Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review

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Abstract

Biological lipids derived from oleaginous microorganisms are promising precursors for renewable biofuel productions. Direct lipid extraction from wet cell-biomass is favored because it eliminates the need for costly dehydration. However, the development of a practical and scalable process for extracting lipids from wet cell-biomass is far from ready to be commercialized, instead, requiring intensive research and development to understand the lipid accessibility, mechanisms in mass transfer and establish robust lipid extraction approaches that are practical for industrial applications. This paper aims to present a critical review on lipid recovery in the context of biofuel productions with special attention to cell disruption and lipid mass transfer to support extraction from wet biomass.

Keywords:
Oleaginous microorganism
Lipid
Wet extraction
Mass transfer
Cell disruption
Biofuel

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1. Introduction

The development of the next generation of renewable liquid transportation fuels is becoming increasingly important due to the depletion of fossil fuel sources and increasing concern over atmospheric CO₂ levels and associated warming trends. The biological production of lipids using oleaginous microorganisms like microalgae, yeast, fungi, and bacteria has been widely studied [1–6]. Lipids accumulate in cell biomass mostly in the forms of triacylglycerides, free fatty acids (FFA), polar lipids, sterols, hydrocarbons, and pigments. Oleaginous microorganisms are historically defined as organisms in which lipid content exceeds 20%, and are thus promising candidates for producing fatty acids as sustainable biofuel precursors. Harvested biomass needs to be fractionated in a downstream process, also referred to as a bio-refinery, in which biofuels, value-added co-products, and energy can be obtained [7]. The bio-refinery concept is a promising pathway for creating a cost-effective biomass-based industry, especially for microbial cell biomass which can be converted to multiple products [8,9], including fuels and value-added petrochemical replacement products.

In a bio-refinery process, the extraction of intracellular lipids has been identified as a crucial element linking the upstream bio-synthesis and downstream upgrading steps. According to a recent microalgal biofuel design report, in a bio-refinery process lipid extraction efficiency has the largest impact on the cost of microalgal biofuel [10]. Thus, it is necessary to develop efficient, scalable, and economical lipid extraction processes.

Compared to lipid recovery from wet biomass, extraction of lipids from dry biomass is usually more efficient. The extraction of lipids from dried biomass has been widely used for analysis and small scale research purposes, e.g. Soxhlet extraction [11], pressurized fluid extraction (PFE) [12], supercritical fluid extraction [13], and in-situ reactive extraction process [14,15]. Although lipid extraction from dry biomass is usually favored due to the higher lipid yields, drying the biomass prior to extraction is economically prohibitive for large-scale fuel applications due to the tremendous energy demand [16,17]. In order to maximize the Energy Returned on Energy Invested (EROEI), it has become necessary to develop a scalable lipid extraction process which can directly extract lipids from wet biomass with a low energy requirement [18,19].

However, the presence of water hampers the efficiency of a solvent-based extraction process, likely because of reduced mass transfer [20–22] and emulsion formation [23], which have not yet been thoroughly investigated or reviewed. Even though various cell disruption technologies have been traditionally applied to expose intercellular lipids to increase mass transfer during the wet lipid extraction [24–26], little is known of the impacts of cell disruption/biomass pretreatment on the lipid extraction regarding lipid accessibility, mass transfer and emulsion formation. Lipid extraction from wet biomass requires intensive research and development in order to understand the complex mechanisms involved and establish robust and economic approaches that can be scaled up to industrial implementation for biofuel productions.

In the context of a scalable biofuel application, this review article highlights the important considerations involved in the development of a practical lipid extraction directly from wet microbial biomass. We introduce first the molecular diversity of lipids found in microorganisms, followed by a detailed discussion of lipid extraction mechanisms and selection of extraction solvents. The physical principles surrounding mass transfer is discussed at length and placed in context with molecular complexity of both the lipid and the biomass matrix. Existing cell-biomass pretreatment methods are also critically reviewed in terms of potential impacts on lipid accessibility, mass transfer and emulsion formation during lipid extraction, indicating effects of microbial biomass pretreatment on lipid extraction efficiency and scale-up potential for biofuel productions. This review is unique in that it presents a first, and critically needed, in depth discussion of lipid extraction from aqueous microbial biomass slurry, which consists of a complex biomass matrix and diverse lipids, to illustrate the challenges and opportunities in this research field.

2. Lipids in oleaginous microorganisms

2.1. Lipid production

In microorganisms, lipid content and composition vary with the organism’s particular metabolic machinery, but can be manipulated by culture conditions like temperature, growth stage at harvest, available nutrients, and pH. The rate of lipid accumulation in oleaginous microorganisms typically increases during nitrogen-limitation when cells still assimilate excess carbon, but cell division is inhibited funneling carbon into triacylglycerides (TAGs) [2,7–9].

Microalgae, such as Chlorella sp., Nannochloropsis sp., and Scenedesmus sp. are promising candidates for biofuel productions as a result of their especially high lipid productivity, and rapid growth compared to other energy crops [7,28]. Lipid content in microalgae can vary widely and examples of microalgal oil production information can be found in [2,30–34]. Although photoautotrophic microalgae grow much faster than terrestrial crops, they grow much more slowly than many heterotrophic oleaginous microorganisms.

Yeasts and fungi are also favorable oleaginous microorganisms, showing rapid growth rates on simple carbon sources such as glucose derived from corn, sugar cane, or cellulose biomass [2,35,36]. Yeast strains, such as species of Rhodosporidium, Rhodotorula, Lipomyces, and Cryptococcus accumulate intracellular lipids (mostly TAGs) up to 70% of their dry biomass comparable in yields to oil-bearing seeds [37,38]. Examples of articles describing yeast and fungal oil production can be found in [39–43].

Oleaginous bacteria, such as Arthrobacter sp. [2], Rhodococcus opacus [44] and Acinetobacter calcoaceticus [45] can accumulate oil content up to 87% of their dry biomass and have high growth rates generating large amounts of biomass during short cultivation times. More descriptions of bacterial oil production can be found in [45–48].
Fig. 1. Overview of the chemical structures of the 12 most common representatives from lipid classes identified in microbial oils. 1. triacylglycerides (1,2-dihexadecanoyl-3-(9Z-octadecenoyl)-sn-glycerol), 2. diglycerides (1,2-di-(9Z-octadecenoyl)-sn-glycerol), 3. monoacylglycerides (1-(11E-octadecenoyl)-sn-glycerol), 4. phosphatidylcholine (1-(6Z-octadecenoyl)-sn-glycero-3-phosphocholine), 5. phosphatidyl ethanolamine (hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine), 6. sulfoquinovosyl lipid (1,2-dihexadecanoyl-3-(6-sulfo-α-D-quinovosyl)-sn-glycerol), 7. galactolipids (Digalactosyldiacylglycerol (18:5(3Z,6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z)), 8. Chlorophyll a, 9. Phytol, 10. Cholesterol, 11. α-tocopherol, 12. β-carotene, 13. Zeaxanthin. Structures derived from www.LipidMAPS.org.

