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REVIEW

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Green microalgae biomolecule separations and recovery

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

Keywords: Microalgae, Enzymes, Biorefinery, Downstream processing

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 (Skjånes et al. 2012; Mercer and Armenta 2011). 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 (Chauton et al. 2015; Benemann 2013). 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

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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 2010). 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* species, 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 Hadi 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. 2009).

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 weight (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 (Packaged Facts 2012). The current source of EPA is mainly fish oil, but *Nannochloropsis* species 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 (Ma et al. 2016; Camacho-Rodríguez et al. 2013; Chen et al. 2013a, b). 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 (Wang et al. 2015; Mourelle et al. 2017). 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 (Rasala and Mayfield 2015; Scranton et al. 2015; Hempel and Maier 2016; Yusibov et al. 2016). *Chlamydomonas*, *Chlorell*a, 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; Scaife et al. 2015; Rasala and Mayfield 2011). 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. 2012). 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 extraction 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 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 (Shiratake et al. 2013; Jegathese and Farid 2014; Show et al. 2015).

Microalgae cell disruption methods can be mechanical or non-mechanical (thermal, chemical, biological)

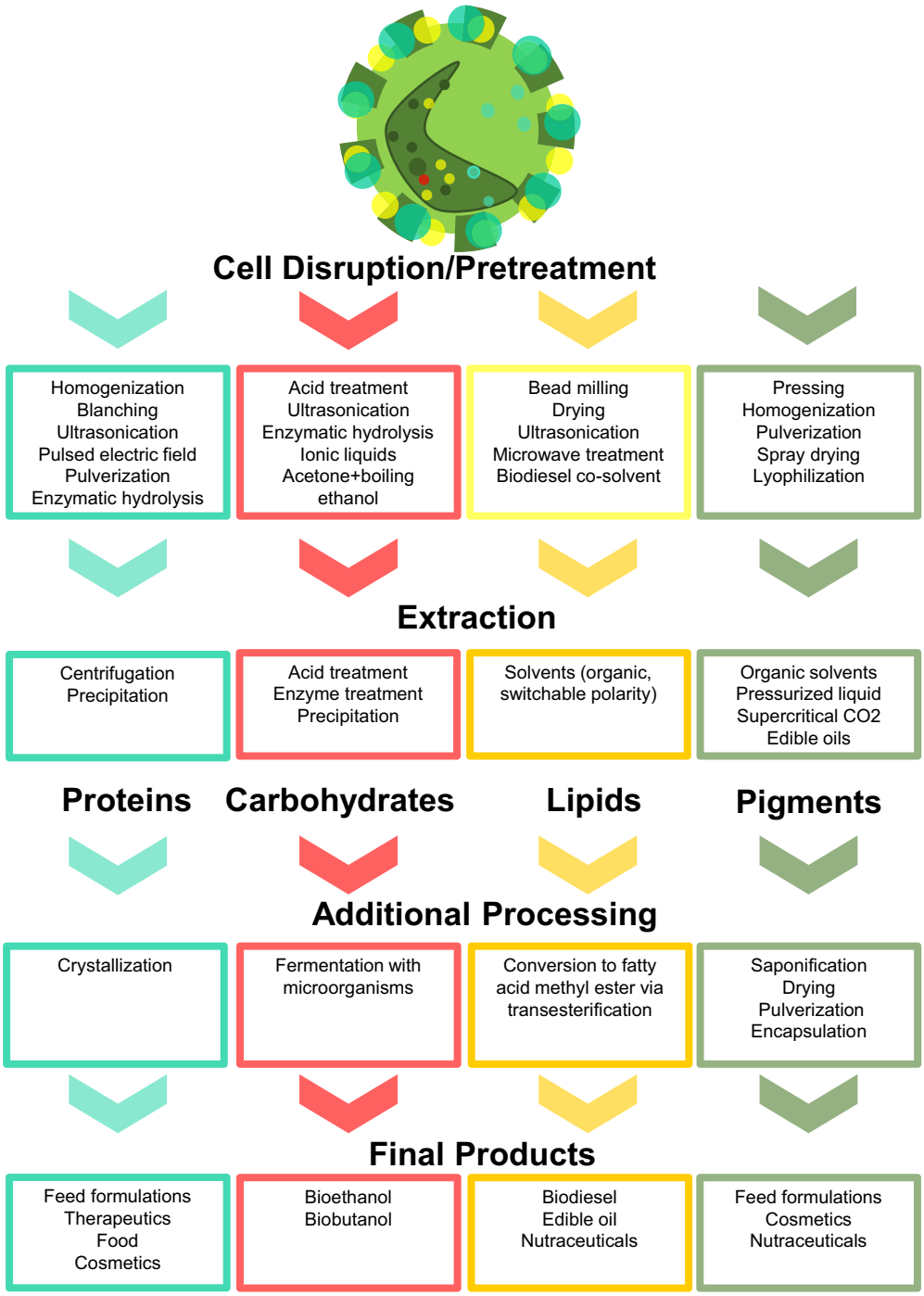

Fig. 1 Downstream processing steps for the recovery of proteins, carbohydrates, lipids, and pigments

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. 2014). 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 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 (Gerardo et al. 2015; Ríos et al. 2013; Beal et al. 2015). 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; Chia et al. 2018; Beal et al. 2015). 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 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 (Barsanti and Gualtieri 2018; t'Lam et al. 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 → 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 1988). Originally thought to be composed of seven layers, further research confirmed five layers within the cell wall (Goodenough 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 (Fig. 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 (Vanthoor-Koopmans et al. 2013; Günerken et al. 2015).

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 (Günerken et al. 2015; Al hattab and Ghaly 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 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 (Show et al. 2015; Günerken et al. 2015). Advantages include scalability and reproducibility and being chemical/enzyme/toxic substance (solvent) free (GEA Niro Soavi 2011). Disadvantages of HPH include the need for

