Algal Cell Disruption and Lipid Extraction: A Review on Current Technologies and Limitations

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Abstract Although numerous laboratory-based analytical techniques were developed and tested over the last five or six decades, industrial-scale algal oil extraction can be considered to be at its infancy.

Cost-effective, industrial-scale algal lipid extraction has been considered only after the advent of the algal biofuel industry. Presently, there is clearly a dearth of literature or reported results from commercial algal extraction technologies. When compared to land-based oil-seed crops, several fundamental differences exist for algal lipid extraction. Starting with the need for cost-effective harvesting and dewatering of dilute algal cultures (with 0.015–0.03 % solids) to differences in cell wall chemistry, and from the unsuitability of standard oil-seed pressing techniques to the need for cell disruption before drying, create unique challenges for microalgal lipid extraction. The present chapter discusses the limitations, challenges, and findings from numerous laboratory-based cell disruption and lipid extraction experiments and analytical techniques developed specifically to characterize or quantify algal lipids for nutraceutical, aquacultural, fine-chemical, or other value-added applications. Some potential industrial-scale, lipid extraction technologies are also discussed.

Keywords Biofuel • Biodiesel • Omega-3 • PUFAs • Algal oils • Solvent extraction • Pressurized lipid extraction • Microwave lipid extraction • Soxhlet • Co-solvent extraction • Sonication • Transesterification

1 Introduction

Commercial algal industry initially focused on harvesting and utilizing the entire cell contents as nutritive supplements and aquacultural feeds (e.g. *Spirulina* as human/animal nutritive supplement and *Chlorella* for aquacultural live feed). In the early

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1950s, the projected world population figures and insufficiency in protein supply triggered a search for unconventional protein (Spolaore et al. 2006). Algal biomass appeared at that time as a good candidate (Becker 2004). Aquaculture industry relies heavily on microalgae, which as a group represent the third-largest aquacultured crop in the world today (after freshwater fishes and mollusks) (Wijkstrom et al. 2000; Wikfors and Ohno 2001). Most aquacultural applications utilize small unicellular algal strains with easy digestibility and with appropriate proteins, fatty acids, and nutrients. In the 1980s commercial production of *Dunaliella salina*, as a source of β-carotene, became the third major microalgal industry (Spolaore et al. 2006). In the following years, the potential for numerous extracts and biomolecules (such as Astaxanthin, Lutein, Polyunsaturated Fatty Acids (PUFAs), etc.) has been identified. As a consequence of the directions of the algal industry to date, commercial lipid extraction did not receive significant attention. Although numerous laboratory-based analytical techniques were developed and tested over the last six decades, current industrial-scale algal oil extraction technology can be considered to be at its infancy. The true industrial-scale algal lipid extraction has been considered only after the advent of the algal biofuel industry. Presently, there is clearly a dearth of literature or reported results from industrial-scale algal extraction technologies. The present chapter discusses the limitations, challenges, and findings from numerous laboratory-based cell disruption and lipid extraction experiments and analytical techniques developed specifically to characterize or quantify algal lipids for nutraceutical, aquacultural, or other fine-chemical applications. Some potential industrial-scale, lipid extraction technologies are also discussed.

2 Background Information

Before discussing the various lipid extraction techniques, it is very important to understand the underlying facts and challenges pertinent to algal cultures, types of algal lipids, cell wall chemistry, differences between oil-seeds and algae, and difficulties in drying and employing oil-pressing techniques. These underlying facts and challenges are critical for understanding and overcoming the limitations of industrial-scale algal lipid extraction.

2.1 Algal Culture Densities and Moisture Content

Unlike land-based oil seeds which are relatively dry at the harvesting stage, algal biofuels start with extremely dilute liquid cultures. High rate open algal ponds typically have algal densities of 150–300 mg-dry/L, which relates to 0.015–0.03 % solids content. Although higher biomass densities are attainable, denser cultures often result in reduced productivity due to light limitations. Presently, Stage-1 harvesting (also known as dewatering) concentrates the dilute cultures to approximately 1–2 %