Table 1
Examples for lipid classes found in oleaginous microorganisms.

| Microorganisms     | Growth conditions                                      | Neutral lipids | FFA | Polar lipids | References |
|--------------------|--------------------------------------------------------|----------------|-----|--------------|------------|
|                    |                                                        | Acylglycerides SE | FFA | PL | GL | |
| Microalgae         |                                                        |                |     |               |            |
| Nitzschia laevis   | Heterotrophic, Lewin’s marine diatom medium with glucose, 23 °C, pH = 8.5 | 79.2           | 11.6 | 8.1 | [62] |
| Pavlova latheri     | Artificial seawater (ASW), 20 °C, 0.3%CO₂, pH = 8.0, light = 20 W m⁻² | 56.5           | 5    | 0.6 | 9.7 | 18.9 | [63] |
| Chlorella sorokiniana | Heterotrophic, Kuhl medium with glucose, 37 °C, pH = 7.0 | 78.9           | 2.7  | 11.2 | 7.1 | [64] |
| Dunaliella viridis  | Inorganic medium, 25 °C, 35–1500 μmol m⁻² s⁻¹, 0.035–1% CO₂ | 0.5–21.5       | 4.1–7.5 | 6.2–13.7 | 31.8–38.2 | [65] |
| Scenedesmus sp.     | Soil extract (SE) medium, 24 °C, 120 μmol m⁻² s⁻¹ | 81.3–82.3       | 6.1–6.7 | 10.9–12.6 | [66] |
| Gymnodinium sp.     | GSe medium, 18.5 °C, 80 μmol m⁻² s⁻¹ | 7.5–28.8       | 1.3–3.0 | 0.7–1.3 | 66.4–84.7 | [67] |
| Schizochytrium      | Heterotrophic, glycerol as carbon source (Seambiotic Let, Tel Aviv, Israel) | 69             | 12.6  | 14 | [68] |
| limacinum           |                                                        |                |     |               |            |
| Nannochloropsis sp. | Not given, obtained from Seambiotic Let (Tel Aviv, Israel) | 41.4           | 9.3  | 37 | [68] |
| Yeast               |                                                        |                |     |               |            |
| Candida 107         | Glucose and NH₄Cl as carbon and nitrogen sources, 30 °C, pH = 5.5 | 66.0–92.0      | 2.0–25.0 | 5.0–21.0 | [69] |
| Rhodosporidium toruloides | Glucose and (NH₄)₂SO₄ as carbon and nitrogen sources, 30 °C, pH = 6.0 | 81–87.7       | 4.0–6.6 | 8.3–12.4 | [70] |
| Fungus              |                                                        |                |     |               |            |
| Cunninghamella      | Tomato wastes hydrolysate(TWH) with glucose, 26 °C, pH = 6.0 | 44.5–87.9      | 1.7–7.7 | 3.6–14.8 | [71] |

The individual lipid content is expressed as% of total lipids. SE: sterol ester; PL: phospholipid; GL: glycolipid.
It should be stated that methods used to measure lipid content vary widely among labs and so comparisons of absolute values taken from different labs must be made carefully.

### 2.2. Lipid composition and distribution

Oleaginous microorganisms contain a vast range of lipid classes, such as acylglycerides, phospholipids, glycolipids, lipoprotein, free fatty acids, sterols, hydrocarbons, and pigments (Fig. 1). These lipid classes have different chemical and physical properties such as polarity, viscosity, solubility, and cellular location which define their availability during extraction [49]. Examples of contents of various lipids in cell-biomass are listed in Table 1.

Lipids accumulate in different locations in the cells and play important roles due to their specific cellular functions. Lipid bodies, consisting primarily of TAGs and sterol esters surrounded by a phospholipid monolayer rich in characteristic lipid body proteins, are present in the cytoplasm as a form of energy storage [50]. These lipid-rich compartments are formed in all eukaryotic organisms, as well as in a few prokaryote genera such as *Rhodococcus* and *Streptomyces* [51]. Acylglycerols such as, diacylglycerol (DAG) and monoacylglycerol (MAG) are key intermediates in the biosynthesis or enzymatic hydrolysis of TAGs. Free fatty acids (FFA) do not typically appear as direct metabolic intermediates in live cells; rather fatty acids involved in catabolic and anabolic processes are typically bound to other molecules such as co-enzyme A and the acyl carrier protein (ACP). FFAs are cytotoxic at higher concentrations and when detected at high levels in microbial biomass are usually the products of lipase-catalyzed acylglycerol hydrolysis. The content of FFAs in microbial biomass has been shown to increase after harvesting due to the enzymatic hydrolysis of lipids [21,52] and, therefore, elevated content of FFA might be expected in microbial biomass during storage [15,53].

Polar lipids in microorganisms mainly consist of phospholipids, glycolipids, lipoproteins, sulfolipids, and acylglycerides. A phospholipid molecule consists of a polar phosphorus-containing moiety (e.g. phosphate, phosphatidylethanolamine, phosphatidylcholine) and fatty acids esterified to a glycerol backbone, whereas a glycolipid contains a polar carbohydrate moiety in place of the phosphor group (Fig. 1). The hydrophilic moieties make the polar lipid difficult to be extracted by nonpolar lipids and they can behave as emulsifiers during a wet extraction. In autotrophic microalgae species, glycolipids and phospholipids can comprise 17–90% of the total lipids (depending on strain and growth conditions) [54] (also see Table 1) and glycolipids mainly occur in chloroplasts [49]. Most carotenoids are naturally occurring in photosynthetic pigment-protein complexes, fitting into or spanning across the lipid bilayer membrane [55]. Secondary carotenoids, which are not related to the photosynthetic apparatus, are harbored in lipid bodies [56].

TAGs and FFAs are usually considered as the favored precursors for biodiesel and hydrocarbon based biofuels such as renewable diesel or renewable jet fuel, while the other sidechains of polar lipids (sugars, proteins, phosphorous-containing molecules) may inactivate downstream processing catalysts [57]. Degumming has been routinely applied in the biodiesel industry to remove small amounts of phospholipids (0.5–0.6%, w/w) from vegetable oil [58]; however, considering the potentially high content of microbial polar lipids (Table 1), a remarkable amount of microbial lipid might be lost in the degumming process [52]. Actually, all lipid compounds containing acyl chains should be considered for biofuel production since acyl chains are the direct biofuel precursors [59,60]. As shown in Fig. 1, the acyl chains in fatty acids, phytol chains in chlorophyll and even hydrocarbon backbones in sterols [61] can be converted into hydrocarbon biofuels via catalytic upgrading processes. Since there are considerable levels of polar lipids in microbial biomass, the total biofuel yield might be remarkably increased if all the lipids (including polar lipids) could be effectively recovered. It is speculated that liberating FFAs from their bonded moieties prior to or after extraction might be a promising approach to increase the renewable diesel and jet fuel yields while at the same time reducing undesired and potentially inhibitory impurities from a lipid stream.

### 3. Lipid extraction mechanisms and challenges

A form of biomass pretreatment, such as cell disruption, is usually necessary to remove or weaken the protective cell walls of microorganisms to make the intracellular lipids more accessible in solvent extraction. Most studies in wet lipid extraction focus on cell disruption, which seemingly to be the sole factor influencing lipid recovery. Actually, lipid recovery from wet biomass is also affected by lipid accessibility, mass transfer, and emulsion. These important factors have been usually overlooked, but can be formidable for the development of economic and sustainable processes. An ideal biomass disruption process cannot only assist lipid extraction by removing cell wall barriers, but should be able to increase mass transfer and simplify downstream processing as well. To better evaluate cell disruption methods, wet extraction mechanisms will be first discussed, introducing the principles in an industrial extraction process to illustrate technical challenges. Then, in the next section, biomass pretreatment technologies will be comprehensively evaluated based on these principles.

#### 3.1. Selection of extraction solvent

An extraction solvent should provide a suitable partition coefficient, forcing the solute to migrate to the solvent phase. The partition coefficient is defined as

\[
K = \frac{[A]_{org}}{[A]_{aq}}
\]

in which \([A]_{org}\) and \([A]_{aq}\) are the solute concentrations in the organic phase and aqueous phase, respectively. Lipids should have high partition coefficient in the selected solvent. The degree of interaction between solvents and lipids can be estimated by their polarities using XlogP value [60] or Hildebrand solubility parameter [72]. Based on the “like dissolve like” principle, hydrophobic lipids (e.g., neutral lipids) will favorably partition into the nonpolar solvent phase with relative ease. In contrast, polar lipids are not extracted so readily with nonpolar solvents due to their bindings with biomass matrix. Co-solvents are traditionally applied in laboratories to break the linkages between the polar lipids and biomass, and to increase the solubility of the polar lipids as well. The well-known Bligh–Dyer [73] and Folch [74] extraction procedures employ chloroform and methanol to improve accessibility and solubility of polar lipids which in turn improves the total lipid yield. Considering the toxicity of chloroform and methanol, various combinations of less toxic co-solvents, such as hexane and short chain alcohols, have been used [75–77].