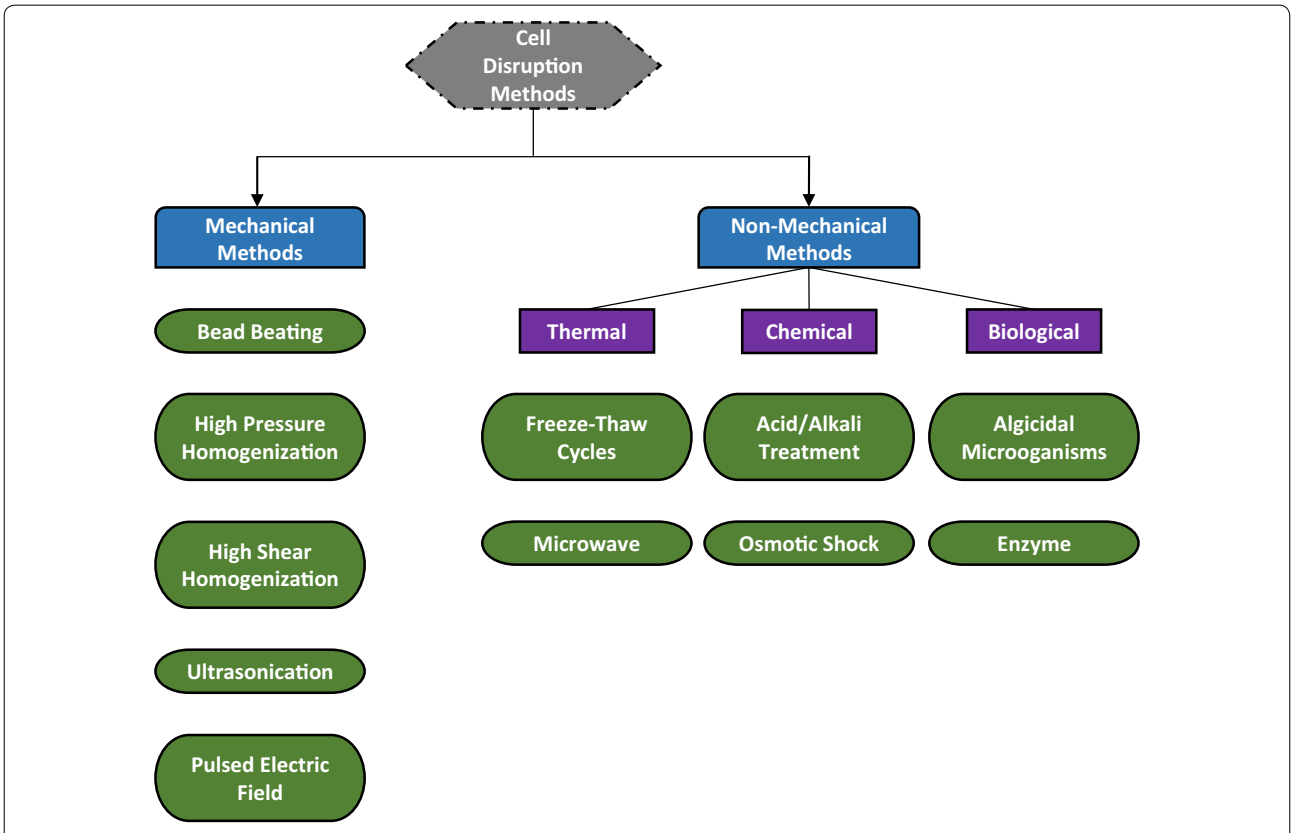

Fig. 2 Cell disruption alternatives

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 *Nannocholorpsis* 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 (Zbinden et al. 2013; Goettel et al. 2013). Above a certain PEF intensity, reversible pore formation become 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 (Günerken et al. 2015; Biller et al. 2013; 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 (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 (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 (Yoo et al. 2012; Bickerton et al. 2016). 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. 2012). 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, b; Munoz et al. 2014; Lü et al. 2013). 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, b; Wang and Yuan 2014; Lenneman et al. 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; Mahdy et al. 2014a; Horst et al. 2012). 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 (Soto Sierra et al. 2017; Dixon et al. 2016). 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 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.

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 (Gong and Bassi 2016; D'Alessandro and Filho 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.

Table 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	References
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 autolysin and 73% total lipid release with trypsin	Soto 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 72 h Improved lipid extraction yield (1.29- to 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)
Haematococcus pluvialis	Carotenoids	Glucanex®, pH 4.5, 55 °C	Ultrasonication	83.9% carotenoid extraction	Machado et al. (2016)
Haematococcus 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)

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. 2014) 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. 2014). 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 2009). In *C. reinhardtii* and *D. salina*, the most abundant LD membrane protein has been termed major lipid droplet protein (MLDP) (James et al. 2011; Davidi et al. 2012) 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 (Yoo et al. 2012; Ghasemi Naghdi et al. 2014). 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 (Huo et al. 2015; Chen et al. 2016; 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 (Soto 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 (Fig. 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

