carbohydrates, lipids, and pigments) from microalgae. Cell morphology and properties of target molecules determine which techniques and methods can be employed at for each of these DSP stages.

Cultivation is the production and accumulation of biomass containing target molecules using a combination of nutrient media, light, mixing/aeration, and CO_2 to promote microalgae growth. Commercially, green microalgae can be cultivated in open raceway ponds, photobioreactors, or hybrid systems. Open raceway ponds are cost-effective but prone to contamination, low biomass productivity, and low utilization of CO_2 due to constant evaporation (Tan et al., 2018). The ponds are also subject to limited light penetration due to dark zones and inadequate mixing. Photobioreactors allow for bulk quantity microalgae production, are less prone to contamination, and require less land for cultivation. Conversely, photobioreactors are much more costly, difficult to clean, and can experience a buildup of dissolved oxygen (Narala et al., 2016; Tan et al., 2018). Cultivation can also occur with a combination or hybrid blend of open raceway ponds and photobioreactors that can reduce cost and improve productivity. With

hybrid cultivation strategies, a dense inoculum is produced in photobioreactors with minimized risk for contamination and then introduced in raceway ponds for continued cultivation and biomass production (Tan et al., 2018). Hybrid cultivation is an interesting strategy for species that accumulate products in nutrient deficient or deplete conditions as photobioreactors can be used for vegetative growth and raceway ponds can be used for bioproduct accumulation (Narala et al., 2016).

Harvesting includes solid–liquid separation by centrifugation, filtration, or gravity sedimentation (Barros et al., 2015) and due to the high volume of algal cultures being processed, this step is often considered to be a processing bottleneck. In fact, harvesting combined with dewatering can account for 20–30% of biomass production costs (Zhu, 2015). To enhance harvesting efficiency via solid–liquid separation, cells can be flocculated by chemical, electroflocculation, and bioflocculation techniques (Chen et al., 2015). For chemical flocculation, salts (e.g., aluminum sulfate and poly aluminum chloride) or polymers (e.g., polyacrylamide and chitosan) are added to change the surface charge of microalgae (Wan et al., 2015). Electroflocculation also modifies surface charge, but the process generates metal ions via a metal electrode instead of metal salts (Pearsall et al., 2011). Bioflocculants are derived from bacteria, fungi, or the microalgae itself (self-flocculation). In this case, the microorganism can be added to the microalgae culture or co-cultured with the microalgae. The flocculants associate with the microalgae cell surface resulting in mixed microalgae–microorganism flocs that can be harvested (Vandamme, 2013).

Secondary dewatering by centrifugation or filtration is often performed to minimize the processing volume, concentrate the algal slurry, and drastically reduce the water content (Barros

et al., 2015). Concentrated biomass slurry is then dried either by air, solar, spray, rotary, or incinerator drying (Jegathese and Farid, 2014; Shiratake et al., 2013; Show et al., 2015).

Microalgae cell disruption methods can be mechanical or non-mechanical (thermal, chemical, biological) depending on the characteristics of the microalgae cell wall/membrane and target molecules. Mechanical cell disruption methods include bead beating, high pressure homogenization (HPH), high speed homogenization (HSH), ultrasonication, and pulsed electric field (PEF) treatment. Thermal cell disruption can occur via microwave treatment, autoclaving, or freezing. Chemical cell disruption methods include organic solvent treatment, osmotic shock, and acid–alkali reactions. Biological cell disruption occurs by microbial degradation or enzymatic hydrolysis. See Green Microalgae "Extracellular matrices and pretreatment strategies for disruption" section for more information on each of these disruption techniques.

After cell walls and membranes have been thoroughly disrupted, extraction and recovery of target products or molecules is the next DSP step. Existing downstream processing operations are primarily designed for the extraction and recovery of one target molecule (proteins, lipids, carbohydrates, pigments, etc.). Soluble protein extraction after cell disruption consists of lysate conditioning (pH and/or conductivity adjustment), centrifugation of cell lysate, and recovery of supernatant. Lipid extraction involves mechanical pressing, homogenization, milling, and lipophilic solvent extraction (Cuellar-Bermudez et al., 2015). Prior cell drying or cell disruption is necessary for current industrial-scale extractions because unruptured cells do not permit solvent access to the internally stored lipids. Carbohydrate extraction requires an enzymatic or acidic pretreatment while pigment extraction occurs via conventional solvents, supercritical fluids, or pressurized liquids (Nobre et al., 2013).

After extraction, clarification, capture/purification, and any necessary polishing steps are performed to obtain the final product. For clarification, crude extracts can be filtered or centrifuged to separate debris from any soluble products into the supernatant fraction. Capture and purification occurs mostly for protein products used for human therapeutic, cosmetic, or nutraceutical purposes (Milne, 2017). Chromatographic operations and/or precipitation steps are commonly used to purify and concentrate proteins.

For carbohydrates and lipids, conversion is necessary. Carbohydrates are converted to simple sugars via saccharification followed by fermentation into various biofuels such as bioethanol and biobutanol (Hernández-Velázquez et al., 2015). Likewise, lipids undergo transesterification to form biodiesel and byproducts (Zhu et al., 2017). Byproducts are separated from biodiesel by taking advantage of density differences or via distillation processes (Zhang et al., 2016).

Economic considerations

Economic evaluation of bioproduct production in microalgae has centered largely on microalgae-derived biofuels with much attention given to the cultivation and harvesting operations (Beal et al., 2015; Gerardo et al., 2015; Ríos et al., 2013). The overwhelming hurdle identified to widespread commercial production of microalgae as a bio-feedstock is the cultivation and harvesting strategy employed (Barsanti and Gualtieri, 2018; Beal et al., 2015; Chia et al., 2018). It has been repeatedly concluded that cultivation in photobioreactors is more costly than open raceway ponds in terms of capital investment, but when downstream processing costs are considered, the higher cell densities achievable in photobioreactors are more economically

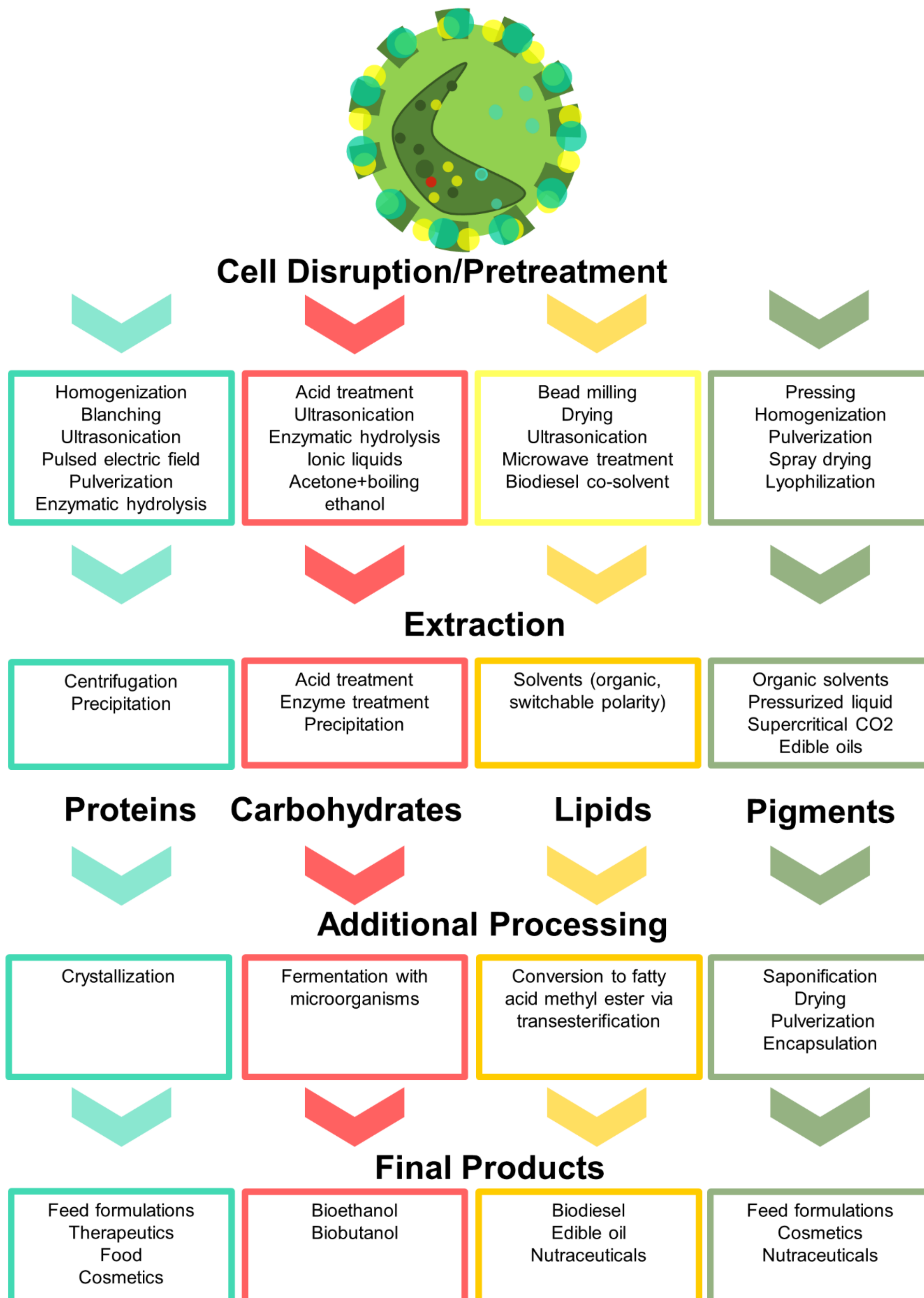

Figure 1.1 Downstream processing steps for the recovery of proteins, carbohydrates, lipids, and pigments.

advantageous (Davis et al., 2011) . Harvesting and dewatering are time and energy-intensive steps and can account for anywhere between 10 and 50% of total operating costs due to the dilute nature of algae suspensions and large process volume ('t Lam et al., 2018; Barsanti and Gualtieri, 2018; Ríos et al., 2013). Operations including microfiltration and centrifugation have been found to cost almost twice that of pH or gravity sedimentation (Ríos et al., 2013). The economics of subsequent DSP steps including cell disruption, extraction, and purification are not readily reported in literature but identified bottlenecks include overall sustainability related to energy costs and environmental impacts (Barsanti and Gualtieri, 2018).

As much of the literature regarding economic evaluation of microalgae production schemes has reported on biofuels, there is a consensus that high value co-products have the potential to increase the total value of microalgae biomass ('t Lam et al., 2018). While a complete microalgae biorefinery process is not yet possible, there have been production facilities attempting to integrate a biorefinery concept into their business model whereby multiple high value compounds are produced in lower volumes rather than using microalgae solely for biofuel production (Barsanti and Gualtieri, 2018). In order for the biorefinery concept to be a profitable enterprise, simple and effective alternatives to the traditional process operations much be employed. As the research into these alternatives is currently occurring in academia and industry, the field as a whole would benefit from economic modeling and evaluation of downstream processing operations and sensitivity analyses to readily assess potential commercial utility of novel approaches to cell disruption and extraction of multiple bioproducts. While downstream processing includes steps such as harvesting, dewatering or drying, cell disruption, extraction, fractionation, and purification, properties (cell morphology, product localization, and

physiochemistry) of the target biomolecule must be considered. Thus, understanding the cell structure and any potential interaction between target molecule and processing equipment and materials is imperative for commercial scale operations.

Extracellular matrices and pretreatment strategies for disruption

The first step for recovery of biomolecules from within the cell is disruption of the extracellular matrix (ECM). The extracellular matrices of cells serve to protect and defend the microalgae cell, are involved in growth and development, and promote adhesion and interaction with other cells and substrates (Domozych et al., 2012). As a structural barrier to release of intracellular molecules, understanding the ECM is imperative for biomolecule extraction and recovery. The following sections begin with a description of the morphology and composition of ECM in various green microalgae species and then highlight traditional techniques for ECM disruption. Finally the emergent use of enzymatic hydrolysis for ECM disruption is discussed with an emphasis on applicable enzyme classes based on ECM composition.

The diversity of extracellular matrices

Within green microalgae, there exists a wide variety of extracellular matrices (Domozych et al., 2012). The major cell wall polymers found in the matrices include cellulose, hemicelluloses (xyloglucan, mannans, glucuronan, $(1 \rightarrow 3)$-β-glucan), and ulvans (Popper et al., 2011). For the six green microalgae discussed in this review, the major ECM groups include cell wall less/deficient species, cellulose-containing (fibrillary) cell-walled species, and multilayered or stratified cell-walled species. Some green microalgae exhibit attributes of several ECM groups such as having a multilayer fibrillary cell wall.

Cell wall less green microalgae include D. *salina*, which lacks a rigid cell wall and is enclosed by a thin plasma membrane. This membrane consists of a glycocalyx-like coating and, therefore, *Dunaliella* is susceptible to osmolar changes in the environment and exhibit flexible cell morphology (Polle et al., 2017).

Multilayered or stratified cell wall green microalgae include *Chlamydomonas reinhardtii*, *Chlorella*, and *Spirulina* sp. The cell wall of *C. reinhardtii* is a cellulose-deficient structure that is primarily composed of carbohydrates, proteins, and hydroxyproline-rich glycoproteins (Imam and Snell, 1987). Originally thought to be composed of seven layers, further research confirmed five layers within the cell wall (Goodenough and Heuser, 1985). The innermost layer is a loose network of fibers that extends into the “central triplet” layers composed of two fibrous layers and a granular layer. Finally, the outermost layer is composed of anastomosing (connected tubular structures) fiber. The cell wall within the *Chlorella* species can vary quite drastically. Species can have a single microfibrillar layer or two possess two layers, one microfibrillar and the other mono- or trilaminar (Gerken et al., 2013). The *Chlorella* trilaminar layer or sheath is composed of sporopollenin as the outermost layer, a secondary wall composed of mannose and chitin-like polysaccharides, and finally an innermost phospholipid bilayer (Kim et al., 2016). Reported polysaccharide composition in C. vulgaris include rhamnose (45–54%), arabinose (2–9%), xylose (7–19%), mannose (2–7%), galactose (14–26%), and glucose (1–4%) (Safi et al., 2014a). *Spirulina* sp. cell walls are composed of four longitudinal layers LI–LIV. LI is not digestible by humans due to the presence of β-1,2 glucan while LII is composed of proteins and lipopolysaccharides, which allows for easy digestion of *Spirulina* by humans (Ali and Saleh, 2012). LII is additionally made of peptidoglycans which provides some rigidity, but overall the overall cell wall is relatively weak (Apogee *Spirulina*, 2012). LIII is thought to contain protein

fibrils and LIV has a structure similar to that of a gram-negative bacteria cell wall (Ciferri, 1983). The walls are reportedly not vulnerable to enzyme digestion although cellular contents are readily available to enzymes following cell lysis (Falquet, 1997).

Green microalgae with characteristics of multiple ECM groups include *Nannochloropsis sp*. and *Haematococcus sp*. *Nannochloropsis sp*. have a bilayered cell wall structure composed of a cellulosic inner wall and outer layer of hydrophobic algaenan (Scholz et al., 2014). Scholz et al. (2014) proposed that the algaenan structure containing long-chain aliphatic hydrocarbons subject to ether cross-linking reactions (Gelin et al., 1997) confers the recalcitrance characteristics observed in *Nannochloropsis sp*. Within different strains, cell wall thickness varies considerably, ranging from 63 to 119 nm (Beacham et al., 2014). Additionally, *Nannochloropsis* cell walls are rich in various polysaccharides. N. *oculata* polysaccharides are 68% glucose with 4–8% of rhamnose, mannose, ribose, xylose, fucose, and galactose (Brown, 1991). Similarly, cell wall polysaccharides in N. oceania are composed of 90% glucose, ~ 3% mannose, traces of rhamnose, fucose, arabinose, xylose, and galactose (Scholz et al., 2014). *Haematococcus pluvialis* possess thick trilaminar cell walls containing cellulose and sporopollenin, which incurs limited permeability and resistance to mechanical treatments (Safi et al., 2014b). In addition to the trilaminar sheath organization, secondary and tertiary cell walls separate the intracellular environment from the outer cell wall. The trilaminar sheath contains algaenan, with cellulose and mannose composing the secondary and tertiary layers (Kim et al., 2016). The above cell wall structure described for *Haematococcus* cells is for the mature red cyst with high astaxanthin content as this is the most common morphology encountered during the processing of *H. pluvialis*.

Traditional ECM disruption techniques

As noted, the ECM or outermost layer must be disrupted to extract and recover biomolecules. Current cell disruption processes involve the use of energy-intensive equipment, high temperature (> 50 °C) treatments, organic solvents, or highly acidic or basic buffers that can potentially decrease product extractability (Wilken and Nikolov, 2016). Cell disruption methods can be largely classified into two groups: mechanical and non-mechanical. Mechanical methods include bead beating, high pressure or high shear homogenization, ultrasonication, and pulsed electric field. Generally speaking, mechanical methods are less specific to the type of microalgae but are more energy-intensive than non-mechanical methods (Lee et al., 2012). The non-mechanical disruption techniques can be further divided into thermal, chemical, or biological methods (Figure 1.2). Chemical disruption while not energy-intensive involves the use of toxic solvents and is not suitable for industrial-scale processing (Show et al., 2015). Likewise, biological disruption is a generally regarded as safe (GRAS) process but is not presently cost-effective (Günerken et al., 2015; Vanthoor-Koopmans et al., 2013).

Mechanical methods

Bead beating

Bead beating is the transfer of energy from small beads made of glass, ceramics, or steel to the microalgae cell with mechanical agitation. The cell disruption occurs via compaction or solid shearing when a collision zone between beads is created. Advantages of bead beating include high disruption efficiency, high throughput, reproducibility, and temperature control (Al hattab and Ghaly, 2015; Günerken et al., 2015). While this mechanical disruption is regarded for its high efficiency, high energy consumption and costs reduce the applicability of bead beating for microalgae cell disruption. Additional disadvantages include the creation of small cell debris

and dispersion of lysate in both the soluble and solid phases, increasing downstream processing costs. Bead beating is most suitable for highly concentrated solutions with products that are easily separated or fractionated after disruption (Show et al., 2015). This technique is applicable for disruption of multiple green microalgae species but particularly advantageous for thick-walled or recalcitrant species such as *Nannochloropsis* and *Chlorella*. Processors should note

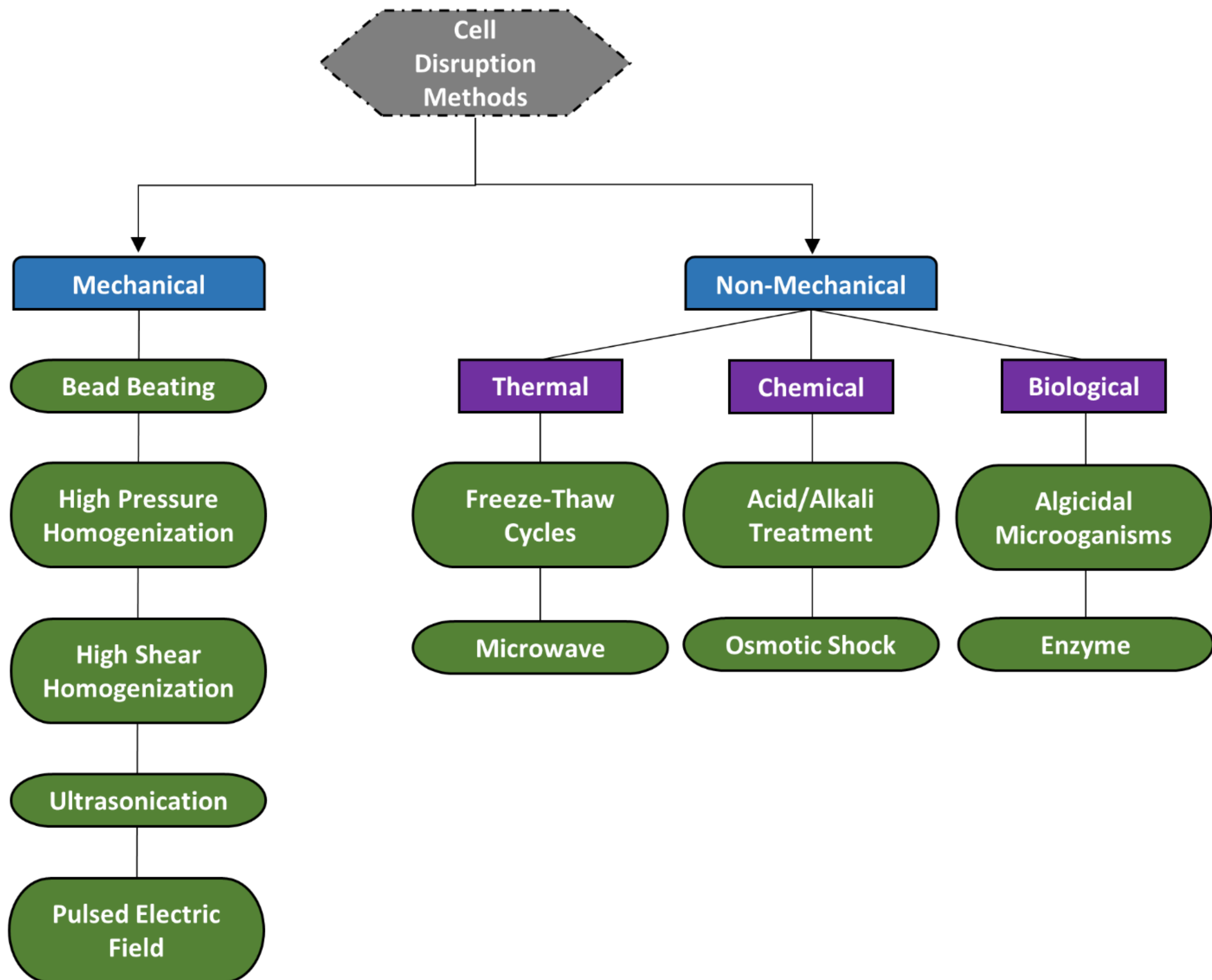

Figure 1.2 Cell disruption alternatives.

that while application of bead beating will disrupt these species, the resultant lysate will be difficult to fractionate as part of a biorefinery model.

High pressure homogenization

High pressure homogenization (HPH) is the pumping or movement of a slurry or cell suspension through a valve at high velocity resulting in shear forces that disrupt cell walls and membranes (Günerken et al., 2015; Show et al., 2015). Advantages include scalability and reproducibility and being chemical/enzyme/toxic substance (solvent) free (GEA Soavi, 2011). Disadvantages of HPH include the need for low dry cell weight concentrations (dilute suspensions) which increase energy demand (Lee et al., 2012) and the creation of small cell debris which increases separation costs (Barba et al., 2015). HPH is commonly used for disruption of *Haematococcus* cells for fish feed formulations (Chisti and Moo-Young, 1986). Additionally, species containing cellulose, algaenan, or sporopollenin such as *Chlorella* and *Nannochloropsis* could be disrupted with HPH. While HPH has proven utility in large-scale operation, high energy demands may limit the technique to recovery of high value products (Yap et al., 2015). HPH could additionally be applicable when cell lysates will be included as part of feed formulation and don't require component fractionation. The technique is also suitable for cells with ECM that are thick-walled, exhibit limited permeability, and are resistant to lower energy mechanical treatments.

High shear homogenization

High shear homogenization (HSH) is the stirring or mixing of cell suspension at high speed. Hydrodynamic cavitation and shear forces at solid–liquid interfaces disrupt cells prior to subsequent processing. The repeated suction of the cell suspension through the apparatus and subsequent exit through holes at the end of the stator tip results in reduced cellular tissue size.

The processing time for HSH is short and high dry cell weight concentrations can be tolerated (Günerken et al., 2015). Disadvantages include the aggressive nature of the cell disruption and the difficulties of scaling HSH rotor–stator apparatus for industrial or commercial scale use. Like HPH, HSH is applicable to multiple green microalgae species but particularly suited for *Chlorella* and *Nannocholorpsis*. These species have multilayered ECM rich in polysaccharides and at present are usually used for one target biomolecule. Application of HSH is not ideal for a biorefinery processing model due to the complexity of the resulting cell extracts, which negatively impacts subsequent separation methods, but could be advantageous for cell disruption prior to protein extraction in *Chlorella sp.* or lipid extraction in *Nannochloropsis sp*.

Ultrasonication

Ultrasonication is the application of high frequency acoustic waves for the disruption of cell walls and membranes. Disruption occurs via two mechanisms: cavitation and acoustic streaming (Gerde et al., 2012). Ultrasound vibrations from the emitting tip can promote cavitation or microbubbles that expand and explode to disrupt surrounding material. In acoustic streaming, the solution is mixed resulting in liquid currents with turbulence that disrupts cellular material. Ultrasonic treatment can disrupt at low temperatures and does not require additional disruption components like beads or chemicals (Al hattab and Ghaly, 2015; Wang et al., 2014). Disadvantages include energy consumption, heat production (protein/metabolite denaturation), and inefficiency of disruption of certain algae species. Ultrasonication has been particularly favored for disruption of *Spirulina* which has an overall weak ECM but can be applied for disruption of *Chlorella*, *Nannochloropsis*, *Chlamydomonas*, and *Haematococcus* sp. In the later cases, ultrasonic treatment is combined with detergent treatment or solvent systems for increased efficiency and decreased cost demands (Günerken et al., 2015).

Pulsed electric field

Pulsed electric field (PEF) treatment is the application of short periods (microseconds) of high voltage electric pulses into cell suspensions situated between two electrodes (Zbinden et al., 2013). Lysed or permeated cell walls and membranes occur through electroporation-associated permeation or pore formation in response to high transmembrane voltage. Pore formation occurs and can be reversible or irreversible depending on PEF intensity (Joannes et al., 2015). The degree of pore formation is dependent on electric field strength, shape/type of pulse, treatment time, and number or pulses (Goettel et al., 2013; Zbinden et al., 2013). Above a certain PEF intensity, reversible pore formation becomes irreversible wall/membrane disruption (Zderic et al., 2013). PEF is considered a minimally invasive or gentle disruption technique with advantages including scalability and combination with other disruption treatments. Disadvantages of PEF treatment include the need to deionize solutions for treatment and a decrease in efficiency upon release of internal cellular compounds (Günerken et al., 2015). PEF has broad applicability within green microalgae species such as *Nannochloropsis*, *Haematococcus*, *Chlorella*, and *Chlamydomonas* as its efficiency can be optimized in terms of energy and electric field strength to permeabilize and/or partially disrupt various ECM composition. As such, PEF is a technique that can be integrated into a biorefinery process, and when combined with other techniques resulting in complete ECM lysis allow for selective bioproduct extraction at later stages.

Non-mechanical methods

Microwave treatment

Microwave treatment is the uniform, non-contact heating of cellular suspensions that results in moisture evaporation and high pressure bubbles that disrupt cells (Barba et al., 2015; Iqbal and Theegala, 2013). Heat in the cell suspension results from rotation and vibration of dipolar molecules and ions in an electromagnetic field (Biller et al., 2013). Advantages include short residence/processing time, scalability, high efficiency, low energy consumption, and low risk of metabolite denaturation (Biller et al., 2013; Günerken et al., 2015; Pasquet et al., 2011). Disadvantages include maintenance costs for industrial-scale treatment and need for cooling to maintain product integrity before continued processing (Al hattab and Ghaly, 2015). Microwave treatment is often combined with other disruption techniques to promote not only disruption but microwave-assisted extraction (MAE) of valuable products. In particular, microwave treatment is commonly applied to *Nannochloropsis* and *Chlorella sp.* for extraction of lipids but is broadly applicable to many species for ECM disruption. The resultant lysate would likely be difficult to fractionate as part of a biorefinery process and thus microwave treatment is primarily used when biomass is intended for extraction of one molecule.

Freezing/unfreezing

Freezing treatment or freeze–thaw cycles allow for the repeated formation of large intracellular ice crystals that promote pore formation in cell walls and membranes that rupture cells, releasing intracellular compounds (Lee et al., 2012). Freezing can occur slowly in a − 15 to − 80 °C environment or rapidly using liquid nitrogen. Thawing is usually a slow process occurring at room temperature (Henriques et al., 2007). Freezing and thawing for the purpose of

cell disruption are associated with high energy consumption and operational/maintenance costs. Additionally, the process is not easily scaled due to treatment or residence times required and the size of freezers necessary for industrial-scale processes. Conversely, this disruption technique does not generate heat and is often utilized at smaller scale for heat-sensitive materials. Freeze–thaw procedures are likely applicable to green microalgae species without a cell wall (*Dunaliella*) or a fibrillary (cellulose) cell wall (*Chlamydomonas*, *Chlorella*, and *Spirulina*). In these instances, a number of freeze–thaw cycles could be employed to disrupt the cell membrane or one or more layers of a stratified cell wall structure while still allowing for selective extraction of biomolecules at a later processing step.

Chemical application

The application of solvents to microalgae is commonly used for lipid or carotenoid extraction, but can be used in coupled cell disruption–extraction procedures. The application of solvents like ethanol, methanol, chloroform, or hexane can have an enhancing effect on cell disruption when cells have been treated with another mechanical or chemical disruption technique. While sparse literature exists on solvent treatment alone, the effectiveness of solvents on disrupted cells is well documented and commonly used method for industrial-scale operations.

Additional chemicals used for cell disruption include acidic solutions (hydrochloric and sulfuric acid), alkaline substances (lime or sodium hydroxide) (Harun and Danquah, 2011), lysine, acetone, methanol, or DMSO (Steriti et al., 2014) which are typically added to cells at high temperatures (120–160 °C). Advantages of chemical treatment of cells for disruption include low energy input and scalability (J. Kim et al., 2013). However, chemical application is not considered to be mild and can have detrimental effects including pigment degradation and

protein denaturation. Additional disadvantages include the need for a continuous supply of chemical/solvent, corrosion of equipment by acids or alkalis, and chemical disposal (J. Kim et al., 2013). As chemical application could include many diverse types of chemicals or solvents, it is applicable to many green microalgae species.

Osmotic shock

Osmotic shock is a disruption technique based on the rapid increase or decrease of salt concentration in solution (Amin, 2009; Parmar et al., 2011). The stress produced can be hyperosmotic in which cells shrink due to fluid diffusion to the exterior of the cell or hypoosmotic in which cells swell and burst in response to fluid diffusion into the cell. Salts commonly used for osmotic shock disruption include sorbitol and sodium chloride (Bickerton et al., 2016; Drira et al., 2017). Osmotic shock has been previously coupled to other disruption techniques for lipid extraction and Ca^{2+} signaling response studies in *Chlamydomonas reinhardtii*, but otherwise has limited utility (Bickerton et al., 2016; Yoo et al., 2012a). Use of osmotic shock for green microalgae cell disruption isn't ECM dependent but freshwater species should be exposed to hypertonic conditions and marine species to hypotonic conditions (Yoo et al., 2012a). For bioproduct extraction, hypotonic conditions are ideal but disadvantages include inefficiency and the high salinity of resulting wastewater.

Algicidal microorganisms

Algicidal microorganisms, previously used for mitigating algal blooms (Bai et al., 2012), can be applied to microalgae cultures for cell wall/membrane disruption and degradation thought to occur via enzymatic reaction (Chen et al., 2013a, 2013b; Lü et al., 2013; Munoz et al., 2014). Microorganisms (bacteria, cyanobacteria, microalgae themselves, and viruses), co-cultured with microalgae, secrete lytic enzymes to disrupt the cell wall. Interestingly, the co-cultured

organisms can be isolated from known microalgae predators and previous work has demonstrated a degree of selectivity and specificity that can be achieved using predator-derived organisms. *Chlorella*, *Nannochloropsis*, and *Dunaliella* species have been successfully disrupted using this technique (Chen et al., 2013a, 2013b; Lenneman et al., 2014; Wang and Yuan, 2014). Advantages of algicidal lysis include cost, elimination of need for external enzyme application during downstream processing, high selectivity, and mild extraction conditions (pH, temperature, etc.) (Demuez et al., 2015). Disadvantages include the need for careful selection of microorganisms, difficulties in establishing optimized co-culturing techniques, and controlling the degree of disruption.

Enzymatic hydrolysis for ECM disruption

The above described mechanical and non-mechanical cell disruption techniques are typically employed as part of a process that focuses on the recovery of one target biomolecule while wasting or inadvertently damaging other potentially valuable biomass components. This highlights the need for selective and targeted disruption of the microalgae cell for recovery of multiple biomolecules. One solution that allows for a biorefinery approach to microalgae biomass processing is enzymatic hydrolysis. Enzymatic hydrolysis of microalgae cell walls and membranes is a mild disruption alternative that allows for processing conditions (mild temperatures, neutral pH, no contact with organic solvent) that maintain the quality and yield of multiple biomolecules. The targeted nature of an enzyme to a specific substrate plays a prominent role in selective disruption and extraction in addition to protecting target biomolecule integrity throughout the disruption process. Potential limitations include the cost of commercial enzymes, lack of knowledge about optimal or compatible enzyme formulations for cell disruption, and the requirement for holding tanks to accommodate long incubation periods

(Günerken et al., 2015). Enzyme immobilization or removal following disruption is also necessary for some high value product formulations. As highlighted in "The diversity of extracellular matrices" section, microalgae have vastly diverse ECM but are generally composed of proteins, carbohydrates, and lipids. Thus, enzymes targeting each of these components have potential for disrupting the microalgae cell.

Proteases

Proteases, an enzyme group that breaks down or cleaves proteins, specifically target amino acid sequence motifs. There are seven families of proteases including serine, cysteine, threonine, aspartic, glutamic, metallo-, and asparagine. Proteases can act near the end of polypeptide chain (exopeptidase) or within the chain (endopeptidase). Protease activity is largely dependent on pH and temperature and varies widely from one enzyme to another. Common proteases with potential for microalgae cell wall/membrane disruption include trypsin, lysozyme, collagenases, papain, and autolysins (Gerken et al., 2013; Horst et al., 2012; Mahdy et al., 2014a). Preliminary screening of enzymes indicated that proteases may catalyze cell wall disruption for *C. vulgaris* cells (Mahdy et al., 2014b). Researchers successfully applied Alcalase® (serine endopeptidase) to hydrolyze the *C. vulgaris* cell wall to enhance biomethane production after anaerobic digestion (Mahdy et al., 2014b). Alcalase® exhibits broad specificity for proteolysis with an optimum pH of 8.5 and incubation temperature of ~ 60°C and has been commercially utilized for detergent and hydrolysate production (Doucet et al., 2003).

The protease activities of Termamyl® 120 L have been used for degradation of glycoproteins in *C. reinhardtii* cell wall (Choi et al., 2010). Termamyl® 120 L contains α-amylase and protease activities with an optimum pH of 7.0 and temperature of 90°C. The enzyme exhibits great thermostability and has many applications in the food, beverage, and

textile industries (Kalegowda et al., 2017; Kłosowski et al., 2015; Raghu and Rajeshwara, 2015). Likewise, autolysins have been extensively studied for their ability to hydrolyze the *C. reinhardtii* cell wall (Dixon et al., 2016; Sierra et al., 2017). In particular, gamete autolysin is a cell wall degrading protease induced by nitrogen-deficient stress conditions during sexual reproduction (Jaenicke and Waffenschmidt, 1981). Gamete autolysin specifically acts on proline-rich residues within the *C. reinhardtii* cell wall thus allowing for selective extractions. The enzyme has an optimum pH and incubation temperature for activity of 7.5 and 35°C, respectively.

Carbohydrases

Carbohydrases catalyze the breakdown or lysis of carbohydrates into simple sugars. This enzyme group includes glucosidases, galactosidases, amylases, cellulases, chitinases, and pectinases among many others. A common application of carbohydrases in microalgae processing is for saccharification prior to fermentation of simple sugars for biofuel production. As the green microalgae cell wall and/or membrane often contain various polysaccharides, carbohydrase cocktails with multiple enzyme activities are employed to break down the complex polysaccharides. Like proteases, optimum conditions for carbohydrases vary widely depending on type and organism source and selected conditions greatly influence enzyme activity. Horst et al. (2012) determined Viscozyme® L and Proteinase K to be candidate enzymes for cell wall disruption of *Nannochloropsis oculata* cells. Viscozyme® L, an enzyme mixture with arabanase, cellulase, β-glucanase, hemicellulase, and xylanase activities with optimum activity at pH 4.0 and 50°C. *N. oculata* cell walls have been similarly disrupted and digested with enzyme mixtures of 4% hemicellulase and 2% Driselase® (mixture of laminarinase, xylanase, and cellulase activities) (Chen et al., 2008). Driselase® digests plant cell walls with optimum activity at pH 4.5

and 37°C. Glucanex®, Lyticase®, and Driselase® have been used as part of an enzyme-assisted cell disruption of H. pluvialis cells. Glucanex® contains β-glucanase, cellulase, protease, and chitinase activities. Glucanex® requires mild conditions for optimum activity including pH 6.0 and 25°C and Lyticase® exhibits optimum activity at pH 7.5 and 25°C.

Lipases/phospholipases

Lipases catalyze the hydrolysis of lipids and substrates including specific positions of the glycerol backbone of lipids. A subset of lipases, phospholipases, hydrolyze phospholipids in fatty acids and include four major classes that catalyze specific reactions at different ester bonds. Phospholipase A1 and A2 cleave the SN-1 and SN-2 acyl chains of a phospholipid, respectively. Phospholipase B cleaves both acyl chains and phospholipase C and phospholipase D cleave before and after the phosphate, respectively. Like most cell membranes, the main lipid component of microalgae membranes are phospholipids. Thus, phospholipases have potential applicability in disrupting microalgae cell membranes. Phospholipase conditions for optimum activity are between pH 7.0–9.0 and 25–37°C. Phospholipase A1 has demonstrated utility in the digestion of *Chlorella* cell walls, but authors were unable to determine if the actual substrate was phospholipids or the structurally similar algaenan (Gerken et al., 2013). It is important to note that if lipids are the target product, application of lipases/phospholipases for ECM disruption could reduce the total lipid yield as lipid substrates in the cell wouldn't be distinguishable to general use lipase/phospholipase cocktails.

Current applications of aqueous enzymatic processing

To date, enzymatic hydrolysis of microalgae cells has been used as a pretreatment for extraction and/or conversion of a single target biomolecule. Table 1.1 presents studies that use enzymes for cell wall pretreatment for the recovery of biomolecules or as part of a bioproduct

conversion process. The enzymatic hydrolysis pretreatment is typically followed by a secondary and/or enhancing treatment or processing technique to fully recover or convert the target molecule. Common target products include cell wall carbohydrates, native proteins, lipids, and carotenoids. To gain access to these intracellular products, cells have been treated with enzyme cocktails that encompass many enzymatic activities which target various components in microalgae ECM. After disruption of the cell, secondary application of organic solvents is commonly used for lipid and carotenoid extraction. Carbohydrates recovered after ECM lysis are often fermented or aerobically digested for biofuel production.

Product-containing organelles and disruption strategies

After lysing the ECM, product-containing organelles must also be disrupted to gain access to target biomolecules. In green microalgae, the chloroplast and lipid droplets are common storage sites of biomolecules and the morphology of each along with cell disruption methods are discussed in the following sections.

Chloroplast structure and disruption strategies

The chloroplasts of green microalgae are the site of photosynthesis and carbon fixation (Engel et al., 2015) and thus, the chloroplast has a great capacity for accumulation of endogenous proteins, starch, lipids, and pigment/carotenoids (Franklin and Mayfield, 2005). Additionally, green microalgae have well-developed genetic engineering toolkits and can be engineered to produce high value "foreign" or recombinant proteins along with high volume products (lipids) within the chloroplast.

Table 1.1 Summary of recent studies employing enzymes for cell wall pretreatment

Microalgae Species	Target Product	Enzyme (with conditions)	Secondary and/or Enhancing Treatments	Yield/Results/Conclusions	Reference
Chlamydomonas reinhardtii	Cell wall carbohydrates	0.005% α-amylase, 90°C, for liquefaction, and 0.2% glucoamylase, pH 4.5,55°C for saccharification	Yeast fermentation for ethanol production	94% carbohydrate hydrolysis 29.2% ethanol production efficiency	(Choi et al., 2010)
Chlamydomonas reinhardtii	Native proteins and Lipids	Gamete autolysin, pH 7.5, 23-37°C;Trypsin, pH 7.8, 37°C	Organic solvent extraction	85% cell lysis and 55% total protein release with gamete autoysin and 73% total lipid release with trypsin	(Sierra et al., 2017)
Chlorella sp.	Cell wall carbohydrates	Cellulases, xylanases, and amylases enzymes, pH 4.8, 50°C	Cells chilled with 95% (v/v) ethanol,cold dried, and ground prior to enzymatic application.	2.9-5.0% hydrolyzed glucose depending on species and 4.8-8.6 total reducing sugars	(Rodrigues and da Silva Bon, 2011)
Chlorella vulgaris	Lipids	Cellulase (Celluclast 1.5 L) β-glucosidases(Novozyme 188), pH 4.8, 50°C	Organic solvent extraction	85.3% cell wall hydrolysis after 72h Improved lipid extraction yield (1.29-1.73 fold) depending on solvent utilized.	(Cho et al., 2013)
Chlorella vulgaris	Cell wall carbohydrates	Alcalase®, pH 8.0, 50°C	Aerobic digestion for methane production	Enhanced methane production (64% increase in yield)	(Mahdy et al., 2014b)
Chlorella vulgaris	Lipids	Snailase (37°C) , lysozyme (55°C), cellulose (55°C), no pH specified	Organic solvent extraction	7% lipids extracted with snailase, 22% lipids extracted with lysozyme, 24% lipids extracted with cellulose	(Zheng et al., 2011)
Chlorella vulgaris, Scenedesmus dimorphus, and Nannochloropsis sp.	Cell wall and lipids	Cellulase, snailase, neutral protease, alkaline protease, and trypsin, pH 4.0, no temperature specified	Ultrasonication	49.82% lipid recovery in *C. vulgaris*, 46.81% lipid recovery in *S. dimorphus*, 11.73% lipid recovery in *Nannochloropsis sp*.	(Liang et al., 2012)
Haemotococcus pluvialis	Carotenoids	Glucanex®, pH 4.5, 55°C	Ultrasonication	83.9% carotenoid extraction	(Machado Jr. et al., 2016)
Haemotococcus pluvialis	Astaxanthin	0.1% Protease K and 0.5% Driselase®, pH 5.8, 30°C	Organic solvent extraction	Low total carotenoid yield after enzymatic treatment	(Mendes-Pinto et al., 2001)
Nannochloropsis sp.	Lipids	Feedlyve® GMA (Fe-GMA)-galactomannanase and Cellulyve® 50LC (Ce-50LC)-β-cellobiosidase/ β-glucosidase, pH 6.0, 45°C	Organic solvent extraction	68.6% lipid extraction	(Zuorro et al., 2015)
Nannochloropsis sp.	Lipids	Ternary mixture of one cellulose and two hemicellulases, pH 5.0, 50°C	Organic solvent extraction	37.2 g lipids per 100 g of dry biomass recovered	(Zuorro et al., 2016)

Understanding morphology and internal structure allows for appropriate selection of disruption techniques for recovery of products from the chloroplast. Microalgae chloroplasts can occupy a large percentage of total cell volume (up to 60%) and are usually cup or basal shaped (Munoz et al., 2014). The chloroplast is generally surrounded by a double envelope membrane and composed internally of thylakoids in bands stacked in irregular patterns. Thylakoids are rich in protein but have membranes dominated by lipids (Simionato et al., 2013). Other components of microalgae chloroplast include photosynthetic pigments, chlorophyll a and chlorophyll b, and carotenoids α- and β-carotene and xanthophylls such as astaxanthin, lutein, zeaxanthin, and neoxanthin (D'Alessandro and Antoniosi Filho, 2016; Gong and Bassi, 2016).