The application of co-solvents in dry biomass is relatively straightforward; however, a complex phase equilibrium will be formed in the presence of water. Solvents, especially polar solvents with high miscibility with water, will partially dissolve, making efficient recycling of the polar solvent via phase separation impossible. Distillation, which is more expensive than phase separation, would then be necessary to recover the dissolved polar solvent from post-extracted slurry after the extraction, making the process not economical. Thus, the selected solvents should be water immiscible and volatile enough, allowing for low-energy phase–separation–evaporation-based recovery from an industrial engineering perspective [78,79]. The physical properties of common solvents in regards to lipid extraction considerations are summarized in Table 2. It is evident that polar solvents such as alcohols are more water-miscible
Physical properties of some common organic solvents.

| Solvent     | Formula | Boiling point (°C) | Viscosity (cP 25 °C) | Density (g/mL) | Solubility in water (g/100 g) | Relative polarity | Enthalpy of vaporization (kJ/kg) |
|-------------|---------|--------------------|----------------------|----------------|--------------------------------|------------------|---------------------------------|
| Methanol    | CH₄O    | 64.6               | 0.54                 | 0.791          | M                              | 0.762            | 1100                            |
| Ethanol     | C₂H₆O   | 78.5               | 1.07                 | 0.789          | M                              | 0.654            | 846                             |
| 1-Propanol  | C₃H₆O   | 97                 | 1.95                 | 0.803          | M                              | 0.617            | 690                             |
| 2-Propanol  | C₄H₈O   | 82.4               | 2.33                 | 0.785          | M                              | 0.546            | 663                             |
| 1-Butanol   | C₅H₁₀O  | 117.6              | 2.54                 | 0.81           | 7.7                            | 0.386            | 592                             |
| Pentane     | C₅H₁₂   | 36.1               | 0.23                 | 0.626          | 0.004                          | 0.009            | 352                             |
| Hexane      | C₆H₁₄   | 69                 | 0.31                 | 0.655          | 0.0014                         | 0.009            | 365                             |
| Cyclohexane | C₆H₁₂   | 80.7               | 0.89                 | 0.779          | 0.005                          | 0.066            | 356                             |
| n-Heptane   | C₇H₁₆   | 98                 | 0.39                 | 0.684          | 0.0003                         | 0.012            | 318                             |
| Dichloromethane | C₂H₄Cl₂ | 39.8              | 0.41                 | 1.326          | 1.32                           | 0.309            | 330                             |
| Chloroform  | CHCl₃   | 61.2               | 0.54                 | 1.498          | 0.8                            | 0.259            | 247                             |
| Ethyl acetate | C₄H₈O₂ | 77               | 0.42                 | 0.894          | 8.7                            | 0.228            | 363                             |
| Ether       | CH₂O₂    | 34.6               | 0.22                 | 0.713          | 7.5                            | 0.117            | 377                             |
| Acetone     | C₂H₆O    | 56.2               | 0.31                 | 0.786          | M                              | 0.355            | 518                             |
| Toluene     | C₇H₈    | 110.6              | 0.56                 | 0.867          | 0.05                           | 0.099            | 351                             |
| p-Xylene    | C₈H₁₀O  | 138.3              | 0.6                  | 0.861          | 0.02                           | 0.074            | 336                             |
| Water       | H₂O     | 100                | 0.89                 | 0.998          | M                              | 1                | 2257                            |

The values were sourced from online and hardbound compilations. Relative polarity data: [82] Enthalpy data: [83].

A M: completely miscible.

3.2. Lipid accessibility and extraction mechanisms using nonpolar solvent

Since nonpolar lipids have very limited solubility in water, most of these lipids exist in the form of tiny oil droplets in an aqueous environment. Nonpolar lipids (TAG and FFA) tend to float to the surface of water phase due to the lighter density, but these lipids may adhere to or be encapsulated by insoluble cellular debris (see Fig. 2). Thus, the mechanism for extraction of these lipids from an aqueous environment by the use of nonpolar solvents is to dissolve the small lipid droplets into the bulk solvent phase, which can then be separated from the aqueous biomass residue by phase separation (Fig. 2).

Changing the pH can alter the partitioning coefficient of many solutes in solvent–water systems. In the case of FFA extraction, the pH should be kept low to keep FFA protonated for high partition coefficient and to avoid emulsion formation by the unprotonated FFAs.

The mechanism for polar lipid extraction is more complex than nonpolar lipids. Polar lipids that are usually constituents of membranes or closely associated with other cellular components are not extracted so readily with nonpolar solvents. These interactions are generally weak hydrophobic or Van der Waals forces, hydrogen bonds, ionic bonds, and covalent bonds (e.g. lipopolysaccharides and lipoproteins) [78,84]. These interactions might still exist even after a cell wall has been broken down. As mentioned above, although co-solvents can be used to increase the recovery of the polar lipids, they are unlikely to be practical for an industrial application due to complex downstream processing. Alternatively, these combined polar lipids can be released by biomass pretreatments, such as changing pH or enzymatic/chemical hydrolysis.
Assuming a polar lipid is liberated from the ruptured cellular debris and exists as a free molecule, a hydration shell exists around the polar lipid due to the electrostatic attraction of the water molecules. Extraction of the polar lipid from an aqueous phase can be envisaged as taking place in two steps, which involve the freeing of an ion or hydrophilic moiety from its associated water molecule followed by transfer into the solvent. This disruption of the water-lipid interaction requires additional energy to free the polar lipid [85]. Thus, extraction of these amphiphilic (or ionic) lipid molecules requires more energy input.

In addition, polar lipids adhere to the solvent-water interface reducing interfacial tension (Fig. 2). These amphiphilic lipids thus behave as surfactants and can readily lead to emulsions. Some polar lipids (e.g. phospholipids and glycolipids) partially dissolve in water to form micelles if the critical micelle concentration (CMC) is reached. The micelles can encapsulate nonpolar lipids and solvent, reducing total lipid yield and leading to solvent loss as well. Amphiphilic lipids can be extracted by solvents but they may form reverse micelles in a nonpolar environment. Reverse micelles have a nonpolar external surface and a polar internal environment, which can hold water, proteins, and even entire cells. The reverse micelles are also known to make the organic solvent phase turbid or even form gels [86], both of which make the solvent and lipid recovery much more difficult.

As discussed above, compared to polar lipids, extraction of neutral lipid (TAG and FFA) is generally preferred, due to higher partitioning in nonpolar solvents, better mass transfer, reduced emulsion problems and improved solvent recovery. Thus, converting polar lipids into FFA in the biomass disruption prior to extraction will be favored from an engineering perspective.

### 3.3. Mass transfer in lipid extraction from wet biomass

The kinetics of lipid extraction from wet microalgal slurry was modeled recently by Halim et al. [72] and described by a first-order equilibrium-driven process. The lipid extraction efficiency was shown to increase with an increase in the speed of agitation, extraction temperature, and the degree of cellular disruption. A modified model has been well validated in our lab by using nonpolar solvent bi-phase extraction systems (data not shown). These kinetic models indicate that lipid extraction can be remarkably improved by increasing mass transfer.

The process for a solvent-aqueous extraction involves lipids diffusing through the feed solution (wet biomass slurry) to the two-phase interface where it transfers across the interface, dissolves into the receiving solvent, and diffuses away from the interface. The most widely accepted model of mass transfer is the two-film theory [87]. This theory presumes that turbulence in the two phases disappears near the phase interface and the resistance to mass transfer only exists within the two films, which are on either side of the interface. Mass transfer is assumed to take place through the two films with concentration gradients in both films and equilibrium at the interface (Fig. 3) [80].

The rate of solute transfer \( \frac{dN}{dt} \) is given by:

\[
\frac{dN}{dt} = r_d A (c - c')
\]

where \( dN/dt \) is solute flux, moles/s; \( r_d \) is dispersion phase mass transfer coefficient, m/s; \( c \) is solute concentration, moles/m³; \( c' \) is solute concentration at equilibrium, moles/m³; and \( A \) is the interfacial area, m² [80].

The solute will experience resistance during transport through the two films. The overall mass transfer coefficient is governed by the mass transfer coefficient of both films [80]:

\[
\frac{1}{R} = \frac{1}{r_c} + \frac{1}{K_{rd}}
\]

where \( R \) is the overall mass transfer coefficient, m/s; \( r_c \) is the continuous phase mass transfer coefficient, m/s; \( K \) is the partition coefficient, and \( r_{rd} \) is the dispersal phase mass transfer coefficient, m/s.