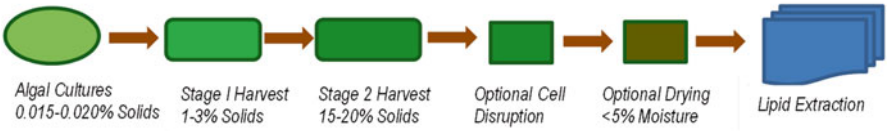

Fig. 1 Unlike oilseeds, significant energy is expended on preparation of algae for lipid extraction and usually involves: (1) stage-1 harvesting, (2) stage-2 dewatering, and an optional (3) stage-3 drying (to <5 % moisture)

solids (Dassey et al. 2014; Cooney et al. 2009). A high-powered centrifuge is usually employed as a Stage-2 system to concentrate the algae to about 15–20 % solids (approximately the consistency of peanut butter) (Fig. 1). The wet paste (with 80–85 % moisture) can be further dried to yield dry algal cake (<5 % moisture). Oil extraction can be performed either on the wet paste or dry algal cake. It is very important to note that significant amount of energy has to be expended to bring dilute algal cultures to ~20 % solids content. Therefore, drying beyond the wet paste consistency is energy intensive (Halim et al. 2011) may not be viable or cost-effective for biofuel or other low-value applications. In fact, this last drying step is known to create negative energy balances.

2.2 Types of Lipids in Algae

Algal lipids can broadly be defined based on the polarity of the molecular head group (Kates 1986a) as: (1) neutral (NL) or non-polar lipids, which comprise of acylglycerols and free fatty acids (FFAs) and (2) polar lipids which can be further sub-categorized into phospholipids (PL) and glycolipids (GL) (Halim et al. 2012a). Acylglycerol consists of fatty acids with ester-bonds to a glycerol backbone. Based on the number of fatty acids, they can be classified as triacylglycerols (TAG), diacylglycerols (DAG), and monoacylglycerols (MAG). FFAs on the other hand are fatty acids bonded to a hydrogen atom. Algal fatty acids range from 12 to 22 carbons in length and can be either saturated or unsaturated (Halim et al. 2012a). Neutral lipids are produced by microalgae for energy storage, therefore are also known as storage lipids. Neutral lipids are bound by relatively weak non-covalent forces (Van der Waals or hydrophobic associations) through their hydrocarbon chains to other lipids and to hydrophobic regions of proteins in the microalgae (Enssani 1990). Due to the weak bonding, neutral lipids are relatively easy to extract. Polar lipids, on the other hand, are part of the molecular building blocks of cell membranes. These polar lipids are harder to extract as they are capable of forming covalent and hydrogen bonds with adjacent molecules. Figure 2 shows the differences in composition of neutral and polar lipids in *Phaeodactylum tricornutum* obtained from two subsequent extractions with 1:1 chloroform: methanol co-solvent mixture (Ryckebosch et al. 2012). Apart from these two main classes of lipids,

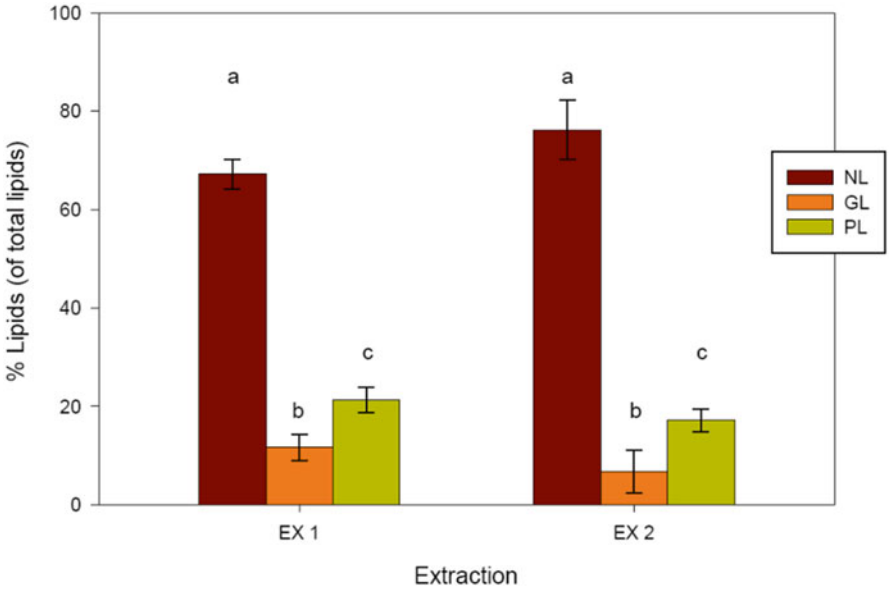

Fig. 2 Compositional variations in neutral (NL) and polar lipids in two consecutive solvent extractions using chloroform and methanol, mixed in 1: 1 (v/v) ratios (Reprinted with permission from Springer (Ryckebosch et al. 2012))