Previous research into disruption of green microalgae chloroplasts has been for purposes including studying chloroplast proteins, exploring chloroplast DNA and protein synthesis processes, and for identifying proteins induced under specific culturing conditions (Balczun et al., 2006; Bayer et al., 2015; Flores-Pérez and Jarvis, 2017). In these instances, researchers isolated intact chloroplasts and then disrupted the organelles using techniques such as freeze–thaw rupture, enzymatic hydrolysis with trypsin and chymotrypsin, and osmotic shock with hypotonic lysis buffers (Bayer et al., 2015; Flores-Pérez and Jarvis, 2017). When the chloroplast is disrupted for the purpose of microalgae biorefining, the proteinaceous nature of the outermost membrane can be targeted using a biological-based disruption technique like enzymatic hydrolysis. While use of enzymes for biorefining microalgae is in the development phase, the selective nature of enzymatic hydrolysis has obvious advantages to current chloroplast lysis techniques such as freeze–thaw rupture and osmotic shock. These methods would likely result in a complex lysate requiring additional fractionation steps.

While the protein composition in the membrane can be diverse, general use proteases or cocktail mixtures can potentially cleave and digest peptide bonds. Trypsin, a general serine protease, can cleave various protein substrates. The relatively mild conditions to achieve optimum activity make trypsin a viable candidate for targeting microalgae organelles, particularly the chloroplast. Metalloproteases, which have reported lytic activity against cell walls (Wu and Chen, 2011), also have potential in enzymatic hydrolysis of organelle membranes. Bacterial proteases with endopeptidase activities in the neutral pH range can cleave proteins from a variety of sources which could likely include the membranes of microalgae chloroplasts.

Lipid droplet structure and disruption strategies

Within the microalgae cell, lipid droplets (LD) are the major site of neutral lipid storage (Goold et al., 2015) and additionally contain valuable products such as carotenoids and pigments. Under nitrogen-replete conditions, green microalgae contain one or two lipid droplets but LD synthesis and accumulation are activated in the presence of stress conditions including nutrient depletion (-N, -Fe, -S, -P), high light, hypoxia, increased salinity, or chemical application (Wang et al., 2009). When stress conditions persist, a metabolic shift or reorganization occurs which leads to the formation of carbon reserves (starch and oil) and a downregulation of photosynthesis and protein synthesis.

The LD comprised a core of triacylglycerols surrounded by a monolayer of polar lipids and proteins interspersed throughout (Goold et al., 2015). For many microalgae species, the major proteins in the LD membrane are generally hydrophobic, maintain the size/structure of LD, and prevent fusion of multiple LD (Moellering and Benning, 2010). In *C. reinhardtii* and *D. salina*, the most abundant LD membrane protein has been termed major lipid droplet protein (MLDP) (Davidi et al., 2012; James et al., 2011) while those in *H. pluvialis* and *Nannochloropsis*

sp. have been termed *Haematococcus* oil globule protein (HOGP) and lipid droplet surface protein (LDSP), respectively (Peled et al., 2011; Vieler et al., 2012). *Chlorella sp.* contain a homolog of caleosin, an oil-body surface protein found in higher plants, as the most abundant protein in their LD (Lin et al., 2012).

Expellers, presses, or lipophilic solvents are used to disrupt LD and access encased lipids or carotenoids but require prior drying of microalgal biomass (Mubarak et al., 2015). These methods are additionally disadvantageous because of slow processing times and the requirement of large biomass quantities (Harun et al., 2010). Lipophilic solvents can be polar or non-polar and include n-hexane, ethanol, 1-butanol, dimethyl ether, and mixtures of chloroform/methanol, n-hexane/ethanol, n-hexane/isopropanol among others (Neto et al., 2013). At an industrial scale, n-hexane is the most used solvent for lipid extraction while chloroform/methanol (1:2 v/v) is common for laboratory scale extractions. Mixing polar and non-polar solvents has been demonstrated to promote solvation and lipid recovery (Ghasemi Naghdi et al., 2014; Yoo et al., 2012b). Although organic solvents have a long and established history in lipid extraction, the handling and toxicity of the volumes required for commercial scale operations have encouraged development of alternatives for disrupting LD membranes and accessing internal products.

As an alternative to conventional techniques of LD disruption, enzymatic hydrolysis could be employed to target the monolayer of polar lipids or the most abundant membrane protein (MLDP, HOGP, LDSP, caleosin) in LDs. Lipases/phospholipases that can be applied to the ECM for disruption exhibit similar potential for lipid droplet disruption. Additional characterization of lipids in the polar monolayer would allow for selection of a candidate phospholipases/lipases for cleavage and digestion of the lipid droplet membrane. For example,

when targeting esters or triglycerides, enzyme preparations with these known substrate specificities could aid in the cleavage of membrane lipids in the lipid droplet.

Future directions of aqueous enzymatic processing

When used for recovery of proteins and oil from microalgae, AEP includes biomass conditioning for maximum enzyme activity, primary enzyme addition, incubation for cell wall disruption and protein solubilization, solvent or detergent-based extraction of biomolecule, and finally centrifugation and biomolecule recovery (Chen et al., 2016; Huo et al., 2015; Wu et al., 2017). An alternative and holistic approach to aqueous enzymatic processing was developed including biomass (biomolecule) production, harvesting, enzymatic degradation of cell wall, enzymatic degradation of organelle membranes, and product separation and fractionation (Sierra et al., 2017). With this process, enzymes were used for cell disruption and for catalyzing product release from internal cellular compartments. There also exists an opportunity for enzymes to disrupt naturally occurring emulsions, facilitating more efficient product separation and fractionation.

After extensive literature review of existing applications of aqueous enzymatic processing, a methodical approach to AEP of various microalgae species was conceived (Figure 1.3) by our research group. This systematic approach focuses on the structural composition of the ECM and any product-containing organelles and allows for the processor to select candidate enzymes to facilitate disruption. Considerations before beginning aqueous enzymatic processing include selection of target products, identification of microalgae species for target product accumulation and corresponding ECM, enzyme targets in the ECM, enzyme targets in product-containing organelles, requirements for additional product processing, and an integration of product streams into final product processing operations.

To assess the applicability of the developed methodology, an evaluation of literature employing enzymatic hydrolysis was conducted with the goal of investigating the methods employed by researchers using microalgae species with similar ECM characteristics. As previously described in "The diversity of extracellular matrices" section, *Nannochloropsis* and *Haematococcus* species share cell wall characteristics that include being cellulose containing (fibrillary) and possessing multilayered organization. Enzymes tested for disruption include various commercial enzyme cocktails of cellulases, hemicellulases, amylases, and glucosidase among others. Carbohydrase activity and specificity have demonstrated potential in targeting the polysaccharide-rich nature of *Nannochloropsis* and *Haematococcus* cell walls (Machado Jr. et al., 2016; Zuorro et al., 2015). Likewise, *Chlamydomonas*, *Chlorella*, and *Spirulina* species possess multilayered cell walls primarily composed of metabolites including proteins, lipids, and polysaccharides (not including cellulose). Proteases, carbohydrase cocktails, and combined carbohydrase–protease cocktails have been used to target the various metabolites in the cell walls of *Chlamydomonas*, *Chlorella*, and *Spirulina* species (Cho et al., 2013; Choi et al., 2010; Liang et al., 2012; Mahdy et al., 2014b; Sierra et al., 2017). As *Dunaliella* species possesses a thin plasma membrane instead of a cell wall, enzymes are not used for pretreatment but rather for biomolecule (carotenoid/pigment) extraction.

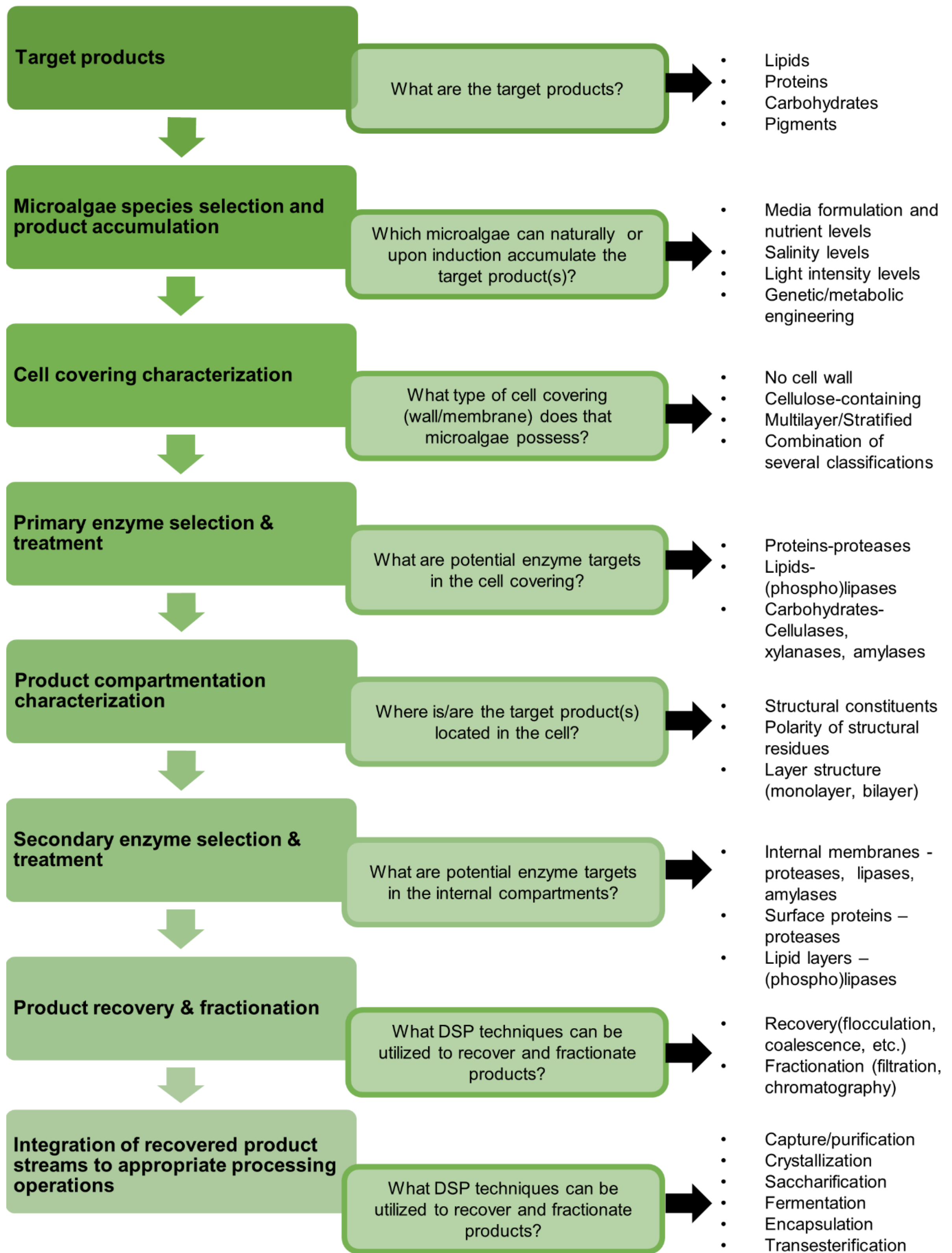

Figure 1.3 Aqueous enzymatic processing scheme workflow and design considerations.

Recently, a methodology for native protein and lipid extraction and recovery from wild-type *Chlamydomonas reinhardtii* was described (Sierra et al., 2017) (Figure 1.4). Candidate enzymes selected to potentially target the cellulose-deficient, hydroxyproline-rich cell wall included lysozyme, trypsin, collagenase, and a *C. reinhardtii*-derived autolysin. Cell wall disruption efficiency was assessed with qualitative and quantitative methods including counting of intact cells after treatment and the application of non-ionic detergents or fluorescent dyes capable of penetrating and staining organelles of cells with disrupted cell walls. Cell wall disruption and native protein release was achieved using gamete autolysin produced by *C. reinhardtii* cells. Analysis revealed that autolysin treatment at 35°C for an extended period solubilized more that 50% of the total protein and resulted in partially disrupted chloroplasts. Following this enzymatic cell wall disruption, remaining proteins and lipid droplet localization in the chloroplast was confirmed. The composition of the chloroplast and lipid droplet membranes was further explored to determine candidate enzymes for cleavage of membrane components and product release. Enzymes include trypsin, Alcalase®, DSM metalloprotease (Maxipro®), and Glucanex®. The highest lipid release (73%) occurred with the application of trypsin to gamete autolysin-treated cells. In summary, a proof of concept study was conducted to enzymatically hydrolyze the cell wall and organelle membranes of *C. reinhardtii* cells. While native proteins and lipids were extracted and/or released from intracellular compartments, optimized conditions for enzyme application and incubation in addition to fundamentally understanding the enzyme–biomolecule interactions are required. Future efforts should include assessing and modeling the enzymatic hydrolysis approach for multiple biomolecule extraction and recovery for large-scale processing operations.

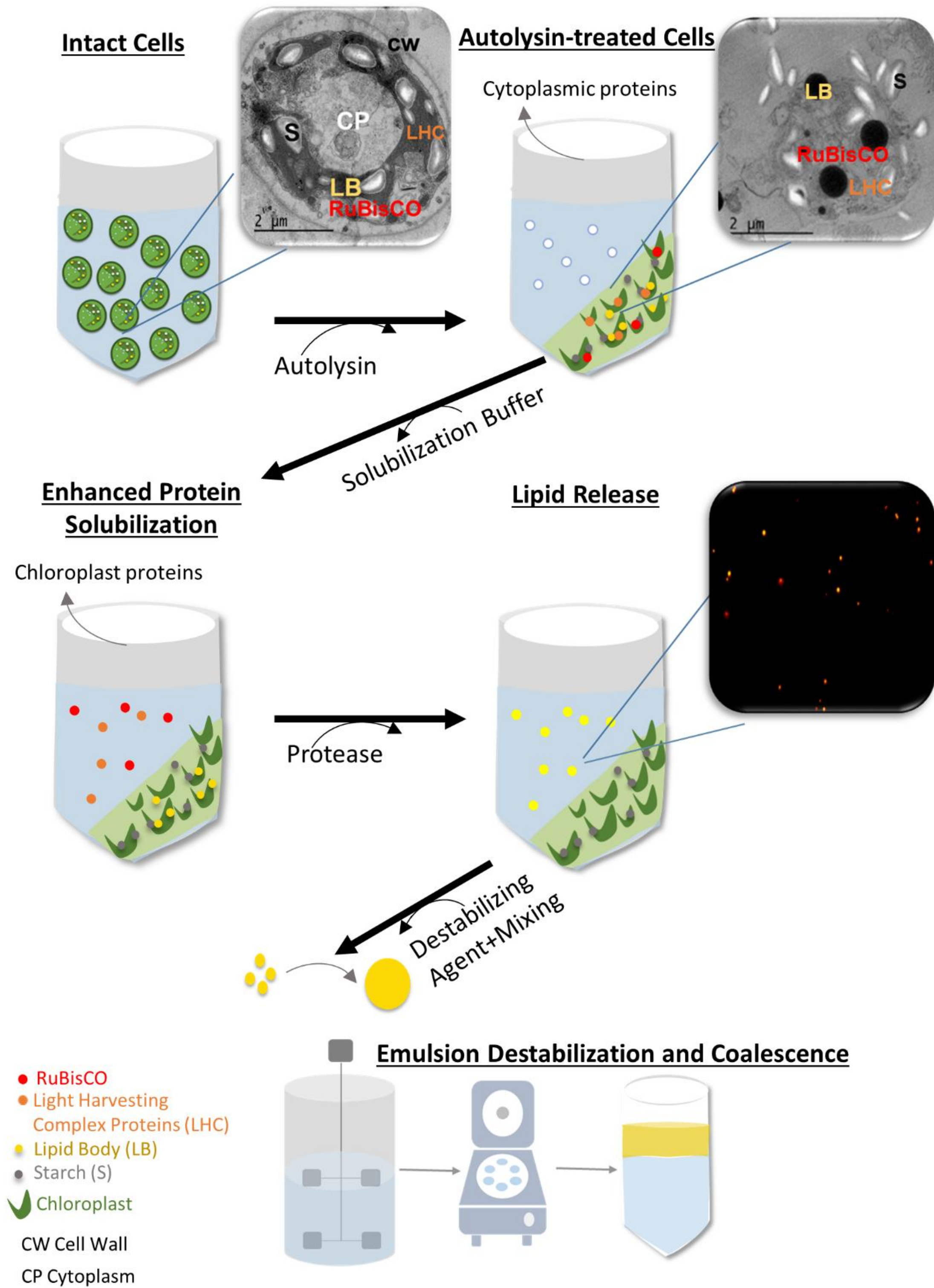

Figure 1.4 An alternative and holistic approach to aqueous enzymatic processing of microalgae for biorefining.

Conclusions

Global interests are increasingly pursuing sustainable and renewable sources of energy and other bioproducts. Microalgae has a demonstrated history as a viable biomass source, but established processing techniques have often proved to be cost prohibitive mainly due to energy investments, scalability, and an underutilization of biomass components. The development of extraction and purification methods have traditionally focused on single product recovery and thus may not be suitable or compatible with multiple bioproduct recovery. Researchers have begun using alternative processing techniques such as enzymatic hydrolysis to disrupt microalgae cells and extract and recover multiple biomolecule product precursors with the goal of improving process economics.

To fully realize the effect of alternative processing strategies such as aqueous enzymatic processing, better characterization of microalgae ECMs, organelle membrane compositions, and resultant biomolecule fractions are necessary. Such characterization will allow for producers to assess the effects of enzymatic treatment on the functionality of target biomolecules. This will provide new opportunities for applications of aqueous enzymatic processing for biomolecule recovery or as an enhancing method for traditional recovery schemes. Additionally, connecting morphological characteristics of microalgae ECM and their organelles to implications on process design can serve to improve bioproduct recovery yield and process economics. Thus, researchers and producers can overcome barriers to commercialization by tailoring the processing techniques to both characteristics of the biomass source and target biomolecules.

Chapter 2 - Fundamental studies on the applicability of aqueous enzymatic processing as part of a microalgae biorefinery[2]

Abstract

Microalgae biomass has the potential to aid scientists and engineers in meeting the challenges associated with a growing global population with finite energy and food resources. The ability to alter the metabolism of microalgae for simultaneous production of multiple biomolecules (bioproducts) for diverse industries has spurred interest in development of microalgae biorefineries. To address the economic and sustainability shortcomings of current biomass processing and biomolecule extraction techniques, a study of the applicability of aqueous enzymatic processing of *Chlamydomons reinhardtii* for native protein and lipid release extraction was conducted. Primary enzymatic treatment with autolysin resulted in complete cell permeation and 60-70% cell lysis. This pretreatment along with an extended higher temperature incubation lead to 54% total protein release with as associated release of 43% of total lipids. Secondary enzymatic treatment of biomass residues with trypsin resulted in release of an additional 30% of total lipids. This demonstration of aqueous enzymatic processing for sequential release of multiple biomolecules is the first step towards realization of a fully enzyme-mediated microalgae biorefinery.

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[2] Partial results have been published as a peer-reviewed journal article. Soto Sierra L, Dixon CK, Wilken LR (2017) Enzymatic cell disruption of the microalgae *Chlamydomonas reinhardtii* for lipid and protein extraction. Algal Res 25: 149-159. https://doi.org/10.1016/j.algal.2017.04.004. As an author of this Elsevier article, I retain the right to include it in a thesis or dissertation, provided it is not published commercially.

Introduction

Catalysts driving the current direction of scientific research include growing energy demands, depletion of fossil fuel resources, climate change, and the ever-increasing global population (Hariskos and Posten, 2014). To address these challenges, scientists must increase energy and food production in an environmentally-friendly and sustainable manner. Sustainable feedstocks are extensively researched and increasingly utilized for generation of biofuels and other valuable bioproducts. Microalgae, a third generation source of biofuels, are considered a promising source of a wide range of bioproducts including functional ingredients, such as polyunsaturated fatty acids, polysaccharides, natural pigments, essential minerals, vitamins, proteins, essential amino acids, and enzymes, as well as bioactive molecules (Phong et al., 2018). Microalgae reproduce quickly, have high productivities in comparison to terrestrial crops and can be cultivated in a variety of water sources (Mata et al., 2010). While microalgae have demonstrated great potential for biofuel generation, the economics of processing the biomass into biofuels are currently unfavorable (Barsanti and Gualtieri, 2018). This is due largely to the cell disruption and extraction techniques singularly employed for recovery of lipids with little interest given to the other products present in the residual biomass components. Energy-intensive biomass drying, harsh cell disruption techniques, and application of toxic petroleum-derived organic solvents increase processing costs and are unsustainable. Researchers have therefore sought to increase the economic value of microalgae biomass by extraction of other valuable biomolecules in addition to lipids for biofuel generation. This strategy of co-extraction of multiple biomolecules from within the same cell has spurred research into microalgae biorefineries.

Biorefinery concept for microalgae biomass: Success and limitations

The term biorefinery first appeared in scientific literature in the early 2000s (González-Delgado and Kafarov, 2011). Generally speaking, a biorefinery includes sustainable biomass processing via an array of technologies to extract carbohydrates, proteins, lipids and other main biomolecule components for direct applications or for transformation into value added products and biofuels. Biorefining serves to maximize valorization of biomass components while minimizing waste. This approach to microalgae biomass processing can improve economic feasibility of the feedstock, reduce greenhouse gas emissions, decrease dependence on fossil fuel usage, and contribute to future global food security (Trivedi et al., 2015).

The major steps of a microalgae biorefinery are: 1) cell wall and organelle disruption using a technique with specificity that retains biomolecule quality and quantity, 2) extraction and fractionation of different biomolecules, and 3) separation and purification of product streams enriched in target biomolecules (Vanthoor-Koopmans et al., 2013). Biomass is usually dried or concentrated into a wet paste prior to processing and then disrupted with a combination of mechanical, chemical, or thermal techniques. Most mechanical, chemical, and thermal disruption techniques are designed with conditions or operating parameters compatible for extraction of one target biomolecule with potential damaging effects to others. Thus, employment of a cell disruption technique that uses mild conditions and specifically deconstructs biomass in a way that retains product quality and quantity is still needed. Microalgae biorefining has generally taken two routes resulting in valorization of one to two products with a resultant residue (Dong et al., 2016; Francavilla et al., 2015; Karemore and Sen, 2016; Nobre et al., 2013). In the first, energy generation is the overall goal. Lipids are extracted for biodiesel production and then hydrogen and carbon in lipid-depleted residues are used for additional energy generation

(Francavilla et al., 2015). The alternative route is to initially extract lipids for biodiesel and then extract other biomolecules (proteins/carbohydrates) for their added value from lipid-depleted residues (Karemore and Sen, 2016; Nobre et al., 2013). In both cases, the processing conditions and toxic solvents used for cell disruption and lipid extraction can lead to loss of co-product functionality and value. This persistent prioritization of lipid extraction from microalgae biomass and its associated drawbacks in terms of biomass pretreatment requirements and detrimental effects on co-product extraction and recovery has led to research into alternative cell disruption and processing techniques as part of an economical and profitable microalgae biorefinery.

Alternative approaches to cell disruption and extraction for microalgae biorefinery realization

While there are reported efforts of microalgae biorefining, recurring limitations include the need to dry biomass prior to extraction, the use of harsh cell disruption techniques resulting in a complex lysate containing a mixture of all biomolecules that can be difficult to separate, and the continued emphasis on lipids as the target biomolecules with valorization of other biomass components (carbohydrates/proteins) via fermentation or pyrolysis rather than as functional ingredients. Alternative approaches to pretreatment, cell disruption, and subsequent biomass processing have the potential to overcome the aforementioned limitations and increase valorization of intracellular biomolecules that retain biological activity and functionality.

As an alternative to drying biomass prior to extraction procedures, wet processing allows for the recovery of both hydrophobic and hydrophilic biomolecules (Zinkoné et al., 2018). During wet processing, proteins and carbohydrates are released into an aqueous phase after cell disruption. The choice of cell disruption technique greatly influences fraction (product stream) yield and purity and additional downstream purification burden. Thus, using wet processing in

conjunction with a mild and selective cell disruption technique could ultimately result in distinct product streams with functional biomolecules.

Mild cell disruption techniques include pulsed electric field, supersonic flow fluid processing, enzymatic hydrolysis, ionic liquids, and explosive decompression (Phong et al., 2018; Vanthoor-Koopmans et al., 2013). These particular techniques employ neutral pH, low temperatures, and low pressures and have demonstrated applicability for a wide range of microalgae species. Table 2.1 summarizes the disruption mechanism, advantages, limitations, and selectivity of each technique.

Table 2.1 Mild cell disruption technique comparison (adapted from Phong et al. 2018 and Vanthoor-Koopmans et al. 2013)

Disruption Method	Pulsed Electric Field	Ultrasonication (stable cavitation)	Supersonic Flow Fluid Processing	Enzymatic Hydrolysis	Explosive Decompression
Disruption Mechanism	Electroporation	Cavitation shear force	Controlled shockwave, electroporation	Enzyme-substrate interaction	Gas expansion, pore formation
Advantages	Minimally invasive Scalability Can be combined with other techniques	Not species dependent; Doesn't' require additional disruption components (beads/chemicals); Can be combined with other techniques	Temperatures don't exceed 35°C; Homogenous disruption	Specificity; Targeted permeation/perforation	Doesn't require chemicals
Limitations	Requires deionized solutions	Overheating is possible (requires cooling); Biomolecule damage/degradation	Limited results in terms of applicability to microalgae	Species dependent; Expensive; Long incubation times	High pressures
Selective Product Recovery	Yes	No	Yes	Yes	No

Cell disruption via enzymatic hydrolysis as part of aqueous enzymatic processing (AEP)

A comparison of the mild cell disruption techniques presented in Table 2.1 shows that mild processing isn't always associated with selective product recovery. Mild disruption methods that cause permeation or perforation of the cell wall and/or membrane such as PEF, supersonic flow fluid processing, or enzymatic hydrolysis allow for selective release of biomolecules from

biomass into an aqueous phase with retention of others in disrupted biomass. Thus, there is a separation of products into distinct fractions or product streams. Enzymatic hydrolysis in addition to mediating selective product release has the advantage of specificity in that components of the cell wall or matrix can be targets for hydrolysis reactions. Depending on the microalgae species used, hemi(cellulose), glycoproteins, or polysaccharides can serve as substrates for enzymatic hydrolysis.

Enzymatic hydrolysis in conjunction with wet processing is aqueous enzymatic processing. Aqueous enzymatic processing (AEP) has been previously studied and applied to the extraction of oils from oil-rich plant material and seeds (de Moura et al., 2008; Latif and Anwar, 2011; Moreau et al., 2009; Wilken et al., 2014). AEP typically involves the application of a combination of enzymes to break the cellular structure and solubilize/release proteins and oil bodies. Selected enzymes for AEP depend on the biomass cell wall structure and cellular localization of desired products. The use of AEP for microalgae has not been extensively explored and is typically focused on the extraction, recovery, and/or conversion of a single biomolecule. When enzymatic hydrolysis is used as part of microalgae processing, the technique serves as a cell wall/membrane pretreatment and secondary treatments with traditional techniques are employed. Table 2.2 summarizes recent studies using enzymes for cell wall/membrane pretreatment and the secondary treatments additionally used for biomolecule extraction. Those secondary treatments involving biomass drying, mechanical disruption, or organic solvents result in product degradation which impacts sustainability and costs of the overall process. It is the requirement of these secondary treatments that leads to additional production cost and loss of viable/functional bioproducts that presents an opportunity for engineering process design and improvement. Development of an aqueous enzymatic process

whereby enzymes are not only used for cell pretreatment but also for organelle disruption and biomolecule liberation serves to facilitate a microalgae biorefinery rooted in enzyme-mediated unit operations.

Table 2.2 Summary of recent studies using enzymes for cell wall pretreatment (adapted from (Dixon and Wilken, 2018)).

Microalgae Species	Target	Enzyme(s)	Secondary Treatments	Reference
C. reinhardtii	Proteins and lipids	Gamete autolysin	Enzyme	(Sierra et al., 2017)
Chlorella sp.	Cell wall	Cellulases, xylanases, and amylases	Chilled, dried	(Rodrigues and da Silva Bon, 2011)
Chlorella vulgaris	Lipids	Cellulase, β-glucosidases	Organic solvent	(Cho et al., 2013)
C. vulgaris	Cell wall	Alcalase®	Aerobic digestion	(Mahdy et al., 2014b)
C. vulgaris	Lipids	Snailase, lysozyme, cellulase	Organic solvent	(Zheng et al., 2011)
C.vulgaris, S. dimorphus, Nannochloropsis sp.	Cell wall and lipids	Cellulase, snailase, proteases	Ultrasonication	(Liang et al., 2012)
H. pluvialis	Carotenoids	Glucanex®	Ultrasonication	(Machado Jr. et al., 2016)
H.pluvialis	Astaxanthin	Protease K, Driselase®	Organic solvent	(Mendes-Pinto et al., 2001)
Nannochloropsis sp	Lipids	Galactomannanase, β-cellobiosidase, β-glucosidase	Organic solvent	(Zuorro et al., 2015)
Nannochloropsis sp	Lipids	Cellulase, hemicellulases	Organic solvent	(Zuorro et al., 2016)

Chlamydomonas reinhardtii

A wild-type *C. reinhardtii* stain was selected as the biomass source due to is versatility in production of lipids, starch, native and/or recombinant proteins. *C. reinhardtii* is approximately 48% protein, 17% carbohydrates, and 21% oil, although composition can vary greatly depending on strain and cultivation conditions (Demirbas, 2011). The cell wall of *C. reinhardtii* is a cellulose-deficient structure that is primarily composed of proteins and hydroxyproline-rich glycoproteins (Imam and Snell, 1987). As an extensively studied model organism, *C. reinhardtii* is known to accumulate triacylglycerols (TAGs) within lipid bodies that are enriched in palmitic, oleic, and linoleic acids under stress conditions (Siaut et al., 2011). In addition to lipid production, *C. reinhardtii* can be used for production of enhanced value co-products, such as recombinant proteins, (Rasala and Mayfield, 2011). Thus, *C. reinhardtii* presents a unique

opportunity to recover high value pharmaceutical proteins along with high volume products such as lipids or starch. An assessment of the applicability of aqueous enzymatic processing on a wild-type strain serves as a proof of concept for later translation of developed processing procedures and strategies to transgenic *C. reinhardtii* strains or other green microalgae species.

Study objectives

The objective of this study is to evaluate the applicability of aqueous enzymatic processing of wild-type *C. reinhardtii* for the extraction and recovery of multiple biomolecules including native proteins and lipids as part of a biorefinery (Figure 2.1). The process will include biomass production and biomolecule accumulation, harvesting, primary enzymatic treatment (PET) for cell disruption, secondary enzymatic treatment (SET) for degradation of organelle membranes and biomolecule extraction, and biomolecule separation and fractionation.

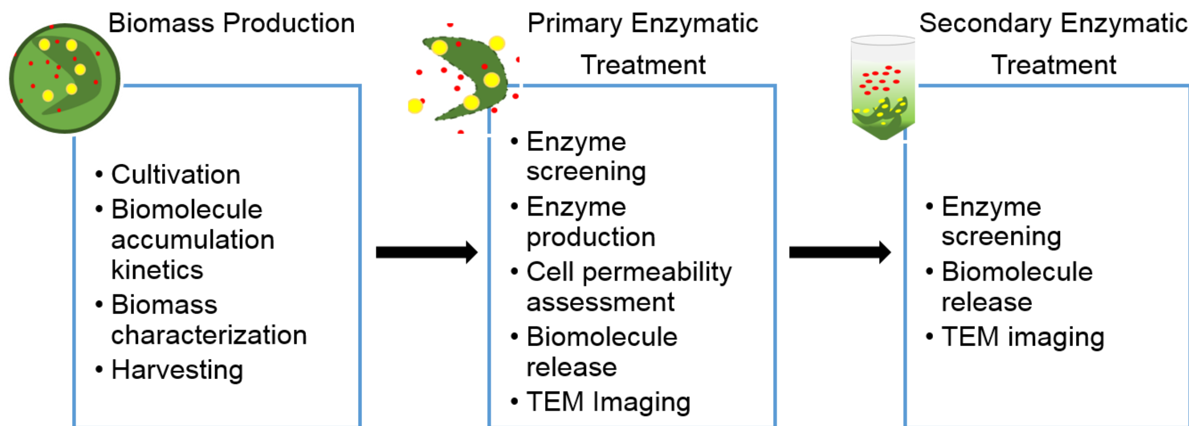


Figure 2.1 Major steps of proposed biorefining procedures.

Materials and Methods

Microalgae source

Production stain

C. reinhardtii (CC-409 mt+) was obtained from the *Chlamydomonas* Resource Center, University of Minnesota. The strain was maintained on solid TRIS-acetate phosphate (TAP) plates for use as inoculum for liquid cultures.

Mating strains

CC 620 mt+ and CC 621 mt- high efficiency mating strains were kindly provided by Dr. Brad Olson of the Division of Biology, Kansas State University, Manhattan, Kansas 66506-4004, United States of America. The strains were maintained on solid TAP plates until use for gamete autolysin production.

Cultivation procedures

CC-409 mt+ was be grown in standard TAP medium at 23°C under 12h/12h light/dark cycles (27 µmol m^{-2} s^{-1}) with continuous shaking at 120 rpm unless otherwise stated. Growth media was autoclaved at 20 psi and 121°C for 40 min. *C. reinhardtii* cultures were streaked on solid TAP agar plates and grown for 5-7 days. Accumulated biomass was then used to inoculate 250 mL of liquid TAP media in a 500 mL Erlenmeyer flask. Cell growth and size was monitored by cell counts with a BrightLine hemocytometer (VWR Scientific). After cells reached mid-exponential phase (5×10^6 -7×10^6cells/mL), cultures were transferred into 1.75 L of fresh TAP media and grown again until the mid/late exponential phase. To induce lipid accumulation via nitrogen deprivation, cultures were centrifuged at 8000 x g for 6 min at room temperature, decanted, and the pellets washed in TAP medium without nitrogen (TAP-N). The washed pellets

were re-suspended in TAP-N medium and after 24, 48, 72, and 96 h, concentrated via centrifugation and frozen at -80°C until required for characterization assays and/or AEP studies.

Characterization of algal biomass

Dry weight determination

The dry weight of *C. reinhardtii* biomass was determined following culture harvesting. Whatman® glass microfiber filters, Grade GF/F were ashed in a muffler furnace at 400°C for 1 h and stored in a desiccator prior to use. All aluminum trays used during dry weight determination were similarly ashed and stored. For each replicate performed the mass of the aluminum tray (W1), filter (W2), and combined tray and filter (W3) were recorded. To begin, the pre-weighed filter was carefully added to the vacuum filtration system. The filter was dampened with 10 mL of 0.5 M Ammonium formate prior to application of the algal culture. Then, 20-30 mL of algal culture was filtered at a vacuum pressure of 35-55 mmHg. Finally, any salts were washed from the filtered biomass by rinsing the filter with 10-15 mL of 0.5 M Ammonium formate. The filter was carefully removed from the filtration system, placed in the pre-weighed tray, covered with foil, and dried in an air oven at 105°C for 1 h. After drying, the sample was cooled in the dessicator for approximately 20 min and a final mass for the combined tray, filter, and dry algae (W4) was determined. The dry weight of the biomass was determined as the difference between W4 and W3.

Total protein quantification

Total protein content of *C. reinhardtii* cells was determined using the Pierce™ BCA microplate assay (bicinchoninic acid assay) after extracting all protein using a combination of chemical and mechanical disruption methods. Algal biomass (10 mL) was centrifuged at 6000 x g for 5 min. The supernatant was decanted and protein extraction buffer (PEB) consisting of 0.75

mM lithium dodecyl sulfate (Amresco), 2.5 mM glycerol (Amresco), 51.4 mM TRIS base (Biosciences), and 0.02 mM EDTA (Alfa Aesar) was used to resuspend biomass. Cells were lysed by sonication (Qsonica) for a total of 2 min at 25% ultrasound amplitude in 30 s increments with rapid cooling in dry ice between sonication cycles to prevent sample overheating and protein denaturation. The lysate was centrifuged at 6000 x g for 5 min and the supernatant containing soluble proteins was transferred to a new tube. To minimize chlorophyll interference with the BCA assay, the supernatant was diluted 10X with ddH_2O. The microplate procedure was followed for the BCA assay using bovine serum albumin as a standard. Results of the BCA assay were used to calculate percent protein content of algae biomass on a dry basis using equation 1.

$$Total\ protein\ content\ DW\ (g/g) = \frac{mass\ extracted\ protein\ (g)}{DW\ (g)} \quad (1)$$

Where,

DW=Dry weight of microalgae biomass (g)

Total lipid quantification

A modified (Bligh and Dyer, 1959) procedure was used for lipid extraction from *C. reinhardtii*. For both nitrogen-replete and deprived *C. reinhardtii*, 90 mL of algal culture was centrifuged at 6000 x g for 5 min and a mixture of chloroform, methanol, water in a 1:2:1(v/v/v) was added to the pelleted biomass. The solvent-biomass mixture was disrupted with a sonicator (Qsonica) at 25% amplitude for approximately 1 min and mixed on a rocking platform for a minimum of 8 h. Chloroform and water in a 1:1 (v/v) ratio were added to the mixture and vortexed briefly. The mixture was centrifuged at 6000 x g for 5 min and the bottom lipophilic phase was extracted and syringe filtered (0.2 μm) into a pre-weighed aluminum tray. Chloroform

(1 volume) was again added to the residual solids and the above extraction and filtration procedure was repeated, combining the extracted lipophilic phases. The filtered lipid extract was evaporated in a fume hood for a minimum of 3 h to eliminate residual solvent and dried in an oven at 105°C for 1 h. The mass of extracted lipid was gravimetrically determined and used to calculate percent lipid content of algae biomass using equation 1.

$$Lipid\ content\ DW\ (g/g) = \frac{mass\ extracted\ lipid\ (g)}{DW\ (g)} \qquad (2)$$

Where,

DW=Dry weight of microalgae biomass (g)

Protein profile analysis

The protein profiles of TAP and TAP-N cultures at various harvesting times (24, 48, 72, 96 h post resuspension) were visualized with gel electrophoresis. Protein extracts from equal amounts of biomass were run under reducing conditions on NuPAGE® Bis-Tris protein gels followed by staining with coomassie blue. SeeBlue™ Plus2 Pre-stained Protein Standard was included to visualize protein molecular weight ranges from 3 to 198 kDa.

Primary Enzymatic Treatment (PET) of microalgae biomass

Candidate enzymes

Various enzymes targeting specific components of the *C. reinhardtii* cell wall were tested for their ability to catalyze cell wall permeability and/or disruption (Table 2.3). Each candidate enzyme was incubated with previously frozen biomass for 2 h prior to determination of cell wall permeability. Three replicates for each treatment were performed.

Table 2.3 Enzymes used for cell permeabilization, cell wall target, compatible conditions and dosage guidelines for incubation. Adapted from [1]Wilken and Nikolov (2011), Nakimbugwe et al., (2006), [2] Yoshida and Noda (1965), [3] Bergmann et al., (1939) and [4] Jaenicke et al., (1987).

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

Production of gamete autolysin

Gamete autolysin was produced by mating CC 620 mt+ and CC 621 mt-. A high volume production process was developed based on the protocol proposed by Jaenicke et al. (1987) including the growth of algal biofilm atop TAP media agar plates followed by solubilization of the biofilm and suspension in liquid TAP-N media. The mating stains developed biofilms after placement under high intensity LED lights for three to seven days. Beginning on day three of biofilm generation mating tests were performed to confirm mating efficiency. 500 µL samples of each strain was mixed and allowed to mate for 15 min and observed using light microscopy. When approximately 95% of the cells were mating, efficiency was confirmed. Each strain was independently transferred into liquid TAP-N media with volume adjustment to achieve a final cell concentration of 1 x 10^7 cells/mL. The liquid cultures were mixed for 4 h under high intensity LED lights for gamete induction and flagella formation. Strains were then mixed in a

clear container and left to sit under high light for approximately 30 min. The mating cells were then centrifuged and the supernatant containing autolysin filtered using a 0.45 μm PES membrane filter and stored in aliquots at -80°C until use.

Enzyme incubation and cell permeability assessment

Quantitative estimation of cell permeability

To quantitatively estimate cell permeability, samples taken before, during, and after the various enzymatic treatments were mixed in 1:1 (v/v) ratio with 1% NP-40 detergent. The mixture was loaded on a hemocytometer and intact cells were counted. Intact cells are insensitive to detergent and indicate the cell wall has not be permeated by enzymatic treatment. Cell permeability/lysis was determined by the following equation 3.

$$\text{Cell Permeability (\%)} = \frac{\text{Intact cells per mL biomass during or after treatment}}{\text{Intact cells per mL biomass before treatment}} \text{x} 100\% \quad (3)$$

Qualitative visualization of cell permeability

To qualitative visualize cell permeability samples taken before, during, and after the various enzymatic treatments were stained with Sytox® green fluorescent dye, which is only able to access and dye nucleic acids of cells with permeable walls and subjected to fluorescence microscopy.

TEM imaging

TEM images of samples collected throughout the enzymatic treatment were taken using Tecnai™ G2 Spirit BioTWIN (FEI Company) at 80 kV acceleration voltages at the Electron Microscopy Facility in the Nanotechnology Innovation Center of Kansas State (NICKS). Biomass samples were fixed in Trump's fixative overnight, post fixed with Osmium tetroxide, dehydrated in graded series of alcohol and embedded into spur resin. Ultra-thin sections were

contrasted with uranyl acetate- lead citrate and observed under a FEI Tecnai 12 Bio-spirit Transmission electron microscope.

Evaluation of protein solubilization following PET alone or with combined chemical/mechanical disruption

Soluble protein released following PET was quantified and compared to soluble protein released after biomass was subjected to PET+Ultrasonication (UTS) or PET+Detergent solution. Autolysin solution was added to biomass for 4 h at room temperature for PET. For PET+UTS, PET lysate was subsequently ultrasonicated for 3 min at 35% amplitude. For PET+Detergent, PET residue separated after crude lysate clarification was resolubilized in protein extraction buffer, vortexed, and allowed to stand to allow for protein diffusion into the aqueous phase. Protein content in the various was quantified with the BCA (Pierce ™) microplate assay and protein solubilized (% of total) for each pretreatment was calculated based on a total protein reference. Total protein was calculated based on the quantification procedures described in the "Total protein quantification" section. Protein solubilized by various pretreatments was calculated using equation 4.

$$\text{Protein solubilized (\% of total)} = \frac{\text{SP(g)}}{\text{TP(g)}} \times 100\% \quad (4)$$

Where,

SP=Protein solubilized by pretreatment (g)

TP=Total protein in biomass (g)

Evaluation of extractable lipid content after PET alone or with combined mechanical disruption

The extractable lipid content from biomass subjected to PET, PET+UTS, (-) control+UTS, or (-) control (no pretreatment) was evaluated using a modified Bligh and Dyer (Bligh and Dyer, 1959) or hexane extraction (Wang and Yuan, 2014).