In a practical extraction process, one phase is dispersed as droplets in the other phase so that mass transfer occurs. According to Eq. (1), a higher concentration difference (Fig. 3) and larger interfacial area are preferred to generate a higher rate of solute transfer. Thus, counter-current extraction and intensive mixing may help to increase the extraction rate. In addition to the two-film theory, more elaborate theories, such as penetration theory, have been developed to describe the mass transfer process [88] and will not be discussed here.

Several major problems arise in the solvent extraction of biologically derived products due to the effects of biomass components and surfactants on mass transfer. When the interface is clean there is little resistance to mass transfer. However, if biomass residue adsorbs to the interface it may create resistance to solute mass transfer, mainly due to the physical obstruction by the adsorbed layer [89]. Biomass is primarily composed of carbohydrates, proteins, and polar lipid compounds, and many molecules have surface activities forming particles that exhibit varying surface polarity causing them to concentrate at the phase interface affecting the mass transfer process [90]. It was reported that the overall mass transfer coefficient and surface tension both decreased with increasing biomass concentration in an extraction process [90] and that the extraction rate was reduced by 86% when concentration of yeast cells increased from 0.02 to 0.1 kg/m³ in the aqueous phase, building up a layer of yeast cells at the interface [91]. In addition to the physical barrier effects, these absorbed solid materials can also reduce mass transfer by dampening of the interfacial turbulence [92].

Since the presence of biomass can impede the lipid extraction by reducing the mass transfer or physical encapsulation, a reduction of non-lipid insoluble cell debris via a physical, chemical, or enzymatic pretreatment prior to extraction might be an effective way to increase extraction efficiency and overall biofuel yield.
3.4. Emulsion formation

Bio-surfactants can promote the formation of a stable emulsion, which is often a problem during extractions of fermentation broth [93]. Even with the application of centrifugal extractors, emulsion formation can limit throughput and may cause losses of product or solvent [94]. Martin (2016) points out that even though the energy requirements of centrifugation is low enough to be feasible, the capital costs of centrifuges would make this approach prohibitive, even taking economy of scale into account [95].

Naturally occurring fatty acid-based bio-surfactants such as monoacylglycerol [96] and diacylglycerol [97] act to stabilize the oil-in-water emulsion, while various phospholipids exhibit excellent surfactant properties [98]. Microbial glycolipids [99], sophorolipids [100,101], and lipopeptides [102,103] have also been reported as powerful bio-surfactants [104]. In addition, certain types of proteins [105,106] and polysaccharides [107] can also act as emulsifiers.

A strategy for breaking emulsions must be developed for an efficient lipid extraction. Emulsions formed by an individual emulsifier are stabilized in specific environments, e.g. certain pH, ion strength, or temperature. However, it does not seem to be practical to break emulsions by adjusting these parameters because of the diversity of emulsifiers in biomass. Alternatively, these emulsifiers can be degraded to smaller units which have no emulsification capability. An example is the hydrolysis of phospholipids which can be degraded to smaller units which have no emulsification capability, improves the extractability of the fatty acids, and eliminates unwanted components such as glycerol and phosphorous (catalyst poisons) in the solvent phase. An example is the hydrolysis of phospholipids which can be degraded to smaller units which have no emulsification capability, improves the extractability of the fatty acids, and eliminates unwanted components such as glycerol and phosphorous (catalyst poisons) in the solvent phase (Dong et al, unpublished data). The overall biofuel yield can also be improved compared to the traditional degumming approach, in which phospholipids are precluded from biofuel production.

4. Effects of biomass pretreatment on lipid extraction

The challenges in wet extraction have been illustrated in the previous section. Lipid extraction efficiency is mainly affected by limited lipid accessibility, blocking effects from insoluble biomass residue and the formation of stable emulsion. An ideal biomass pretreatment or cell disruption prior to wet extraction should not only open cell wall exposing lipids, but help to increase lipid accessibility, improve mass transfer and reduce emulsion formation as well. Meanwhile, the biomass pretreatment process should be energy-efficient and scalable for industrial applications. In this section, we will focus on the cell-biomass disruption and pretreatment methods in biofuel production processes and critically review these approaches based on their analysis methods, lipid recovery, energy consumption, scalability and potential effects on lipid extraction.

4.1. High-pressure homogenization (HPH)

High-pressure homogenization (HPH) has been used to assist lipid extraction from various types of biomass [108–111]. HPH is currently used in industry for high-value protein recovery because it is simple to operate and scale up [24,112].

An overview of the lipid extraction case studies is given in Table 3. Traditionally, cell disruption is quantified by metabolite release, UV absorbance, turbidity, particle sizing and cell counting.

| Disruption methods | Species | Biomass concentration (DCW) | Scale | Energy (MJ/kg) | Analyses and outcome | References |
|--------------------|---------|-----------------------------|-------|----------------|----------------------|------------|
| HPH                | Nannochloropsis oculata | 3.50% | 3 L/h | 46 | Cell counting, 95–100% disintegration | [118] |
|                    | Tetraselmis sp. | 0.0254% | 0.2 L | 407 | Cell counting, 95% disruption | [119] |
|                    | Nannochloropsis sp. | 0.1–25% | 10 L/h | 0.2–144.5 | Cell counting, 70–80% disruption | [115] |
|                    | Nannochloropsis sp. | 25%, 37 °C preincubation | 10 L/h | 2 | Particle size, 100% disruption; hexane extraction, 8784 g for 15 min, 70% lipid recovery | [120] |
| Bead mill          | Chlorella | 6.9–15.8% | 3–62 kg/h | 10.2–36.1 | Cell counting, 85–99% disintegration | [121] |
| Ultrasound          | S. limacinum | 4% | 0.05 L | 2 | Ethanol:slurry = 19:1 (v/v), about 50% lipid recovery | [122] |
|                    | Nannochloropsis sp. | 5% | 0.1 L | 360 | Solvent-less with saline solution and isopropanol to demulsify, 5000 rpm for 10 min, 8.4% lipid recovery | [123] |
|                    | Mixed culture | 0.025% | 0.1 L | 5760 | Bligh method, 46% lipid recovery, Lipid fluorescence density method, lipid recovery increase up to 1 fold | [124] |
|                    | S.dimorphus N. oculata | 0.033% | 0.03 L | 848 | Lipid fluorescence density method, lipid recovery increase up to 1 fold | [125] |
| PEF                | Synechocystis PCC6803 | 0.03% | 25 L | 350 | Cell counting, 99% disintegration | [126] |
|                    | Auxenochlorella protothecoides | 10% | 0.002 L | 2 | Ethanol extraction from freeze-dried biomass, 70% lipid recovery | [127] |
|                    | Ankistrodesmus falcatus | 0.19% | 0.004 L | 22 | Ethyl acetate-methanol extraction, 2-fold more lipid yield | [128] |
|                    | Scenedesmus | 0.46% | 40 L | 24 | Hexane extraction from freeze-dried biomass, 3-fold higher FAME yield, but less than 50% FAME recovery | [129] |
| Microwave          | Scenedesmus obliquus | 7.6% | 0.1 L/min | 9 | Hexane extraction, gravimetric settle, 77% of total lipid recovery | [130] |
|                    | Botryococcus, Chlorella, Scenedesmus | 0.5% | 0.1 L/min | 420 | Bligh method, superior lipid recovery to other tested methods | [108,116] |
| Subcritical water  | Chlorella vulgaris | 20% | 0.005 L | 5 | 77–90% lipid in solid, no lipid extraction | [131] |
|                    | Scenedesmus sp | 1.20% | 0.003 L | 78 | Bligh method, 66% lipid-rich biomass residue yield, with 45% lipid content | [132] |
|                    | C. sorokiniana | 10% | 0.1 L | 6 | No lipid extraction, oil-rich residue was liquefied to bio-crude | [133] |
|                    | C. curvatus | 10% | 0.1 L | 7 | No lipid extraction, oil-rich residue was liquefied to bio-crude | [134] |

Note: The energy available from the combustion of 1 kg of the algal dry mass is estimated to be 26.9 MJ [116].
Spiden et al (2013) evaluated these metrics using *S. cerevisiae*, *Tetraselmis* sp., *Chlorella* sp. and *Nannochloropsis* sp. [114,113]. It was found that turbidity could be affected by aggregation of cell debris; likewise the particle sizing might also not work well if agglomeration occurs. Protein and UV absorbance tests tend to overestimate the cell rupture at low severity, due to the leaking of water soluble compounds prior to fragmentation [114], while cell rupture is likely to be underestimated at high severity because of degradation of released protein. The authors concluded that the cell counting was the most accurate approach to estimate the cell disruption. However, all the methods tested are indirect to evaluate the lipid extraction efficiency. Measuring lipid recovery, rather than the cell rupture degree, is the best method to estimate the impact of cell disruptions on lipid extraction [112].