microalgae also contain neutral lipids without fatty acids, such as hydrocarbons, sterols, ketones, and pigments (carotenes and chlorophylls), which cannot be converted to biodiesels (Halim et al. 2012a). Furthermore, photosynthetically grown microalgae produce high levels of fat-soluble pigments, which hinder the lipid extraction and subsequent biodiesel production (Cooney et al. 2009).

2.3 Variations in Lipid Contents and Compositions

With an estimated 300,000 or more identified species of microalgae, it is impossible to assign a generic lipid composition profile to microalgal lipids. The lipid contents and compositions vary drastically between species and the amounts can range from 15 to 77 % (Chisti 2007). Even for the same species, the lipid content and composition is heavily influenced by culture conditions (light, light/dark ratio, temperature, nutrients, culture densities, etc.). Several researchers have demonstrated that selective nutrient starvation favors metabolic pathways that increase production of storage lipids (Halim et al. 2012a). In general, the oil content (percent) in most microalgal strains selected for biodiesel applications is comparable to or marginally higher than the best land-based oilseeds like rapeseed. However, the real benefit of employing microalgae lies with its potential to produce 10–20 times more lipids than oilseed crops on an aerial productivity basis (L/ha/y).

2.4 Difficulties with Algal Lipid Extraction

The algal cell walls have major variations in their structures, compositions, thickness and chemistry, all of which have a major influence on the choice of the lipid extraction process. For example, several unicellular algal cells with rigid cell walls will not be crushed but will rather flow with water through the thousands of micro-channels that exist in pressing equipment (Cooney et al. 2009). Apart from the cellulosic cell walls that are present for most green and brown algae, diatoms have hard silica frustules that are difficult to break. On the other hand, cells with weak cell walls (like *Dunaliella salina*) can be cracked easily by passing them through a homogenizer or through a pressure expansion valve. "Cell milking", which is a new concept (discussed later), appears to be viable only for cells with weak or porous cell walls. The well-established oil pressing techniques that was perfected for land-based oil seeds may not be practical in all cases for extracting algal lipids as the process requires relatively dry algal biomass with low moisture content (<5 %). Numerous researchers looking into the energy balances have clearly indicated that drying algae to less than 5 % moisture levels, although attempted at an analytical-scale, is impractical as input energy exceeds the energy content of the produced oil. A good and proven alternative to oilseed pressing is the solvent extraction process. However, the extremely low algal cell density and high moisture content in the harvested algal paste (80–85 % moisture after Stage-2 harvesting), coupled with the need for extraction of two different types of lipids (neutral and polar) adds additional complexity to the algal lipid extraction process.

3 Algal Lipid Extraction

Until recently, most algal lipid extraction techniques were based on lipid extraction from wet algal paste or dry algal cake, with or without pre-treatment or cell disruption. Several solvent extraction methods, starting from (Folch et al. 1957; Bligh and Dyer 1959) co-solvent mixture-based extraction to supercritical CO_2 extraction to pressurized lipid extraction have been developed and tested, mostly at laboratory-scale. Various combinations of cell disruptions and lipid extraction techniques have been employed. Some of the commonly used cell-disruption, extraction-augmenting methods, and extraction techniques are presented below.