Modified Bligh and Dyer extraction

Following pretreatment, chloroform, methanol, and water were added to lysates in a volumetric ratio of 1:2:1. PET+UTS and (-) control+UTS samples were ultrasonicated for 1 min at 35% amplitude while PET and (-) control samples received no additional mechanical disruption. All samples were then mixed on a rocking platform overnight. Chloroform and water in a 1:1 (v/v) ratio were added to the mixtures and vortexed briefly. The mixtures was centrifuged at 6000 x g for 5 min and the bottom lipophilic phase was extracted and syringe filtered (0.2 µm) into a pre-weighed aluminum tray. One volume of chloroform was again added to the residual solids and the above extraction and filtration procedure was repeated, combining the extracted lipophilic phases. The filtered lipid extract was evaporated in a fume hood for a minimum of 3 h to eliminate residual solvent and dried in an oven at 105°C for 1 h. The mass of extracted lipid was gravimetrically determined and used to calculate extractable lipid content of the various pretreatments using equation 5. The total lipid reference was calculated as described in the "Total lipid quantification" section.

$$\text{Extractable Lipids (\% of total)} = \frac{EL(g)}{TL(g)} \text{ X } 100\% \qquad (5)$$

Where,

EL=Extracted lipids (g)

TL=Total lipids (g)

Hexane extraction

Hexane was added in a 1:1 (v/v) to PET or (-)control treated lysates with and without the additional sonication step. All samples were mixed overnight and then 1 volume of hexane was added to the mixture and subsequently centrifuged at 2020 x g for 15 min. The top lipophilic layer was removed and residual solvent allowed to evaporate for a minimum of 3 h. The remaining lipids were then dried in an oven at 95°C for 1.5 h. The mass of extracted lipid was gravimetrically determined and used to calculate extractable lipid content of the various pretreatments using equation 4.

Statistical Analysis

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

Secondary Enzymatic Treatment (SET) of microalgae biomass

Quantification of lipid release following secondary enzymatic treatment

After SET, biomass residues were separated from the supernatant via centrifugation at 6000x g for 5 min. Hexane was added in a 1:1 (v/v) to the supernatants and then mixed overnight. An additional volume of hexane was added the following day and samples were centrifuged for 2020 x g for 15 min. The top lipophilic layer was removed and residual solvent allowed to evaporate for a minimum of 3 h. The remaining lipids were then dried in an oven at 95°C for 1.5 h. The mass of extracted lipid was gravimetrically determined and used to calculate lipid release (% of total) based on the total lipid reference.

Results and Discussion

Biomolecule accumulation kinetics

To develop a biorefinery process for *C. reinhardtii*, the conditions necessary for accumulation of target biomolecules (native proteins and lipids) must be determined. As nitrogen deprivation is a well-employed technique for the induction of TAG (triacylglycerol) (Wang et al., 2009) and starch accumulation in microalgae (Wei et al., 2014) but is typically accompanied by a decrease in protein accumulation, the optimum deprivation period was determined. Algal cultures grown to exponential phase were re-suspended in either TAP (nitrogen replete) or TAP-N (nitrogen deplete) media and sampled at 24, 48, 72, and 96 h post-resuspension. At each sample collection point, protein and lipid contents were quantified and the molecular weight profiles were visualized with gel electrophoresis.

Figures 2.2 a and b, show the kinetics of lipid and protein content over the 96 h post-resuspension period. For nitrogen replete cultures, there was no significant increase in lipid accumulation during the 96 h period. The lipid content at 24, 72, or 96 h post-resuspension for TAP and TAP-N cultures was also not significantly different. For nitrogen deplete cultures, 48 h post-resuspension resulted in a significantly different increase in lipid accumulation followed by a sharp decrease in lipid content at 72 h post-resuspension. These results are in contrast with previously reported lipid accumulation patterns following nitrogen deprivation when lipid content steadily increased through 72 h (Valledor et al., 2014) but reports of varied accumulation in different *Chlamydomonas* species has also been reported (Siaut et al., 2011). The reduction in lipid content at the 72 h mark could be due to the fact that lipids are synthesized less rapidly than starch granules upon nitrogen deprivation, but are the prolonged carbon reserve and energy source for the cell during stress conditions (Goncalves et al., 2016).

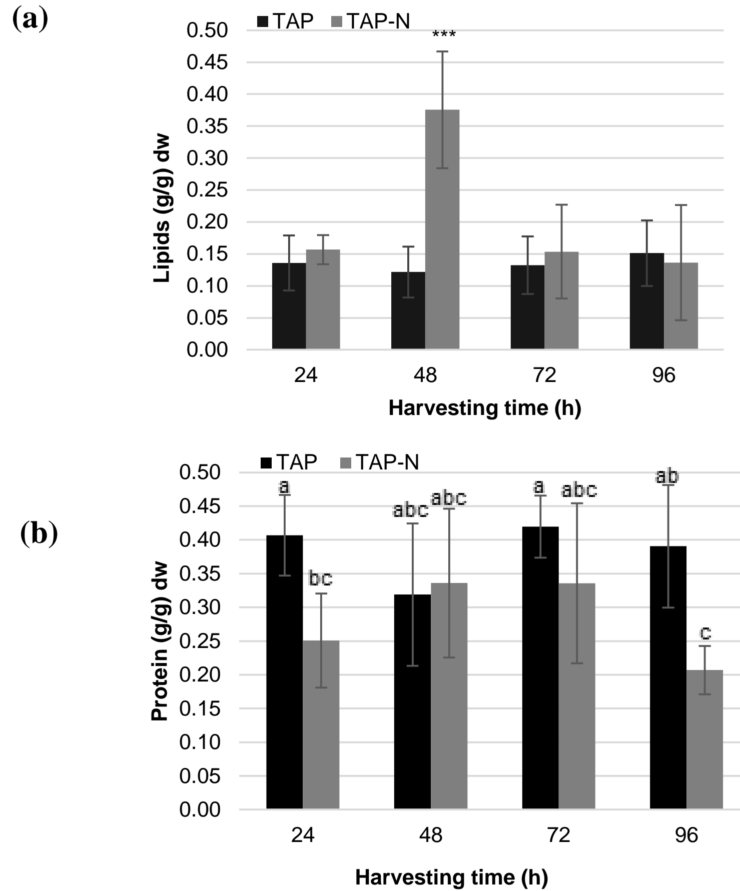


Figure 2.2 (a) Lipid content and (b) protein content (g / g dry weight) for cells grown in nitrogen replete (TAP) media or nitrogen deplete (TAP-N) media. Error bars represent standard error for n>3. Significant differences were corrected for multiple comparisons with Tukey adjustment and a α_{FER} =0.05. Different letters represent significant difference between treatments. Treatments with asterisks (***) are significantly different to those without asterisks. No letter or asterisks above SD bars represents no significant differences.

The effect of nitrogen deprivation on protein content was also assessed. At 48 and 72 h of deprivation, TAP-N cultures had similar protein contents on a dry weight basis as compared to their TAP counterparts and there was no significant difference in the protein content of deprived cultures. By the end of the deprivation period (96 h), there was a significant difference in protein content between TAP and TAP-N cultures. This has been repeatedly observed in previous studies

and is attributed to nutritent recycling via protein degradation to provide carbon for energy reserves such as starch granules and TAGs (Saroussi et al., 2017; Wei et al., 2014). As nitrogen is essential for protein synthesis, depriving cells of this nutrient would expectedly lead to lower overall protein levels.

While no significant changes to protein content were seen between cultures resuspended in TAP and TAP-N for 48 h, the protein profiles were further assessed with gel electrophoresis over the entire 96 h period. Figure 2.3 shows that there is protein degradation as the deprivation period persists (Box C) visualized by the increase in low molecular weight proteins between 3 and 6 kDa with an accompanied reduction in high molecular weight proteins (20-100 kDa) after 48 h. There is additionally a decrease in a ~50-55 kDa protein (Box A) and a complex of proteins ranging from 20-28 kDa (Box B) for nitrogen deprived cultures over time. The ~50-55 kDa protein is likely the photosynthetic chloroplast protein, RuBisCO. As nitrogen deprivation causes a metabolic shift from photosynthesis to energy storage for survival in microalgae cells, the reduction of a photosynthetic proteins such as RuBisCO is expected (Msanne et al. 2012). In fact, it has been reported that RuBisCO can be reduced to as much as 50% of the levels normally seen in cultures grown in nutrient replete conditions in the presence of acetate (Saroussi et al. 2017). Likewise, the complex of proteins ranging from 20-28 kDa are likely light harvesting complex (LHC) proteins (Tokutsu et al., 2004) for which a reduction in transcript abundance for genes encoding LHC1 and LHC2 protein has been reported as early as the first 24 h of nitrogen deprivation (Juergens et al., 2014). Generally speaking, protein band intensities indicate that TAP cultures had a higher protein content than their TAP-N counterparts. While decreases in what appears to be RuBisCO and LHC proteins are evident during the deprivation period, the

overall molecular weight protein profiles are relatively consistent for TAP and TAP-N cultures for the first 48 h of nitrogen deprivation.

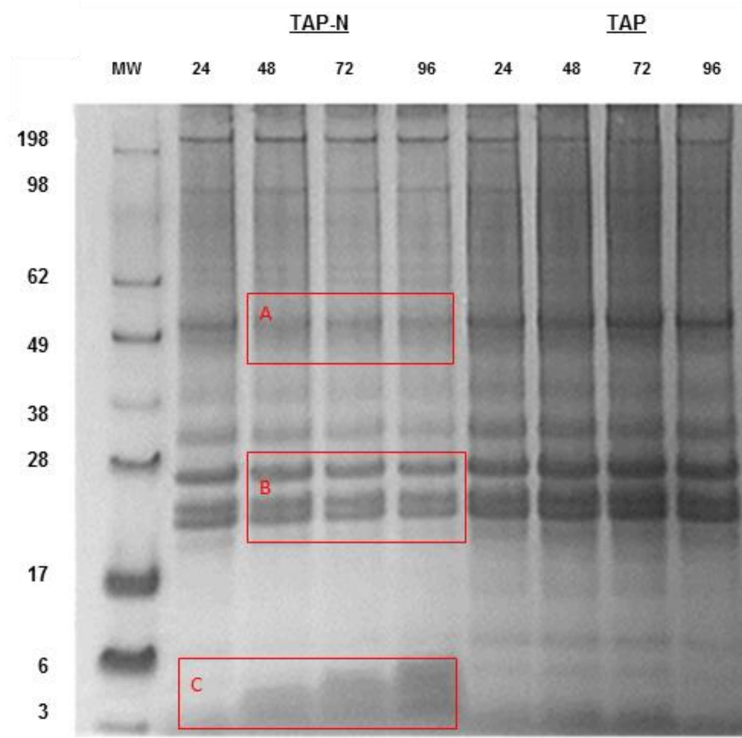


Figure 2.3 Coomassie-stained SDS-PAGE gel of molecular weight protein profiles for TAP and TAP-N *C. reinhardtii* cultures over time in hours. Apparent protein degradation is shown in the box. This gel is a representative sample of 3 replicates.

As this work aims at the development of a biological based biorefinery process wherein multiple biomolecules are extracted and recovered, establishing a deprivation period that allows for balanced target biomolecule content was ideal. As 48 h of nitrogen deprivation resulted in the highest lipid content with no associated loss in protein content and a consistent protein profile, this harvesting time was selected. After the establishment of a 48 h deprivation period (harvest time), process development continued for design of enzyme based cell disruption procedures.

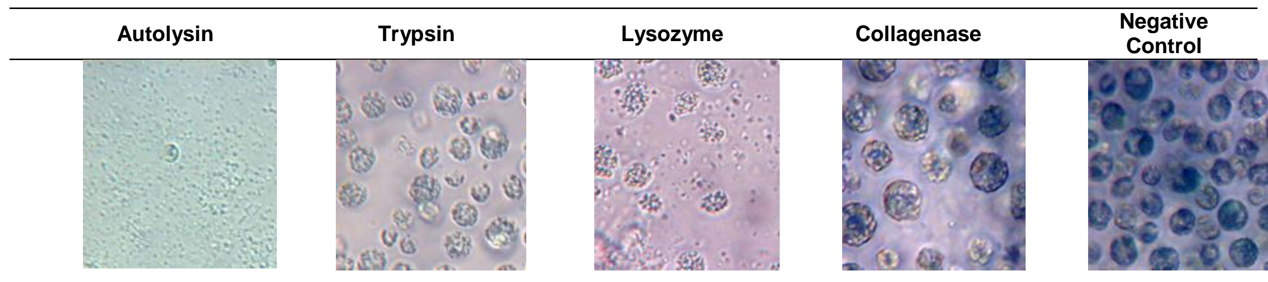
Primary enzymatic treatment of microalgae biomass

Enzyme incubation and cell permeability assessment

After establishing conditions to promote accumulation of target biomolecules, the primary enzymatic treatment step was developed. Four candidate enzymes, selected for their potential to cleave components in the *C. reinhardtii* cell wall, were screened. Initially, cells were treated with each enzyme at conditions known to promote activity for 2 h. NP-40 detergent was added to the enzyme-biomass mixture and visualized by light microscopy. As seen in Figure 2.4a, autolysin resulted in the highest level of cell permeation (% permeable cells) as evidenced by the lack of intact cells following detergent application and cell counting when compared to other enzyme treatments or a negative control that received no application of enzyme. Lysozyme demonstrated some propensity for cell wall permeation while trypsin and collagenase cell permeation was not significantly different from the negative control. As autolysin appeared to be the most effective in creating cells with permeable walls, additional qualitative confirmation of permeability was conducted with Sytox® green fluorescent dye.

Upon addition of Sytox® green fluorescent dye, cells with permeable walls will exhibit fluorescence as the dye can penetrate and stain intracellular nucleic acids. As seen in Figure 2.4b, autolysin-treated cells demonstrated a markedly higher number of fluorescent cells when compared to negative control cells. Thus, autolysin was quantitatively and qualitatively confirmed to permeate >95% of *C. reinhardtii* cell walls within 2 h of application. Based on this preliminary assessment of cell permeation, autolysin-mediated primary enzymatic treatment was further analyzed for its effect on cell disruption and biomolecule release.

(a)



(b)

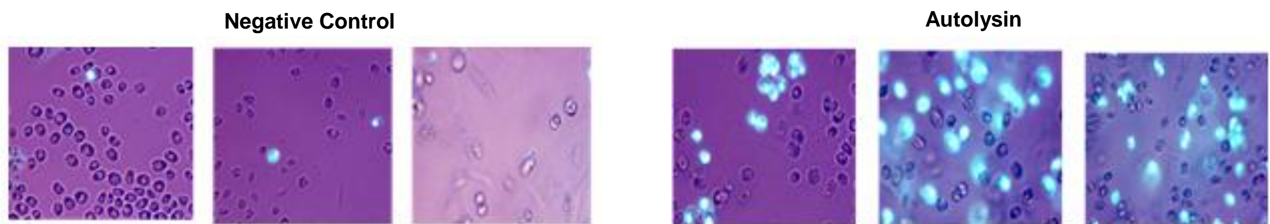


Figure 2.4 Cell permeability imaging assessment (a) using NP-40 to evaluate biomass treated with different enzymes for 2 h and (b) using Sytox® green under fluorescence microscope for negative control samples (no autolysin) and autolysin-treated biomass. Images are representative of > 3 replicates.

Autolysin as a pretreatment technique

Effect of incubation time on cell disruption

Cell disruption is the first step to accessing internally stored biomolecules for subsequent extraction and recovery. While preliminary screening indicated >95% cell permeability with 2 h of incubation with autolysin, the effect of increased incubation time on complete cell disruption and biomolecule release was explored. The incubation time was increased up to 24 h and the difference in cell disruption was visualized with TEM imaging (Figure 2.5).

Incubation Time	Negative Control	Autolysin
2 h (a)	CW S LD 2 μm	S LD 2 μm
2h (b)	10 μm	10 μm
24 h (c)	S LD CW	LD

24 h

(d)

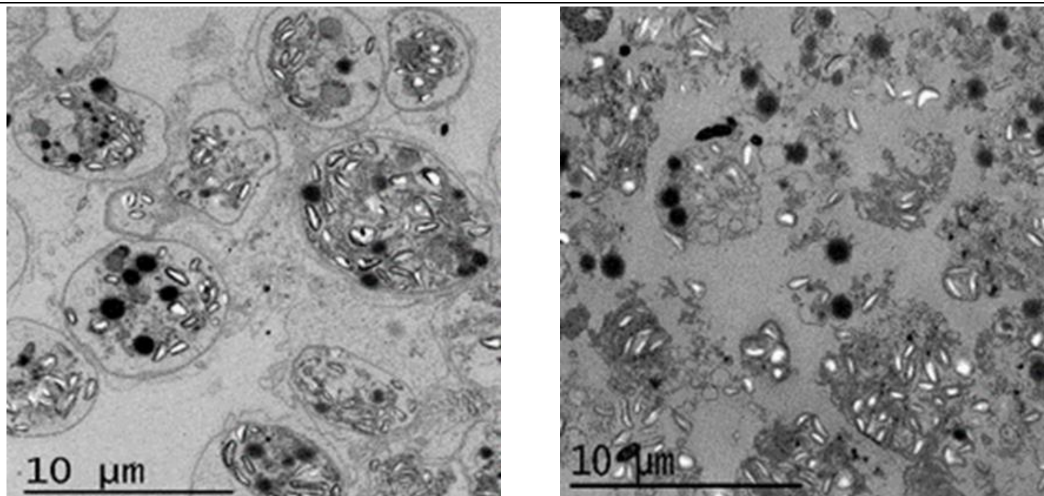


Figure 2.5 TEM images of *C. reinhardtii* cells incubated with TAP-N (negative control) or autolysin for 2 h and 24 h at 890x (a, c) and 2900x (b, d) magnification. Letters indicate cellular components: (S) starch granules, (LD) lipid droplets, and (CW) cell wall. These images are representative of ≥ 2 replicates.

Within 2 h of autolysin treatment, TEM imaging confirmed permeable cells and biomolecules (starch granules) were released (Figure 2.5a-b, right). A defined cell wall also was noticeably absent after 2 h of autolysin treatment when compared to control cells. After 24 h incubation with autolysin, empty lipid and starch sacs indicated that at least some biomolecules were released from disrupted cells (Figure 2.5c, right), while control cells remained intact with an undisrupted cell wall (Figure 2.5c, left). Low magnification images (Figure 2.5d, right) showed that after 24 h of autolysin treatment, although cells were thoroughly disrupted, lipid droplets remained attached to cell or organelle (chloroplast) remnants. Therefore, additional enzymatic treatments are likely necessary for detachment of lipid droplets and coalescence.

Effect of incubation temperature on cell disruption

TEM imaging indicated the ability of autolysin to mediate not only cell permeation but also cell disruption with a 24 h incubation period at room temperature. The effects of increasing temperature up to 37°C during autolysin treatment were evaluated, as autolysin activity has been

reported at temperatures from 20°C to 40°C, and temperature is known to increase the rate of an enzymatic extraction, Cell disruption was evaluated by cell counts at 2, 4, and 24 h of autolysin treatment (incubation and mixing) at 25°C or 37°C. Increasing incubation time from 2 to 4 h resulted in significantly higher cell disruption for both 25°C and 37°C (Figure 2.6). After 4 h incubation, an average of 54±7% of cells were disrupted and there was no significant difference between 25°C and 37°C for either 4 h or 24 h incubation. As conditions ranging from 4 h incubation at 25°C to 24 h incubation at 37°C resulted in cell disruption that was not significantly different, treatment of cells for 4 h at 25°C was selected for autolysin-mediated cell disruption. For all treatment combinations tested, no significant levels of cell disruption resulted in negative control cells (data not shown).

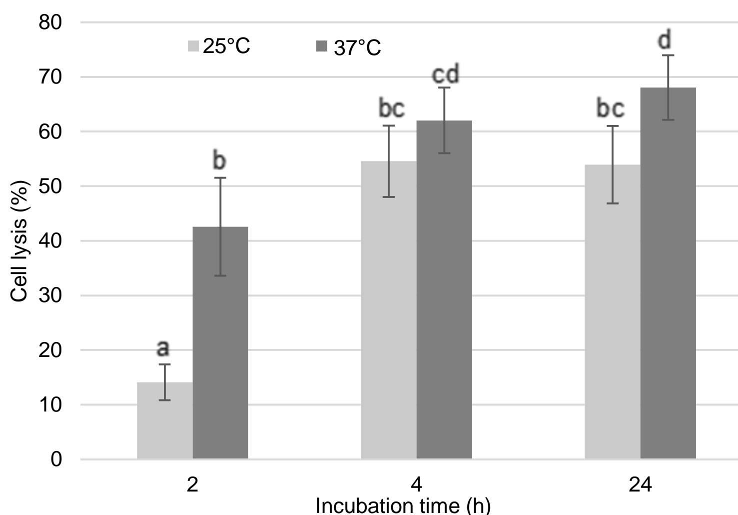


Figure 2.6 Cell lysis (%) after PET with autolysin over time at different temperatures. Error bars represent standard error for n > 3. Significant differences were corrected for multiple comparisons with Tukey adjustment and α_{FER} = 0.05. Error bars represent standard deviations (SD). Different letters above the SD bars indicates significant differences (α_{FER} = 0.05) between treatments. No letter above SD bars represents no significant differences.

Evaluating the effect of autolysin on enhancing lipid and protein extractability

Most biomass pretreatments aim to improve biomolecule extraction efficiency by disrupting biomass to allow extraction buffer and/or solvents to contact intracellular compartments. The effect of PET with autolysin on enhancing conventional pretreatment techniques prior to lipid and protein extraction was evaluated.

To access the impact of pretreating cells with autolysin on lipid extractability, biomass treated with PET (autolysin), PET+Ultrasonication (UTS), (-) control+Ultrasonication (UTS), or (-) control (no pretreatment). The various pretreatment combinations were then subjected to organic solvent extraction with the Bligh and Dyer method or hexane. These two organic solvent systems were used for lipid extraction after the various pretreatment strategies to represent different approaches to lipid recovery currently employed for extractions from lab-scale to industrial scale.

For both extraction methods used, extractable lipids (% of total) were significantly increased in biomass subjected to PET when compared to no pretreatment (Fig 2.7a). When that PET pretreatment was followed by ultrasonication, no significant increase in extractable lipids was observed for either Bligh and Dyer or hexane extraction systems. Thus, PET with autolysin was successful in achieving a level of cell disruption necessary to permit solvent access to lipids and extraction of 90-95% of total lipids depending on the solvent system used. When comparing PET to ultrasonication as a pretreatment, extraction with the Bligh and Dyer solvent system revealed no significant difference in extractable lipids (% of total) as the solvents used are able to diffuse through non-permeable feedstock and contact lipids. Contrarily, comparing hexane extraction of biomass pretreated with PET or UTS, revealed that extractable lipids (% of total) from biomass subjected to PET (~99% extractable lipids) was significantly higher than that of

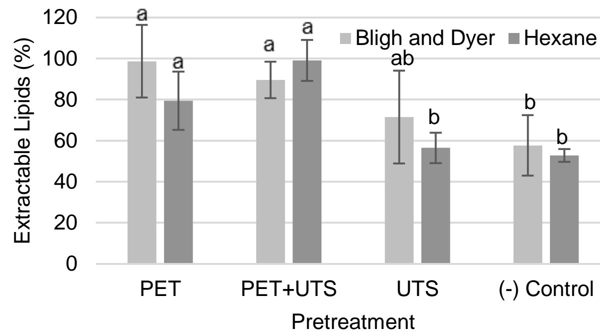
ultrasonication-treated biomass (~72% extractable lipids). The variation in extractable lipids (% of total) observed between these two pretreatments using different extraction solvent systems is likely due to fact that hexane exhibits great extractability from permeable feedstock which would be abundant following PET but perhaps less so after ultrasonication. Therefore, hexane would have more limited access to lipids thus leading to a lower amount of lipid extracted. This behavior is additionally confirmed by the fact that pretreatments of biomass involving PET resulted in on average 30% more extractable lipids in the presence of hexane that ultrasonication alone or no pretreatment.

To assess the impact of pretreating cells with autolysin on protein solubility, biomass was subjected to PET or (-) control treatment followed by ultrasonication or detergent solution application. Application of a buffer with detergent is a common solubilizing agent used for isolation and extraction of membrane-bound proteins in particular. Cytosolic proteins would likely only require cell permeation to diffuse out of cellular material and be soluble in the surrounding aqueous environment. Approximately 80% of total protein was solubilized following PET+UTS or PET+detergent disruption treatment. The level of protein solubilization of these combined disruption treatments was significantly higher than that achieved by ultrasonication of biomass (58% of total) or the application of a detergent alone (53% of total). This indicates that the permeation and disruption of cells during PET treatment can serve to enhance the amount of protein solubilized via traditional mechanical or chemical disruption/solubilizing mechanisms.

Based on the improved extractability of both lipids and proteins from biomass subjected to PET in conjunction with traditional mechanical and/or chemical disruption mechanisms, enzymatic hydrolysis with autolysin is an advantageous pretreatment technique. In the case of

lipid extraction, pretreatment of biomass with autolysin provided sufficient cell disruption that ultrasonication was not necessary to extract nearly all lipids. This could serve as a cost savings at an industrial processing scale in that enzymatic hydrolysis is performed on wet biomass and an energy-intensive mechanical disruption step can be avoided. In terms of protein extraction, the action of autolysin on biomass combined with the addition of a detergent-containing solution lead to 80% solubilization of total protein. Again, elimination of the energy-intensive and potentially costly step of ultrasonication during protein extraction makes enzymatic hydrolysis of biomass with autolysin a promising approach to biomass pretreatment.

(a)



(b)

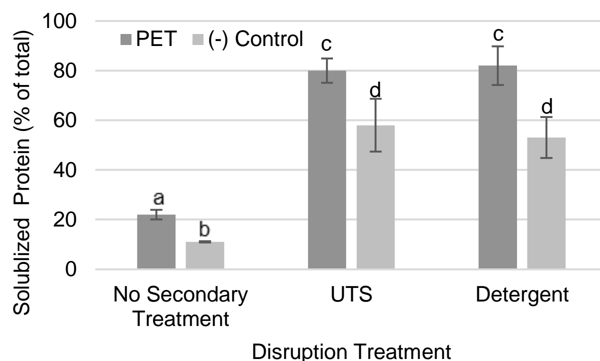


Figure 2.7 Extractable lipids (% of total) via (a) Bligh and Dyer hexane extraction for different biomass pretreatments and (b) solubilized protein (% of total) following PET and negative control with or without secondary disruption. Soluble protein (%) is based on a total protein reference and extractable lipid (%) is based on a total lipid reference. Error

bars represent standard error for n>3. Significant differences were corrected for multiple comparisons with Tukey adjustment and α_{FER} =0.05. Different letters represent significant differences between treatments.

Biomolecule release following Primary Enzymatic Treatment (PET)

Effect of increased temperature and incubation time on protein solubility

Once autolysin was determined to be an adequate cell permeation/disruption agent, release and solubilization of proteins following PET was evaluated. After the initial incubation of biomass with autolysin for 4 h at 25°C for complete cell permeation and ~60% cell lysis, extended incubation at different temperature (25, 37, and 50°C) and time periods (4, 14, 20 h post-PET) was assessed for its effect on protein solubility.

Results indicate a significant increase in protein solubilized at all temperatures for enzyme-treated biomass in comparison to negative control treatment (Figure 2.8a). In particular, increasing incubation temperatures from 25°C to 37°C following PET of biomass resulted in an increase of approximately 10% of solubilized protein while increasing temperatures to 50°C resulted in no significant change. The reduced solubility of proteins at 50°C could be due to protein denaturation and aggregation (Wilken and Nikolov, 2016). Incubation at 37°C for an additional 20 h following PET resulted in an average of 54.2% of total protein released into the aqueous phase and recovered via centrifugation.

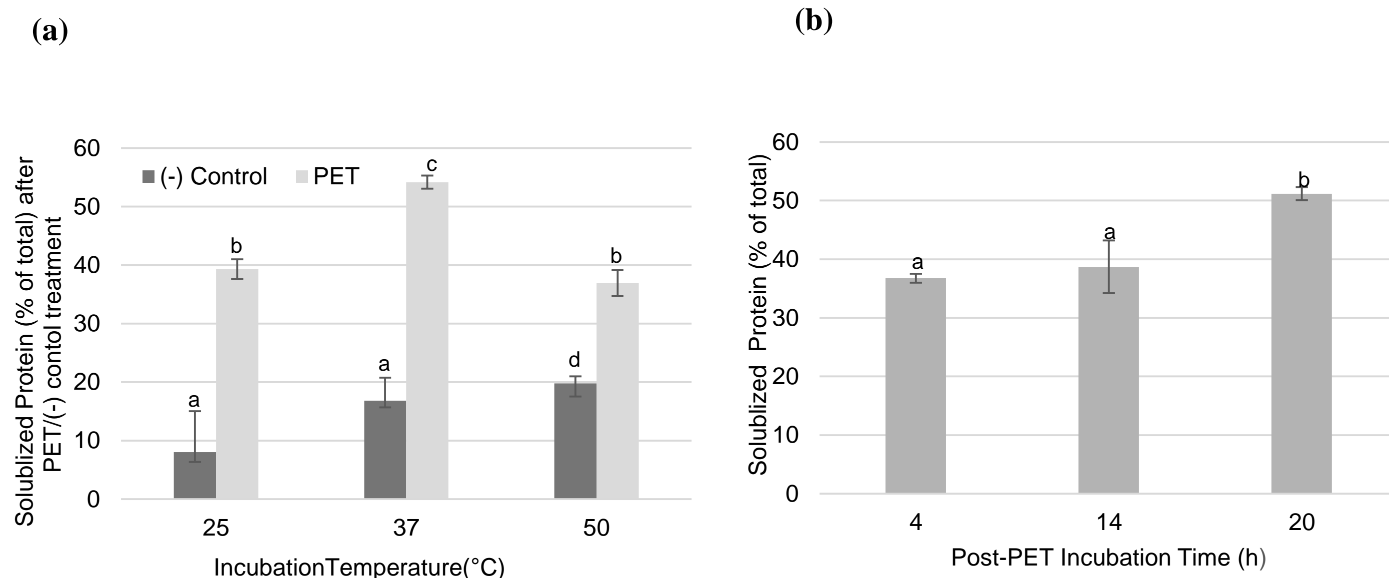


Figure 2.8 (a) Impact of temperature on solubilized protein (% of total) released after PET or negative control treatment with 20 h incubation and(b) impact of time on solubilized protein (% of total) at 37˚C post PET. Error bars represent standard error for n>3. Comparisons were made within and between groups and significant differences were corrected for multiple comparisons with Tukey adjustment and an α_{FER} =0.05. Different letters represent significant difference between treatments.

Assessment of various extended incubation times at 37°C revealed that 20 h resulted in approximately 15% more protein released than 4 h or 14 h incubation post PET (Figure 2.8b). Based on this data, PET at 4 h at 25°C followed by additional incubation at 37° for 20 h resulted in 60-70% cell lysis with an associated release of 54% of total protein. The remaining proteins (RuBisCO and LHCs) were likely stored in the chloroplast remnants and would require addition extraction and recovery procedures at a later stage. Once the majority of proteins were released following PET, the effect of those conditions on lipid release were explored.

Lipid release due to increased temperature and extend incubation

TEM imaging of biomass subjected to PET with the additional 20 h incubation at 37°C for protein solubilization indicated that the majority of lipid droplets were still attached to the disrupted cell solid fraction (Figure 2.9a). Likely, the lipid droplet surface constituents (proteins and/or phospholipids) remain in association with organelle membranes or other proteins and thus aren't released in the aqueous phase of the cell lysate.

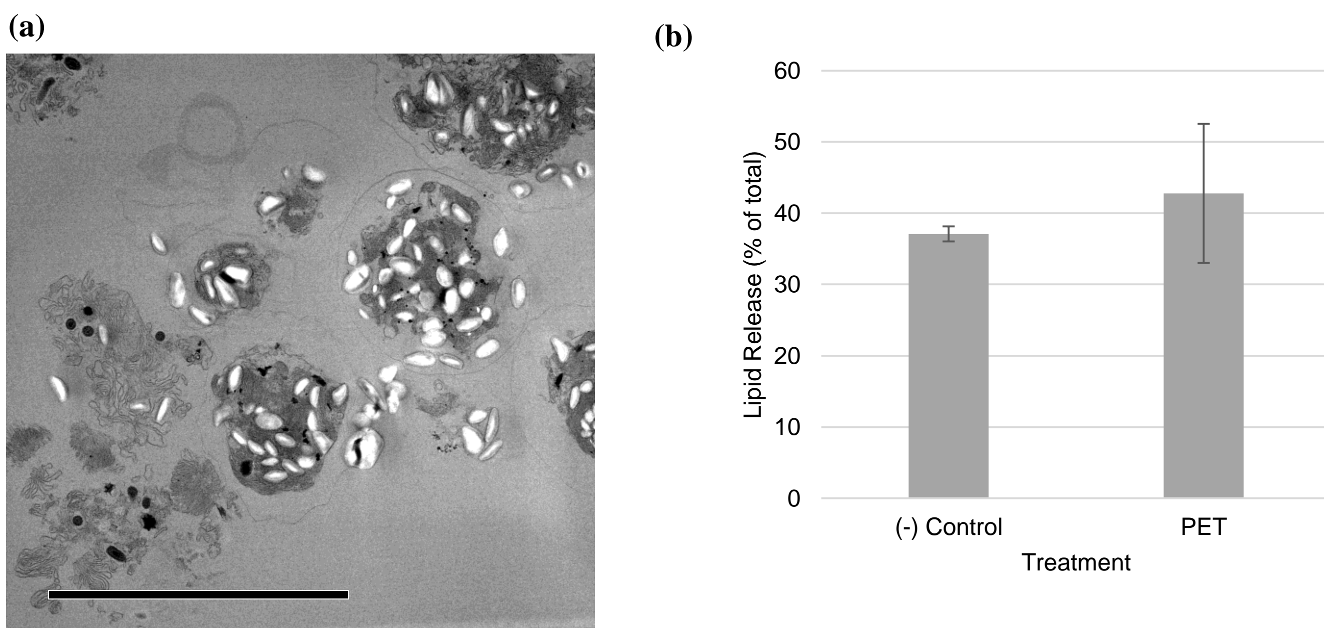


Figure 2.9 (a) TEM image of solid residue after PET and (b) Lipid release (% of total) into aqueous phase (supernatants) of biomass treated with (-) control or PET. Significant differences were found using a P-Value of 0.05. No letter above SD bars represents no significant differences.

To quantify the lipid release, the aqueous phase (supernatants) after (-) control or PET were subjected to hexane extraction. The use of hexane which demonstrates great extractability of permeable feedstock allowed for the separation of only free lipid droplets into the solvent phase while lipid droplet still in association with other cellular components would remain in the aqueous phase. On average, 43% of total lipids were released into the aqueous phase as part of

the PET/protein solubilization procedures which was not significantly different than (-) control treatment subjected to the same conditions without enzyme addition (Figure 2.9b). Thus, the majority of lipids were still contained in the solid fraction following PET and protein solubilization and available for extraction and recovery as part of a distinct product stream.

Secondary Enzymatic Treatment (SET) of Microalgae Biomass

Primary enzymatic treatment of *C. reinhardtii* biomass with an extended high temperature incubation period resulted in up to 54% total protein release with an associated release of 43% of total lipids. Release of the remaining lipid droplets would require cleavage of the linkages or associations between the droplets and partially disrupted chloroplasts. The likely targets of a secondary enzymatic treatment step were the lipid droplet surface protein (LDSP) and other chloroplast proteins. After preliminary screening of enzymes (data not shown), trypsin was selected. Trypsin has reported activity in promoting thylakoid membrane unstacking in green tissues like spinach (Jennings et al., 1981) which could promote release of lipid droplets and additionally could cleave LDSP at up to 20 different points.

Hexane extraction of supernatants following PET only and PET+SET was performed. Results indicate that additional 30% of lipids that remained in solid fraction after PET treatment were released following SET (Figure 2.10a). Figure 2.9b confirms the release of free lipid droplets into the supernatant following PET+SET treatment of biomass as compared to control treatment using Nile Red stain.

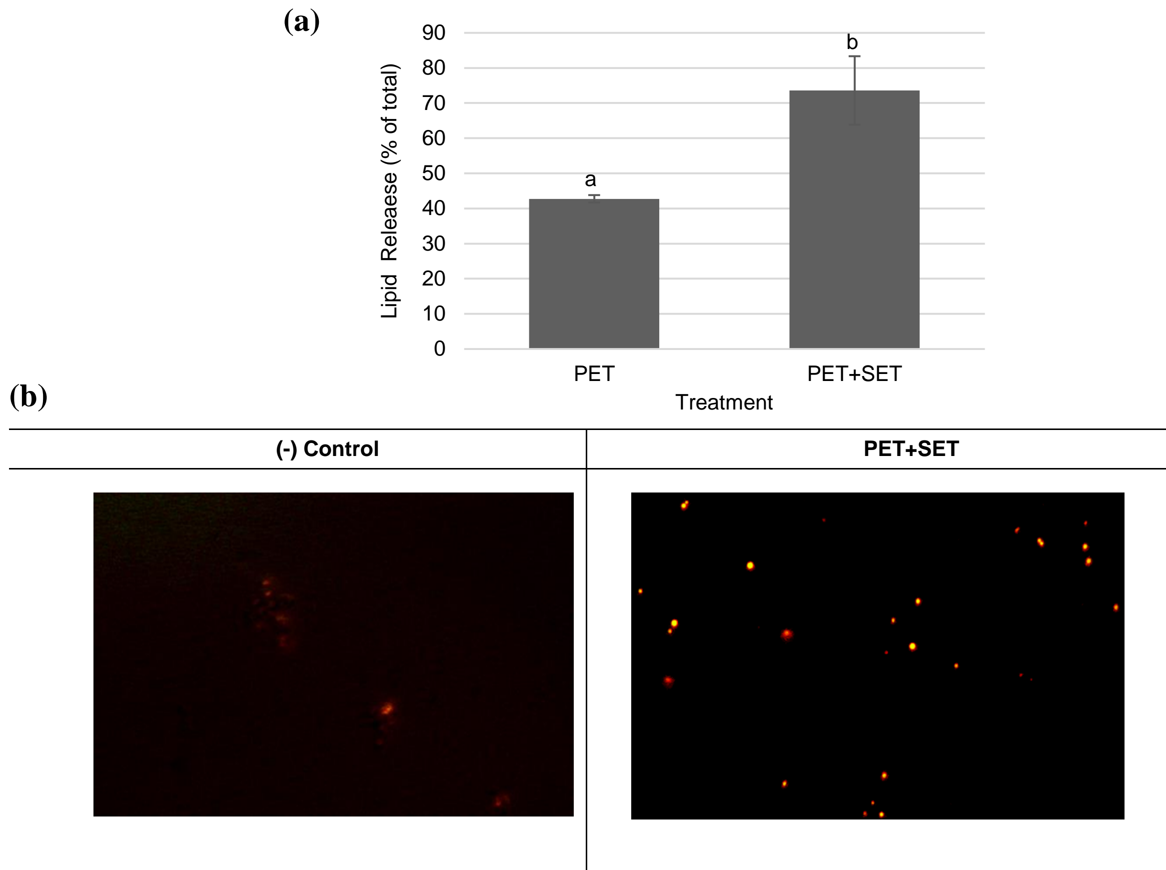


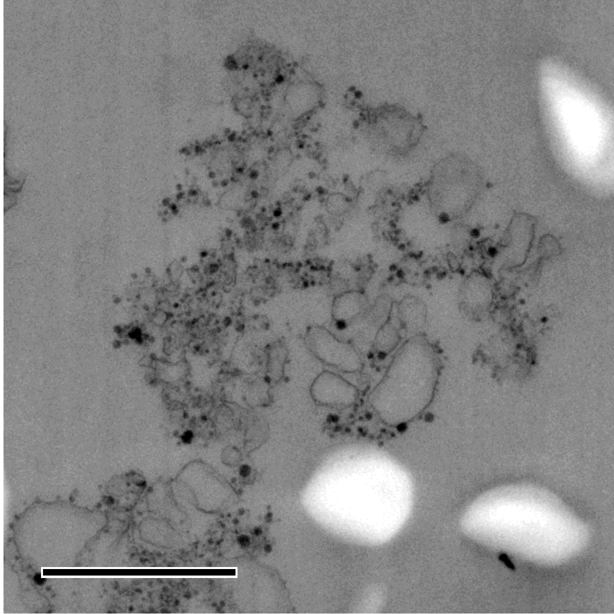
Figure 2.10 (a) Lipid release (% of total) into the aqueous phase (supernatants) after PET and PET+SET and(b) fluorescence microscopy imaging of lipid droplets (yellow fluorescence) stained with Nile Red. Error bars represent standard deviation for n>3. Significant differences determined with α=0.05. Different letters represent significant difference between treatments.

TEM imaging was used to compare biomass subjected to PET only versus PET+SET to explore the effect of trypsin on lipid droplet release (Figure 2.10). Image analysis reveals a high level of cell disruption following PET with an apparent disruption of chloroplast membranes (Figure 2.11a-1). Even with complete cell disruption, lipid droplets remain attached to highly stacked thylakoid membranes (Figure 2.11a-2). Biomass subjected to PET+SET shows “relaxed” and unstacked thylakoid membranes with few small lipid droplets attached (Figure 2.11b). This

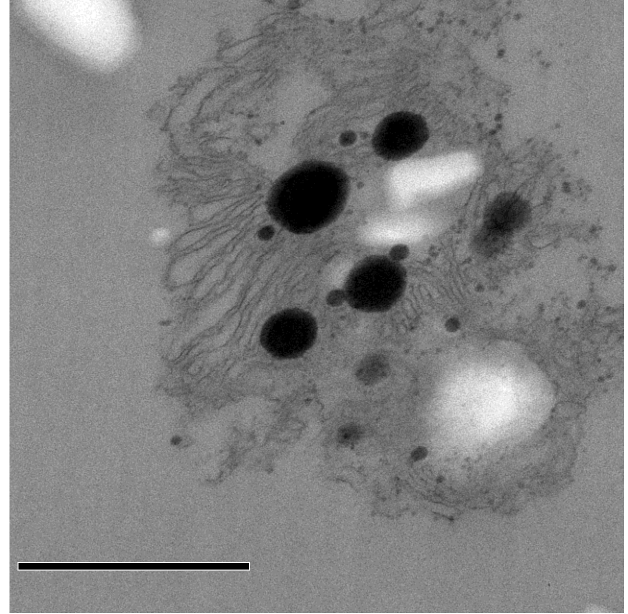
visualization of disrupted biomass and organelles after PET and SET confirms the increase in lipid release quantified via hexane extractions.

(a) Residual solids after PET

1.