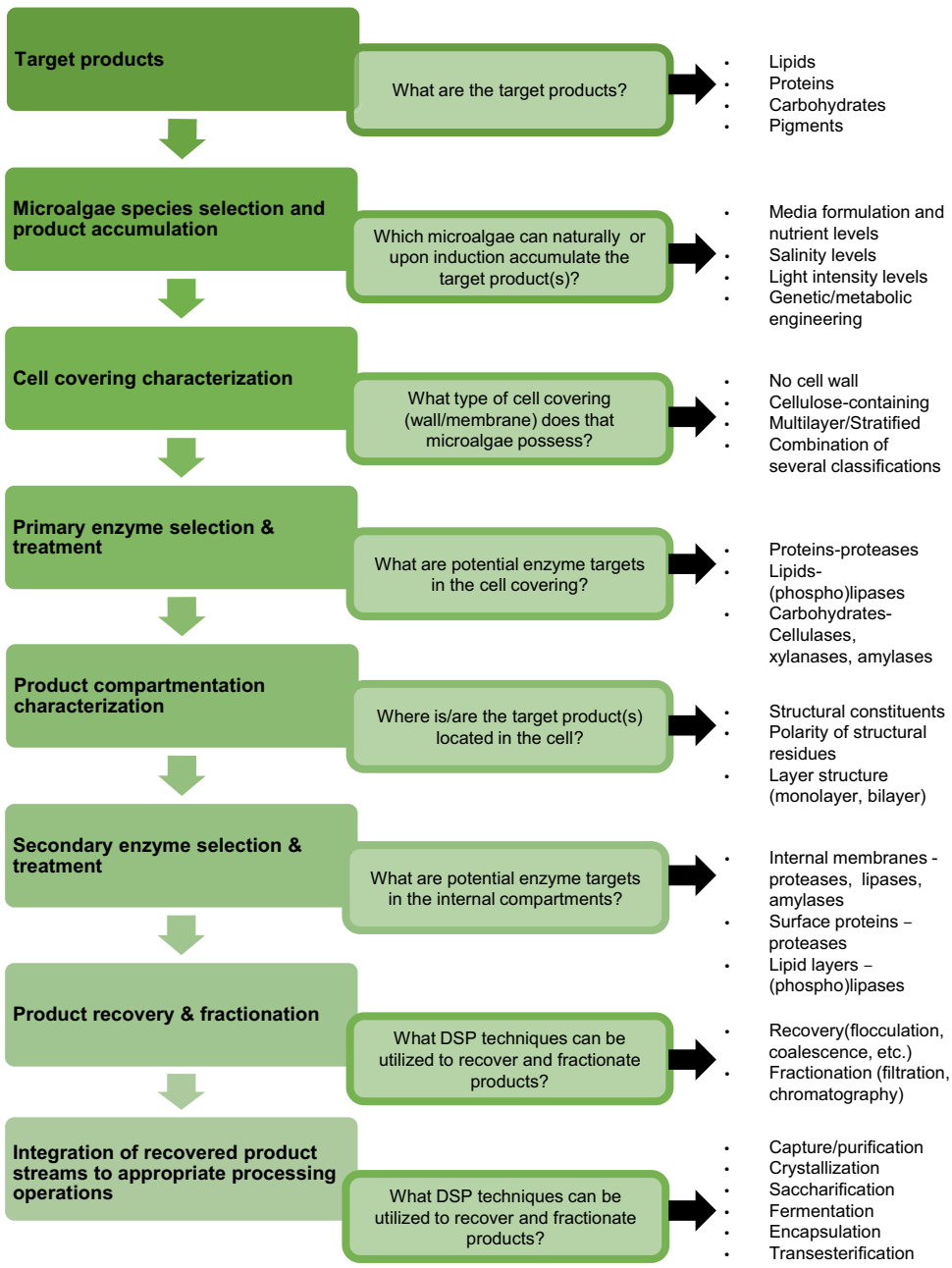

Fig. 3 Aqueous enzymatic processing scheme workflow and design considerations

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

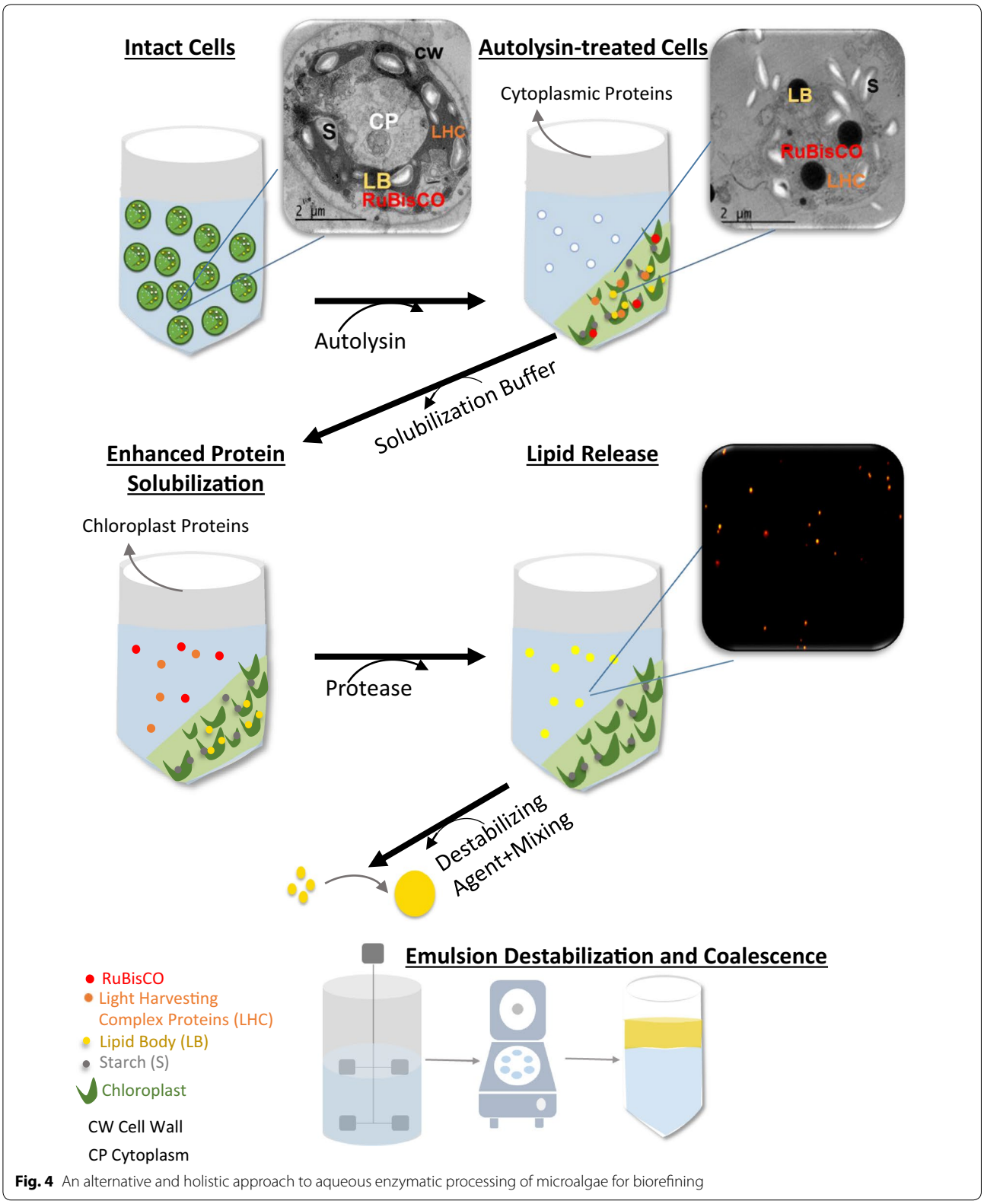

Fig. 4 An alternative and holistic approach to aqueous enzymatic processing of microalgae for biorefining

nature of *Nannochloropsis* and *Haematococcus* cell walls (Machado 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; Soto 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.

Recently, a methodology for native protein and lipid extraction and recovery from wild-type *Chlamydomonas reinhardtii* was described (Soto Sierra et al. 2017) (Fig. 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.

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.

Abbreviations

AEP: aqueous enzymatic processing; CO_2: carbon dioxide; CSFV: classical swine fever virus; DMSO: dimethyl sulfoxide; DSP: downstream processing; ECM: extracellular matrix; EPA: eicosapentaenoic acid; HBsAg: hepatitis B virus surface antigen; HOGP: *Haematococcus* oil globule protein; HPH: high pressure homogenization; HSH: high shear homogenization; IgG: immunoglobulin G; LD: lipid droplet; LDSP: lipid droplet surface protein; MAE: microwave-assisted extraction; MLDP: major lipid droplet protein; NP-1: neutrophil peptide 1; O&M: operational and maintenance; PBR: photobioreactor; PEF: pulsed electric field; RP: recombinant protein; TSP: total soluble protein.

Authors' contributions

CD and LRW developed manuscript concept, review topics, and structure. CD investigated the references and drafted the manuscript. LRW edited the manuscript. Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials
The authors have no data/materials to deposit. The manuscript is based on a literature review of sources included in the reference list.

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