We have tested the effects of HPH on lipid extraction from *Scenedesmus acutus*. The preliminary results showed that fatty acid recovery could not exceed 80% after the HPH, despite that almost all cells were ruptured by using cell counting method (unpublished results). In addition, severe emulsion problems were observed in lipid extraction after the HPH processing, and extra demulsification steps had to be applied to facilitate oil extraction. It is well known that HPH is usually suitable for emulsification process in dairy industry to disperse oil droplets in milk forming the stable product known as homogenized milk. Microbial oil droplets might be extensively dispersed in the slurry after HPH and also probably form stable emulsion with amphiphilic compounds, such as released protein, polar lipids and polysaccharides. Thus, future investigation should use lipid recovery to evaluate cell rupture processes in terms for biofuel application, and the potential emulsion issue should also be taken into account as well.

A recent study suggests that minimal equipment and energy are possible for HPH in an algal biofuel application [115]. The energy consumed by HPH represented between 0.2 and 144.5 MJ/kg dry biomass, depending on cell concentration, HPH pressure, cell wall frangibility and TAG content. Assuming the energy available from the combustion of 1 kg of the algal dry mass is 26.9 MJ [116], this result indicates that HPH might be a promising scalable approach for cell disruption in a biofuel application, provided species with weak cell wall and high oil content was used. The efficiency of HPH on cells varies remarkably across different species and can be growth stage dependent due to different cell wall rigidity [117,113,109], and therefore accurate energy requirement should be evaluated on a case-by-case basis. Though promising, further evaluation of HPH in an industrial scale biofuel production process is needed.

4.2. Bead milling

Cell disruption can be achieved by grinding biomass against the solid surfaces of beads during violent agitation [112]. Bead milling has been applied to microalgae [135], bacteria [112], yeast [136], and fungal biomass [137] to assist lipid extraction. Shen et al. [138] found that for *Scenedesmus dimorphus*, milling followed by hexane extraction gave a lipid recovery 4 times higher compared to Soxhlet extraction. Similarly, bead-milling of *Chlorella protothecoides* followed by hexane extraction recovered 3.4 times more oil than Soxhlet extraction. Bead milling was reported in large-scale applications to allow for the recovery of high-value products, like enzymes [139], however, energy and cooling demands can be higher than HPH because of the additional work and friction involved in moving the bead/cell slurry [112]. A detailed energetic study had been conducted and the power requirements for disintegration of *Chlorella* sp. ranged from 10.2 to 36.1 MJ/kg dry weight (Table 3), which is unlikely to be economical for biofuel production [121,140]. In addition, since cell disruption was estimated by cell counting in this report, the energy requirement might be even higher if oil recovery was applied to evaluate the cell rupture due to the limited polar lipid accessibility and possible emulsion issue. Thus, although bead milling is an efficient cell rupture method in laboratory application, it is not a cost-efficient approach for the industrial biofuel production.

4.3. Ultrasound

Ultrasound-induced cell disruption can improve lipid extraction from microalgae [124,141,125], yeast, and fungi [141,142]. The disruption rate for ultrasonication follows a parabolic relationship with initial cell concentration [119], and lipid yields decreased dramatically when biomass concentration increased in the slurry [123], indicating a major technical challenge to handle slurry with high cell concentration. In the same study it was reported that the mean disruption rate constant for ultrasonication was about seven times lower than that for HPH. As shown in Table 3, the energy consumption for ultrasound is usually high (more than 360 MJ/kg), with the exception of the 2 MJ/kg for *S. limacina*, which is well known as a heterotrophic alga with very fragile cell wall. It should also be noted that, in this report, high volume of ethanol was used to form a single phase (95% ethanol) which could totally dissolve all the lipids in the tested biomass, but only about 50% of total lipids was recovered after the cell disruption. Thus, the low energy requirement in this case could hardly justify the application of ultrasound as a practical approach for biofuel production.

High power ultrasound is a well-known technology to assist the formation of emulsion when the cavitation threshold is attained, even without the presence of emulsifiers [143]. Thus, the ultrasound pretreatment might lead to stable emulsions or extensive dispersion of lipid droplets prior to lipid extraction and therefore reduce the lipid extractability. Although this method can efficiently ruptures cell walls in a lab-scale, it is energy-intensive [144] and difficult to scale up.

4.4. Pulsed electric field (PEF)

Recently, applications of electric fields, such as the pulsed electric field (PEF), has been reported to be promising for intracellular compounds extraction from wet biomass [145] and was shown to be useful for lipid extraction from algae *Synechocystis* PCC 6803 [126] with 70% increase in extraction efficiency, but the total recovered FAME was only 3.03% based on dry cell weight. Eing et al (2013) freeze-dried PEF-treated biomass and extracted with ethanol to obtain 70% of oil yield, which was about 4 times higher than untreated biomass [127]. However, freeze-drying is also a cell disruption, after which cells are usually observed with porous and loose cell wall structures for improved lipid extraction. The effects of PEF on lipid extraction should be evaluated by using treated wet biomass without being further processed.

Notably, PEF is more effective to release small molecular, water-soluble enzymes [146] and ionic compounds rather than compounds with high molecular weight [147] and nonpolar compounds [148]. Eing et al (2013) found that oil could not be released to the medium after PEF due to the large volume of oil droplets [127]. Thus, PEF can facilitate the release of ionic compounds to realize a sequential selective recovery of hydrophilic compounds [149,150]. If this is the case, potential emulsifiers such as amphiphilic protein and polysaccharides might be removed before oil extraction assisting the subsequent oil extraction. However, a secondary cell disruption method, such as HPH or ultrasound, would be needed to further rupture cell wall for better oil extraction [149], and this approach is thus unlikely to be energy-efficient. In summary, the application of PEF for rupturing cell walls for oil extraction is still not practical for biofuel production due to the low oil extraction efficiency and high energy requirement (Table 3).
4.5. Osmotic shock

Sudden change of osmolality, termed osmotic shock, can induce cell rupture due to differing pressures of solutes. Osmotic shock induced by NaCl and sorbitol followed by a lab-scale co-solvent extraction method (hexane: methanol = 7:3) was applied to freshwater wet *Chlamydomonas reinhardtii* biomass to increase lipid recovery. It was found that osmotic shock increased lipid extraction by 2-fold when used on a cell wall-less mutant [20], suggesting a very limited application for cells without rigid cell walls. Osmotic shock has also been applied to freshwater *Botryococcus sp.*, *Chlorella vulgaris*, and *Scenedesmus* sp. [108] by using 10% NaCl, but the results suggested osmotic shock is highly species-dependent and not an outstanding disruption method compared to other options. The recycle of added chemicals and waste water treatment [151] might make this process very expensive.

For seawater algae, the osmotic shock can be performed at hypertonic shift, which is triggered by transferring seawater algae to low salt medium. Halophilic algae *Dunaliella viridis* can release up to 60% of accumulated lipids to medium when they are transferred to low salt medium [152]. The release of lipid microparticles from the cells that are disproportionate to cell lysis was observed under hypertonic drift, indicating the existence of a reversible membrane permeation mechanism in *Dunaliella*. This is an interesting observation and can be potentially useful to recover lipids. However, this technology is still at very early stage and only tested in relatively dilute cell suspensions. In addition, solid/liquid separations to transfer the cells into low osmolarity media will also add to cost.