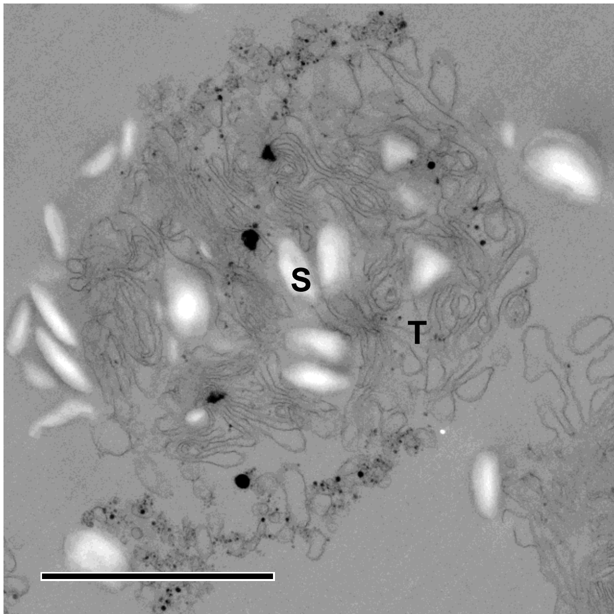


2.



(b) Residual solids after PET+SET

3.



4.

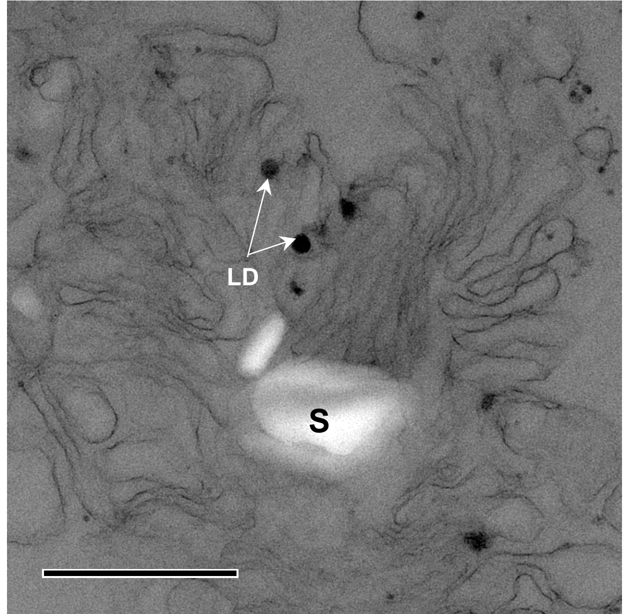


Figure 2.11 TEM images of *C. reinhardtii* biomass subjected to (a) PET only or (b)PET and SET at 6400x (1,2,3), and 2900x (4) magnification. Letters indicate cellular components: (S) starch bodies, (LD) lipid droplets, and (T) thylakoids. Images are representative of >2 replicates.

The lipid release following SET of disrupted biomass with trypsin could be attributed to the enzymes reported relaxation of thylakoid membranes (Jennings et al., 1981) or its digestion of LHC fragments (Steinback et al., 1979).

Summary

This study demonstrates the applicability of aqueous enzymatic processing of microalgae biomass as part of a biorefinery for release of native proteins and lipids from *Chlamydomonas reinhardtii*. Resupension of microalgae cultures in nitrogen deficient media allowed for maximum lipid accumulation within 48 h without detrimental effects to protein content or profile. Results indicate that autolysin produced *in situ* with mating *C. reinhardtii* strains is an appropriate cell wall permeation and disruption agent to mediate primary enzymatic treatment. Additionally, enzymatic pretreatment of cells alone or combined with conventional mechanical and/or chemical disruption mechanisms resulted in improved extractability of proteins and lipids and if integrated at an industrial processing scale, may provide energy and cost savings. Application of autolysin followed by an extended higher temperature incubation resulted in up to 54% of total protein release into an aqueous phase with 43% of total lipids simultaneously released. TEM imaging confirmed attachment of the remaining lipids (in lipid droplets) to cellular or organelle remnants after primary enzymatic treatment. With secondary enzymatic treatment of residual biomass with trypsin which visually relaxed thylakoid membrane stacking and possibly cleaved lipid droplet surface proteins or other chloroplast proteins, an additional 30% of total lipids was released. Together, PET and SET of *C. reinhardtii* biomass has the potential to result in the distinct product streams necessary for a biorefinery but additional process development is necessary.

Further research efforts should focus on addressing the following: 1) Standardized application of lytic activity during PET; 2) Recovery of solubilized proteins from the aqueous fraction after PET; and 3) Recovery (extraction) of lipids without use of organic solvents

At present autolysin is applied in a volumetric ratio to of 0.5 mL solution per mL of culture. This dosage doesn't consider the amount of total protein in the lytic solution or amount of that protein that demonstrates lytic activity. Thus, an opportunity for autolysin solution purification and activity characterization exists which would significantly contribute the robustness and reproducibility of the primary enzymatic treatment step.

Soluble proteins in the aqueous phase after PET would likely have applications in the food and feed industries in which case an enriched solid fraction could be more readily integrated than a dilute protein suspension. Protein recovery or enrichment procedures such as isoelectric precipitation or membrane filtration resulting in protein concentrate or isolate should be explored.

Lipid recoveries and extractions from aqueous streams after PET and SET still require organic solvents. The presence of both lipids and proteins in these aqueous streams has likely led to emulsion formation which would require destabilization before lipid droplets could coalesce and be separated from the surrounding aqueous environment. Applications of high temperatures, acid pH values, and slow mixing should be evaluated for impacts on lipid droplet coalescence.

Chapter 3 - Enhancing protein extraction from *Chlamydomonas reinhardtii* as part of a biological-based biorefinery model[3]

Abstract

Microalgae are increasingly recognized as a sustainable source of protein to help meet the global food demand and can additionally accumulate high levels of other valuable biomolecules. To improve the economics of microalgae processing, researchers have pursued a biorefinery approach, but limitations such as biomass drying and complex lysates still limit biomolecule valorization. Our prior work included development of a biological-based biorefinery approach for protein and lipid extraction. During process development, ~40% of proteins were extracted during cell disruption, and it was desired to maximize protein extraction prior to lipid extraction. To enhance protein release after cell disruption, a design of experiments approach was used to evaluate the effects of extraction variables, pH, time, and temperature. Optimized conditions of pH 12, 4 h, and 45°C resulted in an additional ~33% extracted protein. The enhanced protein extraction step was integrated into the overall bioprocess and protein recovery was further explored.

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[3] This chapter has been submitted for publication as a peer-reviewed research paper to Bioresource Technology.

Introduction

The estimated population growth to over 9 billion people by 2050 coupled with limitations in food supply requires increasing food production and adoption of alternative sources of protein to ensure food security (Alexandratos and Bruinsma, 2012). Microalgae proteins have the potential to address the critical need of a “sustainable protein” source to meet the ever increasing global food demand (Batista et al., 2013; Draaisma et al., 2013). In comparison to conventional protein sources (plant-based/animal), microalgae proteins contain all essential amino acids which humans must obtain from diet but do not contain high levels of saturated fats and cholesterol as compared to animal proteins (Bleakley and Hayes, 2017). As a source of proteins, microalgae are additionally advantageous in that they reproduce quickly, have higher productivities that terrestrial crops, and can be cultivated in a variety of water sources (Mata et al., 2010). Thus, arable lands and freshwater resources would not be additionally burdened to increase dietary protein available for the growing population. To date, proteins extracted from *Spirulina* and *Chlorella sp.* have been used as food supplements for human and animal (poultry, aquaculture) consumption (Becker, 2007). Additionally, protein hydrolysates have been included as food/drink additives (Kose and Oncel, 2015). Although microalgae have been successfully used as protein supplements in feed and food products, a critical protein shortage is still expected in the near future. Thus, alternative and sustainable sources of protein and associated recovery processes are essential.

While microalgae are a promising source of food proteins, they are additionally recognized source of bioproducts for a variety of industries such as energy, feed, nutraceutical, cosmetic, and biopharmaceutical. Enhanced expression of biomolecules (proteins, lipids, carbohydrates, etc.) can occur under environmental stressors or via genetic manipulation, but the current

processing associated with biomass harvesting, biomolecule extraction/fractionation/recovery, and conversion/formulation into a useable bioproduct is cost-prohibitive and limits microalgae product commercialization (Dixon and Wilken, 2018; Günerken et al., 2015). Conventional processing techniques using high temperatures, high pressures, and toxic chemicals are proven in industrial downstream processing but do not provide the mild conditions that allow for extraction and recovery of multiple functional biomolecules. As a means of reducing downstream processing costs and valorizing multiple biomolecules, alternative processing procedures and biorefinery models are increasingly employed (Ansari et al., 2017; Lee et al., 2017; Zhu, 2015). Prior applications of a biorefinery approach to microalgae included valorization of one to two products with a resultant residue. Biomass either previously dried or in the form of a wet paste was subjected to a mechanical (bead milling), chemical (acid hydrolysis), or thermal (microwave-assisted) disruption cell disruption method before subsequent biomolecule extraction (Francavilla et al., 2015; Karemore and Sen, 2016; Laurens et al., 2015; Nobre et al., 2013). Several studies aimed at valorization of multiple products focused efforts on lipid extraction with organic solvents followed by secondary extraction of carotenoids (Nobre et al., 2013) and/or pyrolysis and fermentation of lipid-extracted residue into bio-oils or biogases (Francavilla et al., 2015). While lipids persist as the main biomolecule target of microalgae biorefineries, researchers have demonstrated fermentable sugar and protein extraction prior to lipid extraction (Dong et al., 2016; Laurens et al., 2015; Muñoz et al., 2015). Muñoz et al., 2015 applied alkaline conditions to microalgae biomass for protein extraction followed by lipid extraction using a Soxhlet extraction system. In Parallel Algal Processing (PAP), pretreatment of biomass with acid resulted in fermentable sugars with protein and lipids in algae solids (Laurens et al., 2015). The lipids were subsequently extracted with hexane resulting in a protein-

rich residue with trapped polysaccharides. As an improvement to PAP, Combined Algal Processing (CAP) directly fermented whole acid-pretreated slurry resulting in ethanol and a residue from which lipids were later extracted by hexane (Dong et al., 2016). Development of microalgae biorefineries with iterative design as in the case of PAP and CAP which led a reduction in microalgae biofuel costs demonstrates the impact of valorizing multiple biomass components and justifies further exploration of improved processing strategies for microalgae bioproducts.

In the aforementioned microalgae biorefineries, biomass was commonly dried prior to biomolecule extraction, disrupted using harsh procedures which resulted in a crude lysate containing a complex mixture of all biomolecules, or focused predominantly on lipids as the target molecule with valorization of all other biomass components (carbohydrates, proteins, carotenoids) via fermentation or pyrolysis of lipid-extracted residues. These limitations indicate that further development of microalgae biorefineries is necessary and present opportunities for process design.

To address prior limitations, a biorefinery method was developed for *Chlamydomonas reinhardtii*. With this particular species, processes developed using wild-type strains could ultimately be translated to genetically modified strains expressing recombinant proteins, a potentially high-value microalgae product. As an alternative microalgae biorefinery strategy, our research group developed an enzyme-mediated disruption, extraction, and fractionation processing method for the recovery of multiple *C. reinhardtii* biomolecules into separate process streams (Sierra et al., 2017). This process eliminated prior biomass drying, used an algae produced-autolytic enzyme and serine protease for cell and organelle disruption, and minimized use of extreme (high pH/temperature) processing conditions, energy intensive machinery

(ultrasonication (UTS)/ high shear homogenization), or application of toxic organic solvents. The biological-based process included biomass production and biomolecule accumulation, solid-liquid separation, primary enzymatic treatment (PET) of cells, secondary enzymatic treatment of organelle membranes, and product separation and fractionation. Autolysin, a metalloprotease with specificity for the *C. reinhardtii* cell wall was used to successfully disrupt ≥85% of the microalgae cells with an associated release (extraction) of ~40-50% of the total protein (Sierra et al., 2017). Following cell disruption and partial protein extraction, cellular and organelle remnants were treated with trypsin, which resulted in relaxation of thylakoid membranes in chloroplast remnants and the release of 33% of previously entrapped lipids into an aqueous phase.

After demonstrating an integrative biorefinery with enzyme-mediated cell disruption and protein and lipid release, we aimed to improve overall protein extraction from intracellular compartments prior to enzyme-mediated lipid extraction by exploiting extrinsic factors such as pH, incubation time, and incubation temperature known to increase protein solubility (Kramer et al., 2012). This proposed enhanced protein extraction could serve to separate the majority of total cellular protein from lipid-rich residues, thus resulting in distinct streams enriched in one target biomolecule. Expected outcomes/benefits include: 1) reduced purification burden, 2) increased valorization of individual biomolecules, 3) use of milder protein extraction conditions, and 4) less energy input.

The objectives of this study were to: 1) evaluate pH, time, and temperature impact on protein extraction following PET using design of experiments (DoE)/response surface methodology (RSM); 2) develop a statistical model to determine the effect of these factors; and 3) determine optimal extraction conditions and validate the model. The optimal conditions were

then integrated into the overall bioprocessing procedures and protein recovery with isoelectric precipitation from the resultant aqueous streams was explored.

Materials and Methods

Laboratory equipment and reagents

All chemicals used were reagent grade and were purchased from Sigma Aldrich or VWR Scientific.

Microalgae source

Production strain

C. reinhardtii (CC-409 mt+) was obtained from the *Chlamydomonas* Resource Center, University of Minnesota. The strain was maintained on solid TRIS-acetate phosphate (TAP) plates for use as inoculum for liquid cultures.

Mating strains

CC-620 mt+ and CC-621 mt- high efficiency mating strains were obtained from the *Chlamydomonas* Resource Center, University of Minnesota.

Cultivation procedures

TAP growth media was autoclaved at 20 psi and 121°C for 40 min. *C. reinhardtii* cultures were inoculated on solid TAP agar plates and grown for 5-7 days. Accumulated biomass was then used to inoculate 250 mL of liquid TAP media in a 500 mL Erlenmeyer flask and grown at 23°C under 12h/12h light/dark cycles (27 µmol m^{-2} s^{-1}) with continuous shaking at 120 rpm. Cell growth and size was monitored by cell counts with a BrightLine hemocytometer (VWR Scientific). After cells reached mid-exponential phase (5×10^6 -7×10^6 cells/mL), cultures were transferred into 1.75 L of fresh TAP media and grown again until mid-exponential phase. To induce oil accumulation via nitrogen deprivation, cultures were centrifuged at 8000 x g

for 6 min at room temperature, decanted, and the pellets washed in TAP medium without nitrogen (TAP-N). The washed pellets were re-suspended in TAP-N medium and after 48 h of growth, concentrated via centrifugation and frozen at -80°C.

Autolysin production

Autolysin was prepared using a modified high-volume production protocol based on that proposed by Jaenicke et al., (1987). CC-620 mt+ and CC-621 mt- high efficiency mating strains were grown on solid TAP media agar plates for 3-7 days to generate a biofilm. Beginning on day three of biofilm generation mating tests were performed to confirm mating efficiency. 500 µL samples of each strain was mixed and allowed to mate for 15 min and observed using light microscopy. When approximately 95% of the cells were mating, efficiency was confirmed. The biofilms of each strain were independently solubilized and suspended in TAP-N with volume adjustment to reach a final cell concentration of 1×10^7 cells/mL. The liquid cultures were shaken for 4 h under high intensity LED lights for gamete induction and flagella formation. The two strains were then mixed in a clear glass dish without agitation under high light for approximately 30 min. The mating cells were then centrifuged and the supernatant containing autolysin was depth filtered using a 0.45µm PES membrane, concentrated using tangential flow filtration with a 10 kDa mPES hollow-fiber membrane, and frozen at -80°C until further use.

Biological-based biorefinery approach

The biological-based processing approach used for cell disruption and enhanced protein extraction from *C. reinhardtii* biomass for optimization studies is shown in Figure 3.1a.

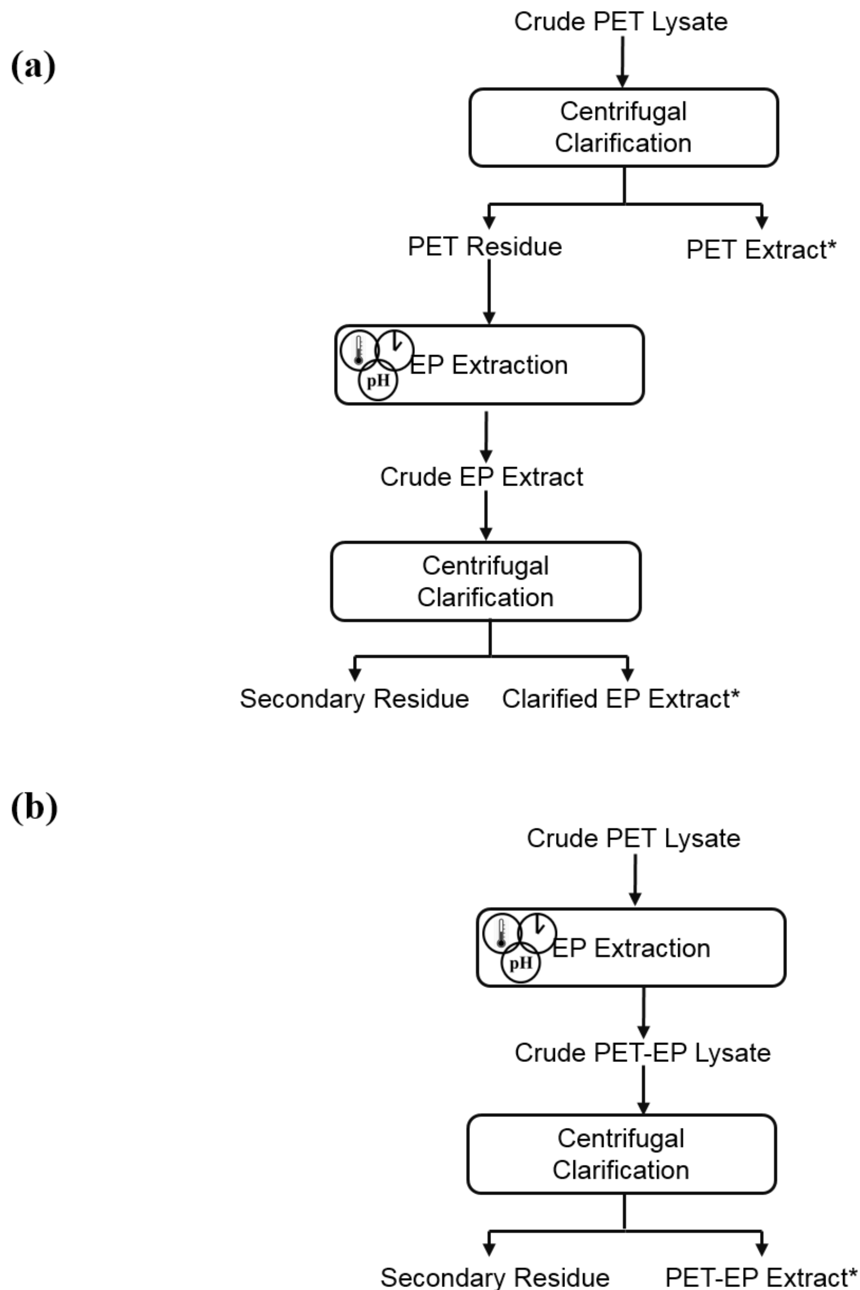


Figure 3.1 Process flow diagram of cell disruption and enhanced protein extraction procedures on (a) PET residue or (b) crude PET lysate. PET, primary enzymatic treatment, of *C. reinhardtii* biomass; EP, enhanced protein extraction, with combination of pH, time, and temperature; PET-EP, primary enzymatic treatment with EP extraction of unclarified PET lysate. Asterisks (*) indicate extracts used for protein recovery by isoelectric precipitation.

Dry weight determination

The dry weight of *C. reinhardtii* biomass was determined following culture harvesting. Whatman® glass microfiber filters, Grade GF/F were ashed in a muffler furnace at 400°C for 1 h and stored in a desiccator prior to use. All aluminum trays used during dry weight determination were similarly ashed and stored. For each replicate performed the mass of the aluminum tray (W1), filter (W2), and combined tray and filter (W3) were recorded. To begin, the pre-weighed filter was carefully added to the vacuum filtration system. The filter was dampened with 10 mL of 0.5 M Ammonium formate prior to application of the algal culture. Then, 20-30 mL of algal culture was filtered at a vacuum pressure of 35-55 mmHg. Finally, any salts were washed from the filtered biomass by rinsing the filter with 10-15 mL of 0.5 M Ammonium formate. The filter was carefully removed from the filtration system, placed in the pre-weighed tray, covered with foil, and dried in an air oven at 105°C for 1 h. After drying, the sample was cooled in the dessicator for approximately 20 min and a final mass for the combined tray, filter, and dry algae (W4) was determined. The dry weight of the biomass was determined as the difference between W4 and W3.

Primary enzymatic treatment (PET)

Previously harvested biomass was defrosted and separated from liquid media via centrifugation at 8000 x g for 6 min. Autolysin solution was added to the pelleted biomass at a ratio of 50 mg protein/g dry biomass. The enzyme-biomass solution was mixed end over end or with an orbital shaker at 200 rpm for 4 h initially at 23°C and then at 37°C until cell disruption was ≥85%. Once the desired cell disruption was achieved, the PET crude lysate was clarified via centrifugation (8000 x g for 6 min) to separate the PET extract (supernatant) containing autolysin and any native proteins released during the cell disruption step from the PET residue (solid

pellet) containing cellular and organelle remnants with entrapped biomolecules (proteins, lipids, starch, etc.). The PET extract was collected and protein concentration was determined. For each replicate, a tandem sample was terminated after the PET step. This sample was used for mass balance calculations and determining the amount of protein entrapped in the PET residue after lysate clarification.

Enhanced protein (EP) extraction after PET of microalgae

Native proteins from the PET residue were initially extracted under 8 different treatment combinations of the independent variables pH (7-12), time (1-4 h), and temperature (23-45°C) as shown in Table 3.1. Because a wild type microalgae strain was used for this study and any native proteins would likely be incorporated into a food or feed product, the range of variables were chosen to promote solubility while maintaining any potential functional properties such as emulsification, foaming, or gelation (Benelhadj et al., 2016; Jiang et al., 2009; Ursu et al., 2014). Neutral pH water was added to the PET residue at a 1:10 biomass-to-water ratio (w/v) and pH adjusted by addition of 0.5 M sodium hydroxide (NaOH). The solution was mixed either end over end or with an orbital shaker at 200 rpm at the treatment conditions. Throughout the incubation period, pH was monitored and adjusted as necessary. After the enhanced protein (EP) extraction step was completed, the soluble protein in the EP extract were separated from insoluble material (secondary residue) by centrifugation at 8000 x g for 6 min. The EP extract was collected for subsequent protein content quantification.

Table 3.1 Variables and levels considered for protein extracted (%)

			Coded levels		
Factors	**Variables**	**Unit**	**Low (-1)**	**Mid (0)**	**High (1)**
X_1	pH	---	7	9.5	12
X_2	Time	h	1	2.5	4
X_3	Temperature	°C	23	34	45

Protein content quantification

Protein content of the various extract and residue fractions after PET and EP steps were determined using the BCA microplate assay (bicinchoninic acid assay) with bovine serum albumin as a standard. To minimize chlorophyll interference with the BCA assay and ensure samples fell within the working range of the standard curves (25-1500 μg/mL), samples were diluted. Resulting concentrations were used to calculate protein extracted (%) via EP treatment combinations with equation 1.

$$\text{Protein extracted (\%)} = \frac{\text{protein in EP extract (μg)}}{\text{protein in PET residue (μg)}} \times 100\% \qquad (1)$$

Experimental Design

Two level full factorial design

Three independent variables (pH, time, and temperature) were selected to model the protein extracted (%) from PET residue. The range and levels in coded and uncoded terms can be seen in Table 3.1. Minitab 17 (Minitab Inc., State College, PA, USA) statistical software was used to design a two level, full factorial consisting of 10 randomized runs (8 factorial with 2 center) to assess error within the model and detect the presence of curvature as seen Table 3.2. Each of the treatment combinations were performed in triplicate and the average protein extracted (%) used as the response. Analysis of variance (ANOVA) was performed on the responses to identify model parameters including coefficient of determination (R^2), adjusted coefficient of determination (R^2 adj), predictive coefficient of determination (R^2 pred), lack-of-fit, and significant difference.

Design augmentation to face centered central composite design

Upon detection of curvature in the statistical analysis of the two level full factorial design, Minitab 17 was used to augment the design with 6 axial runs and 2 additional center runs

to essentially create a face centered central composite design (FCCD) (Table 3.3) with α=1. The FCCD allowed for the protein extracted (%) to be modeled by a second order polynomial function. The generalized second order polynomial function is given in equation 2.

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i X_i+\sum_{i=1}^{k}\beta_{ii}X_{ii}^2+\sum_{j=1}^{k}\beta_{ij}X_iX_j \qquad (2)$$

Where Y is the response; X_i and X_j represent variables (i and j range from 1 to k where k is the number of independent variables); β_0 is the model intercept coefficient; β_i, β_{jj}, β_{ij} represent regression coefficients for each linear, quadratic and interaction term, respectively. For this study k=3 as variables studied include pH, time, and temperature. Analysis of variance (ANOVA) was performed on the responses to identify model parameters including R^2, R^2 (adj), R^2 (pred), lack-of-fit and significant difference.

Optimization of protein extracted (%) and model validation

The Minitab Response Optimizer tool was used to calculate the optimal conditions for protein extracted (%) using the previously determined model. As the goal of this work was to model within a design space that was scalable and industrially applicable for microalgae processing, the response optimizer tool was also used to determine conditions that maximized protein extracted (%) under processing constraints such as economics, safety, or sustainability. The processing constraints included reducing the pH to minimize the amount of NaOH used, reducing temperature to minimize likelihood of protein denaturation or proteolysis, minimizing heating between unit operations, and minimizing processing time of this specific extraction step. The optimized conditions for protein extracted (%) and maximized conditions for industrially attractive processing constraints were used to assess the validity of the model.

Integration of enhanced protein extraction step into overall bioprocessing procedures

The optimized conditions for protein extracted (%) were evaluated on crude PET lysate following enzymatic cell disruption as a means of integrating the enhanced protein extraction step directly in the overall bioprocessing procedures and minimizing the number of solid-liquid separation steps employed during protein extraction (Fig. 3.1b). After ≥85% of cells were disrupted via enzymatic hydrolysis, crude PET lysate was pH adjusted as necessary via addition of 0.5 M NaOH and incubated according to the previously determined optimized incubation time and temperature. Following the enhanced protein extraction, this lysate was separated via centrifugation at 8000 x g for 6 min into PET-EP extract and secondary residue. Thus, total protein distribution between the PET-EP extract and secondary residue was compared to the distribution obtained in the PET extract, EP extract, and secondary residue to determine utility of optimized extraction conditions on PET crude lysate as opposed to PET residue.

Protein recovery via isoelectric precipitation

Soluble proteins in the PET and PET-EP extracts were recovered via isoelectic precipitation (IEP). Proteins extracted via UTS into comparable background buffers (ie. pH and conductivity) were additionally recovered with IEP to serve as a control reference. All extract precipitations were conducted in triplicate. HCl (0.25 M) was added to the extracts until the desired pH (3.0-5.0) was reached. The samples were then centrifuged at 12,500 x g for 15 min. The resultant supernatants were collected and the precipitated protein (solid pellet) was resolubilized in a solution of equal volume and pH to the initial extract. Protein content quantification was performed as previously described.

Protein profile analysis

Equal volumes of various total protein, UTS, PET, EP, PET-EP extracts and secondary residues were run under reducing conditions on NuPAGE® Bis-Tris protein gels followed by staining with coomassie blue. SeeBlue™ Plus2 Pre-stained Protein Standard was included to visualize protein molecular weight ranges from 3 to 198 kDa.

Statistical analysis

One way analysis of variance (ANOVA) was conducted for statistical analysis of the experimental data using Minitab 17 software. To compare significant differences between treatments, a Tukey adjustment was made for a family wise error rate of 0.05 (α_{FER}=0.05).

Results and Discussion

Statistical analysis and model fitting

The experimental design for protein extracted (%) generated 18 experimental runs (8 factorial, 4 center, and 6 axial), which resulted in extraction of 11.8±1.8 to 58.9±2.3% of protein from PET residue (Table 3.2 and 3.3). The adequacy and fit of the model was tested with multiple regression analysis and an ANOVA table with degrees of freedom (df), adjusted sum of squares (Adj SS), adjusted mean squares (Adj MS), F-values, and P-values was generated (Table 3.4). The full quadratic model was significant (P=0.000) and the R^2=0.9883 and R^2 (adj)=0.9755 were satisfactory. Additionally, the predictive power of the model for new observations was R^2(pred)= 0.8741. The validity of the model was confirmed with a lack-of-fit p-value >0.05, which was not significant and provided no evidence that the model doesn't represent the data at a 95% confidence level. The full quadratic model included linear effects, quadratic effects, and

two-way interactions between the factors of interest and can be used with factors expressed in coded units. The resultant value has percentage units.

The full quadratic model is given in coded units in equation 3:

$$Y(\%)=17.763+13.503X_1+3.401X_2+3.843X_3+11.26X_1X_1+1.28X_2X_2-0.80X_3X_3 \quad (3)$$

$$+2.731X_1X_2+3.296X_1X_3+1.391X_2X_3$$

where Y is the protein extracted (%); X_1 is pH; X_2 is time; X_3 is temperature.

Table 3.2 Experimental matrix for 2 level full factorial design with center runs and experimental and predicted responses

Standard Order	Run Order	X_1 (pH)	X_2 (Time)	X_3 (Temperature)	Y= Protein Extracted (%)	
					Experimental	Predicted
1	6	-1	-1	-1	15.5	14.7
2	7	1	-1	-1	32.0	29.6
3	4	-1	1	-1	16.8	16.0
4	8	1	1	-1	39.7	41.9
5	2	-1	-1	1	16.0	15.8
6	5	1	-1	1	41.1	43.9
7	3	-1	1	1	18.3	17.1
8	9	1	1	1	58.9	56.2
9	1	0	0	0	19.4	17.9
10	10	0	0	0	18.5	17.9

Table 3.3 Augmented FCCD with axial runs and additional center runs and associated experimental and predicted responses

Standard Order	Run Order	X_1 (pH)	X_2 (Time)	X_3 (Temperature)	Y= Protein Extracted (%)	
					Experimental	Predicted
11	12	-1	0	0	13.0	15.9
12	15	1	0	0	42.7	42.9
13	13	0	-1	0	15.5	14.5
14	16	0	1	0	20.4	22.0
15	17	0	0	-1	11.8	14.0
16	18	0	0	1	19.9	21.7
17	14	0	0	0	19.9	17.9
18	11	0	0	0	17.8	17.9

Based on the ANOVA table for the full quadratic model, terms with p-values less than 0.05 were removed one-by-one as they were not significant at the 5% level of significance.

Removed terms included the quadratic effects of time, quadratic effects of temperature, and the interactive effects of time and temperature. Once terms were removed, they were no longer considered as part of the regression analysis. The ANOVA table of the resulting reduced quadratic model is shown in Table 3.4.

The reduced quadratic model is given in coded units in equation 4:

$$Y(\%)=17.883 + 13.503\ X_1 + 3.401\ X_2 + 3.843\ X_3 + 11.52\ X_1{*}X_1 + 2.731\ X_1{*}X_2 + 3.296\ X_1{*}X_3 \quad (4)$$

where Y is the protein extracted (%); X_1 is pH; X_2 is time; X_3 is temperature.

Table 3.4 Analysis of variance (ANOVA), test of significance for protein extracted (%) for reduced quadratic model

Source	df	Adj SS	Adj MS	F-Value	P-Value	
Model	6	2823.14	470.52	96.04	0.000	Significant
Linear	3	2086.66	695.55	141.97	0.000	
pH	1	1823.31	1823.31	372.16	0.000	
Time	1	115.67	115.67	23.61	0.001	
Temperature	1	147.69	147.69	30.14	0.000	
Quadratic	1	589.88	589.88	120.40	0.000	
pH*pH	1	589.88	589.88	120.40	0.000	
2-Way Interaction	2	146.60	73.30	14.96	0.001	
pH*time	1	59.68	59.68	12.18	0.005	
pH*temperature	1	86.92	86.92	17.74	0.001	
Error	11	53.89	4.20			
Lack-of-fit	8	51.22	6.19	7.18	0.066	Not significant
Pure Error	3	2.67	0.89			
Total	17	2877.03				

The reduced model included all three linear effects, one quadratic effect, and two, two-way interactions. As all coefficients for the model were positive, an increase in all variables contributes to an increase in protein extracted (%) from PET residue. Again, the model was found to be significant (P=0.000) and the model validity was confirmed with a lack-of-fit p-value>0.05. As all included terms were statistically significant, the reduced quadratic model was used for response estimation (Table 3.2 and 3.3) and for optimization of protein extracted (%).

While the R^2 and R^2 (adj) were reduced slightly in the reduced model compared to the full model, the predictive power of the model increased from 0.8741 to 0.9053.

Effect of independent variables

The resultant protein extracted (%) from PET residue varied widely from 11.8±1.8% to 58.9±2.3% with different levels of the independent variables of pH, time, and temperature. To illustrate the main and interactive effects of these variables, contour plots and three dimensional (3D) response surface plots were generated by maintaining one variable at its central level and varying the other two variables within their respective ranges (Figure 3.2).

Effect of pH

pH is a factor known to improve protein solubility, especially at extreme acidic or alkaline values (Cavonius et al., 2015). The effect of pH on protein extracted (%) was studied and the results are shown in Figure 3.2. Increasing the pH of the solution was associated with increased protein solubility and protein extracted (%) with a markedly sensitive response to higher pH values as evidenced by the steep curvature of the response surface plots (Figures 3.2a and b). Treatment combinations involving pH 7 resulted in 15.5-18.3% of protein extracted (depending on levels of time/temperature) while combinations involving pH 12 resulted in significantly higher protein extraction ranging from 32.0-58.9%. With the addition of NaOH at alkaline pH values, the distribution of net charges on disrupted biomass and any exposed proteins is affected and leads to increased solubility. As the PET step prior to enhanced protein extraction results in partially disrupted organelles, peripheral membrane proteins are exposed to the surrounding aqueous environment and are likely solubilized by the increased Ph, contributing to enhanced protein extraction (Pieper et al., 2009). Additionally, any proteins still attached to

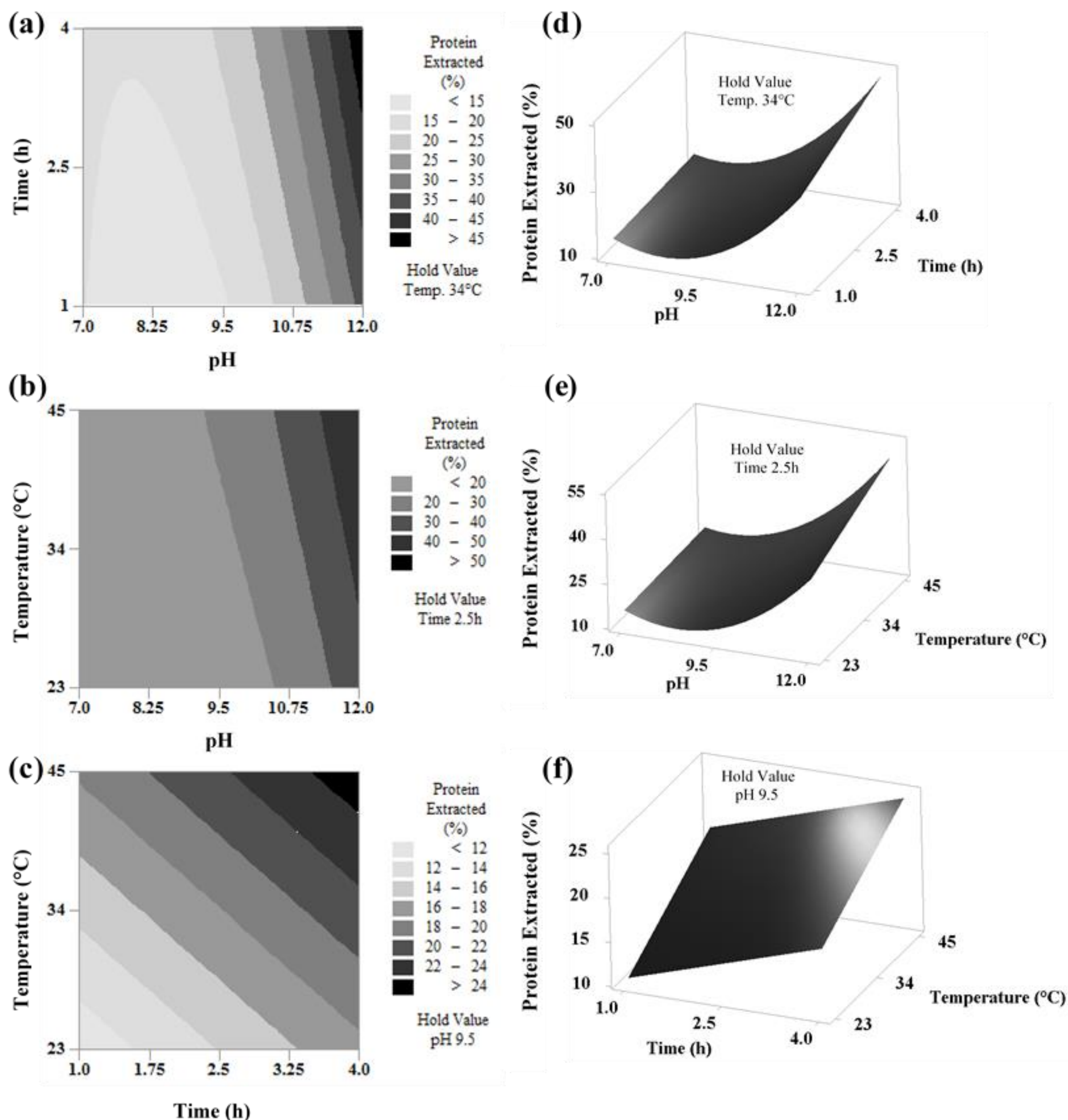


Figure 3.2 (a-c): Contour plots for protein extracted (%) as a function of a) time and pH, b) temperature and pH, and c) temperature and time. (d-f) 3D Response surface plots for protein solubilized (%) as a function of d) time and pH, e) temperature and pH, and f) temperature and time.

cell remnants via electrostatic or other noncovalent bonds following PET are likely solubilized by the increase in pH (Stillwell, 2016). The interactive effects of pH and time and pH and temperature are markedly pronounced at the highest levels of pH (12) in conjunction with the mid-to-high levels of time (2.5-4 h) and temperature (34°C-45°C) when the contour plot regions are uneven in size. As pH increased from 9.5 to 12, protein solubility increased by upward of 30% with prolonged incubation times or increasing temperatures. This variance in the contours plots indicates significance of the two-way interactions and confirms the ANOVA analysis for inclusion of the interactive effects in the regression model.

Effect of time

The effect of time on protein solubility and subsequent protein extracted (%) was studied and the results are shown in Figure 3.2. Like pH, increasing the incubation time of the enhanced extraction step was associated with an increase in protein extracted (%). The ascending slope is evident over the time range from 1 h to 4 h in Figure 3.2f and demonstrates the sensitivity of the response to this particular variable. An increase in time allows for protein diffusion from cellular or organelle remnants of the PET residue into the aqueous phase and also allow soluble proteins to reach an equilibrium within the aqueous environment (Kramer et al., 2012). An analysis of the impact of pH and time revealed that at lower pH values (pH 7.0-9.5), increased time was not associated with a statistically significant increase in protein extraction (1.3-4.9%, depending on temperature) while at higher pH (pH 12) values, increased time significantly enhanced protein extraction between 7.7-17.8% (depending on temperature). Conversely, an analysis of the interactive effects of time with temperature (Figure 3.2c) demonstrated that the interaction of the two variables is not significant as evidenced by a contour plot with parallel straight lines and evenly spaced contours. Thus, the contour plot further confirms the non-significant p-value

observed in the full quadratic model with this two-way interaction (time and temperature) and justifies its removal from the reduced regression model for protein extracted (%).

Effect of temperature

The effect of temperature on protein solubility and protein extracted (%) was studied and the results are shown in Figure 3.2. As with the other two independent variables, increasing temperature was associated with increasing protein extracted (%). At pH 7.0, the impact of temperature was minimal while at pH 12, 9.1-19.2% more protein was extracted with increasing temperature (with time held constant).This is consistent with reported literature that protein solubility and extractability generally increases with temperatures up to 50°C (Guimarães Pelegrine and de Moraes Santos Gomes, 2008). It has been theorized that temperature likely affects the crystal structure of proteins and/or the dielectric constant of the aqueous solution (Arakawa and Timasheff, 1985). The positive effect of temperature (increased protein yield of 34 % (DW) has also been reported as part of protein extraction procedures from other green microalgae species like *Dunaliella salina*, *Chlorella ovalis*, and *Nannochloropsis oculata* albeit the hot alkaline extraction was performed on previously lyophilized and/or mechanically disrupted biomass (Slocombe et al., 2013). The cell pretreatment strategy of aqueous enzymatic processing used in this study that dispenses with prior biomass drying or mechanical disruption is thus capable of generating comparably disrupted biomass for which solubilizing factors such as incubation temperature can positively affect protein extraction.

Optimization of protein solubilized (%) and model validation

By employing the Minitab Response Optimizer, the optimal conditions to maximize protein extracted (%) were calculated. According to the response optimizer tool, the highest level of each factor, pH 12.0, 4 h incubation, and 45°C, would maximize protein extracted at a

predicted value of 56.2%. Further evaluation was not pursued as higher pH or temperature may impact protein quality and functionality and longer time was previously demonstrated to have no significant effect on extractability (data not shown). The optimizer tool was additionally used to determine conditions which would maximize protein extracted under constraints for one of more variables. A minimum of three independent replicates were run at each model validation point (VP) to compare the experimentally obtained value protein extracted (%) to model-predicted values. VP1 corresponds to conditions that optimize protein extracted (%) from PET residue. VP2 is an industrially relevant set of conditions whereby a processor could increase pH to an alkaline value while minimizing incubation time of the enhanced protein extraction step without having to change incubation temperatures from the prior PET step. This would serve to minimize overall processing time and energy inputs. VP3 is another industrially relevant set of conditions where the pH is held at 11, a value on the upper end of extraction buffer pH used in techno-economic studies involving proteins and/lipids from microalgae (Sari et al., 2016, 2015). VP4 is a set of conditions that fall within with the active pH and temperature ranges of our secondary enzymatic treatment. The predicted and experimental protein extracted (%) for each validation point is shown in Table 3.5. For VP1, VP2, and VP3 the model prediction was slightly higher than the experimentally obtained protein extracted (%) while the model prediction for VP4 was nearly identically to experimentally obtained values. The variation between model predicted and experimentally obtained values for VP1-VP4 (4.6%, 7.2%, 13.5%, and 0.7% respectively) was acceptable and demonstrated the predictive capabilities of the model.