3.1 Cell Disruption and Extraction-Augmenting Methods

Numerous researchers have employed cell-disruption prior to lipid extraction. Cell disruption techniques shatter the cell wall and facilitate better lipid extraction. Some of the most commonly employed cell-disruption/pre-treatment techniques include: bead beating (or bead milling), sonication, high pressure homogenization, heat

Table 1 Cell disruption and/or extraction augmenting techniques

Bead beating/Bead mill/Dyno mill	Bead beating uses grinding balls and high speed spinning (or agitation) to disrupt the cell walls. This is a well-established and widely used method, which has been used both on a laboratory as well as an industrial scale.
High pressure homogenization	This method was originally employed in the dairy industry, therefore is a well-established technology, both at the laboratory and industrial scale. High pressure pumps (positive displacement pumps) are used to force cells through a valve seat, which can be tightened for a narrower orifice and extreme pressures (up to 2500 bar). The cells are sheared as they are forced through a narrow orifice. As the pressurized fluids (with sheared cells, intact cells, liquids) exits the orifice, they are subjected to sudden expansion, causing an explosion that causes additional dell disruption.
Sonication	Ultrasound in the frequencies of 20 kHz and higher are employed for cell disintegration. As the high intensity waves propagate through the liquid, it creates alternating high-pressure and low-pressure cycles. These cycles create micro-bubbles that collapse violently in a process called as cavitation. These implosions cause very high localized temperatures (5000 °K) and pressures (2000 atm.), which facilitates cell-disruption. Although this technology is not as established as the earlier two methods or was proven economically viable for algae at the commercial scale, the technology lends itself ideally to a continuous-flow pre-treatment process, therefore, was grouped with other established methods.

disruption (including autoclaving, boiling, microwave heating), osmotic shocking, lyophilization (freeze drying), liquid nitrogen, lipolysis, alkaline/chemical pre-treatment, enzyme pre-treatment, and anti-oxidant addition. It is important to note that some of these methods are not always employed at the pre-treatment stage, but also in conjunction with solvent-extraction process to augment the extraction efficiency (e.g. simultaneous sonication during solvent extraction). A brief description of two mature and one promising industrial-scale cell-disruption methods, along with a short description for each are listed in Table 1. One important but neglected step in algal cell disruption and lipid extraction is the critical assessment of industrial viability of cell-disruption/extraction-augmenting technologies. Improvements in these areas can lead to significant cost-savings in the overall lipid extraction from microalgae.

The true benefit of cell-disruption is heavily dependent on the employed extraction method and/or algal strain. For example, if effective co-solvent mixtures (such as chloroform and methanol) are employed in sufficient volumes or if cells with thin or weak cells walls (ex. *Dunaliella salina*) are used, pre-treatment or cell disruption may not be necessary. Ryckebosch and co-workers (Ryckebosch et al. 2012) demonstrated that cell-disruption techniques (fresh algae-control, lycophilization, lycophilization and sonication, lycophilization and liquid nitrogen, lycophilization and bead beating) had no significant effect on the amount of total lipids extracted using 1:1 chloroform: methanol mixture, when compared to total lipids extracted from fresh algae. However, when ethyl ether alone was employed, lycophilization with bead beating performed significantly better than all other methods and extracted 92 % of non-polar lipids in the very first extraction. They indicated that petroleum ether could not sufficiently penetrate the cell wall or dissolved the components in the cell wall of intact cells.

In contrast to the findings of Ryckebosch and coworkers, Lee at al. (1998) reported almost twice the crude lipid yield by employing mechanical disruption of *B. braunii* with their chloroform/methanol (2/1 v/v) co-solvent extraction. More recently, Lee and co-workers (Lee et al. 2010) worked with aliquots of 0.5 g dry cell biomass blended with 100 ml distilled water, which was subjected to cell disruption using five different methods (autoclaving, bead beating, microwave, sonication, and osmotic shock). Their results indicated marked differences between the different cell-disruption methods, with bead beating and microwave treatments delivering consistently high lipid yields. However, it is very important to note that the reported results for various methods were not standardized against the energy consumed during the cell-disruption (e.g. per kWh). Such standardized comparisons are crucial for assessing the viability of an industrial-scale cell-disruption process.

Another recent comparative microalgal cell-disruption study was undertaken by Prabakaran and Ravindran (2011) who tested the efficacy of sonication, osmotic shock, microwave, autoclave, and bead beating on lipid extraction efficiency of three microalgal species (*Chlorella* sp., *Nostoc* sp., *and Tolypothrix* sp.). Their results indicated that all pre-treatment methods for all three species had a marked improvement over lipid extraction from the controls (no disruption). Among the tested methods, sonication, microwaves, and bead beating had the best extractions (Fig. 3).