4.6. Microwaves

Microwave-induced heating is very selective to polar solvents such as water and can generate steam, which will rupture the cell wall releasing intercellular contents [153] and potentially lead to an effective oil extraction procedure. Microwave pretreatment followed by the Bligh–Dyer extraction method demonstrated the best oil extraction from wet *Botryococcus sp.*, *C. vulgaris*, and *Scenedesmus* sp. [108] compared to other methods, such as autoclaving, bead milling, sonication and osmotic shock. However, Yu et al. (2015) showed controversial results, indicating that microwave treatment was only slightly better than autoclaving, but remarkably inferior to other methods, such as sonication, bead milling and acid hydrolysis [142]. Balasubramanian et al. (2011) processed *Scenedesmus* obliquus biomass in a continuous microwave and showed a 77% of total oil recovery using a subsequent hexane extraction [130]. The energy requirement for this experiment was about 9.5 MJ/kg DCW, which is promising as an early stage investigation. However, very few reports with detail energy utilization in this field have been published since.

Microwave treatment at atmospheric pressure is unlikely to remarkably reduce the insoluble solid amount or emulsifier contents to improve lipid extraction. Previously, severe emulsion formation was observed in a lipid extraction from *C. sorokiniana* after microwave treatment at ambient pressure using hexane as solvent (Dr. Shulin Chen’s Bioprocessing and Bioproducts Engineering Laboratory, Washington State University, unpublished data). It was speculated that the gelatinized starch could assist the formation of emulsion by forming stable lipid-starch complex, adversely affecting the lipid recovery. Although microwaves can be applied in an industrial scale, the efficiency on lipid recovery and energy requirement is still questionable.

4.7. Subcritical water hydrolysis

Subcritical water is defined as liquid water in the temperature range from the boiling point to near the critical point (100–374 °C). The ionic products of water, H+ and OH−, are relatively high in the subcritical range and suggest that acid or base-catalyzed reactions (e.g. biomass hydrolysis) can be effectively accelerated [154,155]. Levine et al. [131] applied subcritical water to remove non-oil biomass leaving an oil-rich biomass cake for biodiesel production. Similarly, a flash hydrolysis process was performed at subcritical water conditions to release nitrogen-containing compounds (amino acids and peptides) to the water phase [132,156], producing an oil-rich biomass residue [132]. In addition, Chao et al. [157,134] applied subcritical water extraction to microalgae and yeast biomass to recover polysaccharides and protein-derived products from aqueous phase. This approach requires moderate-to-high energy inputs (5–78 MJ/kg DCW), based in large part on the concentration of biomass (Table 3). The energy consumption might be reduced by improving heat recycling efficiency during the cooling process.

This method might be promising to assist lipid recovery due to the cell wall hydrolysis capability of subcritical water. Also, phospholipids can be hydrolyzed to FFA under subcritical water condition [131], indicating better extractability by nonpolar lipid due to the resulting increase in target hydrophobicity and the concomitant decrease in emulsification properties. Considerable amounts of biomass (protein and polysaccharides) can also be hydrolyzed into water soluble compounds to potentially improve mass transfer during lipid extraction. However, it is not clear if the application of subcritical water hydrolysis can assist oil extraction, because biomass residues usually tend to coagulate [132] and therefore entrap oil after the high temperature treatment leaving more uncertainties for the approach. Nevertheless, it is worth studying the lipid extraction efficiency from the oil-rich residue after subcritical water hydrolysis.

This technology is capable to be scaled up for industrial applications. However, the major drawback of this method is the relatively high capital cost for the high pressure reactor, as well as need for large scale heat exchangers.

4.8. Enzymatic hydrolysis

Enzymatic hydrolysis is a promising, non-destructive pretreatment and can prevent thermally sensitive components from degrading. Enzymatic treatment has been proven to be successful in facilitating the extraction of oil from plant seeds [158] and has been demonstrated on microorganisms. Cho et al. [159] reported that lipid extraction yields by organic solvents improved by as much as 70% after enzymatic hydrolysis of *C. vulgaris*. Fu et al. [160] applied enzymatic hydrolysis to hydrolyze *Chlorella* to increase sugar and lipid recovery.

As shown in Table 4, the biomass concentration in reported enzymatic hydrolysis is usually low, leading to a relatively high energy consumption despite the lower temperature compared to subcritical water treatment. Pretreatment prior to enzymatic hydrolysis might be able to increase the biomass concentration and improve the lipid recovery as well. Jin et al. [161] reported that lipid recovery could be increased by 5-fold by pretreating biomass with microwaves prior to enzymatic hydrolysis, leading to a lipid recovery up to 96.6% with ethyl acetate extraction (Table 4). The
| Species                | Biomass concentration | Condition & scale                                                                 | Energy a (MJ/kg) | Analyses and outcome                                                                 | References   |
|-----------------------|-----------------------|-----------------------------------------------------------------------------------|------------------|-------------------------------------------------------------------------------------|--------------|
| **Enzymatic**         |                       |                                                                                   |                  |                                                                                     |              |
| C. vulgaris           | 1%                    | Cellulase, β-glucosidase, 50 °C, 72 h                                             | 10.4             | Bligh method, 73.1% lipid recovery; 85.3% sugar yield                                | [159]        |
| Chlorella             | 2%                    | Immobilized cellulase, 50 °C, 72 h                                               | 5.2              | Hexane extraction 56% lipid yield; 62% sugar yield;                                  | [160]        |
| *R. toruloides*       | 12.8%                 | Microwave pretreat 1 min, β-1,3-glycomannanase, 37 °C, 2 h, 0.01 L               | 8.4              | Ethyl acetate extraction, 6200 g for 5 min, 96.6% lipid recovery                    | [161]        |
| *Mortierella alpina*  | 3%                    | Pretreat at 80 °C 30 min, pectinase: papain (5:3, v/v), 60 °C, 6 h, 0.02 L,       | 4.9              | Hexane extraction, 6000 rpm for 5 min, 100% lipid recovery                           | [165]        |
| *Scenedesmus sp.*     | 0.25%                 | Cellulase:pectinase:hemicellulase ratio of 1:1:1, 30 °C, 60 h, 0.03 L              | 8.4              | Hexane extraction, 5000 rpm for 5 min, 86.1% lipid recovery                         | [183]        |
| **Chemical hydrolysis** |                       |                                                                                   |                  |                                                                                     |              |
| *C. curvatus*         | 2.5–5%                | 0.17–0.3 M HCl, 78 °C 2 h, 0.02 L                                                 | 4.4–8.8          | Modified Bligh method, 87.5% lipid recovery                                          | [142]        |
| *Scenedesmus, Chlorella, Nannochloropsis* | 7.5%                  | 2% H,SO₄, 145 °C, 10 min, 0.005 L                                                 | 6.7              | Hexane extraction, 8437 g for 10 min, 97% lipid recovery                              | [184]        |
| *S. acutus*           | 25%                   | 2% H,SO₄, 155 °C, 10 min, 4 L                                                     | 2.3              | Hexane extraction, 2000 g for 10 min, 87% lipid recovery                              | [176]        |
| *C. vulgaris*         | 2%                    | 1% H,SO₄, 150 °C 8 min, 0.1 L                                                     | 26.0             | Hexane extraction, 88% fatty acid lipid recovery                                     | [185]        |
| *Chlorella sp.*       | 2%                    | 41 mM FeCl₂ or 29 mM Fe₂(SO₄)₃, 2% H,SO₄, 120 °C, 90 min                         | 19.8             | Hexane extraction, 89.5–94.5% lipid recovery                                        | [186]        |
| *Chlorella sp.*       | 1.7%                  | 2 mM K₂S₂O₇ or 0.5% H,SO₄, 90 °C, 60 min, 0.01 L                                | 15.9             | Chloroform extraction, 1500 rpm for 5 min, 95% lipid recovery                       | [181]        |
| *Autrariochytrium*    | 1.5%                  | Poly-dimethylaminomethylstirene (pDMAMS) membrane, 0.015 L, room temperature      | None             | Hexane extraction, 4000 rpm for 10 min, 25.6% lipid yield compared to 0.77% yield in control (total lipid content in biomass is not given) | [174]        |
| *Chlorella sp.*       | 1%                    | Organic-nanoclay, 20% based on dry biomass weight, 1 L                            | None             |                                                                                      | [175]        |
| **Other methods**     |                       |                                                                                   |                  |                                                                                     |              |
| *Chlorella sp*        | 2%                    | TiO₂ for harvest and then rupture cell by UV irradiation, 365 nm, 3 h, 0.2 L      | 40.5b            | 95% damage/disrupted by cell counting using Nile red                                  | [182]        |
| *H. pluvialis*        | Not given             | Induce germination of cell to weaken cell wall                                    |                  | 1-Ethyl-3-methylimidazolium ethylsulfate, 1 min extraction, 82% astaxanthin recovery | [172]        |
| *H. pluvialis*        | Single cell           | Crystalline gold nanoscalpel (Au-NS) to incise cells to release astaxanthin       |                  | Astaxanthin leaks from injured cell, which healed later accumulating more astaxanthin | [187]        |

a Energy is needed for the elevated temperature during the pretreatment.
b The energy is required for UV irradiation.
improved lipid extraction was attributed to the physical changes of cell wall, which demonstrated a rough and barbed surface after microwave pretreatment. The damaged cell wall was expected to be more susceptible to enzyme hydrolysis. Similar observations have also been reported recently [162–164]. However, the total cost for this combined process could be prohibitive. It should be said the choice of solvent might also have played an important role in this case, because ethyl acetate could extract about 8-fold more oil than hexane extraction, probably due to its higher affinity to polar lipids (including non-fuel lipids). However, the solubility of ethyl acetate in water is about 8.7% (Table 2), indicating an expensive distillation process is needed for the solvent recycle. Thus, the general applicability and practicality of this approach are uncertain.