Table 3.5 Validation points for protein extracted (%) at optimized and industrially relevant conditions

ID	pH	Time(h)	Temperature (°C)	Predicted Protein Extracted (%)	Experimental Protein Extracted (%) (Mean±SD)
VP1	12.0	4	45	56.2	53.6 ± 8.5
VP2	12.0	1	37	38.7	36.0 ± 2.6
VP3	11.0	4	45	41.0	35.4 ± 2.6
VP4	8.0	2	40	14.4	14.5 ± 2.9

Integration of the EP extraction step into biorefinery process

As the optimization study previously described resulted in conditions that maximized protein extracted from isolated PET residue only, it was desired to translate and integrate those extraction procedures into our biorefinery. Thus, a solid-liquid separation step could be eliminated between PET and EP steps, potentially minimizing overall processing time and costs. To assess that applicability of the optimal conditions for enhanced protein extraction as part of a step integrated into the overall processing procedures, the PET crude lysate following enzymatic cell disruption was pH adjusted to 12 with NaOH and the lysate incubated for 4 h at 45°C. At the conclusion of the 4 h, the lysate was clarified resulting in PET-EP extract and secondary residue. A comparison of the percentage of total protein content distributed in the PET-EP extract and its secondary residue to that obtained when extraction conditions were applied to isolated PET residue can be seen in Figure 3.3. In Case 1 where enhanced extraction occurred on isolated PET residue, an average of 40±8.2% of total protein was in the PET extract. On average, optimized enhanced extraction conditions resulted in an additional 33±7.1% of total protein released into EP extract. The remaining 27.7±4.8% of total protein was not extracted and thus remained in the secondary residue. In Case 2 where the PET crude lysate was directly subjected to the optimized enhanced extraction conditions, an average of 65.3±6.9% of total protein was present in the PET-EP extract and approximately 34.7±4.5% of total protein was not extracted and thus remained in the secondary residue. While the total protein extracted in Case 2 was marginally lower (65% as

compared to 73%) than that observed when protein was extracted in two distinct steps as in Case 1, the reduction in time and energy inputs associated with eliminating a solid-liquid separation step could prove more cost effective to the process than the small increase in total protein extraction. Thus, subsequent development of the biological-based processing procedures included direct subjection of PET crude lysate to EP extraction procedures.

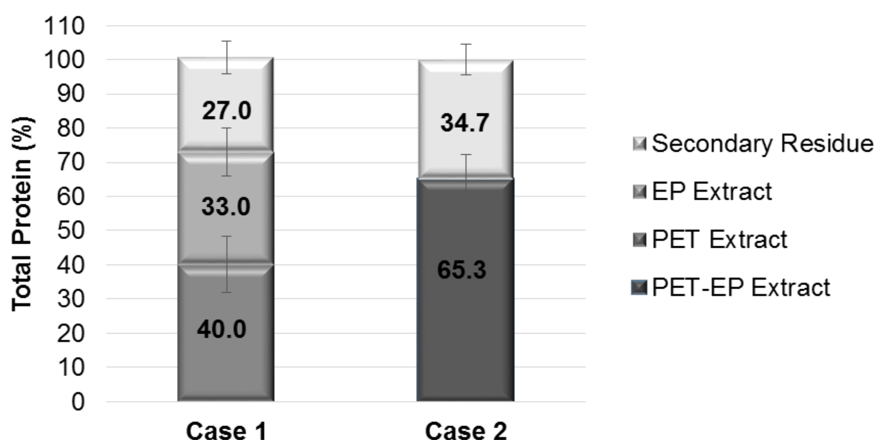


Figure 3.3 Distribution of total protein in extracts and residues resulting from separated PET and EP extraction procedures (Case 1) and integrated PET-EP extraction procedures (Case 2). Error bars represent standard deviation for n>3.

Protein recovery via isoelectric precipitation

As substantial amounts of total protein could be extracted into aqueous phases during PET and PET-EP procedures, recovery of those proteins via IEP was further explored. Known factors affecting protein recovery include solution pH, conductivity, as well as protein concentration, size, and structure. Our biological-based process exposes extracted proteins to various enzymes and operating conditions which could potentially affect protein size and structure and thus all recovery procedures were compared against a control reference protein extract obtained after ultrasonication of *C. reinhardtii* biomass. The various pretreatment strategies resulted in UTS extracts with concentrations ranging from 3.0-5.0 mg/mL and PET

and PET-EP extracts with concentrations of 0.5-1.5 mg/mL. These extract concentrations are similar to those used as part of protein precipitation studies previously reported. Precipitation pH values ranging from pH 3.0-5.0 were tested for extracts resulting from UTS, PET, and PET-EP pretreatment of biomass and there was no significant effect on protein precipitation for the individual extracts between pH values. While precipitation pH had no significant effect on precipitation efficiency on the individual extracts tested, a comparison of precipitation efficiencies between PET, PET-EP, and US protein extracts (Figure 3.4a) revealed that factors other than precipitation pH were likely affecting protein recovery efforts. On average, 95.6±0.9% of proteins in the UTS extract precipitated with 94.3±14.0% of those proteins recovered. This was significantly higher than the 48.6±1.7% of proteins precipitated from PET extract (with 73.0±11.5% of those recovered) and 32.1±5.7% of proteins precipitated from PET-EP extract (with 66.4±24.5% of those recovered). To determine if extract concentration or buffer composition (i.e. conductivity) was affecting precipitation efficiency, extracts resulting from UTS or PET of biomass adjusted to have comparable initial concentrations and/or buffer compositions were compared (Table 3.6).

Table 3.6 Comparison of protein precipitation from UTS and PET extracts with comparable protein concentrations and/or buffer composition after pH adjustment to 4.5

Pretreatment	Extraction Buffer	Extract Conductivity (mS/cm)	Extract Concentration (μg/mL) (Mean±SD)	Protein Precipitated (%) (Mean±SD)
UTS	Neutral pH ddH_2O	0.25	5.0±0.14	95.0±0.7[a]
UTS	Neutral pH ddH_2O	0.25	1.5±0.25	89.7±3.2[b]
UTS	TAP-N	1.4	1.5±0.15	88.6±1.7[b]
PET	TAP-N	1.6	1.4±.0.07	48.6±1.7[c]

Different letters denote significant differences in parameters by Tukey's test at $P<0.05$.

Upon dilution of the protein extract after UTS of biomass (in a neutral pH water) to a protein concentration comparable to that of extracts after PET of biomass a significant difference

(6-7% reduction) in precipitation efficiency was observed when compared to precipitation from a more concentrated extract. Interestingly, precipitation efficiency of protein extracts after UTS of biomass into buffers with different compositions (neutral pH water vs. TAP-N buffer) but with similar initial concentrations was not significantly different. These results would indicate that initial extract concentration does likely play a role in precipitation efficiency but doesn't explain the reduction in precipitation efficiency of ~45% observed in protein extracts after PET of biomass compared to protein extracts after UTS.

Observations after centrifugation of precipitation solutions revealed that UTS extracts separated into a pellet adhered to the centrifugation tube and a supernatant while PET and PET-EP extracts separated into a "loose" pellet that although visually distinct from the supernatant was not adhered to the tube wall (Figure 3.4b). Thus, some of the precipitated protein from in these "loose" pellets was inadvertently collected as part of the supernatant which could account for the lower precipitation efficiencies and higher standard deviations observed in PET and PET-EP extracts. Previous reports of "loose" pellets during protein precipitation were attributed to the possibility of DNA or polysaccharides present in the extracts leading to increased viscosity and slowed sedimentation (Burgess, 2009).

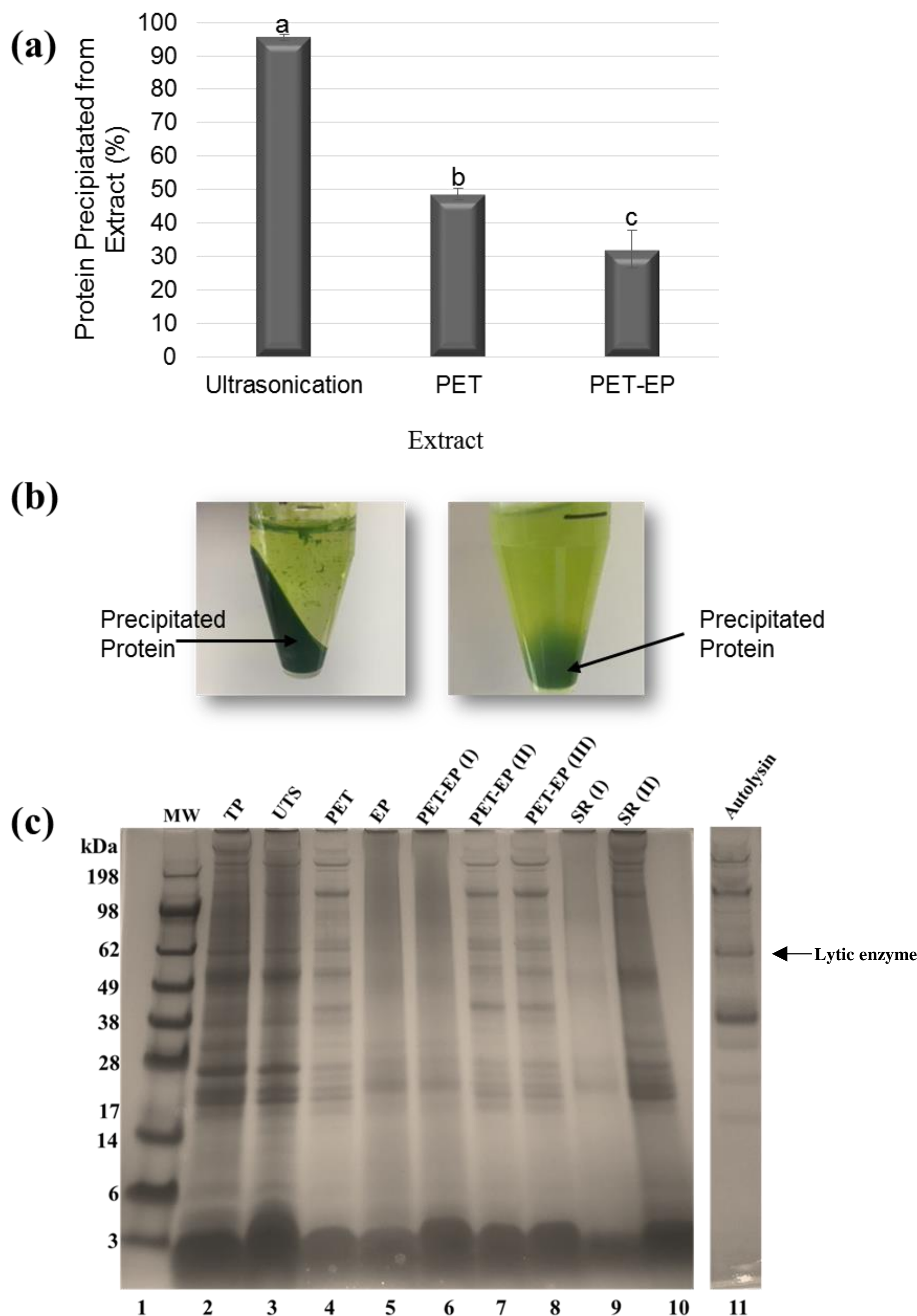


Figure 3.4 Protein recovery from UTS (ultrasonication), PET (primary enzymatic treatment), and PET-EP (PET plus enhanced protein). (a)Precipitation efficiencies after isoelectric precipitation at pH 4.5. (b) Qualitative comparison of supernatant and pellet of UTS extract (left) and PET or PET-EP extract (right). (c)Protein profiling with gel electrophoresis. Lane 1 (MW marker), lane 2 (total protein), lane 3 (UTS extract), lane 4 (PET extract), lane 5 (EP- extract, pH 12, 4h, 45°C), lane 6 (PET-EP (I) extract, pH 12, 4h, 45°C); lane 7 (PET-EP (II) extract, pH 7, 4h, 45°C); lane 8 (PET-EP (III) extract,

conditions: pH 7, 4h, 23°C); lane 9 (SR(I)-secondary residue after PET-EP(1)); lane 10 (SR(II)-secondary residue after PET-EP(II)); and lane 11 (autolysin solution)

As the "loose" pellet of precipitated proteins from PET and PET-EP extracts could indicate compositional differences in the extracts leading to an inability of proteins to aggregate, gel electrophoresis was used to explore the impact of cell disruption/extraction method on soluble protein profile (Figure 3.4c). The molecular weight profile of total protein in the biomass is shown in lane 2. In lanes 3-5, proteins extracted (released) after UTS, PET, or PET-EP (cond. I: pH 12, 4h, 45°C) can be seen. Ultrasonication (lane 3) of biomass alone resulted in a protein profile similar to that obtained from of our total protein reference. The protein profile of PET extract included proteins in the autolysin solution and those solubilized during PET treatment. In the UTS extract, there were low molecular weight proteins (≤3kDa) as well as proteins ranging from 17 to 198 kDa. Analysis of the profile of PET extract revealed that following PET, larger molecular weight proteins in the extract are readily visualized in addition to lower molecular weight proteins. Interestingly, upon exposure of crude PET lysate or PET residue to EP extraction procedures at pH 12 for 4 h at 45°C, less large molecular weight proteins are evident within the resultant extracts (lanes 5-6). To assess which of the optimized conditions of the EP extraction procedures could be contributing to the shift in size to lower molecular weight proteins, PET lysate was treated at pH 7 for 4 h at 45°C (lane 7) and at pH 7 for 4 h at 23°C (lane 8). In both of these extracts, higher molecular weight proteins ranging from 17-198 kDa were again evident along with low molecular weight proteins (≤ 3kDa). In lanes 9 and 10, proteins remaining in residues after PET-EP cond. I (lane 9) and PET-EP cond. II (lane 10) were visualized. Finally in lane 11, proteins in the autolysin solution applied as part of PET of *C.*

*reinhardti*i can be seen. In particular these proteins were found in PET and PET-EP extracts in addition to any protein extracted as part of those processing steps.

From analysis of extracts with enhanced protein extraction conducted at lower pH values or lower temperatures, it is likely that the increased pH at which optimized EP occurs somehow contributes to the reduced size of proteins in EP extracts. The increase in pH likely extracts proteins/enzymes with proteolytic activities or itself contributes to protein degradation. While PET extract does not show the same reduction in protein size as PET-EP (pH 12, 4h, 45°C) extracts, the cell disruption that occurs at this step could lead to release of native proteases capable of proteolysis and structural changes on extracted proteins upon pH increase. As TEM image analysis of biomass after PET previously indicated (Sierra et al., 2017) complete cellular and partial organelle disruption, chloroplast and mitochondrial proteases in *C. reinhardtii* are likely released. Many of these proteases are active in the neutral/alkaline pH ranges (Derrien et al., 2012; Lang et al., 1979) and could be cleaving exposed proteins during the PET or EP incubations. Additionally, the precipitation procedure itself could be causing additional structural changes to extracted proteins to the determent of aggregation. Future work should aim at assessing the impact of PET and EP extraction conditions on structural changes in extracted proteins and/or explore other methods of protein recovery such as membrane filtration. Using EP extraction conditions less conducive to native protease activity or protein degradation, while perhaps decreasing overall extraction, could improve subsequent recovery efforts and protein valorization in the designed biorefinery. While this study focused on maximizing extracted protein, efforts in protein recovery and functional characterization of extracted proteins continue.

Conclusions

The results of this study demonstrate the integration of an optimized enhanced protein extraction step between two enzyme-mediated unit operations as part of a designed biological-based biorefinery for microalgae. The extraction was adequately modeled using DoE/RSM and allows flexibility in terms of pH, time, and temperature when solubilizing proteins from disrupted biomass. The step can be directly implemented following cell disruption, thereby minimizing solid-liquid separations and associated processing time/energy inputs. Continued efforts are needed to assess the impact of EP extraction on lipid release to further demonstrate applicability of a biological-based biorefinery model for microalgae processing.

Chapter 4 - Evaluating the effects of a biological-based biorefinery model on lipid release from *Chlamydomonas reinhardtii*

Abstract

Microalgae biomass is a potential feedstock for energy and other valuable products to meet the ever growing food and energy demands. Lipids, carbohydrates, and other biomolecules can be used to produce various biofuels which can reduce global reliance on petroleum-based fossil fuels. In an effort to maximize valorization of microalgae biomass, a biological-based biorefinery process was developed for release and recovery of native proteins and lipids from *Chamydomonas reinhardtii*. Following enzyme-mediated cell disruption and high temperature, alkaline incubation of disrupted biomass for protein extraction, some lipids are released into the aqueous environment. To determine the effect of the developed process on lipid profile, compositional and characterization studies were conducted. Lipid accumulation procedures were confirmed to enrich biomass in triacylglycerols. Pore formation upon freezing of biomass was observed to aid in lipid release when compared to freshly harvested biomass. In comparison to conventional mechanical cell pretreatments, enzymatic cell disruption and enhanced protein extraction procedures resulted in evidence of degradation to free fatty acids, diacylglycerols, and some polar lipids. Finally, a preliminary prediction of the biodiesel potential of released lipids indicates suitability, favorable combustion behavior, and high oxidation stability.

Introduction

With energy demands expected to increase by 50% or more by 2030, alternative fuel/energy sources are of increasing interest to scientists, governments, and citizens around the world (Shuba and Kifle, 2018). Driving factors for increasing demand include increased consumption, fluctuating price, and availability of fossil fuels (Cheng, 2017). Renewable energies in the form or hydroelectric, geothermal, wind, solar, and biomass are being explored and implemented to reduce current and future reliance of petroleum-based fossil fuels. Biomass energy currently accounts for a small market share of total energy production and exists largely in the form or bioethanol (corn and sugarcane), biodiesel (plants and waste oils), biogas (organic wastes) and electricity/heat generation (wood chips) (Cheng, 2017).

As the biomass energy market is chiefly dependent of the cost of feedstock, biomass processing and conversion must be cost effective and environmentally friendly. Additionally, persistent challenges of biomass-based energies include the use of feedstocks that are also food commodities and the requirement of land/water resources (Shuba and Kifle, 2018).

Microalgae biomass is increasingly recognized as a biomass source from which energy and other valuable products can be derived. Additionally, several microalgae chemical intermediates can be converted into biofuels including hydrogen, lipids, hydrocarbons, and carbohydrates (Randrianarison and Ashraf, 2017) and thus produce multiple types of biofuels from constituents of the same cell (Gutiérrez-Arriaga et al., 2014). As a feedstock, microalgae possess several advantages over other biomass sources including rapid growth (Harris, 2009), accumulation of oil as up to 60% of dry weight (depending on species), cultivation in a variety of water sources water sources (Randrianarison and Ashraf, 2017) and on non-arable lands thus eliminating competition for resources with food commodities (Horst et al., 2012).

Prior work by our research group has aimed at development of a biological-based biorefinery for extraction and recovery of multiple biomolecules from *Chlamydomonas reinhardtii*. Of interest are native proteins and triacylglycerols (TAGs) to be included in food and feed products or transformed into biodiesel, respectively. The biorefinery model includes primary enzymatic treatment (PET) of biomass with autolysin for cell disruption followed by a high temperature, alkaline incubation for enhanced extraction of native proteins. A secondary enzymatic treatment (SET) of residual biomass with trypsin can also be employed for the recovery of lipids still attached to cellular/organelle remnants after PET and enhanced protein (EP) extraction procedures. Our prior research relied on gravimetric analyses for evaluation of lipid accumulation and release. However, additional investigation into changes in lipid profile and fatty acid composition following nitrogen deprivation of biomass and of extracts resulting after conventional and/or biological biorefinery pretreatment methods serves to inform processor decisions. For example, comparing lipid profiles after mechanical disruption of cells versus enzymatic hydrolysis or high temperature, alkaline incubation of cells could determine any bias toward release of specific lipid classes due to the pretreatment used. For example, preferential release of TAGs or polar lipid species could affect the suitability of any biodiesel produced. Additionally, evaluating lipid profiles of extracts when pretreatments are applied to freshly harvested cells or to previously frozen cells could reveal the effects of pore formation (in cell walls and/or membranes) due to freezing on lipid content/composition or extractability. The following study evaluated the effects of cell disruption and protein release/extraction conditions on concomitant lipid release, fatty acid profile, and lipid stability.

The objectives of this study were to: 1) evaluate lipid composition by thin layer chromatography (TLC) and GC-FID following nitrogen deprivation of *C. reinhardtii*; 2)

characterize (using TLC and CG-FID) lipid release with respect to biomass state (fresh or frozen after harvest) and cell pretreatment (enzymatic hydrolysis, mechanical disruption, high temperature/alkaline, or combination thereof); and 3) evaluate the biodiesel potential of lipids released by enzymatic hydrolysis and enhanced protein extraction (high temperature/alkaline) conditions using Biodiesel Analyzer© Version 2.2 software.

Materials and Methods

Microalgae Source

Production stain

C. reinhardtii (CC-409 mt+) was obtained from the *Chlamydomonas* Resource Center, University of Minnesota. The strain was maintained on solid TRIS-acetate phosphate (TAP) plates for use as inoculum for liquid cultures.

Mating strains

CC-620 mt+ and CC-621 mt- high efficiency mating strains were obtained from the *Chlamydomonas* Resource Center, University of Minnesota.

Cultivation procedures

CC-409 mt+ was grown in standard TAP medium at 23°C under 12h/12h light/dark cycles (27 µmol m^{-2} s^{-1}) with continuous shaking at 120 rpm unless otherwise stated. Growth media was autoclaved at 20 psi and 121°C for 40 min. *C. reinhardtii* cultures were streaked on solid TAP agar plates and grown for 5-7 days. Accumulated biomass was then used to inoculate 250 mL of liquid TAP media in a 500 mL Erlenmeyer flask. Cell growth and size was monitored by cell counts with a BrightLine hemocytometer (VWR Scientific). After cells reached mid-exponential phase (5 x 10^6 to7 x 10^6 cells/mL), cultures were transferred into 1.75 L of fresh

TAP media and grown until the mid/late exponential phase. To induce lipid accumulation via nitrogen deprivation, cultures were centrifuged at 8000 x g for 6 min at room temperature, decanted, and the pellets were washed in TAP medium without nitrogen (TAP-N). The washed pellets were re-suspended in TAP-N medium for 48 h, concentrated via centrifugation and used immediately (fresh) or frozen at -80°C.

Dry weight determination

The dry weight of *C. reinhardtii* biomass was determined following culture harvesting. Whatman® glass microfiber filters, Grade GF/F were ashed in a muffler furnace at 400°C for 1 h and stored in a desiccator prior to use. All aluminum trays used during dry weight determination were similarly ashed and stored. For each replicate performed the mass of the aluminum tray (W1), filter (W2), and combined tray and filter (W3) were recorded. To begin, the pre-weighed filter was carefully added to the vacuum filtration system. The filter was dampened with 10 mL of 0.5 M Ammonium formate prior to application of the algal culture. Then, 20-30 mL of algal culture was filtered at a vacuum pressure of 35-55 mmHg. Finally, any salts were washed from the filtered biomass by rinsing the filter with 10-15 mL of 0.5 M Ammonium formate. The filter was carefully removed from the filtration system, placed in the pre-weighed tray, covered with foil, and dried in an air oven at 105°C for 1 h. After drying, the sample was cooled in the dessicator for approximately 20 min and a final mass for the combined tray, filter, and dry algae (W4) was determined. The dry weight of the biomass was determined as the difference between W4 and W3.

Enzyme source

Autolysin production

Autolysin was prepared using a modified high-volume production protocol based on that proposed by Jaenicke et al. (Jaenicke et al., 1987). CC-620 mt+ and CC-621 mt- high efficiency mating strains were grown on solid TAP media agar plates for 3 to 7 days to generate a biofilm. Beginning on day 3 of biofilm generation mating tests were performed to confirm mating efficiency. Samples (500 µL) of each strain was mixed and allowed to mate for 15 min and observed using light microscopy. When approximately 95% of the cells were mating, efficiency was confirmed. Biofilm of each strain was independently solubilized and suspended in TAP-N to reach a final cell concentration of 1×10^7 cells/mL. The liquid cultures were shaken for 4 h under high intensity LED lights for gamete induction and flagella formation. The two strains were then mixed in a clear glass dish without agitation under high light for approximately 30 min. The mating cells were then centrifuged and the supernatant containing autolysin was depth filtered using a 0.45 µm PES membrane, concentrated using tangential flow filtration with a 10 kDa mPES hollow-fiber membrane, and frozen at -80°C until further use.

Biological based biorefinery procedures

Primary Enzymatic Treatment (PET)

Previously harvested biomass was separated from liquid media via centrifugation at 8000 x g for 6 min. Autolysin solution was added to the pelleted biomass at a ratio of 50 mg protein/g dry biomass. The enzyme-biomass solution was mixed end over end or with an orbital shaker at 200 rpm for 4 h initially at 23°C and then at 37°C until cell disruption was ≥85%. Once the desired cell disruption was achieved, the PET crude lysate was clarified via centrifugation (8000 x g for 6 min) to separate the PET extract (supernatant) from the PET residue (solid pellet) containing cellular and organelle remnants with entrapped biomolecules (proteins, lipids, starch, etc.). The PET extract was collected for lipid extraction and characterization.

Enhanced Protein (EP) extraction

Neutral pH water was added to PET residue after crude PET lysate clarification at a 1:10 biomass-to-water ratio (w/v) and pH adjusted to 12 by addition of 0.5 M sodium hydroxide (NaOH). The solution was mixed end over end at 45°C for 4h and pH adjusted as necessary. After the 4h incubation period, the solution was clarified via centrifugation (8000 x g for 6 min) to separate the EP extract (supernatant) from the secondary residue. The EP extract was collected for lipid extraction and further characterization.

Combined PET-EP

Harvested biomass was subjected to the PET procedures described in the Primary Enzymatic Treatment (PET) section. Following cell disruption, the crude PET lysate was pH adjusted to 12 by addition of 0.5 M NaOH and incubated at 45°C for 4 h. After the incubation concluded, the solution was clarified via centrifugation (8000 x g for 6 min) to separate the PET-EP extract (supernatant) from the secondary residue. The PET-EP extract was collected for lipid extraction and further characterization.

Lipid Extraction

Modified Bligh and Dyer extraction

A modified Bligh and Dyer (Bligh and Dyer, 1959) procedure was used for extraction of released lipids in PET, EP, and PET-EP extracts. Chloroform and methanol in a 1:2 (v/v) ratio was added to the aqueous extracts and mixed overnight. The following day, chloroform and water in a 1:1 (v/v) ratio were added to the mixture and vortexed briefly. The mixture was centrifuged at 6000 x g for 5 min and the bottom lipophilic phase was extracted and syringe filtered (0.2 µm) into a pre-weighed glass tube. Chloroform (two volumes) was again added and the above extraction and filtration procedure was repeated, combining the extracted lipophilic

phases. The filtered lipid extract was dried under a stream of nitrogen and dissolved in chloroform at 10 µg/µL or 25 µg/µL.

Hexane extraction

Hexane was added in a 1:1 (v/v) to PET, EP, and PET-EP extracts. All samples were mixed overnight and then 1 volume of hexane was added to the mixture and subsequently centrifuged at 4800 x g for 5 min. The top lipophilic layer was extracted, and syringe filtered (0.2 µm) into a pre-weighed glass tube. Another volume of hexane was added, and the extraction and filtration procedures were repeated. The filtered lipid extract was dried under a stream of nitrogen and dissolved in chloroform at 10 µg/µL or 25 µg/µL.

Lipid Profile Analysis

Thin layer chromatography (TLC)

Lipid extracts were spotted on TLC Silica gel 60 plates (Millipore Sigma, Burlington, MA USA) and separated using a hexane/diethyl-ether/acetic acid (70:30:1) solvent system and visualized with iodine vapor or under UV light after staining with 0.075% Dichlorofluorescein in 95% methanol. Thirty µg of C15:0 (pentadecanoic acid) was spotted on the center of each band of interest to serve as an internal standard. Lipid bands were scrapped and transferred to a clean glass tube with a Teflon-lined screw cap. Toluene (500 µL) was added to each tube as co-solvent during transmethylation.

Preparation of fatty acid methyl esters (FAMEs)

FAMEs were prepared by acid-catalyzed transmethylation. 1 mL of 5% (v/v) sulfuric acid in methanol and 25 µL of a 0.2% BHT solution was added to the scrapped lipid bands. After vortexing, the mixtures were heated at 95°C for 1 h. After cooling to room temperature, 1.5 mL of 0.9% potassium chloride (KCl) was added and the FAMEs were extracted with 2 mL of

hexane. The FAME extracts were dried under a stream of nitrogen and dissolved in 300 µL of hexane.

GC-FID analysis

FAMEs were analyzed and quantified by GC with a flame ionization detector. Each sample (1 µL) was injected into the gas chromatograph (Shimadzu GC-2010) equipped with a DB-23 capillary column operated in split mode (1:10 ratio). The carrier gas was helium and the column was heated as follows: initial increase to 130°C, increased by 6°C min-1 to 170°C, increased by 2.8°C min^{-1} to 215°C, and then held at 215°C for 1 min. FAMEs were identified by comparing their retention time to Supelco® 37 Component FAME Mix.

Fatty acid quantification

Raw data for each FAME peak identified in the chromatogram was used to quantify molecular lipid classes in each sample. The peak area was normalized to the number of carbons in the corresponding fatty acid and the hexane dilution used to prepare the GC-FID samples. The resultant area was normalized to either nmoles or µg of the 15:0 internal standard added to each sample. Molar or mass fraction attributable to each FAME was determined and masses of specific lipid classes were calculated by normalizing total FAME mass in a sample to number of fatty acids in TAG (3 FA) , DAG (2 FA), or polar lipids (2FA).

Biodiesel potential analysis

Using the FAME composition resulting from GC-FID analysis, biodiesel properties including cetane number, degree of unsaturation, cold flow properties (cloud point/cold filter plugging point), oxidation stability, higher heating value, kinematic viscosity, and density were predicted using BiodieselAnalyzer© Version 2.2 software.

Results and Discussion

Evaluating lipid composition following nitrogen deprivation of *C. reinhardtii*

Separation of neutral lipid species in total lipid extracts of nitrogen replete (TAP) and deprived (TAP-N) cells using TLC indicated relatively similar species found under the two growth conditions (Figure 4.1). Of note is an unidentified band that migrated between (diacylglycerols) DAGs and free fatty acids (FFAs) in TAP cultures that isn't present in TAP-N. Additionally, TAG band intensity (boxed) in TAP-N cultures is greater than TAP cultures and could serve as qualitative confirmation of TAG enrichment.

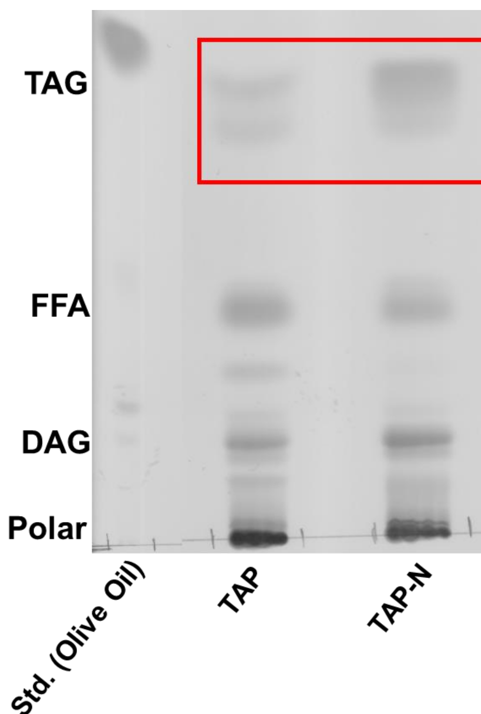


Figure 4.1 TLC separation of lipids from nitrogen-replete (TAP) and nitrogen-deprived (TAP-N) cultures.

To quantitatively assess TAG enrichment and any changes in distribution of lipid classes due to nitrogen deprivation, the fatty acid composition of polar lipids, DAGs, FFAs, and TAGs

was determined (Figure 4.2a). Lipid extracts from TAP cultures were enriched in polar lipids and FFAs with TAGs accounting for less than 10% of the total fatty acids on a molar basis. Upon nitrogen deprivation, TAGs accounted for greater than 45 mol % of the total fatty acids with all other lipid classes reduced in comparison to TAP cultures. Under replete growth conditions, the microalgae stored 95% less TAGs, ~0.12 μg/ (10^6 cells), compared to cells under 48 h of nitrogen deprivation that stored 2.4 μg/ (10^6 cells) (Figure 4.2b). When comparing total lipid accumulation, nitrogen deprived biomass accumulated 2 to 2.5 times more total lipids per gram dry biomass than nitrogen replete biomass which was consistent with our prior gravimetric analysis.

A comparison of the fatty acid composition of TAG species in TAP and TAP-N cultures used in this study matched previous reports of compositional changes in *C. reinhardtii* cultures. upon nitrogen deprivation (Moellering and Benning, 2010; Siaut et al., 2011). TAGs in TAP cultures were enriched largely in palmitic acid and while TAGs produced during nitrogen deprivation were enriched in palmitic, oleic, and linoleic acids (Figure 4.3a) as previously reported. De novo TAG biosynthesis can be inferred as oleic acid was the fatty acid increased

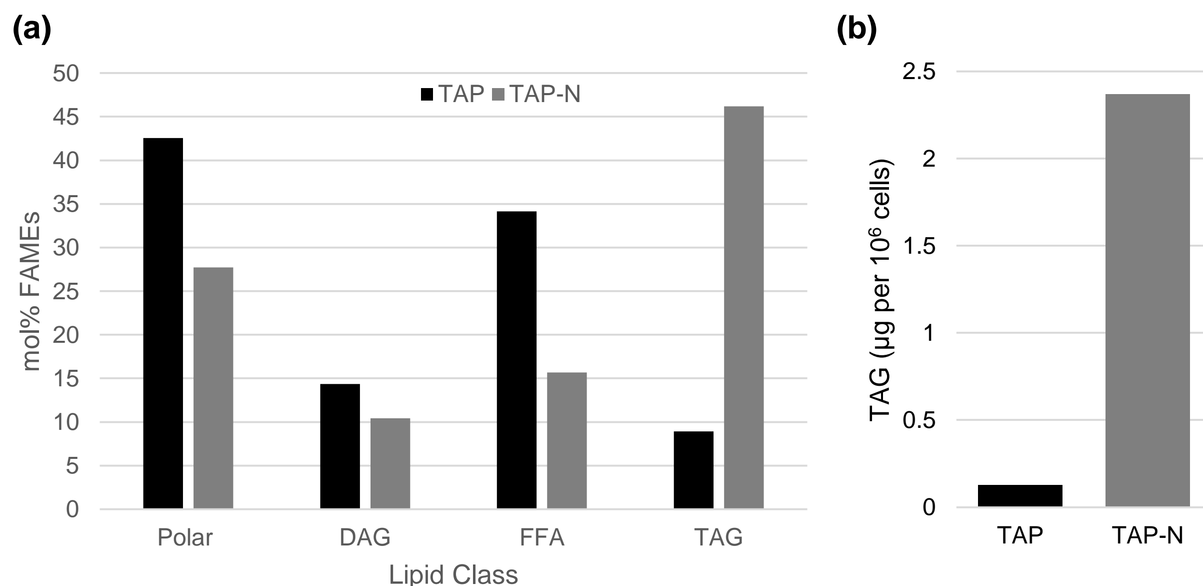


Figure 4.2 (a) Comparison of lipid classes in TAP and TAP-N cultures and (b) TAG accumulation in TAP and TAP-N cultures.

most upon nitrogen deprivation (Li et al., 2012), but other synthesis pathways such as plastid membrane lipid degradation and extraplastidic membrane lipid modification and turnover are possible due to the presence of 16:4, 18:3 (9,12,15), 18:1(11), 18:3 (5,9,12), and 18:4 fatty acids in TAP-N TAG species and their reduction upon nitrogen deprivation in polar lipids (Figure 4.3b).

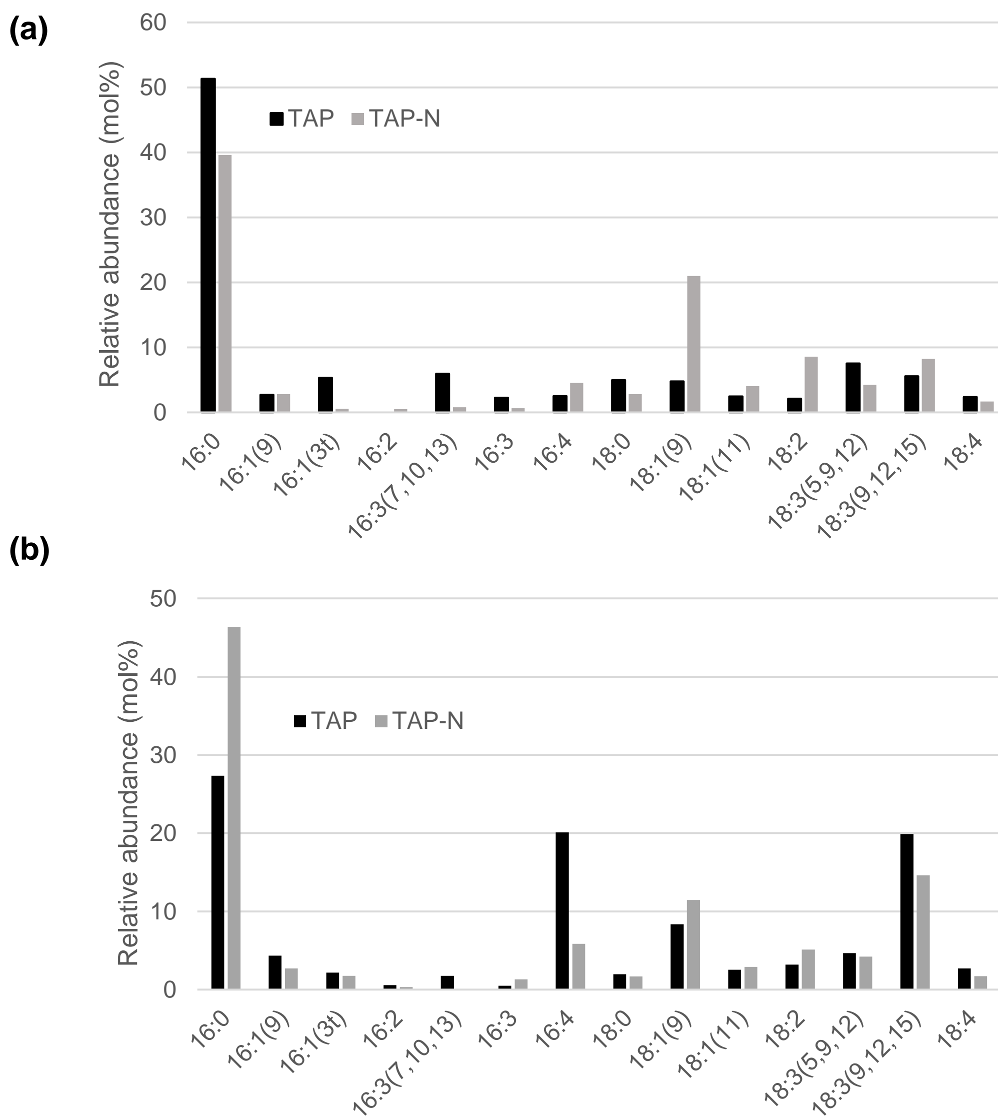


Figure 4.3 Comparison of FAME composition in (a) TAG and (b) polar lipids of TAP and TAP-N cultures.

The fatty acid profiles of various lipid species upon nitrogen deprivation, but most importantly TAGs confirms that the cultivation procedures result in a biomass feedstock enriched in TAGs that can then be extracted by our enzyme treatments and/or high temperature/alkaline conditions.

Characterizing lipid release due to biorefinery procedures

Effect of biomass state on lipid release after PET or EP step

Biomass used in the development of our biological based microalgae biorefinery is frozen upon harvesting at -80°C and prior to experimentation and thus is likely susceptible to pore formation in the cell wall and/or membrane during freezing. Freezing biomass was performed for convenience of the work capacity of the research laboratory and additionally allowed for not only temporary permeation but also complete cell disruption after application of autolysin. Prior reports on the effect of freeze-drying biomass on lipid extractability have found increased yields in comparison to undisrupted, wet biomass (Grima et al., 2003) but lipid extractability from previously frozen wet biomass has not been readily explored. Thus, the effect of biomass state, fresh (non-frozen), or frozen biomass on lipid release during primary enzymatic treatment and enhanced protein extraction was investigated.

Visualizing polar and neutral lipid classes with TLC revealed that fresh biomass incubated under PET negative control conditions (TAP-N buffer, room temperature) led to release of polar lipids and possibly TAGs (Figure 4.4 lane 1) while frozen biomass incubated under the same conditions led to release of polar species and FFA (Figure 4.5 lane 1). TLC of lipids in the aqueous extract of fresh biomass after PET revealed polar lipids, some DAGs, and TAGs while the extract of frozen biomass after PET revealed intense polar lipids, DAG, FFA, and TAG bands. The effect of biomass state on lipids released during the EP extraction step was

similarly explored. Fresh and frozen biomass subjected to TAP-N (PET negative control) or autolysin (PET) was subsequently exposed to EP extraction conditions (pH 12 incubation in ddH_2O for 4 h at 45°C). Lipids in the aqueous EP extracts were separated and visualized using TLC. Similar lipid profiles including polar lipids, DAG, FFA, and TAG were present in all EP extracts with lipids bands for extracts from autolysin-treated biomass being more intense for both fresh and frozen conditions (Figure 4.4, lanes 3 and 4; Figure 4.5, lanes 3 and 4). In particular, the presence of such intense lipid bands in the EP extracts of fresh biomass could indicate that the conditions used for enhanced protein extraction contribute significantly to lipid release.

Evaluation of the PET and EP extracts of fresh and frozen biomass indicated that the permeation of fresh biomass with autolysin led to limited lipid release but for autolysin treatment of biomass that has been previously frozen, more lipids and lipid classes are present in the extract. Previous visualization of freshly-harvested versus frozen biomass using TEM imaging showed apparent chloroplast membrane disruption after freezing (Sierra et al., 2017). Thus, the cell wall and/or membrane permeation upon freezing biomass is likely contributing to lipid release into aqueous extracts after PET and EP steps. If it is desired to process freshly harvested biomass and achieve the same lipid release, additional, permanent cell permeation mechanisms could be explored in conjunction with autolysin treatment. Using pulsed electric field (PEF) technology with autolysin could create permanent pores to allow for protein and lipid release from biomass into aqueous extracts. PEF applied after PET of fresh biomass would likely require a lower electric field strength, treatment time, and number of pulses as compared to application to undisrupted or not permeated biomass as pore formation would have already been initiated by autolysin.

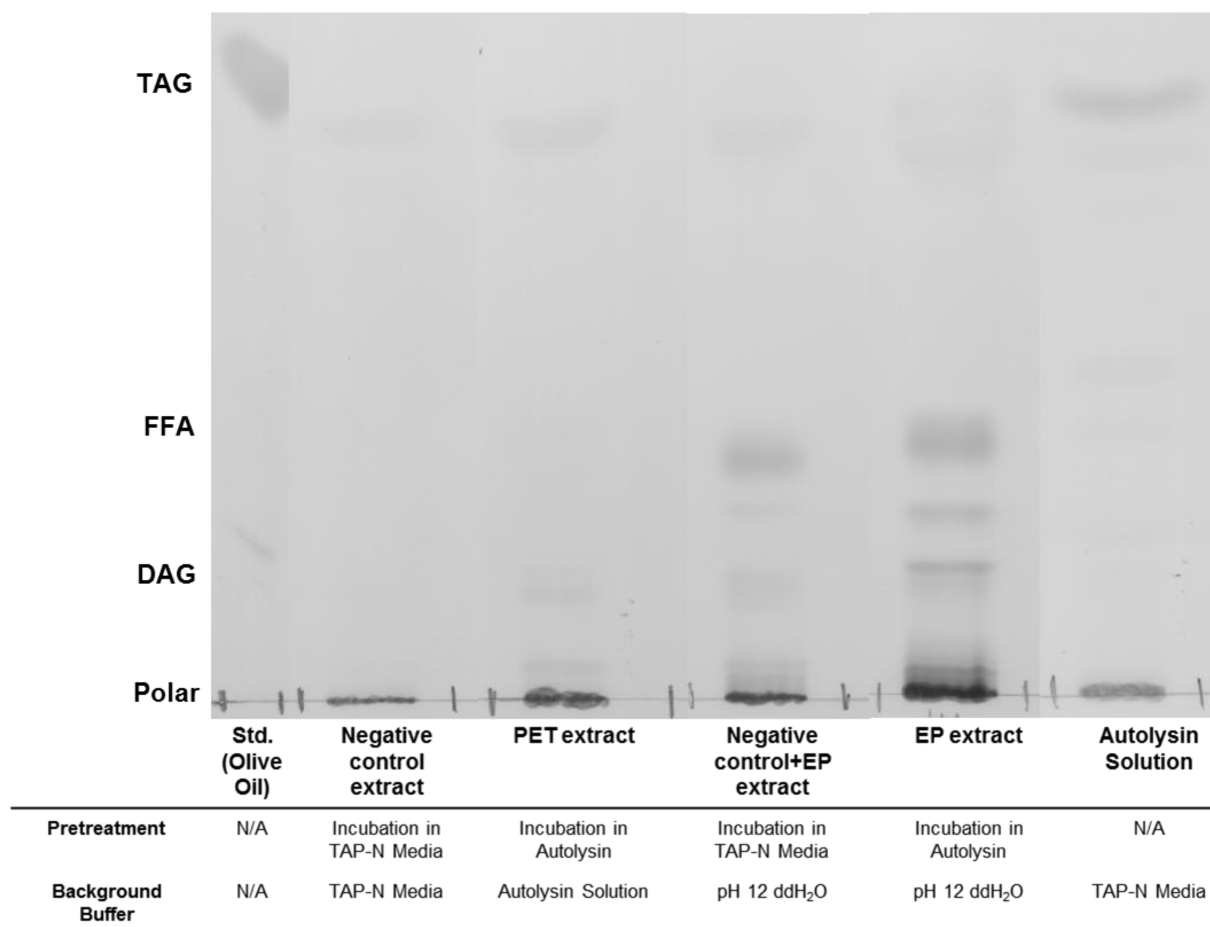


Figure 4.4 TLC separation of lipids in aqueous extracts after PET (- control), PET, and EP of fresh biomass and lipids in autolysin solution.

As the autolysin solution is a result of mating *C. reinhardtii* strains that secrete the enzyme upon shedding their cell wall, lipids naturally present in the solution was also separated and visualized with TLC (Figures 4.4 and 4.5 lane 5). Lipids present in the autolysin solution as visualized with TLC appear to include polar spp., FFAs, and TAGs. Since TAGs are of interest for microalgae biodiesel and were readily identified in fresh and frozen biomass PET extracts and in the autolysin solution, a comparison of the fatty acid profiles was conducted (Table 4.1). This comparison allowed for the determination of whether TAGs present in the PET extracts are

already present in the autolysin solution or if they were released from the biomass as a result of cell disruption.

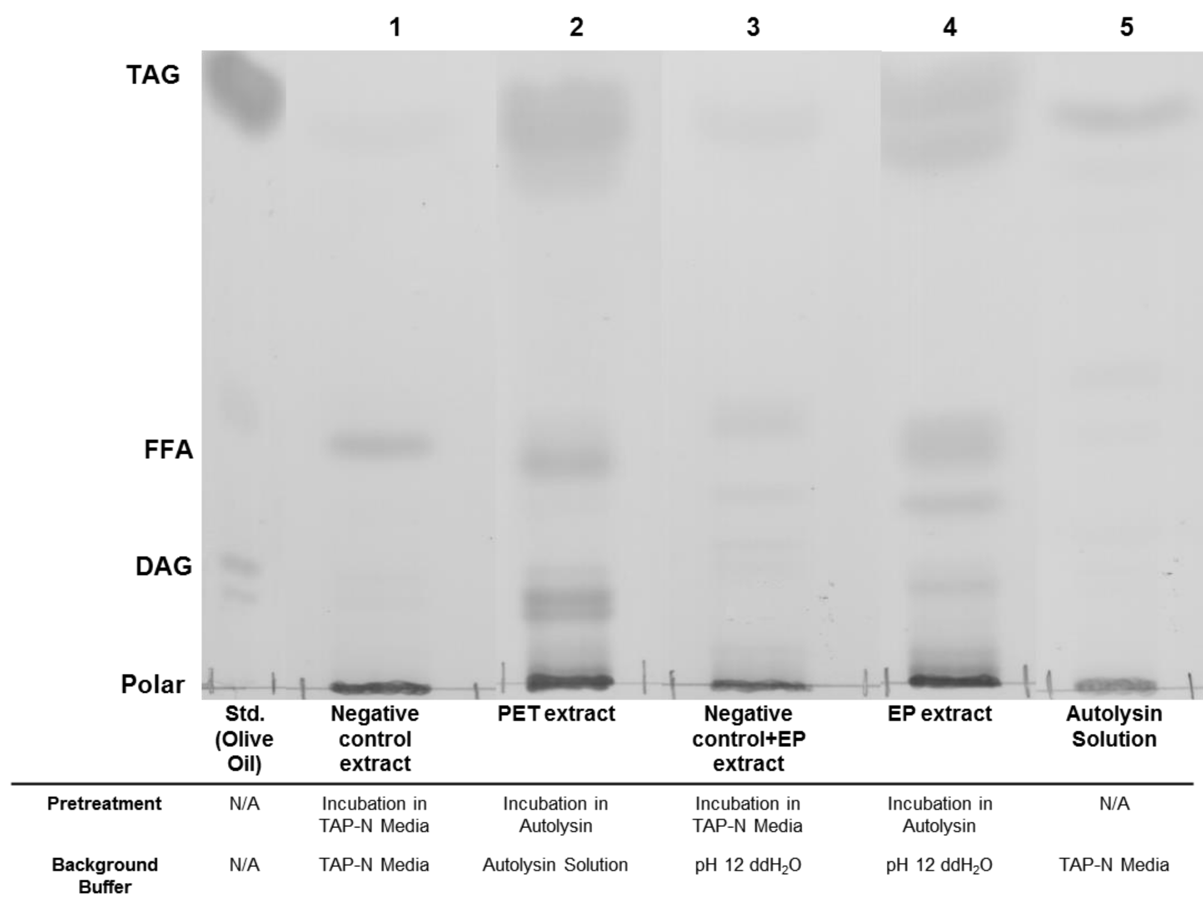


Figure 4.5 TLC separation of lipids in aqueous extracts after PET (- control), PET, and EP of frozen biomass and lipids in autolysin solution.

Overall, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) accounted for a greater molar composition (%) in the TAGs of PET extracts of fresh and frozen biomass while the autolysin solution was enriched in saturated fatty acids (SFA). Fatty acids including 16:4, 18:1 (9), 18:1(11), 18:2, 18:3 (5,9,12), 18:3 (9,12, 15), and 18:4 were found in extracts following PET of fresh and frozen biomass but not detected in autolysin solution. In particular, linoleic (18:2) and α-linolenic acid (18:3 (9, 12, 15)) each accounted for approximately 9 mol% of TAGs in both PET extracts. This comparison of the fatty acids of

TAGs in PET extracts to that in the autolysin solution indicates the release of TAG species from the biomass distinct from the TAG species added to biomass-enzyme solution mixture already in the autolysin solution added during PET. Thus, cell disruption mediated by primary enzymatic treatment with autolysin results in some lipid release into the surrounding aqueous environment regardless of whether cells are frozen or not prior to treatment.

Table 4.1 TAG fatty acid composition in PET extracts and autolysin solution

		Frozen biomass PET extract	**Fresh biomass PET extract**	**Autolysin Solution**
Fatty Acid	**Nature**	**mol%**	**mol%**	**mol%**
16:0	SFA	39.55	36.96	61.27
16:1(9)	MUFA	2.89	3.01	2.78
16:1(3t)	MUFA	0.45	0.74	6.16
16:2	PUFA	n.d	0.59	n.d
16:3(7,10,13)	PUFA	n.d	0.33	10.19
16:3	PUFA	n.d	0.93	7.05
16:4	PUFA	4.75	5.68	n.d
18:0	SFA	2.93	2.82	4.47
18:1(9)	MUFA	22.09	19.82	8.08
18:1(11)	MUFA	4.17	3.90	n.d
18:2	PUFA	8.71	9.03	n.d
18:3(5,9,12)	PUFA	4.23	5.08	n.d
18:3(9,12,15)	PUFA	8.51	9.21	n.d
18:4	PUFA	1.72	1.92	n.d
	ΣSFA	42.48	39.78	65.74
	ΣMUFA	29.60	27.46	17.03
	ΣPUFA	27.92	32.76	17.23

n.d not detected

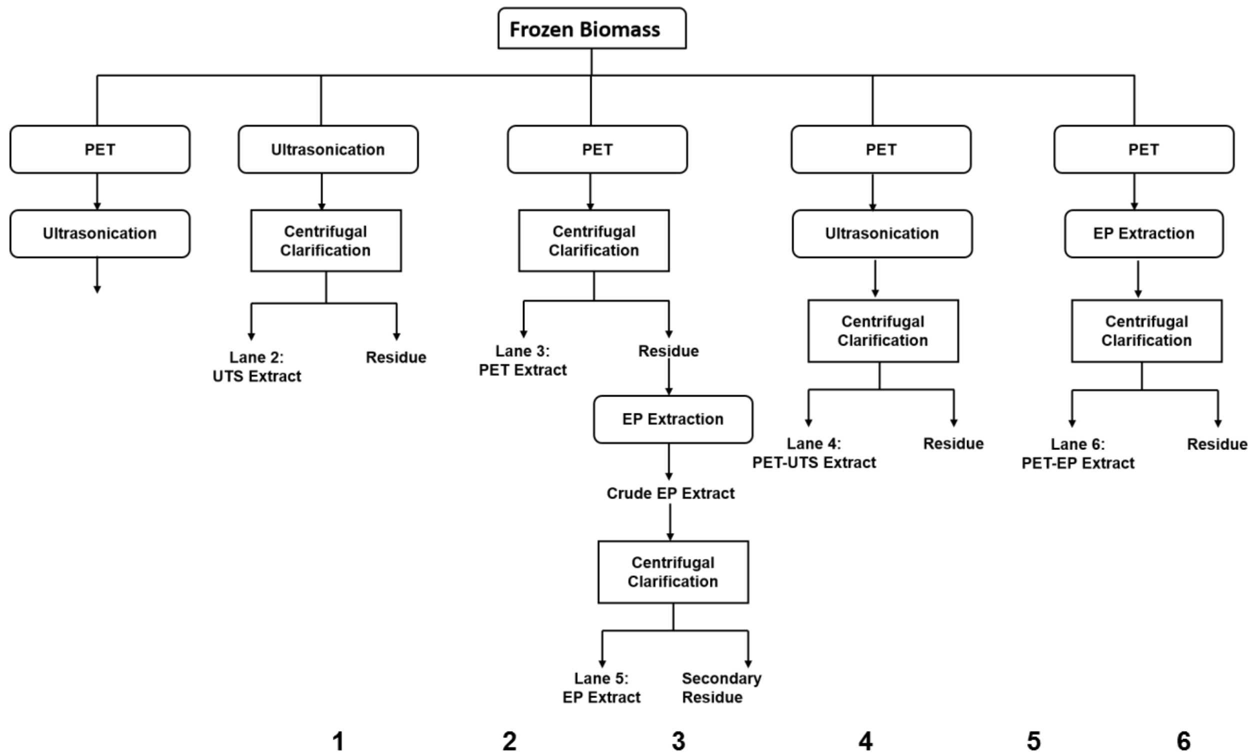
Effect of cell pretreatment on lipid release

Following confirmation of lipid release during enzymatic cell disruption and enhanced protein extraction, the effect of enzymatic hydrolysis and high temperature alkaline conditions on released lipids was evaluated. A conventional pretreatment method, ultrasonication, was evaluated alone and in conjunction with PET procedures to compare conventional and alternative disruption mechanisms used in microalgae lipid release and recovery. Figure 4.6 depicts the process flow and TLC separation of lipid classes released into the aqueous phase after

pretreatment of biomass with ultrasonication (UTS), PET, PET followed by ultrasonication, EP following PET with a solid-liquid separation and buffer addition step, and PET followed by EP procedures with pH adjustment and no solid-liquid separation or buffer addition.

An analysis of the profiles of lipids released reveal that polar lipids, DAG, FFA, and TAG are present in all the aqueous extracts after all pretreatments. Of note is the apparent reduction in DAG in both EP and PET-EP extracts (boxed in Figure 4.6). In particular, DAG species appear reduced with a possibly more intense FFA band when comparing lanes 3 and 6 (PET and PET-EP extracts, respectively). Similarly, less DAGs and an increase in FFA was quantified when comparing PET and PET-EP extracts (Table 4.2). This could indicate DAG degradation when biomass is exposed to the EP extraction conditions. Such degradation implies that endogenous lipases could be retaining activity during biomass freezing or be active throughout PET and/or EP extraction. Further comparison of the fatty acid composition of TAGs, FFAs, DAGs, and polar lipids was conducted to determine any changes in relative abundance (mol%) in the extracts resulting from the various cell pretreatments (Figure 4.7).

The fatty acid composition of TAGs did not vary much across pretreatment procedures as all were similarly enriched in palmitic, oleic, and linoleic acids (Figure 4.7a). Likewise, FFA composition was relatively similar between pretreatments except slight reductions in relative abundances of 16:4 in EP and PET-EP extracts (Figure 4.8a). DAG fatty acid composition was fairly consistent across pretreatments except for reductions in 16:1 (3t), 16:4, and 18:3 (9, 12, 15) in EP and PET-EP extracts (Figure 4.8b). Finally, comparison of polar lipid fatty acid composition across pretreatments revealed a reduction in relative abundance of 18:3 (9, 12, 15) in both EP and PET-EP extracts (Figure 4.8c).



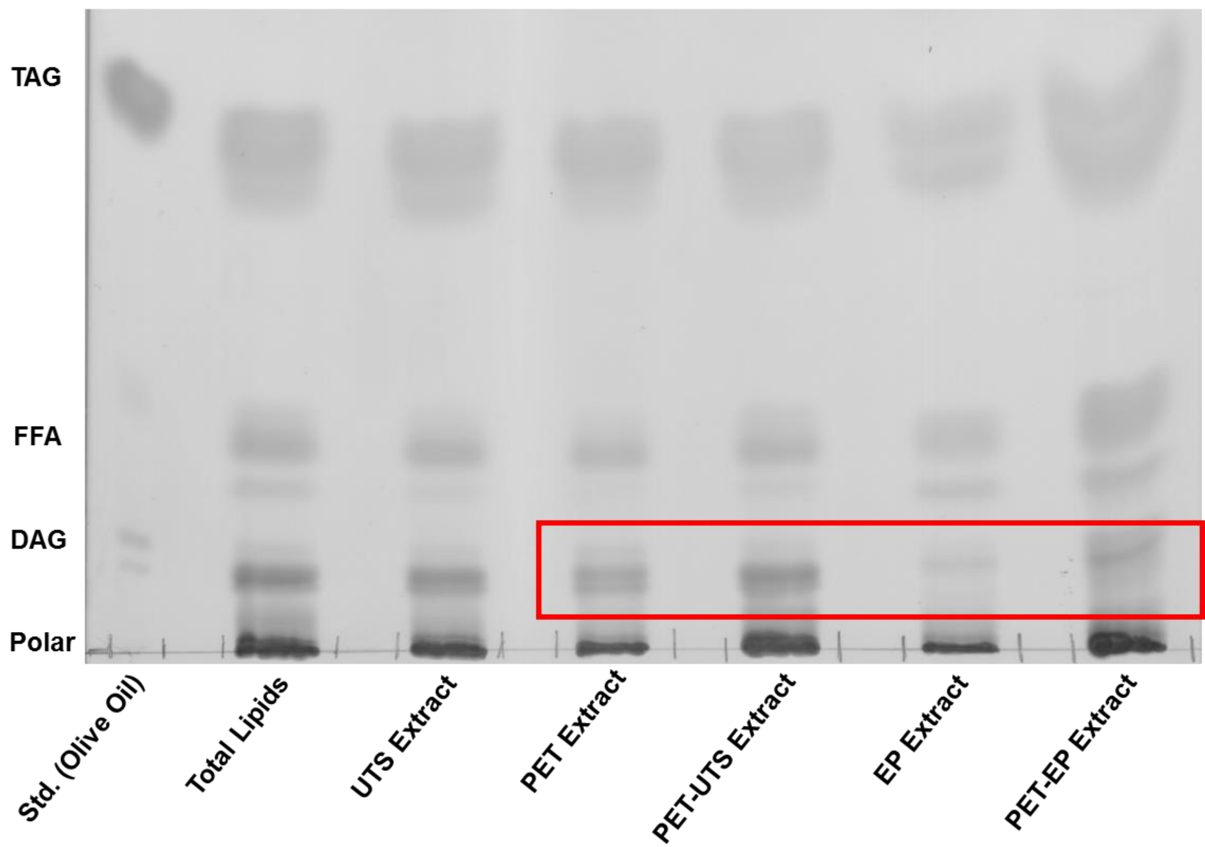


Figure 4.6 Process flow and TLC separation of lipids in aqueous extracts after various cellular pretreatments.

Table 4.2 Lipid class mass measurements in aqueous extraction after various cellular pretreatments

		Extract					
		Total Lipids	UTS	PET	PET+UTS	EP	PET-EP
Lipid Class (µg)	TAG	109.52	36.91	73.85	109.65	39.06	274.74
	FFA	69.81	76.01	62.14	85.36	83.49	168.3
	DAG	37.36	36.73	22.89	39.72	3.88	20.21
	Polar	136.87	90.97	67.29	141.39	3.06	13.88

The reduction in specific fatty acid species could be attributed to naturally occurring lipases that are released from intracellular compartments of the biomass during PET or EP procedures leading to fatty acid degradation. That degradation, especially if TAGs are impacted, could counteract the TAG enrichment of the biomass and reduce biomass yields and/or quality. To guard against degradation due to lipase activity, a method of lipase inactivation could be explored. Biomass lyophilization or use of isopropanol during lipid extractions have been reported to deactivate lipases but these strategies would circumvent the advantages of enzyme-mediated cell disruption and multiple product extraction from wet biomass. Thus, while processing wet biomass and minimizing use of organic solvents serve to improve the sustainability and process economics of a microalgae biorefinery, such conditions could also negatively impact the profile and content of released lipids. Kinetics studies of the composition of released lipids during the incubation periods of PET, EP, or combined PET-EP steps could further elucidate where in the process that fatty acid degradation is occurring and allow processors to adjust procedures to minimize degradation. A potential mitigation strategy for minimizing lipid degradation is reducing the pH during the EP extraction step which could reduce overall extraction of lipases as protein. A balance would therefore need to be established between protein extraction conditions and their effects on lipid stability.

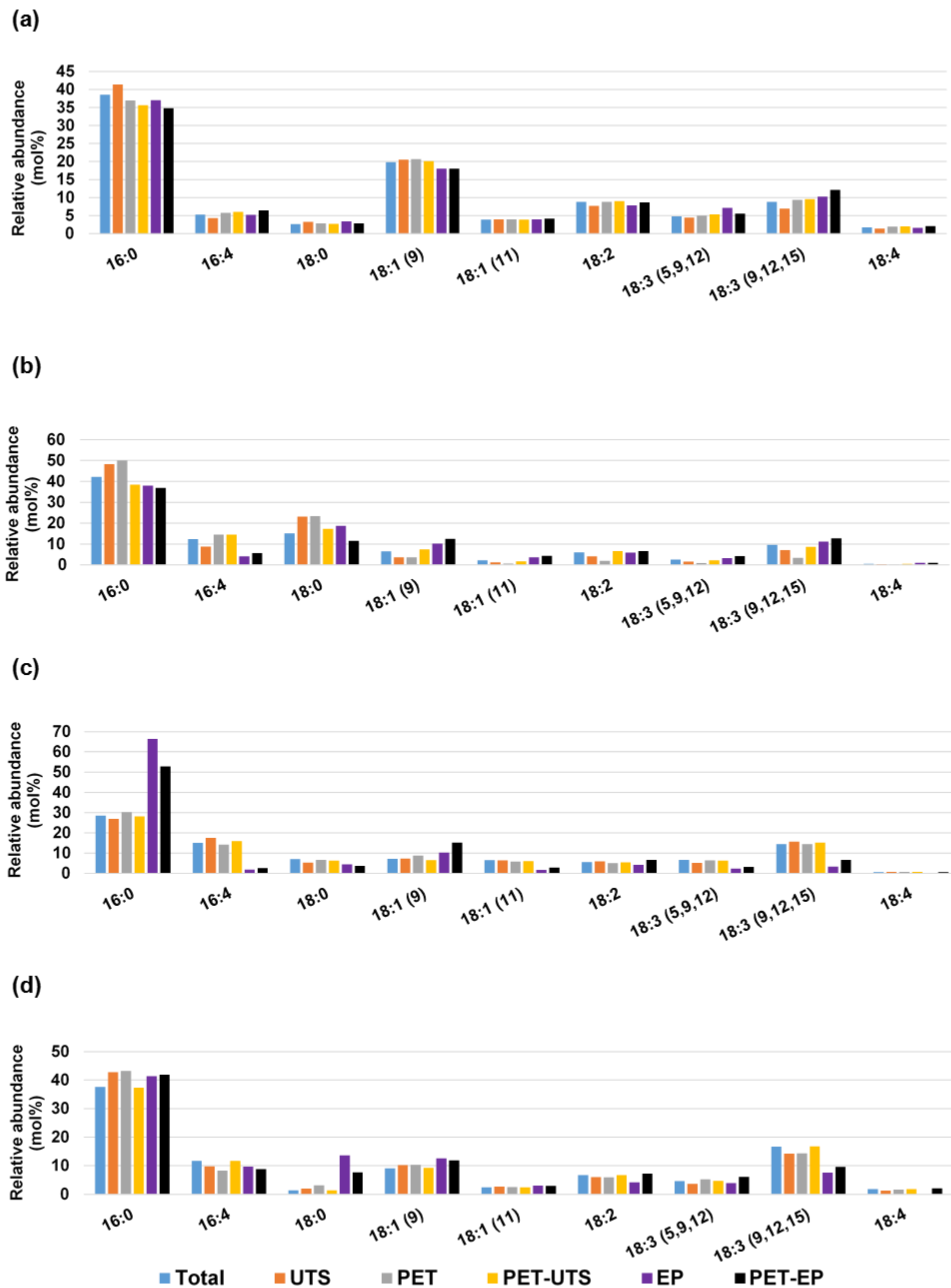


Figure 4.7 Comparison of fatty acid composition in (a) TAG, (b) FFA, (c) DAG, and (d) polar lipids released into aqueous extracts after various cell pretreatments.

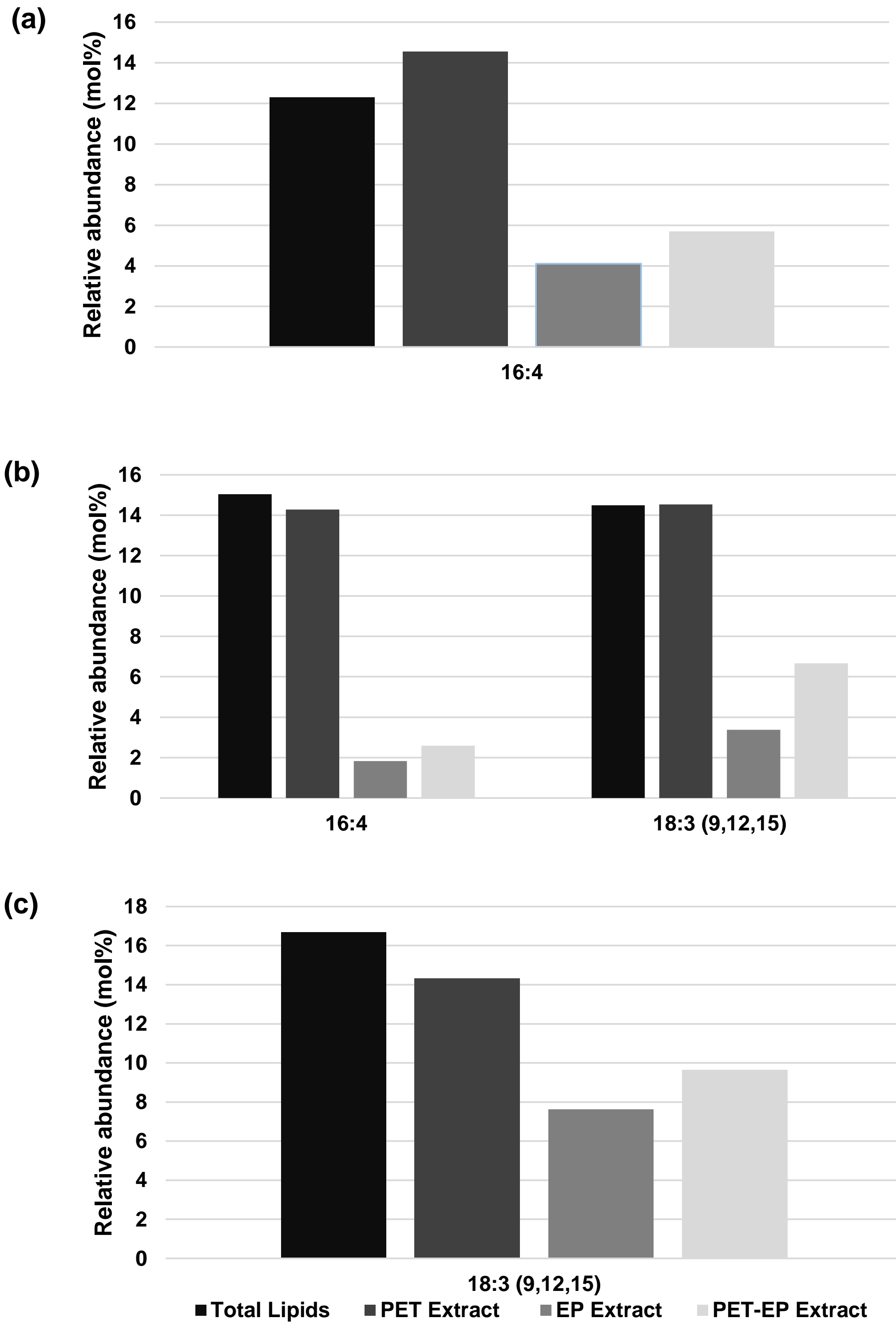


Figure 4.8 Comparison of selected (subset of Figure 4.7) fatty composition in (a) FFA, (b) DAG, and (c) polar lipids released into aqueous extracts after various cell pretreatments.

Evaluating biodiesel potential of lipids released during biorefinery procedures

Processing procedures used aimed to recover both native proteins and lipids from *Chlamydomonas reinhardtii* for food/feed and bioenergy applications, respectively. Specifically, TAGs released during PET or PET-EP steps could potentially be used for biodiesel production and thus, their biodiesel potential was assessed. Based on the fatty acid compositions of TAGs released from biomass into the PET extract or the PET-EP extracts, biodiesel properties including cetane number (CN), iodine value (IV) cold flow properties (cloud point/cold filter plugging point (CFPP)), oxidation stability (OS), kinematic viscosity (υ), and density (ρ) were predicted using BiodieselAnalyzer© Version 2.2 software (Table 4.3). The predicted properties were compared to the specifications for biodiesel in the European standard for biodiesel: EN 14214 Automotive fuels - Fatty acid methyl esters (FAME) for diesel engines - Requirements and Test Methods (CEN, 2009) and the American standard for biodiesel: ASTM D6751-02 Standard Specification for Biodiesel Fuel (B100) Blend Stock for Distillate Fuels (ASTM, 2002).

Saturated fatty acids and unsaturated fatty acids both impact biodiesel properties, so having a balance of both allows for improved biodiesel quality (Islam et al., 2013). Specifically, increases in SFA increase the cetane number (CN) of a fuel and a higher CN is associated with improved combustion behavior and shorter ignition delay time (Gopinath et al. 2009). Conversely, degree of unsaturation greatly effects IV value, kinematic viscosity (υ), density (ρ), and cold filter plugging point (CFPP).

Table 4.3 Biodiesel properties calculated from the fatty acid profile of TLC-separated TAGs in UTS, PET and PET-EP Extracts

	TAG Extract Source			Biodiesel Standard	
Properties	**UTS**	**PET**	**PET-EP**	**EN 14214 (CEN, 2009)**	**ASTM D6751-02 (ASTM, 2002)**
SFA (%)	44.65	45.97	48.83	---	---
MUFA (%)	23.92	22.06	22.08	---	---
PUFA (%)	20.48	23.25	20.99	---	---
IV (g I_2/100 g oil)	72.07	77.99	71.98	<120	---
CN	59.42	57.36	58.45	>51	>47
CFPP (°C)	1.65	1.71	2.79	<5 to <-20	---
OS (h)	8.77	8.09	8.68	≥6	---
υ(mm^2/s)	3.17	3.24	3.29	3.5-5.0	1.9-6.0
ρ (g/cm^3)	0.78	0.80	0.80	0.86-0.90	---

The CN value for TAGs in both PET and PET-EP extracts are above the acceptable minimum values for EN 14214 and ASTM D6751-02 and their respective IV values are below the maximum limits of EN 14214. Both extracts are slightly below the kinematic viscosity limits of the EN 14214 standard but within range of the ASTM D6751 standard. Additionally, both extracts were slightly below the acceptable density range of the EN 14214 standard.

TAGs released after PET or PET-EP procedures are predicted to produce biodiesel with favorable combustion behavior and high oxidation stability. Contrarily, below minimum acceptable levels of predicted kinematic viscosity and density could indicate problems with adequate fuel supply reaching injectors or unfavorable stoichiometric ratios of air and fuel in engine combustion chambers (Ng et al., 2012, 2011). While *Chlamydomonas reinhardtii* is not typically used for lipid production for biofuels for cases where lipids are the primary products, this preliminary prediction of biodiesel properties is encouraging in that lipids released from microalgae biomass via biological bio-refinement procedures have the potential to be a high-

volume co-product alongside high value products such as native or recombinant proteins from the same cell.

Conclusions and recommendations

The results of this study demonstrate the utility of *Chlamydomonas reinhardtii* as a bioenergy feedstock and the application of biological bio-refinement procedures for release of TAGs. Nitrogen deprivation lead to more than twice the amount of total lipids per gram of dry biomass with a 95% increase in TAG content as compared to nitrogen replete biomass. Freezing biomass prior to processing was shown to enhance lipid release and the class of lipids present in various extracts after pretreatment. Lipids release during primary enzymatic treatment (cell disruption), enhanced protein extraction, and combined primary enzymatic treatment-enhanced protein extraction steps was confirmed and degradation of free fatty acids, diacylglycerols, and some polar lipid species was observed visually with TLC and quantified with GC-FID. Finally, the biodiesel potential of TAGs released during primary enzymatic treatment and combined primary enzymatic treatment-enhanced protein extraction steps was predicted to be favorable.

Results from this study indicate that additional re-evaluation of the enzymatic treatment and enhanced protein extraction steps of the microalgae biorefinery are necessary to protect the integrity of both released lipids and proteins. Freezing biomass prior to harvesting followed by autolysin treatment results in complete cell disruption, but in doing so, potentially releases lipases into the aqueous extract. The moderate temperatures and aqueous environment used during enzymatic cell disruption apparently allows released lipases to retain lytic activity. Additionally, the high pH conditions and extended incubation period used to promote overall protein extraction are likely extracting lipases. While extraction of these lipases contributes to

the protein release/recovery goals of the microalgae biorefinery, lipid integrity and stability is impacted. Therefore, a more targeted approach to cell permeation and extraction and/or a change in the order of biomolecule extraction is necessary. Enzyme-mediated permeation rather than complete cell disruption could provide access to intracellular biomolecules without their release into the aqueous environment. Subsequent processing steps could be designed for specific protein release or lipid release. If proteins are the desired biomolecule following biomass permeation, extraction buffer conditions promoting native protein extraction with careful consideration given to minimizing lipase solubility could be explored. Alternatively, lipid release could follow cell permeation with secondary enzymatic treatment of biomass. In both cases, the resultant extracts should be assessed for signs of lipid degradation. Continued signs of degradation could indicate that the developed biological biorefinery could benefit from changes in target biomolecules or the intended applications of recovered biomolecules. For example, processing procedures for lipids and starch as primary extraction targets with native protein solubilization and extraction as a secondary goal could allow for conditions (i.e. high temperatures greater than 50°C) that inactivate lipases but come with the added risk of protein denaturation.

Additional work should also include exploration of mechanisms for recovery of lipids and proteins out of aqueous extracts. In this study, lipids were extracted with organic solvents for characterization purposes. When aiming to recover functional proteins and lipids from the aqueous extracts after primary enzymatic treatment or combined primary enzymatic treatment-enhanced protein extraction steps, organic solvent use, especially at relatively high temperatures could denature proteins and reduce their suitability for inclusion in food or feed products. Inducing lipid coalescence with application of an emulsion destabilizing agent in conjunction

with protein recovery via isoelectric precipitation or membrane filtration could provide adequate yields of both target molecules while retaining their integrity and value.

Chapter 5 - The impact of six critical impurities on recombinant protein recovery and purification from plant hosts[4]

Abstract

Molecular pharming of plants for recombinant protein expression has undergone several shifts in focus since inception. Initially, achieving commercially viable protein expression levels was the primary focus without giving much attention to the downstream processing (DSP) and critical plant-derived impurities. Thus, there is an increasing need to further develop robust and efficient DSP operations following recent successes of plant-based recombinant proteins in clinical trials and in the FDA regulatory approval process. There are several nuanced challenges that arise during DSP of seed-, leaf, and bioreactor-based expression platforms. These challenges or critical impurities are proteases, native host proteins, phytic acid (salts), polysaccharides, phenolics, and pigments. While mitigation strategies to circumvent the effects of these impurities have been independently developed on a case-by-case basis, this chapter describes the critical impurities and proposes measures whereby impurities can be minimized during extraction or removed during further downstream processing.

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Introduction

Evolution of molecular pharming

In the early 1980s, the field of molecular pharming was ushered in with the proof of concept that plants could be genetically modified to express recombinant proteins. The use of plants as platforms for the production of recombinant proteins (RP) has since undergone an evolution that included addressing concerns over expression level and the lack of regulatory framework for production and manufacturing of plant-made pharmaceutical (PMP) products. In the past, industry concerns centered on upstream production and the successful expression of the pharmaceuticals in plants. As these issues were addressed, government groups and the pharmaceutical industry's interests piqued and investments were made in building manufacturing facilities for PMPs (Tuse et al., 2014). In 2010, the Defense Advanced Research Projects Agency (DARPA) awarded Medicago a grant to support the development of PMP manufacturing capabilities for accelerating the scale-up of PMP infrastructure and producing antigens for pandemics like influenza. The field of molecular pharming has since experienced a resurgence of attention because of pioneering successes of select leading-edge pharmaceutical products. The achievement of commercially-viable expression levels has led to an ever increasing number of PMP products successfully traversing clinical trials, shifting the focus to the development of robust and efficient downstream processing (DSP) for commercial-scale production. For plant expression platforms to reach fruition as an alternative to traditional recombinant expression platforms, development of efficient extraction and purification processes is critical. With over 80 % of pharmaceutical manufacturing costs attributed to DSP, the importance of developing cost-effective operations is essential. This chapter identifies and describes the critical plant-derived impurities and proposes measures whereby impurities can be minimized during

extraction or removed during further DSP. The progress that is being made in addressing plant-specific impurities is underscored by the commercial success of several PMP products.

Recent successes in plant made recombinant proteins

In 2012, the FDA approved Elelyso™, a recombinant glucocerebrosidase cultured in carrot cells for the treatment of Gaucher's disease (Holtz et al., 2015; Sabalza et al., 2014; Stoger et al., 2014). As the first PMP approved for use in humans, Elelyso™ represented a landmark success for molecular pharming. Several other plant-made products such as enzymes, antibodies, and vaccines are in various phases of clinical trials (Yao et al., 2015). Molecular pharming received another noteworthy nod to utility and acceptance by the pharmaceutical industry and the wider public with the success of ZMapp™ in combating the 2014 Ebola virus outbreak. ZMapp™, a monoclonal antibody cocktail produced in tobacco leaves, was used to treat seven infected patients including two American aid workers (Wong and Kobinger, 2015). ZMapp™ had been under development since 2004 with demonstrated efficacy in chimpanzees, but had not been tested on humans before the outbreak (Choi et al., 2014). In February 2016, ZMapp™ was granted fast track status by the FDA and the drug developer, LeafBio, Inc., announced promising results from the Prevail II clinical trial (LeafBio, 2016). Other therapeutic candidates used during the outbreak included Favipiravir (RNA polymerase inhibitor) and TKM-Ebola (small interfering RNA (siRNA) drug cocktail) but tobacco-derived ZMapp™ animal study data was more favorable and, thus, created an opportunity for demonstrating the potential of PMPs. Although at the time ZMapp™ had not yet and still has not received approval by the FDA, the potential for rapid response from the plant platform in producing the monoclonal antibodies demonstrated the advantages of PMPs. The plant platform and more specifically, tobacco, was afforded an opportunity to gain public acceptance as a therapeutic production platform.

With the successes of ZMapp™ in human clinical trials and the current sale of Elelyso™ as an injectable treatment, the role of DSP is more evident than ever in terms of products requiring high final purity. Thus, downstream processors must develop a series of unit operations compatible to the unique characteristics of the plant platform while considering the idiosyncrasies of product release, process containment, and process-related impurities. By recognizing the challenges associated with DSP of PMPs, mitigating actions can be implemented, resulting in cost-effective product extraction and purification processes.

Even more recently, in June 2016, Protalix Ltd., the company that developed and manufactured Elelyso™ began Phase III trials for PRX-102 for the treatment of Fabry Disease (Protalix BioTherapeutics, Inc). The recombinant human α-galactosidase-A enzyme is an enzyme replacement therapy (ERT) as an alternative to existing ERT treatments. PRX-102 has improved activity toward target organs and longer half-life and stability. Researchers have also successfully developed and begun large-scale production of an edible protein drug, CTB-FIX (FIX fused with a transmucosal carrier, CTB), expressed in the chloroplast of lettuce leaves for the treatment of hemophilia B (Su et al., 2015). The leaves are grown in a cGMP facility, freeze dried, and powdered for oral delivery by capsule. This product represents the first of its kind demonstration of commercial viability of a PMP made from whole plants. The quantities produced could provide 24,000–30,000 doses per year, enough necessary to proceed with human clinical trials. This is a step toward proving clinical utility of whole plant products.

Trends in recombinant protein products

Recent evaluation of trends in molecular pharming reveal a focus on targeting niche markets, developing oral vaccines and topical products, expressing RP with purification tags, and using whole plants or minimally processed plants. In addition to these recurring trends, there has

been a consensus of goals for the industry to meet in order to support more PMP process engineering research and development, product commercialization, and public acceptance. One of these goals is to continue to refine and standardize DSP. The industry needs to simplify and streamline DSP (Yao et al., 2015) and address concerns over optimization and new technology development (Chen and Davis, 2016; Gronemeyer et al., 2014) associated with currently used DSP operations. Steps to standardize DSP include continued development of process engineering along with statistical design of experiments and process scale-up/scale-down modeling and simulation (Santos et al., 2016). It is only after adoption and implementation of these strategies that DSP can influence the commercial viability and industry attraction of PMPs.

The niche markets include products that need to be manufactured on a short timescale, benefit from simplified purification steps, require massive or minute volumes, are suitable for growth in region-specific areas, or cannot be produced in other platforms (Stoger et al., 2014). For example, in the event of a pandemic or bioterrorist threat, PMP products expressed transiently provide scalability and timely production that cannot be matched by mammalian or microbial production platforms.

Oral vaccines are advantageous over traditional vaccines for reasons including immunogenicity, administration, product stability, and long-term storage. To date, the most common plants used to produce oral vaccines include rice, corn, potato, and lettuce (Chan and Daniell, 2015; Merlin et al., 2016). Compared to traditional vaccines, oral vaccines retain antigen immunogenicity and biological activity (Takeyama et al., 2015). When produced via a plant platform, the oral vaccine is naturally protected from the acidic stomach environment of the patient with bioencapsulation in seeds or the plastids of freeze-dried leaf material (Chan and Daniell, 2015; Gorantala et al., 2014). Administering oral vaccines does not require qualified

health workers and there is no risk of contaminated needles or syringes, which often pose an additional environmental safety problem (Levine, 2010). The minimized DSP costs associated with oral vaccines could be less than 50 % of the total production cost (Wilken and Nikolov, 2012a), which is additionally considered by manufacturers. Furthermore, oral vaccines do not require cold storage, making them especially attractive to developing countries. In the next 10–15 years, oral vaccine production is expected to become an increasingly attractive social and fiscal opportunity for developing countries that serve to benefit from the protection of vaccines made possible by oral delivery rather than injectable form. Characteristics of oral vaccines including minimized DSP costs and infrastructure, ease of administration (including raw consumption of plant material), and no requirement for a cold storage chain, all benefit the medical and production environment in developing countries (Waheed et al., 2016).

Expressing RP with purification tags to facilitate an affinity purification step is a practice that is used in all expression platforms and increasingly utilized in plant hosts. Purification tags can be large molecules used to enhance expression or affect solubility of the RP (Waugh, 2005) while smaller tags are used solely to help bind the RP in an initial capture step. Small tags such as multi-histidine (HIS-tag) and Strep-tag II are popular choices that can be added at the N- or C-terminus depending on where they are more likely to be accessible. The FLAG purification tag is very popular because it contains a built-in protease cleavage site (enterokinase) that allows it to be specifically removed after purification. Other tags can be linked with a protease cleavage site to facilitate their post-purification removal so that the tag does not interfere with biological activity.

Using whole plants or minimally processed plants for recombinant protein production and administration is an alternative that minimizes or eliminates DSP operations (Łojewska et

al., 2016). To date, minimal processing has been mainly explored for veterinary applications and industrial enzymes where less regulatory rigor is exercised (Buyel et al., 2015). With continued success in higher RP expression levels, whole fruits, leaves, or seeds eaten raw can serve as host for oral vaccines and topical treatments. The trend of utilizing whole or minimally processed plants is ideal for new manufacturing operations as the initial capital costs associated with DSP infrastructure can be reduced. Additionally, whole and minimally processed plants can be a cost-effective addition to current pharmaceutical manufacturers as there is little to no competition for square footage on the already crowded manufacturing plant floor. Whole and minimally processed plants for recombinant protein production readily allow for traditional (mammalian or microbial) recombinant protein-derived pharmaceutical operations to continue without the addition of new or specialized equipment for DSP.