Enzymes present a unique challenge in that, in designing an efficient enzymatic procedure for hydrolyzing microbial cell walls, one must first determine the composition of the cell walls. Typically, enzymatic hydrolysis requires a cocktail formulation of several diverse enzymes to effectively hydrolyze the cell walls [165,166]. Although enzymatic hydrolysis is a scalable approach, economic viability due to the relative high cost of enzymes impedes the implementation in biofuel production. Immobilization of enzyme could reduce the overall cost [160], but mass transfer between insoluble cell wall and immobilized enzyme might be very slow and result low cell wall disruption efficiency. In addition, algal cell walls vary to a remarkable degree based on species and growth stage and therefore enzyme cocktails might need to be customized for a specific process and perhaps fine-tuned depending on the variable quality of biomass harvested throughout the year.

4.9. Autolysis and germination

Autolysis is a natural self-digestion process causing disruption of cell structures and releasing cytoplasmic and cell wall compounds [167]. It has been widely observed in fungi, yeast [168,169] and bacteria [170,112]. Autolysis usually takes place at the end of the stationary phase and is associated with cell death [171] and increased autolysis-related protease and chitinase activities [169]. Autolysis can be induced by temperature, osmotic pressure, detergents, pH, or other stressors.

Incubation of algal biomass prior to HPH seems to be effective to weaken cell wall (Table 3), increasing cell disruption efficiency in the following HPH. The cell wall weakening by incubation has been tested [23,120], and lipid recovery of 25% and 70% was obtained from N-replete and N-deplete biomass, respectively. Autolysis is a promising approach to weaken or break down cell walls with very low energy requirement [95] to improve lipid extraction, though there are costs associated with long term incubation.

The recovery of astaxanthin from the Haematococcus pluvialis cyst cells remains an energy-intensive process, because the accumulation of astaxanthin in is accompanied by the formation of a rigid cell-wall consisting of trilayers which are remarkably resistant to physical and chemical cell disruptions. Recently, a novel strategy utilizing a short-period germination based on the natural life cycle of H. pluvialis was developed as an energy-efficient pretreatment for the extraction of astaxanthin [172]. The germination resulted in damage and deconstruction of the cyst cell wall, and thereby facilitated the extraction of astaxanthin by ionic liquids at room temperature. About 82% of astaxanthin yield could be obtained after this natural pretreatment along with sequential 1-ethyl-3-methylimidazolium ethylsulfate extraction. This research opens a new avenue to simplify cell disruption by utilizing algal biology.

4.10. Chemical hydrolysis

Oleaginous biomass can also be efficiently hydrolyzed by chemical pretreatment. Acid hydrolysis has been reported to be an effective method to disrupt yeast cell walls for lipid extraction [173] and using a suitable solvent system for lipid recovery can be adapted to the pilot-plant scale [173]. Yu et al. [142] for the first time conducted a comparative investigation using several different species of oleaginous yeast, fungi, and microalgae to report lipid recovery yields after various cell disruption methods. They concluded that acid hydrolysis prior to extraction was the simplest yet effective method to extract lipids from oleaginous microorganisms compared to bead beating, microwaving, autoclaving, or sonication. Heterogeneous solid acid catalysts have also been reported for acidic hydrolysis of biomass to improve lipid extraction [174,175].

Laurens et al. (2015) reported that dilute acid pretreatment can assist lipid extraction to recover up to 97% of total fatty acids using hexane. The microalgal cell wall was ruptured and degraded, Fig. 4. Morphological changes of algae’s cellular structure after acid pretreatment relative to the original biomass (A) Nannochloropsis before treatment, (B) Nannochloropsis after acid treatment, (C) Scenedesmus before treatment, (D) Scenedesmus after acid treatment.
Chemical pretreatment may be a promising approach to facilitate lipid extraction from oleaginous microorganisms for two reasons: (1) it effectively ruptures cell walls and the linkages between lipids and the biomass matrix thus making lipids more accessible to the solvent and (2) amphiphilic polar lipids can be hydrolyzed to liberate fatty acyl chains leaving the hydrophobic moieties, which might be toxic to downstream catalytic upgrading, in the aqueous phase (Dong et al., unpublished observation), generating a cleaner lipid stream with increased FFA content.

### 4.11. Other emerging methods

Recently, with the fast development of nano-technology and ionic liquids, new cell disruption methods are emerging rapidly.

Hydroxyl radicals that are generated from semiconductors (TiO₂) under UV light irradiation can function as non-selective and strong attackers of cell surfaces [182]. Photocatalytically-active amineclay-conjugated TiO₂ was applied for both algal harvest and sequential UV-initiated cell disruption. Within 10 min, the injection of amineclay-conjugated TiO₂ into 1.5 g/L-concentration microalgal feedstocks produced an 85% harvesting efficiency through adsorption. Subsequent UV-irradiation resulted to 95% of cell disruption based on cell counting (Table 4). Although UV energy consumption is relatively high (40.5 MJ/kg dry biomass), the method might be promising because it simplified harvesting and cell disruption process by using multifunctional TiO₂. However, the UV light could not penetrate concentrated biomass and this might adversely affect the disruption efficiency in a large scale application. Also, the recycle and regulation of nanoparticle in algal processing should be further investigated.

In a proposed milking process, target compounds are continuously extracted from cells which keep accumulating the compounds. Recently, PraveenKumar et al. (2015) reported extraction of astaxanthin from a single cell of H. pluvialis through incision of the cell wall by a novel gold nanoscalpel (Au-NS), which allows single-cell analysis of wound healing and reaccumulation of astaxanthin [172]. The results indicate that upon the Au-NS incision, the injured cell could reaccumulate astaxanthin at a rate two times faster than the control cells. The demonstration of regenerative extraction of astaxanthin at a single cell level hints toward the potential of a milking process for continuous recovery of target compounds from microalgae while keeping the cells alive. Even though this is a proof-of-concept research at very early stage of development, this kind of novel idea might spark new thoughts for lipid extraction using non-traditional approaches.

### Table 5
Comparison of biomass pretreatment methods in the context of an industrial biofuel production process.

| Disruption methods | Scalability | Energy consumption for acceptable disruption | Ability to improve lipid accessibility | Ability to improve mass transfer |
|--------------------|-------------|---------------------------------------------|--------------------------------------|---------------------------------|
|                    |             |                                             |                                      | Reduce insoluble biomass | Reduce emulsion |
| HPH                | H           | L-H                                        | L                                    | L                              | L                 |
| Bead mill          | H           | H                                          | L                                    | L                              | L                 |
| Ultrasound         | L-M         | H                                          | L                                    | L                              | L                 |
| PEF                | L-M         | H                                          | L                                    | L                              | L                 |
| Microwave          | M-H         | M-H                                        | L                                    | L                              | L                 |
| Subcritical water  | H           | L-H                                        | M-H                                  | M-H                            | M-H               |
| Enzymatic          | H           | L-M                                        | M-H                                  | M-H                            | M-H               |
| Chemical hydrolysis| H           | L                                          | M-H                                  | M-H                            | M-H               |

H: high; M: medium; L: Low.
4.12. Comparison of biomass pretreatment methods for industrial lipid extraction

The well-established biomass pretreatment methods are summarized in Table 5 for better comparison regarding scalability, energy consumption and effects on lipid extraction. The prime function of biomass pretreatment is to expose lipids for better extraction. As discussed above, high lipid recovery results have been often observed after pretreatment methods such as HPH, bead mill, ultrasound, enzymatic and chemical hydrolysis. Screening by energy consumption, it is obvious that pretreatment methods such as HPH, enzymatic and chemical hydrolysis are more attractive due to the relatively lower energy requirements. All these methods can be scaled up to industrial application and these methods are probably the most promising approaches from the perspective of cell disruption.