Although some of the emerging production environments for PMPs seek to minimize DSP, established expression platforms are becoming increasingly used for the production of high purity human therapeutics. These focused avenues intend to serve specific segments of the pharmaceutical industry rather than replace or displace traditional production platforms. As a whole, molecular pharming has undergone a "self-evaluation" of sorts and concentrated efforts of the industry seek to match the expression platform or unique plant characteristics to complement physiochemical properties of the recombinant protein product versus producing all products in only a small number of expression systems (Buyel et al., 2015). Additionally, the aforementioned focus areas encourage additional focus on DSP and the appropriate and cost-effective application of extraction and purification procedures. While niche markets, oral vaccines and topicals, expressing RP with purification tags, and whole and minimally processed

plants may not require a full DSP scheme, any unit operation employed must be efficient and ideally translatable between different plant platforms.

In addition to the trends previously discussed, the early recombinant protein products that entered the commercial market as industrial enzymes, nutraceuticals, and research/diagnostic reagents still persist. These established industries are generally less regulated with fewer requirements for rigorous clinical trials or the applications require a low final purity.

For the intended purposes, early recombinant protein products benefitted from fewer DSP operations and cost-conservative purification procedures to maintain the low cost, high yield product models. Even today with the progression of therapeutic drugs derived from recombinant proteins, those non-pharmaceutical industries still readily use plant-derived recombinant protein products. For example, Ventria Bioscience produces many non-therapeutic products for use as cell culture reagents (Fischer et al., 2012). Likewise, leaders in the life science/biotechnology space, such as Sigma Aldrich produce and sell plant-derived recombinant proteins for reagent use. In academia, researchers continue to actively pursue RP expression in plants with intended applications outside of therapeutics. Tobacco plants have recently been used for the production of recombinant bovine chymosin for potential use in the milk and cheese industry (Wei et al., 2016). Cellulases have been expressed in corn for use as reagent enzymes or in textile or pulp processing (Hood et al., 2014). The breadth of applications for plant-derived recombinant proteins in both therapeutic and non-therapeutic industries demonstrates the far-reaching utility of molecular pharming.

As the recombinant protein product industry matured and turned its focus to therapeutics, the demand for high final purity products (>99 %) intensified. This demand provided downstream processors the opportunity to design and implement extraction and purification

operations that were perhaps more costly but produced a higher quality product. The new high cost–low yield product model allowed for the implementation of more complex DSP operations but also highlighted the need for efficiency in mitigating recurring challenges in DSP.

Plant expression platforms and implications on downstream processing

The plant platform for recombinant protein products

Generally speaking, the plant platform is considered disruptive, that is, outside the realm of accepted technologies for the production of pharmaceutical products. However, recent PMP successes have encouraged public acceptance and manufacturer inclination for new PMP products. Plants have traditionally been used as bioreactors for lower value, non-pharmaceutical products such as industrial enzymes and secondary metabolites. With the commercialization of these non-pharmaceutical products, academic researchers and companies demonstrated competency and encouraged investments to pursue new pharmaceutical and therapeutic products. Increased development of RP for therapeutic applications such as plant-based vaccines instead of mammalian-derived vaccines additionally emphasized the role of DSP for molecular pharming as a whole. Major plant-based expression systems fall within three categories: leafy tissues, seeds, and bioreactor-based systems. Tobacco is the most common leafy tissue used for PMPs but alfalfa, soybean, and lettuce have also been used (Liew and Hair-Bejo, 2015; Spök and Karner, 2008). Common seed expression systems include corn, rapeseed, rice, and soybean while bioreactor-based systems include rice, tobacco, and carrot cell culture, *Lemna* fronds, and plant-like microalgae (Huang and McDonald, 2012; Wilken and Nikolov, 2012a, 2012b; Xu et al., 2011).

Leafy tissues, seeds, and bioreactor-based systems each have advantages and disadvantages regarding recombinant protein expression and DSP. As a whole, plant-based

systems are inexpensive and scalable. They also benefit from transient expression and natural bioencapsulation to retain RP integrity. However, each expression system presents unique DSP challenges due to a variety of native plant molecules that may interfere with extraction, stability, and purification, of a recombinant protein. For example, leafy tissues naturally contain numerous proteases and phenolics/pigments that interfere with extraction, affect recombinant protein activity, and/or reduce binding capacity or resin lifecycles of chromatography-based capture steps. Similarly, seeds can contain high levels of phytic acid that complicate chromatography-based capture or purification steps in DSP. Bioreactor-based expression systems can likewise present with the aforementioned impurities but also contain polysaccharides or host cell proteins (HCP). Polysaccharides are known to increase the viscosity of the cell suspension or lysate during DSP while HCP can cause adverse or antigenic effects if not completely removed during product purification (Farrell et al., 2015). Thus, while some of these challenges including proteases, polysaccharides, phenolics and pigments, phytic acid, and proteins derived from the host are not unique to the plant platform, strategies for addressing these critical impurities in the DSP efforts of plant products are largely absent from existing literature. The following sections investigate the advantages and disadvantages of each plant platform for the expression of recombinant proteins and highlight recent successes.

Leaf-based systems

Leafy tissues are often utilized as RP expression platforms because they are not a feed or fuel source, with the exception of lettuce, which is currently being explored for administration of an oral vaccine (Su et al., 2015). Furthermore, the agricultural infrastructure, gene transfer, and protein expression constructs for leafy tissue are well established and tissues experience rapid growth and multiple growing cycles. Additionally, leafy tissues have significant advantages with

regard to transient expression over other plant-based systems. In general, RP yields in leafy tissue are much higher than other platforms like seed crops (Twyman et al., 2003). Conversely, in terms of expression, RPs expressed in leafy tissues are prone to instability. Expressing RP in leafy tissue can cause leaf cells to experience necrosis (Phoolcharoen et al., 2011), minimizing expression of functional proteins. After harvest, RP are subject to proteolytic attack (Yao et al., 2015), and thus harvested leaves require immediate desiccation or freezing to retain the biological activity of the expressed proteins (Ahmad, 2014; Obembe et al., 2011). Because leaves must undergo a stabilizing treatment upon harvest, long-term storage prior to DSP can be challenging and expensive.

Tobacco, the most commonly used leafy tissue platform, is advantageous in molecular pharming because there are established transformation methods that result in high soluble protein accumulation. Additionally, tobacco plants produce high biomass per land area and are not a feed/food source (Abiri et al., 2015). Recent PMPs produced in tobacco include monoclonal antibodies (mAb) (Ma et al., 2015; Madeira et al., 2016; Sack et al., 2015), antigens (Gottschamel et al., 2016), and therapeutic enzymes (Abdoli Nasab et al., 2016). While most products like HIV-neutralizing human monoclonal antibody 2G12 (Ma et al., 2015), a protective antigen vaccine against anthrax (Gorantala et al., 2014), and human extracellular superoxide dismutase (hEC-SOD) for treatment of skin disease and arthritis (Park et al., 2016) were produced in tobacco leaves, other organs are also being used. For example, a functional mAb against hepatitis B was expressed in tobacco seed (Hernández-Velázquez et al., 2015) and mAb M12 has been expressed in the tobacco hairy root (Häkkinen et al., 2014).

Seed-based systems

Seeds are an attractive expression platform for RP. Seeds experience a high level of protein accumulation, naturally express less native proteins and phenolic compounds, and confer long-term protein stability during harvest, storage, and transportation (Yao et al., 2015). These characteristics maximize extractable RP while minimizing exposure to potentially degrading proteases. For example, Ventria Bioscience has successfully expressed recombinant human serum albumin (rHSA) at levels of ~10 % TSP that after extraction can be increased to over 80 % of TSP (Huang et al., 2012; Sheshukova and Wilken, 2018). Seeds also have a naturally lower bioburden than leafy tissues. The dormant nature of seeds allows for the decoupling of upstream and DSP and physical separation of the production fields and processing operations (Boothe et al., 2010; Wilken and Nikolov, 2012b).

Seed expression platforms like corn, rapeseed, rice, and soybean have been used for the production of many PMP products including vaccine subunits (Moravec et al., 2007), cytokines (Fujiwara et al., 2016; Kudo et al., 2013), mAbs (Rademacher et al., 2008), and other therapeutic molecules. The endosperm of rice and corn are common sites of protein expression and accumulation. In rice, the endosperm is composed of two sites for accumulation and storage of RPs: the endoplasmic reticulum-derived protein bodies (PB-1) and protein storage vacuoles (PB-II) (Wakasa et al., 2015). HIV-neutralizing antibody 2G12, recombinant AAT (alpha-antitrypsin), a serine protease inhibitor (Zhang et al., 2013), and fibroblast growth factor (FGF-2) (An et al., 2013) have been expressed in the rice endosperm. Interestingly, rice has also been used for the expression of cedar pollen allergens for use as an oral allergy vaccine (Wakasa et al., 2015). In corn, the β subunit of the heat-labile enterotoxin of *Escherichia coli* (LT-B) has been expressed in the endosperm (Juárez-Montiel et al., 2015). Other seed platforms like rapeseed

have been used for the expression of interferon gamma (IFN-γ) (Bagheri et al., 2010) and a hepatitis C virus core protein (Mohammadzadeh et al., 2015). Soybean protein storage vacuoles have been used for the expression of human growth hormone (hGH) and human coagulation factor IX (hFIX) to treat Type B Christmas disease (da Cunha et al., 2014).

Bioreactor-based systems

Bioreactor-based expression platforms are the most similar to the traditional mammalian/microbial cell lines currently used to produce therapeutic RPs. Additionally, this platform does not have many of the regulatory restraints associated with terrestrial plants. Thus, from a regulatory stance, RPs expressed in these platforms could translate most readily into marketable products. The production schemes are cGMP-compatible and provide consistency between batches that is not always feasible between harvests of leafy tissue or seeds (Xu et al., 2012). The media used to culture plants or microalgae do not contain many of the proteins or serum components that can complicate DSP. Bioreactor platforms allow for genetic engineering to secrete the expressed RP into the culture media, thus eliminating the extraction and clarification steps of DSP. Conversely, the main disadvantages of bioreactor expression systems are low yields due to the genetic instability of the cell culture and the very low or dilute concentration of any secreted RPs. Thus, any expression of PMPs in bioreactor systems would benefit from stable transformation lines and standard operation procedures for culturing.

Bioreactor-based expression systems including tobacco BY-2 suspension cells (Reuter et al., 2014; Santos et al., 2016), rice cell culture (Bandehagh et al., 2016; Kuo et al., 2013), carrot cell culture (Huang and McDonald, 2012; Xu et al., 2011), Lemna fronds (Firsov et al., 2015; Nguyen et al., 2012), and microalgae (Gimpel et al., 2015; Specht and Mayfield, 2014) have been used to express vaccine components and other therapeutic molecules. Tobacco BY-2

suspension cells have expressed interleukin-10 (Bortesi et al., 2012; Kaldis et al., 2013), neutralizing anti-HIV-1 antibodies (Sack et al., 2007), and human serum albumin (Sun et al., 2011) while rice cell culture has been used to express interleukin-12 (Shin et al., 2010) and human serum albumin (Liu et al., 2015). Carrot cell culture is most widely known for its expression of recombinant glucocerebrosidase, which was the first FDA-approved PMP for human use. *Lemna* fronds have expressed IgG1 and recombinant IFN-α2b for the treatment of Non-Hodgkin's lymphoma and hepatitis C, respectively (De Leede et al., 2008; Wilken and Nikolov, 2012b). Additionally, avian influenza H5N1 hemagglutinin (HA) protein has been expressed in a *Lemna* isoleucine autotroph (Nguyen et al., 2012). The microalgae, *Chlamydomonas reinhardtii* is the most extensively studied for the expression and accumulation of RPs due to the vast knowledge about its genetic manipulation and metabolism (Rasala and Mayfield, 2015; Yusibov et al., 2016). Expressed proteins in *C. reinhardtii* include the E7 protein of Human Papillomavirus vaccine (Demurtas et al., 2013), *Plasmodium falciparum* surface protein (Pfs25) fused to the β subunit of the cholera toxin (CtxB) (Gregory et al., 2013), Angiotensin II fused to Hepatitis B virus capsid antigen (HBcAg) for hypertension vaccine (Soria-Guerra et al., 2014), and the C-terminal antigenic region of the Pfs48/45 antigen for a malaria vaccine (Jones et al., 2013).

The examples of RPs previously mentioned are certainly not an exhaustive list of the expression capability of plant platforms. The diversity and extensive application of PMPs are growing with the successful transformation of new plant species and development of new delivery methods for treatment purposes. The expansive nature of molecular pharming and PMPs only further necessitates new developments in DSP for purification and recovery of products.

Although the different plant expression platform categories are defined, they each require an individually designed and executed DSP operation scheme.

The downstream process

While the field of molecular pharming addressed the upstream challenges of plant transformation and product yield, the DSP of PMPs was not given the same consideration. A typical downstream process includes recovery (harvest, pre-processing, extraction, clarification, and pretreatment and conditioning) and purification (capture and polishing) (Figure 5.1). The recovery portion of DSP for the three major systems (leaf, seed, and bioreactor) is the most varied part of the process because methods employed depend primarily on the properties of the expression platform. The overall goal of recovery in DSP is to reduce process volume and remove critical impurities (impurities that impact RP stability or have biological significance). This includes minimizing the feed volume between different unit operations and separating the target protein from impurities like host cell proteins, DNA, viruses, and endotoxins.

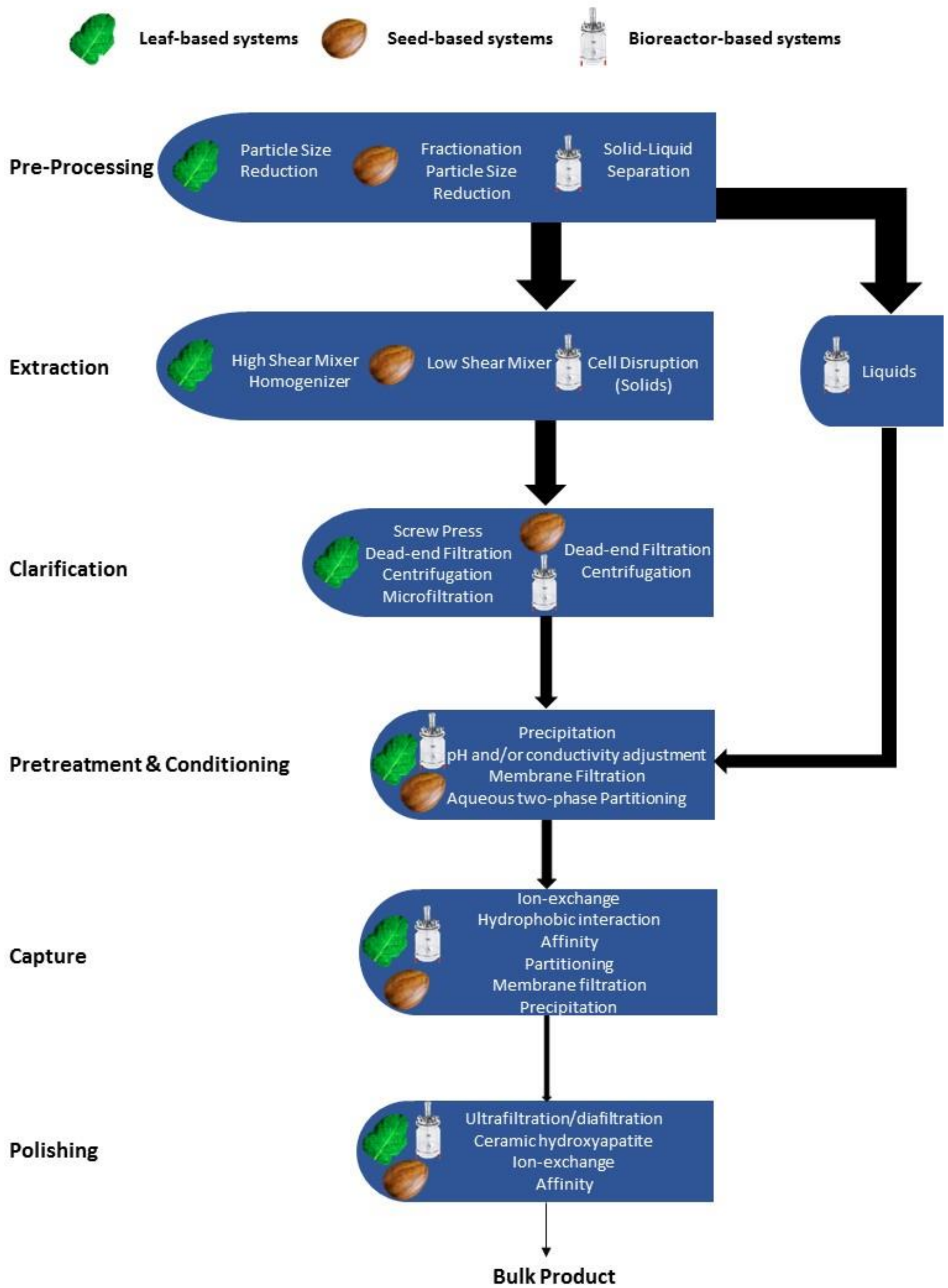

Figure 5.1 Downstream processing diagram with unit operations for recombinant protein purification from leaf-based, seed-based, and bioreactor-based systems.

In general, the first step in the DSP recovery phase is a particle size reduction in leaf and seed-based systems or solid–liquid separation in bioreactor-based systems. These pre-processing strategies are used to isolate the leaf/seed tissue or cell culture containing the recombinant product. Pre-processing is followed by extraction via mixing, homogenizing, or another disruption technique and clarification using centrifugation and/or filtration to produce a clarified extract. The extraction step aims to release the maximum amount of target protein while retaining biological activity and minimizing critical impurities. The downstream processor must consider both the physiochemical properties of the RP as well as those of the plant platform in order to achieve both purposes. Clarification via centrifugation or filtration serves to remove any insolubles or particulates with properties markedly different than the desired product. Clarified extract is conditioned for compatibility with the subsequent capture step by adjustment of pH and/or conductivity and if necessary partitioning and precipitation of the target protein occur before beginning the purification phase of DSP.

Capture steps further reduce the feed volume thus concentrating the target product and increasing purity. Depending on the operation employed, the capture step can also serve to remove critical impurities. Chromatographic (ion-exchange, hydrophobic interaction, affinity) and non-chromatographic (aqueous two-phase partitioning, membrane filtration, and precipitation) methods are employed to purify plant-made RPs. The final step of purification is polishing with the most common processes including chromatography followed by ultrafiltration/diafiltration. Polishing is for achieving the final required product purity and for final product formulation. Recombinant protein products are then commonly crystallized or lyophilized for long-term storage and transportation.

Recurring challenges in downstream processing

Although the general unit operations for DSP of biomolecules like RPs have been defined since the 1980s with the advent of recombinant DNA technology, recurring challenges were only realized with the expansion of expression platforms. For the downstream processor of plant made pharmaceuticals, it was realized by empirical process development that the plant itself presents challenges for purification, and generally speaking they are one of the following six Ps: proteases, proteins derived from the host, phytic acid, polysaccharides, and phenolics and pigments. This complexity can be addressed in upstream processing with secretion of the product into a culture/hydroponic medium or downstream with carefully selected extraction conditions to minimize the presence of interfering impurities. The actual plant can also be genetically modified to co-express stabilizing molecules (protease inhibitors, etc.) (Kim et al., 2008; Komarnytsky et al., 2006) or for subcellular targeting of RP expression to specific cell compartment (Pillay et al., 2013). In *Nicotiana tabacum* (BY-2) cell suspensions, antisense RNA directed toward each of the four classes of proteases reduced proteolytic degradation of IgG1(κ) antibody 2F5 (Mandal et al., 2014). Interestingly, if high expression can be achieved, the cell can naturally produce fewer impurities due to finite metabolic resources. If extraction of interfering impurities cannot be avoided, other mitigation strategies can be employed during DSP. Independent of the type of impurity, strategies include formulating buffers to stabilize the RP, conditioning/pre-treatment of extract for removal of impurity, or removal by selective purification.

Because plants are being used for an increasing number of potential products for pharmaceutical applications, DSP must be able to provide protein that is of the appropriate quality for administration to animals and humans. Since plants are highly complex systems, many unique challenges had to be overcome in order to meet the goal of safe and efficacious

products. To date, downstream processors at both the academic and industry level have performed primarily case-by-case studies that led to the identification of certain molecules or critical impurities that interfere with product extraction and purification. To develop remediation strategies for the identified DSP challenges, individual unit operations were optimized rather than taking a holistic approach to the entire DSP scheme. The general descriptions of DSP communicated in the current literature do not readily convey the wealth of technical experience that has been accumulated from these distinct extraction and purification studies.

In the following section, we distill the challenges into six areas: proteases, proteins derived from the host, phytic acid, polysaccharides, and phenolics and pigments. Each of these areas is presented separately even though these categories may sometimes overlap. Simplifying these challenges into six types of biochemical compounds represents progress in this maturing industry.

The 6Ps

Proteases

The progress that has been made in developing plant-made RP products required overcoming many hurdles. One obvious hurdle is protein expression level. Since expression of a given protein is based not only on how much the plant can make but also how susceptible it is toward degradation, the presence of plant proteases, enzymes which cleave proteins at specific sites, is a potential limitation to high expression of some proteins in plant hosts. From a downstream perspective, proteases can also cause degradation of RPs in extracts and during downstream process steps reducing the yield of intact purified protein. Since proteolytic activity varies with the plant host, the location, and the maturity of the tissue, there is no one size fits all

approach to managing proteolytic activity. Despite this variation, there have been many successes demonstrating that the challenge posed by plant proteases is not insurmountable.

Proteases are named for the amino acid that dominates the active site and are therefore instrumental to their catalytic activity (Barrett, 1994). Over 800 proteases have been identified in the *Arabidopsis* genome (Van der Hoorn, 2008). This genome comprises at least five catalytic types of proteases: serine, cysteine, aspartic, threonine, and metalloproteases (Schaller, 2004). Plant cysteine proteases tend to be most active at neutral pH and are the best known since papain isolated from papaya latex was the earliest plant protease studied (Drenth et al., 1968). Aspartic proteases are most active at low pH (Laing and Christeller, 2004) and thus also tend to be well-recognized in plants since working at pHs near 5 where RuBisCO is less soluble can reveal problems due to these proteases (Stevens et al., 2000). Serine proteases that include trypsin- and chymotrypsin-like activities actually outnumber cysteine and aspartic proteases (Schaller, 2004) . Metalloproteases are less common than the serine, aspartic, and cysteine proteases in plants but show the most diversity in terms of functional roles (Schaller, 2004).

Although they are ubiquitous within plant tissue, certain locations within the plant are known to host a number of proteases. For example, a number of regulatory proteases are found in chloroplasts (Van der Hoorn, 2008), a popular targeting location for expressing some non-glycosylated RPs. Vacuoles contain a great deal of physical space in plant cells so these can also be popular destinations for expressing some RPs despite high proteolytic activity associated with these lytic vacuoles (Palaniswamy et al., 2016). Another type of vacuole, protein storage vacuoles (PSV), have some proteases but they tend to not degrade protein stored there until germination (Schaller, 2004). Apoplastic targeting can result in degradation due to exposure of the RP to proteases all along the secretory pathway and finally in the apoplastic space (Goulet et

al., 2012). Many proteins are targeted to the apoplastic space due to the post-translational processing that occurs along the pathway resulting in proteins that have the full complement of modifications required for proper function. An alternative is to express the protein with the KDEL sequence that targets the protein for retention in the ER. This strategy often results in high levels of protein expression (Ma et al., 2003) but for some proteins, cleaved forms have been reported (Badri et al., 2009; Benchabane et al., 2009a). Many proteins expressed transiently using agroinfiltration are also targeted to the apoplastic space. Agrobacterium, being a plant pathogen, may stimulate higher levels of proteases to be expressed in the plant during transient infection. Aspartic, cysteine, and serine protease activities have been identified in leaf intracellular fluid of *Nicotiana tabacum* (Delannoy et al., 2008). In cell culture systems, proteases are secreted into media where they can degrade some secreted proteins (Doran, 2006; Hellwig et al., 2004).

Proteases can be problematic both during deposition of the protein in plant tissue *(in planta*) as well as during extraction when proteases are released from cellular compartments (*ex planta*). Numerous examples of low expression due to protein instability *in planta* have been reported and the variation that occurs in expression when the same protein is made with different targets within the same plant host is a testament to the fact that the local environment is key to expression strategy (Doran, 2006). Proteolytic degradation is particularly problematic when proteins are secreted in cell culture media (Doran, 2006) by either suspension cells, hairy roots, or whole organisms such as algae, moss, or *Lemna*.

Ex planta protein degradation of RP from homogenized plant tissue is less well-appreciated than *in planta* degradation but generally recognized as problematic especially during the extraction and subsequent purification of protein from green plants. Green tissue is more

proteolytically active compared to seed and therefore must be processed right away or frozen if not used immediately after harvest. In one report, the method of recovering an antibody expressed transiently in *Nicotiana benthamiana* affected the extent of degradation observed (Lombardi et al., 2010). Degradation is not commonly reported in seed systems but some monoclonal antibodies are particularly susceptible and the development of a degraded form during DSP of an antibody from maize seed extract has been reported (Ramessar et al., 2008).

One limitation of using purification tags in a plant host is the presence of protease activity that may cleave the tag prematurely from the RP. If the researchers do not use tag-specific methods to confirm the presence of the tag, the loss of the tag may not be obvious. The loss of purification tags will lead to low recoveries for the affinity chromatography purification step. This was the case reported for a recombinant human asialo-erythropoietin (EPO) *Strep*-tag linked via a TEV protease cleavage sequence at the C-terminus (Kittur et al., 2015). Cleavage occurred despite the use of a plant protease inhibitor cocktail and 1 mM EDTA in the buffer. The authors speculate that the cleavage was related to the presence of an amino acid that is typically cleaved in other hosts based on similar findings by others expressing a multi-sialylated version of human EPO with a C-terminal IgG Fc fragment (Castilho et al., 2013). Likewise, the expression of a single variable antibody domain fragment of camelid origin, called V_HH, was greatly improved through the use of a C-terminal Fc fusion. However, the source of the Fc was critical in determining how much intact fusion protein resulted (De Buck et al., 2013).

There are likely many instances where the commonly used HIS-tag is also removed in plant expression systems. A hook worm vaccine candidate with a C-terminal HIS-tag expressed in *Nicotiana benthamiana* displayed partial loss of the tag when extracted on a kilogram scale (Seid et al., 2015). Western blots specific for both the protein and the tag were performed due to

the fact that a doublet was observed on SDS-PAGE for the starting extract. These subtle observations can easily be overlooked but should be further investigated when low purification recoveries are obtained in tag-specific affinity purifications.

Many strategies have been used to prevent degradation in a variety of different hosts and modes of expression (see reviews by (Mandal et al., 2016) and (Benchabane et al., 2008)). These include the use of protein-stabilizing polymers such as PVP or gelatin in media from plant cell culture or hairy roots (Häkkinen et al., 2014; LaCount et al., 1997; Madeira et al., 2016; Magnuson et al., 1996; Sharp and Doran, 2001; Wongsamuth and Doran, 1997), and the use of buffering proteins such as BSA (James et al., 2000), or HSA (Baur et al., 2005) in culture media. Genetic approaches to reduce the impact of proteases include co-expression of different protease inhibitors in a wide variety of platforms including plant cell culture systems (Kim et al., 2008; Komarnytsky et al., 2006) and transiently agroinfiltrated *Nicotiana benthamiana* (Goulet et al., 2012; Robert et al., 2016).

Just as improvements made to promote expression through the use of protease-deficient strains of *E. coli* occurred some 20 years ago (Meerman and Georgiou, 1994; Murby et al., 1996), modifications are now being made to various plant hosts to reduce proteolytic activity. In one example, antisense RNA directed toward one of each of four different protease families was used to improve monoclonal antibody accumulation in BY-2 cells (Mandal et al., 2014). In tobacco, several cysteine protease genes were identified as possible candidates preventing high accumulation of IL-10 in tobacco. Downregulation of one cysteine protease that localized to the ER improved IL-10 expression somewhat (Duwadi et al., 2015). Progress in identifying the proteases responsible for proteolytic degradation of monoclonal antibodies as well as the

sequences susceptible to proteolysis will aid continued successes in expressing high levels of monoclonal antibodies in plant hosts (Hehle et al., 2015; Magy et al., 2014; Niemer et al., 2014).

Although co-expression of a protease inhibitor or the use of protease deficient host plants might help to prevent some proteolysis during downstream unit operations, these approaches are unlikely to fully prevent degradation of susceptible RPs. The co-expression strategies listed above for preventing *in planta* proteolysis can also target activities responsible for degradation during DSP (Benchabane et al., 2009b; Rivard et al., 2006). In many cases, the activities that caused instability *in planta* are likely to also cause degradation in extracts.

Many of the practices that have become routine for handling plant tissue derive from the desire to prevent proteolytic degradation. Working quickly and at low temperatures to slow enzymatic reactions and inclusion of protease inhibitors are recommended in the extraction of plant proteins (Jervis and Pierpoint, 1989). Seeds have the advantage of not only being a less metabolically active tissue, but they also have moderate levels of endogenous protease inhibitors designed to protect storage proteins so these practices are usually not needed for purification from seeds. In fact, when surveying extraction methods used for RPs expressed in plants, one difference that was apparent was the inclusion of protease inhibitors in the extraction buffer used for the majority of proteins expressed in leaf tissue and suspension cell culture but not for those expressed in seeds (Wilken and Nikolov, 2012a, 2012b).

Many different types of protease inhibitors have been used to reduce proteolytic degradation during the purification of RP from plants. Inhibitors range from those targeting cysteine, serine, aspartic, and metalloprotease-specific inhibitors with those targeting the combination of cysteine and serine proteases such as phenylmethylsulfonyl fluoride (PMSF) and chymostatin being recommended (Benchabane et al., 2008). PMSF is toxic but quickly

inactivated in aqueous solution making it useful in the case of material that needs to be held for a period of time before it can be processed. It does irreversibly inhibit the serine and cysteine proteases it targets. A review of many plant expression publications shows most work being done with PMSF at a concentration range of between 0.1 mM (Shaaltiel et al., 2007) and 2 mM (Millán et al., 2003). Leupeptin, which also targets serine and cysteine proteases is another popular choice, typically used at 10 µg/mL (Fulton et al., 2015).

The use of macromolecular inhibitors such as aprotinin (also called bovine pancreatic trypsin inhibitor or BPTI) during DSP can be tricky due to the need to remove the inhibitor later. The use of protease inhibitor cocktails designed specifically for use with plant extraction is quite common in bench scale work. One limitation in using these cocktails is that they may contain macromolecular inhibitors in addition to small molecule reversible and irreversible inhibitors. When they are effective at preventing degradation, it might be difficult to dissect which components were critical and which were not needed. This information is important in adopting inhibitor use in a manufacturing strategy where each additive must be justified and then removal during purification validated.

Some protease inhibitor cocktails contain EDTA which is effective in inhibiting metalloproteases. Use of a protease inhibitor cocktail or simply 5mM EDTA were found to be effective at preserving a full-length plasminogen activator from vampire bat (DSPAα1) expressed in tobacco BY-2 cells (Schiermeyer et al., 2005). The use of EDTA is feasible on a manufacturing scale and may be justified in certain cases. Bench-scale use is common with reported concentrations ranging from 0.5 mM (Millán et al., 2003) to 20 mM (Shaaltiel et al., 2007).

Despite the concerns with proteolytic activity that may impact the expression and/or DSP of RP, there are many examples of successful expression of RPs targeted to compartments within cells that are known for their proteolytic activity. For example, recombinant β-glucocerebrosidase is made in storage vacuoles of carrot cells (Shaaltiel et al., 2007). The stability of this enzyme is probably due to the fact that it normally functions in lysosomes which could be considered the mammalian cell equivalent of plant lytic vacuoles. Vacuolar targeting was used in the case of the heat labile enterotoxin B (LTB) of *E. coli* where expression reached 12% of TSP (Streatfield et al., 2003). A chloroplast expression success story is that of human serum albumin. This protein is known to be susceptible to proteolytic degradation and has been difficult to express in plant systems at high levels. When targeted to chloroplasts however, expression as high as 11 % of TSP was obtained because it formed inclusion bodies protecting it from proteolytic degradation (Millán et al., 2003).

Proteolytic activity can also be advantageous. For example, bovine trypsin expressed in maize seed as a zymogen to prevent plant health problems could not be identified unless extracted at very low pH (Woodard et al., 2003). This finding was true for other protease zymogens expressed in maize and not unique to trypsin. There are no doubt other cases where processing of proteins by plant proteases could be exploited.

Proteins derived from the host

HCP, also known as native proteins are bioprocess-related impurities of great consequence in the extraction and purification of plant-derived RPs. The protein content of plant species varies widely from approximately 2 to 40% (w/w) (Menkhaus et al., 2004). These proteins exhibit a wide range of immunological and physiochemical properties. Immunologically, HCP can cause adverse or antigenic effects when ingested by the patient or

end consumer (Farrell et al., 2015). Physicochemically, the diversity of HCP can be observed in varied molecular mass, isoelectric point, hydrophobicity, and structure (Gronemeyer et al., 2014). Separation of host cell proteins from the RP is a primary focus of extraction method development and clarification procedures. Key challenges to address regarding native proteins are the abundance of such impurities and the similar physicochemical properties of these impurities to the RP.

Although each of the plant platforms described in this chapter naturally produce HCPs, the effect of HCP on the target product is largely dependent on the abundance and location of the impurity in the host tissue, required extraction conditions to solubilize the RP, and the order of operations within DSP for which the reduction and/or removal is attempted. Operations impacted by HCP include filtration, capture, and purification. In particular, the capture step of DSP including various chromatographic operations are prone to HCP interference. The main mechanisms by which HCP are known to persist into the product stream of chromatographic operations include product association or interaction with the target protein or co-elution after binding to the chromatographic ligand or resin backbone (Levy et al., 2014).

In leafy tissues, the best known and most widely studied HCP is Ribulose-1,5-bisphosphate carboxylase/oxygenase or RuBisCO. In plant tissue, RuBisCO comprises 30–50% of TSP (Buyel et al., 2013; Carmo-Silva et al., 2015). Extraction and purification of RP expressed in leaves often suffers from interference by RuBisCO due to its high protein content. In DSP, filtration operations can be impacted as HCP can cause aggregation of target products like mAbs (Tarrant et al., 2012). Additionally, RuBisCO affects capture and purification, primarily through a reduction in chromatography column binding capacity (Buyel and Fischer, 2014).

In seeds, including corn, soybean, and rice, the most common HCP are albumins, globulins, prolamins, and glutelins. It is important to note that although these proteins can be considered HCP, in the case of albumins in particular, recombinant forms are often expressed as the target protein product in plant seeds. Since DSP deals largely with extraction buffers with varying concentrations of sodium chloride, water soluble and salt soluble proteins pose the greatest risk of interfering with target product extraction and purification. Albumins and globulins are globular proteins soluble in salt-free water and dilute salt-water solutions, respectively (Menkhaus et al., 2004).

Recombinant protein expression in bioreactor-based systems is unique in that exposure and risk of interference of HCP to the target protein depends on the expression construct. Target RP expressed intracellularly are subject to HCP interactions much the same as in leafy tissues. When the RP in a bioreactor system is constructed to secrete into the media, HCP exposure can be markedly reduced. In the case of secreted RP, additional threats such as proteolytic degradation must be considered. Secreted RP can be more unstable or susceptible to protease degradation as stabilizing proteins are not present or are in limited quantities. It is in the presence of degrading proteases that the stabilizing effect of native or HCPs is advantageous. For additional information regarding protease properties and controlling for proteolytic degradation of RP products, refer to the “Proteases” section.

HCP is often a critical consideration for the development of optimal RP extraction methods and/or subsequent clarification steps. In particular, reduction of HCPs in aqueous extracts can be achieved by careful selection of extraction pH, ionic strength, temperature, and buffer composition, which is critical to process efficiency (Wilken and Nikolov, 2012b). Several studies have shown that pH has a particularly strong effect on protein extractability from

transgenic plant tissues and, in general, the amount of host cell protein extracted increases with an increase in extraction pH. For example, the amount of TSP (native protein plus the recombinant protein, human lysozyme) in transgenic rice extracts increased from 5–6 mg/g flour at pH 4.5 to 7 mg/g at pH 6.5, 8 mg/g at pH 7.5, and 15–16 mg/g at pH 10 (Wilken and Nikolov, 2010). For extraction of total protein from sugarcane, increasing the pH from 4.5 to 7.5 increased the amount of TSP in extract by nearly 10-fold (Barros et al., 2013). In another study, careful selection and control of extraction conditions resulted in significant reductions (4–5-fold) in extracted, indigenous protein from *Lemna minor*.

Previous work has demonstrated that for optimal recovery and purification of RPs expressed in transgenic plant tissue, protein extraction at a pH of 4 to 6 is desired with or without the salt. The specified pH range is ideal because the isoelectric points of a majority of soluble, native plant proteins fall within this range, which minimizes their solubility. Thus, the resulting extract has a greater RP (fraction) concentration expressed as percent of TSP. Thus, selection of the optimal extraction conditions is often based on the ratio of RP concentration to TSP concentration. Increasing salt, most commonly sodium chloride, can increase the presence of native protein, especially for plants with high content of globulins. In some cases, use of high ionic strength buffers is required to solubilize a RP from the plant tissue. However, the efficiency of subsequent DSP operations can be reduced. For example, ionic strength can drastically reduce the binding capacity for a subsequent ion exchange capture chromatography step. Also, if precipitation of native proteins (from high pH extracts) is needed, the presence of high levels of salt can prevent protein aggregation and impede precipitation of native protein (Barros et al., 2013). However, ionic strength can be reduced through dilution or through buffer exchange using membrane filtration.

Independent of the plant platform used for RP production, HCP removal is most commonly accomplished via a pretreatment strategy either early on in DSP operations or by separation during chromatography operations. HCP concentrations can be reduced by >90 % by isoelectric precipitation, polyelectrolyte precipitation, aqueous two-phase separation/partitioning, blanching, or heat precipitation (Buyel et al., 2015). It is important to note that the RP must be thermostable for use of blanching and heat precipitation methods. Membrane filtration is an alternative strategy for removal of HCP prior to further purification.

Common precipitation methods for HCP removal include polyelectrolyte precipitation (with polyethylene glycol (PEG) or polyethylenimine (PEI)), isoelectric precipitation, and ammonium sulfate precipitation. PEG precipitation at pH 5.0–6.0 allowed for the selective removal of plant HCP which were then filtered out of the product stream (Giese et al., 2013). An additional study by Arfi *et al.,* (Arfi et al., 2016) similarly depleted RuBisCo using 12–16% w/v PEG resulting in 70–92% HCP removal. In both instances, increasing PEG concentration was determined to be correlated with decreasing HCP concentration. Similarly, treatment of plant extracts with diafiltration and charged polyelectrolyte precipitation has been demonstrated for the removal of native proteins from *Nicotiana benthamiana* (Fulton et al., 2015). Diafiltration with a 10 kDa MWCO prior to PEI precipitation also reduced the amount of polyelectrolyte necessary for reduction and removal of host cell impurities. For example, diafiltration prior to precipitation removed 80% of native proteins using 0.36 mg PEI/mg TSP compared to 60% removal using 800 mg PEI/mg TSP without prior diafiltration.

Isoelectric precipitation is another effective technique for reduction of HCP from transgenic plant extracts, but requires that the isoelectric point of the RP be sufficiently different from that of HCP. Based on the isoelectric points of native proteins, adjustment of high pH

extracts to pH <5.0 has been suggested for the precipitation of native proteins from transgenic extracts (Azzoni et al., 2002; Buyel et al., 2015; Buyel and Fischer, 2014). TSP decreased up to 80 % in transgenic sugarcane extract (Barros et al., 2013), >70% in transgenic rice extract (Wilken and Nikolov, 2010), and >70% in transgenic *Lemna minor* extracts (Woodard et al., 2009) using isoelectric precipitation with minimal or no loss of RP. Precipitation has also been shown to improve subsequent depth filtration through a reduction in filtration time and also decrease backpressure during loading of chromatography columns (Woodard et al., 2009).

Low saturation (25–30%) ammonium sulfate solutions have been used for pre-treatment to reduce concentrations of native plant proteins, protein aggregates, and cell debris (Garger et al., 2000; Lai et al., 2010). Munjal *et al.* (Munjal et al., 2015) evaluated ammonium sulfate concentrations from 0.5 to 2 M for removal of host cell protein (primarily RuBisCO) from transgenic *Chlamydomonas reinhardtii* cell lysates and demonstrated a linear increase in HCP removed (approximately 10–95% removal) with increasing ammonium sulfate concentrations up to 2.0 M. However, precipitation with 1 M ammonium sulfate was selected as a compromise between HCP removal and product loss (approximately 50% loss at higher concentrations of ammonium sulfate). In addition, chlorophyll was reduced almost 70% with 1 M ammonium sulfate. Park *et al.* (Park et al., 2015) used ammonium sulfate for precipitation of native protein from transgenic tobacco leaf extract (with expressed colorectal cancer vaccine candidate, GA733-FcK). The concentration of ammonium sulfate was varied (15–80%) and impact on TSP and RP was measured. Concentrations of 40–60% precipitated the most protein but the amount of GA733-FcK that co-precipitated increased with ammonium sulfate concentration.

Additional solutions used beyond those described above include protamine sulfate (PS) and phytate (Buyel et al., 2015). PS precipitation of supernatant obtained from freeze dried and

homogenized soybean leaves determined that a 0.1% protamine sulfate was adequate for depleting RuBisCo and enriching the content of other target proteins (Kim et al., 2013).

Further strategies for removal of HCP include heat precipitation of leaves and low pH conditions combined with ion-exchange chromatography (Buyel et al., 2014; Menzel et al., 2016). Blanching tobacco leaves between 62°C and 80°C followed by homogenization and clarification removed more than 90% of HCP (Buyel et al., 2014). Likewise, heat precipitation of tobacco leaves expressing malaria vaccine candidates prior to extraction reduced HCP levels more than 80% and maintained the stability of target proteins at temperatures up to 80°C (Menzel et al., 2016).

Partitioning of native or host cell proteins from target proteins via an aqueous two-phase system (ATPS) can also be used for protein characterization or selective extraction when followed by gel filtration and chromatographic operations. ATPS partitioning efficiency is largely influenced by the hydrophobicity and charge of proteins (Gu and Glatz, 2007). With hydrophobicity dominating the partitioning effect, ATPS have been employed to recover transgenic proteins in corn, tobacco, alfalfa, and soybean (Aguilar and Rito-Palomares, 2008; Ibarra-Herrera et al., 2011; Lee and Forciniti, 2010; Ross and Zhang, 2010). Transgenic proteins in corn have been recovered to levels of 90–95% with up to 14-fold purification (Gu and Glatz, 2007). Likewise, tobacco and soybean RPs have experienced a level of recovery greater than 60% with greater than 4-fold purification increases. In the ATPS, native proteins precipitate to the phase interface while target proteins partition into the top phase. This product separation allows for simplified DSP operations and reduced burden during purification.