As discussed in Section 3, lipid extraction is also affected by limited polar lipid accessibility, biomass interfacial blocking, and emulsion formation. By using enzymatic and chemical hydrolysis, the accessibility of polar lipids might be improved by breaking the bonds between lipids and biomass matrix. Hydrolysis of polar lipids to produce FFA with more hydrophobicity is favored for better extraction. The content of solid biomass can also be reduced via enzymatic and chemical treatment to expose more lipids for better mass transfer. Moreover, emulsifiers such as polar lipids, polysaccharides, and protein can be simultaneously hydrolyzed, leading to a simpler downstream process and more efficient solvent recycle. Thus, considering the potentials in these areas, enzymatic and chemical treatments are very promising to further improve lipid extraction efficiency. However, the cost of enzyme and chemicals should be taken into account. In addition, we found that discussions on emulsion formation in wet extraction processes have been usually overlooked. Centrifugation has been routinely applied to assist phase separation after the extraction (Table 3 and 4); however, readers could not get sufficient information to estimate the stability of emulsion. It will be helpful to report the stability of emulsion and demulsification approach in a wet extraction for a more comprehensive evaluation of biomass pretreatment and lipid extraction process.

As shown in Table 5, subcritical water hydrolysis seems to have the similar capabilities to reduce solid biomass content, hydrolyze emulsifier, and increase polar lipid accessibility. The technology is scalable and can be energy-efficient with concentrated biomass. Despite all these facts, there is not yet enough lipid extraction work in this field to verify if the subcritical water hydrolysis is an efficient biomass pretreatment approach to assist lipid extraction. This might be worth of further investigation.

Although HPH is not likely able to reduce biomass and emulsifier contents, it is still a very promising approach provided emulsion could be suppressed by combined methods. Also, HPH and subcritical water hydrolysis could be cost-advantageous compared to enzymatic and chemical pretreatment, because no additional chemicals or enzymes are needed. A detail techno-economic analysis (TEA) will be helpful to determine the best methods in an industrial biofuel production process.

5. Implementation of microbial lipid extraction in pilot and industrial scales

To date, microbial products for use as biofuels other than fuel ethanol have not been commercialized due to their relatively high production cost; however, high-value microbial products, such as ω-3 and ω-6 fatty acids [188,189] have been produced at pilot and industrial scales. Extraction of these high-value products is typically from dehydrated biomass powders or flakes at high purity. There are only a few reports on pilot and industrial scale microbial oil extraction from wet biomass. In this section, we discuss several widely cited reports for lipid extraction from oleaginous yeast, algal, and fungal biomass without extensive dehydration.

During 1990s the production of single cell oil as a cocoa butter substitute was developed at a pilot-scale using the oleaginous yeast, *A. curvatum* ATCC 20509 (*Candida curvata* D) [190]. At the pilot-scale level, a 250 m³ bubble fermenter was used for yeast biomass production producing 50 kg of yeast slurry. A continuous ball-mill was used to break the cell walls and the ruptured yeast biomass at 15–20% dry weight. The ruptured biomass slurry was extracted with a mixture of isopropanol/hexane. The extraction was implemented by a two-stage process. The first stage consisted of extraction of the slurry with a 60:40 (w/w) mix of IPA: hexane. The cell sludge was recovered by nozzle separator and re-extracted with pure hexane. The combined organic phases provided a recovery of up to 97% of the total oil. It was reported that hexane could be used as the sole solvent for extraction in a single stage, though this required longer extraction times. The addition of polar solvents likely reduces the activities of surfactants, increases mass transfer and reduces emulsion formation. However, the residual IPA then must be recovered from the aqueous cell sludge phase due to its high solubility in water. In the same study, a techno-economic analysis was carried out and it was identified that extraction of oil from yeast concentrate while still in the wet state is a key step for a successful industrial scale production of single cell oil, even for high-value cocoa butter substitute production. It is important to note that solvent recovery and energy inputs are of less importance for high value products than they are for biofuel production.

Pilot-scale production of arachidonic acid by the oleaginous fungi *Mortierella alpina* was carried out by Lion Corporation (Japan) [191]. A 500 L fermenter was used to culture 300 L of biomass broth. After 16 days of cultivation, cells were centrifuged and the wet cell mass was milled. The lipids were extracted with the co-solvents hexane and ethanol and were then esterified to methyl esters with further purification to enrich for ARA.

Martek Biosciences Corporation filed a patent using a countercurrent extraction process for oil recovery from a wet biomass slurry [192]. Dinoflagellate cell mass was harvested via centrifugation to produce a slurry of 14–20% w/w solids. This slurry was then ruptured by a HPH and pumped into the top of a 5 foot tall, 6 in. diameter column containing 5/8-in. metal disks as baffles. Hexane was injected from the bottom of the column allowing the aqueous phase to settle to the bottom while the hexane/oil phase would rise to the top. Multiple columns increased the oil yield up to 81% using 6 of these counter-current columns in series, when only water immiscible solvent was applied.

The above pilot-scale oil extraction and recovery examples were defined by the food-grade nature of the products. The physical cell rupture and use of food grade solvents were applied to protect the quality of the products. However, as food-grade quality is not necessary for biofuels production, alternative cost-effective cell rupture and extraction strategies can be used for more efficient and economical oil recovery. Future microbial lipid extraction researches need to demonstrate effectiveness, scalability and economic viability to show feasibility in an industrial biofuel production configuration.

6. Summary and conclusion

Since the high energy input needed for extensive dehydration of microbial biomass for lipid extraction precludes its use in biofuel applications, effective methods for extraction of wet biomass are
required for competitive process economics. However, lipid extraction from wet microbial biomass faces several major challenges such as limited lipid accessibility, reduced mass transfer, and formation of stable emulsions.

Although co-solvents are effective for total lipid extraction laboratory scale, they are not likely to be practical for industrial biofuel production. In such a setting, nontoxic nonpolar solvents with minimal miscibility in water and with minimal latent heat of vaporization are preferred, allowing for economical solvent recycle. Future research on industrial biofuel production should include solvents with these characteristics to evaluate lipid recovery from microbial biomass.

However, polar lipids are not effectively extracted by nonpolar solvents due to limited partitioning in the solvent and strong affinity to the biomass matrix. Amphiphilic polar lipids and other surface active compounds can also serve as emulsifiers that might complicate the extraction process by promoting stable emulsions which will reduce extraction efficiency, impede phase separation and result in solvent loss. In addition, solid residues can also reduce lipid extraction efficiency by blocking the solvent contact or accumulating on solvent-water interfaces to reduce mass transfer. All these challenges have to be tackled in a practical industrial biofuel production process.

A proper biomass pretreatment process can mitigate these problems to increase lipid extraction efficiency. In microbial biofuel production configurations, cell disruption processes are not only necessary to break down cell walls, but have the added benefits of providing a means to liberate combined lipids for better extraction, reduce insoluble solid residue to increase mass transfer, and diminish emulsifiers to improve solvent recovery. In this way, amphiphilic polar lipids can be converted into hydrophobic FFA for better extraction and utilized as preferred biofuel precursors with reduced toxicity in a downstream catalytic upgrading. Solid residue and emulsifiers can also be reduced to improve mass transfer and phase separation. Thus, future biomass pretreatment research should be comprehensively evaluated regarding lipid extraction efficiency, energy consumption, scalability and compatibility with downstream processing. A thorough techno-economic analysis will also be helpful to the overall cost of the extraction process and to provide guidance for improvements including a cost/benefit analysis of process modifications that increase lipid yields.

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