Membrane filtration of pre-processed plant biomass has also been used for the reduction of native protein content and enrichment of target proteins. Selectivity and protein fractionation

using ultrafiltration depends on operating conditions such as transmembrane pressure (TMP) and flow rate, membrane properties such as pore size, configuration, and material (commonly polyethersulfone (PES), polysulfone (PS), or cellulose acetate), and solution properties (pH, ionic strength, and composition). Ultrafiltration is easy to scale and generally less expensive than some other HCP removal strategies such as ATPS and less denaturing than some precipitation methods (Aspelund and Glatz, 2010). Additional advantages are the possibility of RP concentration and buffer exchange for conditioning purposes prior to additional downstream processes like chromatography. This method can be used when the molecular weight of RP is sufficiently larger in relation to a majority of the native protein (HCP removed through permeate stream) or when significantly smaller (HCP retained while RP recovered in permeate) as highlighted by the following collagen and bovine lysozyme cases. Aspelund and Glatz (Aspelund and Glatz, 2010) separated recombinant human collagen (265 kDa) from corn HCP using a 100 kDa PES flat-sheet membrane and measured the resultant sieving coefficients (concentration in the permeate/concentration in the retentate) for collagen and HCP. The impact of corn tissue (germ and endosperm) extracted on ultrafiltration performance was also evaluated. Results indicated that TMP and crossflow rate did not have a significant effect on the sieving coefficient of HCP. The authors did not observe a significant effect of filtration pH on HCP sieving but did observe an effect between filtration pH and extracted corn tissue (germ vs. endosperm). A reduction in HCP sieving from germ extracts, as compared to endosperm extracts, was due to the greater concentration of large molecular weight proteins in the germ extract. HCP removal of 96% and 91% from endosperm and germ extracts, respectively, were reported with purification factors from 4–6-fold. Additional observations include that filtration at a pH near the HCP isoelectric points resulted in fouling, which reduced flux.

Barros *et al.,* (Barros et al., 2013)used membrane filtration to separate sugarcane HCP from bovine lysozyme (BvLz) and for product concentration. Ultrafiltration was evaluated with pH 4.5 and pH 6.0 extracts with 150 mM NaCl using a 100 kDa PES flat-sheet membrane. BvLz yields were similar for both extracts indicating membrane filtration was not significantly affected by pH and extract composition. However, a 2-fold purification was observed for pH 6.0 extract while little to no purification was achieved with pH 4.5 extract. Additional HCP removal and BvLz concentration was possible by processing the 100 kDa permeate stream using a 3 kDa regenerated cellulose membrane. Approximately 65% of HCP was removed from both 4.5 and pH 6.0 permeates (from previous 100 kDa filtration).

The presence of host cell proteins or native proteins at the DSP stage of plant-made pharmaceuticals poses a great challenge to the stability and integrity of RPs. Dependent on HCP location in the plant and abundance, processors must consider upfront pretreatment (removal) strategies or techniques to reduce HCP impact if extraction is unavoidable. Many methods for leafy, seed, and bioreactor (cell culture) HCP removal have been studied and analyzed for clearance efficiency including precipitation (polyelectrolyte, isoelectric, heat, ammonium sulfate, etc.), aqueous two phase partitioning, and membrane filtration. Leafy HCP have also been addressed using heat precipitation or blanching techniques but these techniques require the RP to be thermostable. Bioreactor-based HCP removal techniques are similar to those of leafy tissue HCP except in the case of RP secretion into the media. In these cases, processors must recognize additional challenges such as proteolytic degradation and a lack of stabilizing proteins on subsequent RP recovery.

The vast diversity of HCP in plant species has encouraged the use of powerful tools in designing efficient and effective extraction and purification operations. Design of experimental

approaches and high throughput screening efforts serve to determine conditions maximizing RP recovery and identify HCP properties or interactions causing RP instability or degradation.

Phytic acid (salts)

As indicated in the "Protein derived from the host" section, extraction in acidic buffer is often ideal to minimize native protein solubility which reduces the subsequent purification burden. This extraction strategy works well with acidic, neutral, and basic RPs expressed in green leafy tissue such as tobacco and *Lemna minor* but not as well when RPs are expressed in transgenic seed. Seeds (cereal and oilseeds alike) contain significant amounts of phytic acid (phosphate storage) which is easily co-extracted with the protein in this pH range. Phytic acid (1,2,3,4,5,6 hexakis (di-hydrogen phosphate) myo-inositol) is the primary storage form of phosphate that accounts for a significant percentage of total phosphorus. For example, phytic acid accounts for 80% of the total phosphorus content of brown rice (Juliano, 1980). Structurally, phytic acid is the phosphoric ester of inositol (hexahydroxycyclohexane) with 12 ionizable protons having pKa values from 1 to 12 (Turner et al., 2002). Therefore, negatively charged phytic acid can electrostatically bind positively charged metal and non-metal cations, amino acids, and proteins (Weaver and Kannan, 2002) or indirectly associate with negatively charged proteins, minerals, and starch through polyvalent cations (Crea et al., 2008; Dendougui and Schwedt, 2004). Cereals and oilseeds typically contain 1–2% phytate by weight but some varieties can contain as much as 3–6% (Cheryan and Rackis, 1980). Except for soybean, phytic acid is concentrated within crystalloid substructures called globoids (Cheryan and Rackis, 1980) that are localized within specific components of seeds. Corn contains 0.89% phytic acid (dry basis) with 88% concentrated in the germ (O'dell et al., 1972) while brown rice typically contains 0.84–0.99% of phytic acid which is concentrated in the bran and aleurone layer (Reddy,

2002). Phytic acid exists primarily as potassium, magnesium, and calcium mixed phytate salts (Crea et al., 2008). Monovalent cation-phytic acid complexes, such as potassium phytate and sodium phytate, are soluble over a wide pH range while those of divalent cation-phytic acid complexes are least soluble at neutral pHs. Solubility is also impacted by the cation to phytate ratio (more soluble at low cation to phytate ratio). Binding of cations depends on ionic strength and, thus, selected extraction conditions (pH and ionic strength) will greatly influence the amount of phytic acid solubilized during extraction. The selected buffer ions and composition are also important as they can promote or suppress precipitation, especially with pH adjustment.

Phytic acid is well studied in food systems since it chelates micronutrients and prevents bioavailability of these nutrients in humans and animals which lack phytase in their digestive tract (Gupta et al., 2015). More recently, new extraction and purification techniques have been investigated based on its beneficial effects on human heath (Canan et al., 2011). These techniques can be exploited to reduce phytic acid concentrations in process streams during purification of RPs from seed-based systems and to minimize the impact of phytic acid on process efficiency. It has been demonstrated that the presence of phytic acid or phytate in extracts interferes with designing optimal purification processes of basic and acidic proteins by limiting the efficiency of ion exchange capture and purification steps; a type of resin that is frequently used in the biotechnology industry. Phytic acid can also impact chromatography operations by formation of complexes with proteins or minerals that can lead to blockage of resin pores, chromatography columns, and increased backpressure. In fact, a reduction of 85% of dynamic binding capacity has been observed. Clearly, this drastic impact on column capacity, which is directly linked to manufacturing costs, and purification could greatly impact overall process efficiency. Since phytic acid is compartmentalized in most cereals and oilseeds, it is

possible to minimize the presence of this compound through fractionation (i.e. removal of the aleurone layer of rice seed) or targeted expression of the RP to a separate compartment (i.e. endosperm of corn). However, detrimental impacts of phytic acid are likely not sufficient to be the primary justification for modifying the expression strategy.

Genetic improvements through generation of low phytic acid mutants and pre-treatment methods, such as milling and soaking, are potential methods for the reduction of phytic acid in plant systems prior to RP extraction. Phytate concentrations in the extracts depend on its concentration in the seed which can translate to up to 1–2 mg/mL in aqueous extracts. Once extracted, phytic acid can be removed through precipitation (as phytate salts) or by using disposable anion exchange resins specially designed for adsorption of small molecules. Various methods to minimize the detrimental impacts of phytic acid during processing proposed in the literature and from prior experiences are discussed.

In some cases, the amount of phytic acid can be reduced prior to RP extraction through fractionation of seed to remove portions where phytic acid is prevalent. Abrasive milling of rice is an effective strategy for removal of phytic acid from rice since phytic acid is concentrated in the outer portions, the bran and aleurone layer, of the rice seed. The degree of milling greatly impacts the minerals and phytic acid remaining in rice seed as more than 98% of total phytic acid can be located in 20–25% of the outer portion of the kernel (Liang et al., 2008). The duration of milling can be optimized for phytic acid and mineral removal. Liang *et al.* (Liang et al., 2008) achieved 87–91% phytic acid removal after 3 min and >95–99% after 5 min of milling three different rice cultivars. Protein accumulates in the germ so the bran and aleurone removal is a feasible method to decrease phytic acid.

Phytic acid can also be leached out of the starting material, such as corn germ, prior to protein extraction. Wilken and Nikolov (Wilken and Nikolov, 2016) evaluated phytic acid removal by soaking dry milled corn germ as part of a Germ Wet Milling (Lohrmann et al., 2015) process, designed to produce high purity germ for food protein products by releasing or leaching undesirable impurities such as phytic acid and starch. The removal of phytic acid during soaking would reduce the amount of phytic acid co-extracted during the subsequent protein extraction step. The impact of pH, temperature, and time on phytic acid and protein leaching was investigated as retention of protein would be desired to retain the product within the germ while separating the phytic acid in the leachate. Depending on soak time, the amount of phytic acid leached was up to 23% of the initial phytic acid content at pH 4.5 and up to 20% for soaking at pH 7 over 6 h in lab-scale experiments. Also, soaking at high temperature (50°C rather than 25°C) enhanced phytic acid extractability. The ratio of TSP to phytic acid in the soak water was also determined for both soak conditions. To retain protein (to minimize RP loss) while effectively leaching phytic acid, the ratio of phytic acid to total soluble protein in the soak water should be maximized. Increasing the soak time increased the phytic acid to TSP ratio for pH 4.5 soak but decreased the ratio for pH 7 soak. Thus, pH 4.5 appears to be the best choice (compared to pH 7) unless soak time is limited to 1.5 h or less.

Phytate salts can be removed from low pH extracts by precipitation using pH adjustment. For example, >80% of calcium phytate is soluble at pH 4.5 but when pH adjusted to pH 6, 5–35% is soluble and at pH 7, 0–5% is soluble, depending on the equivalent ratio of metal to phytate phosphorus (Cheryan and Rackis, 1980). Magnesium phytate is highly soluble (>80%) below pH 7 but is <10% soluble at pH 9. Canan *et al.* (Canan et al., 2011) evaluated the precipitation of phytic acid from rice bran extract by pH adjustment to pH 7–9 (with 0.5 pH unit

increments) using 1.5 M Na_2CO_3 or 4 M NaOH. The best conditions for recovery of phytic acid was from pH 8 to pH 9 using 1.5 M Na_2CO_3 for pH adjustment or pH 9 using 4 M NaOH for pH adjustment. Since phytic acid exists as a mixture of phytate salts and some, such as sodium and potassium phytate, remain soluble over a wide pH range, pH adjustment alone may not precipitate sufficient quantities of phytic acid. To precipitate additional phytic acid, addition of calcium hydroxide for pH adjustment rather than or in addition to NaOH can enhance removal. To keep phytate salts soluble in alkaline environments (if formation of precipitate is not desired), an alternative strategy is to add chemical compounds such as ethylene diamine-tetra-acetic acid (EDTA) to an extract. EDTA is a chelator that competitively binds to the metal cations. The formed metal cation-EDTA complexes are soluble, which decreases the presence of insoluble phytate salts. Use of citrate buffer (and other chelating buffers) for extraction can also prevent precipitation of phytic acid with pH adjustment to neutral or basic pH.

An alternative strategy for phytic acid is to use inexpensive anion exchange resins designed for small molecule removal. Phytic acid maintains a negative charge at typical working conditions for extraction and purification as the most acidic pKa of phytic acid is approximately 1.8–2.2 and the most basic pKa is between 9.2 and 9.7 (Barre et al., 1954; Evans et al., 1982). Thus, anion exchange chromatography is commonly used for adsorption of phytic acid from plant extracts. Raboy and Dickinson (Raboy and Dickinson, 1984) used Dowex 1-X8 resin for adsorption of phytic acid from soybean flour extract. Thiel *et al.* (Thiel et al., 2015) used Purolite A200, an industrial grade, polystyrene crosslinked with divinylbenzene strong anion exchange resin with a quaternary ammonium functional group, for adsorption of phytic acid from rapeseed meal extract. Adsorption at pH 2 and pH 12 were evaluated and pH 12 resulted in a higher

percent of phytic acid binding (>90%) compared to that at pH 2 (approximately 55%). Adsorption at pH 12 drastically reduced the amount of protein that was co-bound to the resin.

Our previous experiences working with RP purification using transgenic seeds (rice and corn) highlights various phytic acid challenges and strategies to alleviate interference. The purification of rice-seed-expressed human lysozyme (basic protein) was significantly impacted by phytic acid. Initial work independently optimized extraction and cation exchange adsorption. High purity human lysozyme (95%) was achieved in a single-step cation exchange purification process by combining pH 4.5 extraction (highest human lysozyme to native protein ratio) with pH 6 cation exchange adsorption. However, the resin binding capacity was reduced 80% compared to that for pure human lysozyme while other adsorption conditions only reduced the binding capacity by 30% (Wilken and Nikolov, 2006). Phytic acid interference was caused by complex formation with the basic protein (as verified by RP-HPLC) and also by formation of insoluble phytate salts with pH adjustment. By binding phytic acid, the overall surface charge of lysozyme was reduced which reduced the extent of lysozyme interaction with the negatively charged cation exchange resin. The insoluble phytate salts formed with pH adjustment caused immediate precipitation on top of the resin during loading which resulted in column plugging. Removal of phytate by enzymatic hydrolysis with phytase eliminated precipitation and demonstrated that phytate was the cause of fouling and reduced lysozyme adsorption. If sodium phosphate buffer was used to raise the pH in combination with NaOH, no precipitation was observed and the binding capacity was increased slightly but was still unacceptably low. Wilken and Nikolov (Wilken and Nikolov, 2010) proposed a method to reduce phytic acid interference including using positively charged buffer anions (TRIS) to reduce electrostatic interactions to reduce the effective negative charge of phytic acid and consequently, the formation of binary

lysozyme-phytic acid complexes. This strategy resulted in a 3-fold increase of the dynamic binding capacity of human lysozyme and high lysozyme purity (95%). Another successful approach to reduce the impacts of phytic acid was to extract human lysozyme at pH 10, precipitate native protein at pH 4.5, adjust the pH to 6 using 1 M NaOH, and purify human lysozyme using cation exchange chromatography. At pH 10, the amount of phytic acid extracted was significantly lower than all other pHs evaluated (pH 2, 4, and 6) and lysozyme-phytic acid electrostatic interactions were reduced due to the near zero net charge of the RP. This strategy resulted in high purity lysozyme and yield and equally high lysozyme binding capacity as the TRIS-mediated purification method.

Another example of phytic acid interference occurred during purification of cellobiohydrolase I (CBH-I) from transgenic corn seed (Wilken et al., 2012). The RP was extracted between pH 3 and 4 to minimize native protein solubility, which resulted in CBH-I being approximately 15% of TSP. Prior to purification, the extract was adjusted to pH 7.5 with NaOH and TRIS (tris(hydroxymethyl)aminomethane) to provide buffering capacity. However, a very low dynamic protein binding capacity was observed when CBH-I was purified by anion exchange chromatography (CaptoQ). Because low pH extraction was used, phytic acid was co-extracted with CBH-I and the negatively charged ions interacted with the anion ligand and competed with negatively-charged, acidic proteins. Keeping phytate in solution (not allowing the formation of insoluble calcium phytate) by using either TRIS or citrate did not mitigate the interference. To reduce the impact of phytic acid, we investigated the effect of extraction buffer on phytate removal and compared several inexpensive pretreatment methods for phytate removal prior to anion exchange purification. Effective methods developed for either partial or complete removal of phytic acid included pH adjustment, adsorption with an inexpensive and disposable

anion exchange resin such as IRA-402, and addition of a divalent cation. IRA-402 was highly effective and removed >97% of phytic acid from low pH (3–4) extract. Precipitation of phytic acid using pH adjustment was also evaluated for phytic acid removal. The selected buffer (citrate vs. acetate) impacted phytic acid removal because citrate is a chelator and reduced the amount of phytic acid precipitated with pH adjustment. With TRIS and NaOH, little to no phytic acid was precipitated when extract in citrate buffer was pH adjusted but 65% of phytic acid was removed from extract prepared in acetate buffer. To increase phytic acid removal efficiencies in both cases, addition of calcium as calcium hydroxide was necessary. By using 10 mM calcium during pH adjustment with acetate-based extract, phytic acid removal was increased to 99%. The percent of CBH-I bound to the anion exchange resin increased from 20% to greater than 90% by phytic acid removal and controlling adsorption conductivity and the adsorption capacity at least 6-fold. This increase of CBH-I binding capacity will result in operating cost reduction. It should be noted that neutral proteins to be purified by anion exchange chromatography would also be inhibited by phytate, if present in the extract.

Polysaccharides

Polysaccharides are high molecular weight polymers of sugars that vary in size and structure. There is a wide range of types of polysaccharides of plant origin. The best known plant polysaccharide is cellulose; the fibrous component of primary plant cell walls. Cellulose is insoluble and therefore easily separated from protein extracts. Extracted plant tissue can be clarified by centrifugation to pellet insoluble fiber or by pressing wet extract against a coarse barrier such as cheesecloth or nylon mesh to filter pieces of tissue.

Several forms of hemicellulose are also present in plant cell walls. Hemicellulose is considered the cross-linking polymer that holds the cellulosic fiber together in plant cell walls

(Carpita and McCann, 2000). Unlike cellulose which consists of linear glucose chains, hemicelluloses consist of different sugars and are branched. Xyloglucans and glucuronoarabinoxylans are the major hemicelluloses found in flowering plant primary cell walls (Carpita and McCann, 2000). Arabinoxylan is a hemicellulose abundant in cereal grains. Hemicelluloses have limited solubility in aqueous solutions but can be solubilized using temperature or highly acidic or basic pH values.

Where hemicellulose serves a cross-linking function in plant cell walls, pectins tend to surround the cellulose and hemicellulose of the primary plant cell wall. Pectins are yet even more structurally complex polysaccharides as they consist of a galacturonic backbone with two alternating types of branched rhamnogalacturonans along the backbone (Carpita and McCann, 2000). Pectic polysaccharides are gel-like and typically isolated from apple or orange peel. They are also abundant in green plant tissues, but less so in grasses. Pectins are water soluble and therefore potentially more problematic than other polysaccharides with regard to DSP processing. Pectin-like polysaccharides will be negatively charged at neutral pH, making them ideal candidates for binding to anion exchange columns where they can prove difficult to elute (Cheng and Kindel, 1995).

Another well-known polysaccharide worth mentioning is starch, the primary carbohydrate storage form for plants. Starch is mostly insoluble in water so typically not a concern for DSP for PMPs. Like cellulose, starch can be removed by centrifugation of plant extracts.

Although they are often mentioned as a potential source of concern to DSP of PMPs, few specific examples of their impact and description of how their impact was mitigated are specified in the literature. The impact of polysaccharides is discussed most often in the context of plant

cell culture systems (Hellwig et al., 2004; Wilson and Roberts, 2012) although there are very few reports regarding how polysaccharides secreted from plant cells, hairy roots, and whole plant cell cultures such as moss, algae, and *Lemna* impact bioprocessing. Pectin-like polysaccharides are secreted into cell culture media where they contribute to a higher viscosity of solutions (Georgiev et al., 2009). It is commonly reported that polysaccharides from cell culture media coat the walls of a vessel causing cells to accumulate on surfaces.

These viscous polysaccharides coat membrane surfaces causing decreased flux in different types of filtration processes (Hellwig et al., 2004; Raven et al., 2015). To circumvent problems with filtration, expanded bed adsorption (EBA) chromatography is favored for purification of protein from plant cell culture media (Hellwig et al., 2004; Raven et al., 2015). EBA chromatography was designed for use with unclarified media so it is a logical choice. The types of media that are designed for use in EBA chromatography are limited however so this may limit its adoption.

Where the impact of polysaccharides on filtration and purification processing might be more obvious in a plant cell culture context, that impact is less recognized when it comes to extracts from leafy tissue and seeds where polysaccharides are likely to be a component of complex extracts. The degree to which the two types of soluble polysaccharides in plant tissue pose difficulties in DSP can vary with the sample type and processing conditions. As an example, extracts made from transgenic *Lemna minor* expressing a monoclonal antibody were very difficult to filter when prepared at neutral pH but filtered easily when dropped to pH 4.5, spun to precipitate insoluble material, and then raised back to neutral pH or when extracted at pH 4.5 (Woodard et al., 2009). *Lemna* is known to contain an acidic pectic polysaccharide of 100 to 300 kDa called lemnan (Popov et al., 2006). It is not known if lemnan was a contributing

factor in filter clogging in the case of these neutral pH *Lemna* extracts but it is plausible that the negatively charged pectic polysaccharide associated with some protein drops out of solution at the lower pH where it would be uncharged.

Neutral pH extracts from *Lemna* also contained more color and left a buildup of colored material at the top of the protein A column used to capture the monoclonal antibody-containing extract (Woodard et al., 2009). The filtering difficulty and resin fouling were largely attributed to phenolic compounds, but HPLC profiling showed very little difference in the phenolic composition. One possible explanation is that *Lemna* polysaccharide was associated with phenolics. A recent analysis of the content of *Lemna minor* cell wall polysaccharides identified phenolic compounds associated with some of the pectic polysaccharide that comprises more than 50% of the carbohydrate found in *Lemna minor* (Zhao et al., 2014).

In grains, polysaccharide-linked phenolics of the cell wall hemicelluloses arabinoxylans and arabinogalactans are well-known. These molecules can also interfere in downstream purification steps. We found that pretreatment of low pH (2–3) extracts from defatted ground corn expressing the thermophilic cellobiohydrolase, CBH-I, with a polymeric resin known to absorb phenolic compounds through hydrophobic binding also improved performance in tangential flow filtration and subsequent anion exchange chromatography (Woodard et al., 2010). We believe that in addition to removing free phenolic compounds, the resin was also removing phenolic-linked polysaccharides that were detrimental to the filtration process since higher overall fluxes were able to be maintained in the case of pretreatment.

Phenolics and pigments

Before transgenic plants became candidates for protein production, plant tissue was used for years as a source of enzymes and proteins (Andersen and Sowers, 1968; Loomis, 1974), and

phenolics were identified as an impediment in the purification of proteins. Phenolic compounds are secondary metabolites that are important for growth, reproduction, and defense of plants. They also contribute toward the color and sensory characteristics of fruits and vegetables. Structurally, phenolic compounds comprise an aromatic ring, with one or more hydroxyl substituents, ranging from simple phenolic molecules (C6) to the highly polymerized condensed tannins $(C6–C3–C6)_n$. Despite this structural diversity, the group of compounds is often referred to as polyphenols (Balasundram et al., 2006).

Phenolic compounds are a complex group of substances that have gained intense attention due to their biological properties, natural antioxidant capacity, physiological health benefits from anticarcinogenic or antimicrobial activities, and other properties related to food quality (Galanakis, 2015; Ozdal et al., 2013; Valdés et al., 2015; Williamson and Manach, 2005). Because phenolics impart color and flavor to food products and possess properties beneficial to health, there are several studies relating phenolic compounds to dietary protein activity or isolation and the strategies used to extract them from plant material (Castro-Muñoz et al., 2016; D'Alvise et al., 2000; Loomis and Battaile, 1966; Shchekoldina and Aider, 2014; Weisz et al., 2013).

Each plant host contains a combination of different phenolics which belong to various classes, such as phenolic acids, flavonoids, flavones, anthocyanins, catechins, or tannins (Dimitrios, 2006; Dykes and Rooney, 2007; Naczk and Shahidi, 2006), and the same is true for each extract from transgenic plants expressing RPs. Along with the RP, the extracts of transgenic *Lemna*, tobacco, and other leafy plants typically contain a mixture of hydrocinnamic acids and flavonoids (Barros et al., 2011). Extracts from grains like rice, wheat, and sorghum primarily contain phenolic acids and flavonoids, while anthocyanins are present in pigmented grains

(Dykes and Rooney, 2007). The major phenolic compound in corn extracts is p-coumaric acid (Bacchetti et al., 2013; Žilić et al., 2012) while phenolic acids and coumarins are the predominant phenolics in vegetables (Naczk and Shahidi, 2006). Ferulic acid is widely present in all types of plant extracts because it is a component of cell wall lignocelluloses, serving as the link between polysaccharides and lignin (Kumar and Pruthi, 2014; Smith and Hartley, 1983).

It is believed that colorants with a molecular mass above 200 kDa are browning products of cell wall polysaccharides and comprise polysaccharides bonded to phenols and their oxidation products (San Miguel Bento, 2009). Phenolic compounds were found to be involved in the enzymatic browning of sugarcane, as they were substrates for polyphenol oxidase, which converts phenolics to polymeric colorants. Increases in the color of sugarcane juice was observed to be proportional to a decrease in the concentration of chlorogenic acid and other flavonoids (Patton and Duong, 1992).

Phenolic compounds can react with proteins and alter some of their physicochemical properties such as solubility, electrophoretic behavior, hydrophobicity, molecular weight, and secondary and tertiary structure (Ozdal et al., 2013; Rohn et al., 2002). The presence of phenolics in protein production is a concern, as they can interact with proteins through a number of mechanisms including hydrogen bonding, oxidative coupling, and ionic and/or hydrophobic interactions (Jervis and Pierpoint, 1989; Loomis, 1974). A study of the influence of different classes of phenolic compounds on functional properties of soy proteins (Rawel et al., 2002) brings to light the interaction mechanisms. Binding of phenolic compounds, within transgenic plant extracts, to a RPs active site can be particularly detrimental if a RPs functional activity is decreased.

Although the interaction of phenolics with native proteins can be considered beneficial, as during rice fermentation by fungus (Schmidt et al., 2014), impurities in transgenic plant extracts present obstacles in DSP. By interacting with the target proteins, they can negatively impact precipitation processes. Another undesirable impact resulting from the presence of phenolics is membrane fouling during filtration. Phenolics, polysaccharides, and proteins are the sugarcane components believed to be responsible for fouling separation membranes (Du Boil, 1997; Godshall et al., 2001; Saha et al., 2007).

Polyphenols are also responsible for fouling of chromatographic resins during purification. In plant extracts containing monoclonal antibodies (mAbs), the presence of phenolics that could build up over time on a protein A-type affinity resin increases the probability of shortening resin lifetime (Barros et al., 2011; Woodard et al., 2009).

Choosing the plant tissue low or deficient in phenolics to target the expression of the heterologous protein is one strategy that can be applied to minimize drawbacks brought by phenolic compounds during extraction and purification operations. For example, advantages for using seed-based expression systems for plant-made protein products extend beyond the long-term stability benefit and include the ease of storage and transportation, fewer native proteins and phenolic compounds, and secondary metabolites when compared to leaves (Yao et al., 2015). Alternatively, if one decides to take advantage of the large amount of biomass leaves that can be produced quickly, but wants to avoid using tobacco due to high concentrations of phenolic impurities, producing RP in lettuce by agroinfiltration has been demonstrated to be a viable option (Chen and Lai, 2015). Lettuce produces negligible quantities of phenolics and alkaloids and its use as a host for protein production may overcome the challenge of their removal during DSP. Transient expression of a truncated mutant version of human glutamic acid

decarboxylase using a construct that reduced membrane association enabled effective extraction without detergents (Merlin et al., 2016). Elimination of detergent from the extraction buffer is desirable as it can interfere with DSP by reducing the functional capacity of filters and chromatography resins as well as increasing the concentration of soluble contaminants in the extract, including phenolic compounds.

For improvement of RP extraction from diverse plant tissues, the optimization of pH and ionic strength levels has been investigated (Azzoni et al., 2005; Farinas et al., 2005; Merlin et al., 2016; Munjal et al., 2015; Wilken and Nikolov, 2006). In addition to the concentration of the desired protein, these studies also looked at the extracted impurities such as native proteins, phenolic compounds, oil, and carbohydrates, which can negatively affect subsequent DSP. In corn extracts, extraction of phenolics – predominantly phenolic acids – is maximized using aqueous extraction under alkaline conditions. Lower concentrations of phenolics are obtained when the pH and the ionic strength of the solutions are at their lower levels. If high pH values need to be used, increasing the ionic strength of the solution should be applied to minimize extraction of phenolic contaminants (Azzoni et al., 2005; Farinas et al., 2005).

Similar optimization has been carried out to improve RP extraction from algae (Munjal et al., 2015), and in the same way, the extraction of chlorophyll was evaluated together with the concentration of the target protein. A combination of low pH buffer and low ionic strength buffer was shown to be effective in reducing 70% of chlorophyll content in algae extracts. The absence of detergent in the buffer also minimized the pigment extraction. In the case of transgenic *Lemna* expressing a human mAb, the amount of IgG extracted was shown to be similar regardless of whether the extract was prepared using a salt-containing pH 4.5 or pH 7.5 buffer (Woodard et al., 2009). The extract prepared at pH 4.5 had a ferulic acid form that was not extracted at pH 7.5,

resulting in 22% greater phenolic content in the pH 4.5 extract. For sugarcane, preparing extracts by high shear homogenization instead of juice using a pressing mill resulted in lower TSP concentration of sugarcane extracts (52 µg/mL) than sugarcane juice (490 µg/mL) and less colored impurities (Figure 5.2).

Until recently, few publications addressed the topic of phenolic removal methods for the purification of RPs from plant extracts. Approaches for phenolic removal from plants include aqueous two-phase partitioning (Gecchele et al., 2014; Hooker, 2004; Platis and Labrou, 2006) , membrane filtration (Akbari and Wu, 2015; D'Alvise et al., 2000; Xu and Diosady, 2002), and adsorption (Naik et al., 2012; Payne and Shuler, 1988; Woodard et al., 2009). Aqueous two-phase partitioning has been used to reduce the amount of phenolics, alkaloids, and pigments in leaf extracts (Hasmann et al., 2008; Platis et al., 2008). When a system of PEG and salt (sodium citrate) was used for extraction of phenolics from wood, it was found that polyphenols partition to the PEG-rich phase (Xavier et al., 2015). Using ATPS to extract RP from tobacco was demonstrated to be effective for reducing the concentration of phenolic compounds in the extract. Most of the phenolics (approximately 76%) in tobacco leaf extract were concentrated in the aqueous phase, while the RP of interest partitioned to the detergent phase (Gecchele et al., 2014).

In the DSP of RPs, membrane filtration is used as a clarification step to remove impurities such as pigments, phenolic compounds, oil, and native plant proteins (Nikolov et al., 2009) and to concentrate the final product. Several applications of pressure-driven processes like ultrafiltration (UF) and nanofiltration (NF) have been used to separate small organic molecules from proteins (Akbari and Wu, 2015; Galanakis, 2015; Loginov et al., 2013). When intending to separate polyphenols and proteins, filtration is usually applied in association with an acidic

precipitation step. For flaxseed hull extract, Loginov *et al.* (Loginov et al., 2013)found that membrane fouling, which was more severe at higher pH values, increased the separation selectivity and, thus, produced a better quality filtrate. For canola meal, an acidic wash removed approximately 40% of phenolic compounds before protein alkaline extraction and UF (Akbari and Wu, 2015). A less common approach is the use of membrane filtration before precipitation. This sequence of operation was used to remove pigments and a majority of phenolics using a 10 kDa molecular weight cut-off (MWCO) membrane to avoid the interference from polyphenols during polyelectrolyte precipitation of HCP from transgenic tobacco extract containing a monoclonal antibody (Fulton et al., 2015).

Amid the various approaches used to alleviate the problems caused by the presence of impurities in plant extracts, inexpensive resins have been tested for phenolics removal before protein purification. Prehydrated polyvinylpirrolidone (PVPP) has been used in a variety of applications ranging from the fundamental isolation of plant enzymes (Loomis and Battaile, 1966) to RP purification from transgenic tobacco (Holler and Zhang, 2008). Relevant applications of adsorption using inexpensive resins for food processes, removal of phenolic compounds, detoxification of fermentation media, color removal, and purification of sugar solutions and microbial metabolites have been reviewed (Soto et al., 2011). Phenolic compounds present in *Lemna* extracts can effectively be removed using inexpensive adsorption resins prior to the affinity step (Barros et al., 2011). Activated charcoal and dextran-coated charcoal was used for the removal of pigments and phenolic compounds from *Lemna* extract without reducing the antibody concentration (Naik et al., 2012). Pre-treatment using a contact time of 10 min was sufficient to remove around 80% of the phenolic compounds from the plant extract.

Because phenolics comprise a variety of compounds and several polymeric adsorbents are available for removal of pigments and phenolics, understanding the type of interactions that govern phenolics adsorption would facilitate the choice of resin to be applied to a specific plant extract. Hydrophobicity of studied adsorbents played an important role in adsorption of *Lemna* phenolics; the dynamic binding capacities (DBCs) of the resins with hydrophobic matrices (XAD-4 and IRA-402) were 3- and 10-fold greater than the DBC of agarose-based resin (Q-Sepharose). The cost of adding a phenolics removal step to a mAb purification train was determined (Barros et al., 2011), and the economic analysis indicated that the addition of a phenolics adsorption step would increase mAb production cost by only 20% for IRA-402 and 35% for XAD-4 resin. The cost of the added adsorption step could be offset by increasing the lifespan of protein A resin from 20 to 30 or more cycles and an actual reduction of mAb production cost can be achieved by using disposable IRA-402. In addition to increasing the lifespan of protein A resin without increasing the production cost, the inclusion of a phenolics removal step has the potential to generate a product of uniform quality, an important factor for a pharmaceutical protein. Although the outcomes were obtained for *Lemna* extracts, the approach for deciding which would be the most suitable styrene-based resins for the extract pretreatment can be extended for other green tissues, whose phenolic compounds can be an obstacle in the purification of plant-made proteins.

Equilibrium adsorption isotherms of model phenolic solutions at pH 4.5 and 7.5 described the interactions between five phenolic compounds commonly found in plant extracts (chlorogenic acid, ferulic acid, rutin, syringic acid, and vitexin-2-O-rhamnoside) and three commercial types of polymeric resins (IRA-402, PVPP, XAD-4) (Barros et al., 2010). The chosen compounds belong to four classes of phenolic compounds (hydroxycinnamic acids,

hydroxybenzoic acids, flavonols, and flavones) that are typically found in the plant extracts and therefore relevant to work on RP purification from transgenic plants. Regardless of the adsorption mechanism, hydrophobic-matrix resins XAD-4 and IRA-402 exhibited a greater affinity than PVPP for the five representative phenolic compounds. At pH 4.5, phenolic acids had 10-fold lower partition coefficients than flavonoids with XAD-4, indicating that XAD-4 would not be suitable if plant extracts contain large quantities of phenolic acids, but it would be adequate for capturing flavonoids such as rutin and vitexin. Adsorption isotherms generated with ferulic and chlorogenic acids on IRA-402 resulted in partition coefficient values higher than or closer to those of rutin and vitexin-2-O-rhamnoside. In the case of vitexin, it appears that XAD-4 would be a better adsorbent at pH 4.5 and IRA-402 at pH 7.5. At pH 7.5, phenolic acids had higher affinities for the charged resin than the non-charged one.

To minimize the impact of phenolic compounds and pigments on purification, it may be necessary to examine the phenolics profile and evaluate corresponding removal strategies on a case-by-case basis due to the diversity of plant hosts and expression strategies for making RP in plants. The increasing body of knowledge on strategies for the removal of phenolics and pigments provides a good foundation to guide the selection of a method that is likely to work for the expression host and tissue used for a specific RP product. The effective removal of these compounds is essential for the development of robust processes for producing recombinant products in plant hosts.

Figure 5.2 Sugarcane juice (left) and sugarcane extract (right).

Summary and conclusion

Many of the challenges in developing RP products from plant hosts have been addressed resulting in commercial products. However, strategies for addressing critical plant impurities have not been comprehensively reported despite experiential know-how by those in the field of DSP of plant-derived RPs. Trends for this industry include using PMPs at the extremes of the marketplace either where very large or small volumes are needed, when product is needed quickly, or for products with unique features derived from the flexibility of plant expression systems. These trends are fueling the need for efficient DSP strategies.

Proteases, polysaccharides, phenolic compounds and pigments, phytic acid, and host cell proteins present potential challenges as more host systems and expression strategies are developed. Basic downstream unit operations can be impacted by the presence of these

impurities, some of which are unique to plant hosts. Progress is being made in identifying the components responsible and mitigating their impact. Degradation by proteases is being addressed through down-regulation of specific proteases implicated in degradation, co-expression of or exposure to stabilizing molecules, and through the use of small molecule protease inhibitors in extraction of plant tissue. The impact of polysaccharides on filtration can be reduced through use of a low pH precipitation step. Likewise, phenolic compounds and some phenolic-linked polysaccharides can be removed using adsorption to inexpensive polymeric resins. Phenolics and pigments can also be removed through ATPS extraction or membrane filtration. Processing conditions and use of buffer additives can have a major impact on the co-extraction of pigments and influence the phenolic content of starting feed material. Phytic acid is a concern for seed-based RP production systems, especially with low pH extracts from these hosts. The detrimental impacts of phytic acid can be alleviated by considering extraction conditions and by implementation of removal methods such as precipitation and resin adsorption. The impact of HCP can be minimized through the judicious choice of extraction conditions or precipitation strategies. ATPS and membrane filtration are also beneficial in reducing HCP in particular cases.

In summary, the increasing base of knowledge regarding critical plant-based impurities and effective strategies to circumvent the challenges they pose can aid in and accelerate process development. Although these challenges presented limitations previously for using plant systems for the production of RPs, development of these methods will allow for plants to be more competitive with traditional production platforms. With that, the field can continue to advance toward more plant-based products and greater acceptance of plant platforms for their ability to deliver quality products for a variety of applications.

Chapter 6 - Conclusions and recommended future work

Conclusions

Microalgae and other plants hosts are a promising source of renewable and sustainable energy and bioproducts. In particular, the use of microalgae biomass for multiple high value and high volume biomolecules is of interest to address global concerns regarding food security and energy availability. The current processing techniques to obtain useful and functional biomolecules from microalgae are cost prohibitive and unsustainable largely due to high energy investments, scalability, and incomplete valorization of biomass components. Thus, there are numerous scientific and engineering opportunities to employ a biorefinery concept to microalgae biomass, whereby product use is maximized and waste is minimized. The studies presented in this dissertation evaluated the development of a biological-based biorefinery for the extraction and recovery of multiple biomolecules from the freshwater microalgae *Chlamydomonas reinhardtii*.

To begin, a literature review was conducted that focused specifically on microalgae in the division of Chlorophyta, or green algae and their extracellular matrices (ECM), potential for commercial products, and finally discussed traditional and emerging approaches to biomolecule extraction and recovery. The review connected the morphological characteristics of microalgae ECM or organelle membranes to implications on separations and purifications technologies and described an approach that focuses on the structural composition of not only the microalgae ECM but also any product containing organelles to facilitate selection of candidate enzymes for disruption.

Fundamental studies were conducted to demonstrate the applicability of aqueous enzymatic processing of microalgae biomass as part of a biorefinery. A nitrogen deprivation

period of 48 h was determined to maximize lipid accumulation without detriment to protein content or profile. Autolysin, a metalloprotease with specificity to the structure of *C. reinhardtii* cell walls, was successfully used to not only permeate but also mediate complete cell lysis within 4 h. A subsequent high temperature incubation resulted in 54% total protein release into an aqueous phase with 43% total lipids simultaneously released. Application of trypsin as part of a secondary enzymatic treatment, led to release of an additional 30% of total lipids. This proof of concept study showed the potential of aqueous enzymatic processing and additional optimization of procedures followed.

A second study was conducted after refinement of autolysin production procedures and primary enzymatic treatment conditions. To create distinct product streams, a design of experiments optimization study was done to evaluate the effects of pH, time, and temperature on protein extraction from the cellular and organelle remnants of enzymatically disrupted microalgae biomass prior to enzyme mediated lipid release. Optimized conditions for enhanced protein extracted were determined at pH 12 for 4 h at 45°C and resulted in greater that 65% total protein release. The enhanced protein extraction step was directly integrated following cell disruption, thus minimizing solid-liquid separations and reducing processing time/energy inputs.

In the third study, lipids released during primary enzymatic treatment and enhanced protein extraction steps of the biorefinery process were characterized. Similar triacylglycerol fatty acid profiles were found in enzymatically-released lipids as compared to mechanically-(ultrasonication) released lipids. Possible degradation was observed in diacylglycerols, free fatty acids, and polar lipid species after PET and EP procedures but preliminary prediction of biodiesel potential of TAGs in both PET and PET-EP extracts indicated that the resultant biodiesel would have favorable combustion behavior and high oxidation stability.

Finally, a comprehensive review of molecular pharming of microalgae and other plant hosts was completed. *Chlamydomonas reinhardtii* was specifically chosen for the studies previously described for its ability to express and accumulate recombinant proteins in addition to native biomolecules such as TAGs. Thus, the biomass could serve as source of high value and high volume bioproducts. The exploration of challenges encountered when processing microalgae and plants hosts for therapeutic proteins such as proteases, native host proteins, phytic acid (salts), polysaccharides, phenolics, and pigments indicate that while in some processes these would be considered critical impurities, in the case of a biorefinery, they could be valuable co-products.

Future work

While the work herein described demonstrated the application of a biological-based biorefinery concept to *Chlamydomonas reinhardtii* for the release and recovery of native proteins and lipids, biomass used in the various studies was previously frozen for convenience of the work capacity of a research laboratory. As the proposed biorefinery process when adapted to pilot or industrial scale operations would likely be capable of continuous operation, freshly harvested microalgae biomass would be the feedstock. Thus, any morphological changes that occur during the freeze-thaw process such as permanent pore formation in the cell wall and/or membrane that could be contributing to the total cell disruption observed after PET of frozen biomass would be eliminated. To bolster the temporary permeation (pore formation) of fresh biomass by autolysin during PET and achieve a similar level of cell disruption as seen in frozen biomass, combination of autolysin and an additional mild disruption mechanism should be explored. Pulsed electric field technology in combination with autolysin could to create permanent pores and allow for biomolecule release from fresh biomass. Lipids released

following PET, EP, or PET-EP procedures still required solvent extraction for quantification and characterization. As the overall goal of this work is to apply biological-based techniques for release and recovery of proteins and lipids, future work should focus on lipid recovery with destabilization of the polar lipid monolayer surrounding lipid droplets. Enzymes (proteases and phospho(lipases)) and bioactive molecules (phosphatidic acid) should be evaluated for their ability to destabilize emulsions and along with slow controlled mixing, promote coalescence.

Starch granules observed with TEM imaging of biomass present an opportunity for additional biomolecule recovery to further improve the process economics of *C. reinhardtii* as a bioproduct platform. Integration of starch granule release, extraction, and fractionation should be explored and assessed for potential to further valorize *C. reinhardtii* biomass. Additionally, functional characterization of recovered lipids and proteins is necessary. The emulsifying activity, emulsion stability, antioxidant activity, and protein digestibility of recovered proteins should be tested. For lipids, biodiesel properties that were predicted as previously described should be assessed using test methods specified in the EN 14214 and ASTM D6751-02 standards.

Finally, process efficiency and commercial feasibility by modeling autolysin production and the proposed biorefinery procedures should be evaluated. SuperPro Designer® software can be used for cost estimates and sensitivity analyses and to identify bottlenecks and major cost contributors in the proposed biological-based biorefinery.

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