If one were to base their decision on the well-established Folch or Bligh and Dyer methods, which do not require cell disruption and drying for achieving extremely high lipid extraction efficiency from most algal cells, it appears as though cell disruption can be avoided with a careful selection of the choice of solvents, co-solvent ratios, and solvent: sample proportions. Increasing the duration of the solvent interaction, agitation, or solvent temperature may also be of crucial importance

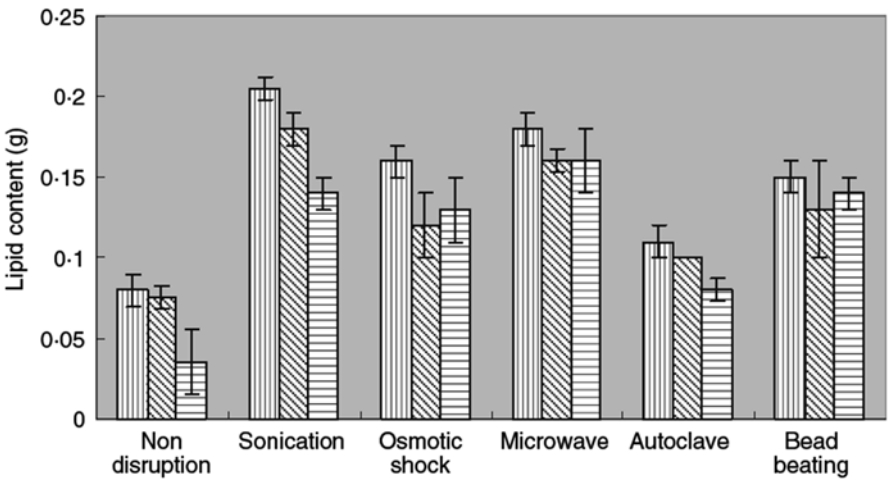

Fig. 3 Among the tested cell-disruption methods, sonication, microwave, and bead beating resulted in consistently high lipid contents. The three bars in each method represent *Chlorella* sp., *Nostoc* sp., and Tolypothrix sp., respectively (Reprinted with permission from Wiley (Prabakaran and Ravindran 2011))

for effective lipid extraction without cell-disruption. However, if one were to consider algal lipid extraction on process and economic viability, introducing a low-energy demanding cell disruption technique that will lower the solvent usage and solvent recovery costs sounds logical. Due to these mixed and contradicting results and numerous unanswered questions, better clarity on the role and need for cell-disruption or pre-treatment techniques is needed. New experiments that will not only quantify the improvements in lipid yields for each pre-treatment, but also account the energy/economic burden of each pre-treatment is very critical. Future research should also quantify lipid yield improvements for each cell-disruption method and standardize it against energy consumption.

3.2 Physical Extraction Techniques

3.2.1 Expeller or Mechanical Pressing

Oil presses or expellers are the most common method employed for extracting oils from oilseeds and nuts. The oily materials are mechanically squeezed under high pressures, which causes the material to heat up due to friction. The higher temperatures facilitate better oil recovery. Despite the simplicity of the unit and suitability for continuous operation, the extraction efficiency for commercially viable expellers is usually around 75 %. The same technology can also be used for microalgae if algae can be subjected to cell-disruption and cost-effectively dried to <5 % moisture levels. As mentioned earlier, drying algae to 5 % moisture content (or less) can induce negative energy balances (Halim et al. 2011; Scott et al. 2010), therefore, may not be a viable alternative for biofuel or other low-value applications. Another hurdle to overcome with the drying process is linked to the need for cell-disruption under moist conditions (Halim et al. 2012a; Cooney et al. 2009). In other words, the wet algal paste has to be subjected to cell-disruption prior to drying.

3.3 Solvent Extraction Techniques

3.3.1 Single Solvent Extraction

Organic solvents such as benzene, cyclo-hexane, hexane, acetone, and chloroform have been used for extracting lipids from microalgae. Solvent destroys the algal cell wall, and extracts oil from aqueous medium because of their higher solubility in organic solvents than water (Singh and Gu 2010). The oils may be extracted after subsequent distillation or solvent evaporation. Although hexane is reported to be one of the best solvents for lipid extraction, the overall lipid efficiency is relatively low as a portion of the neutral lipids are held in the cytoplasm as a complex with polar lipids. Releasing lipids from this complex is not easy as this complex is

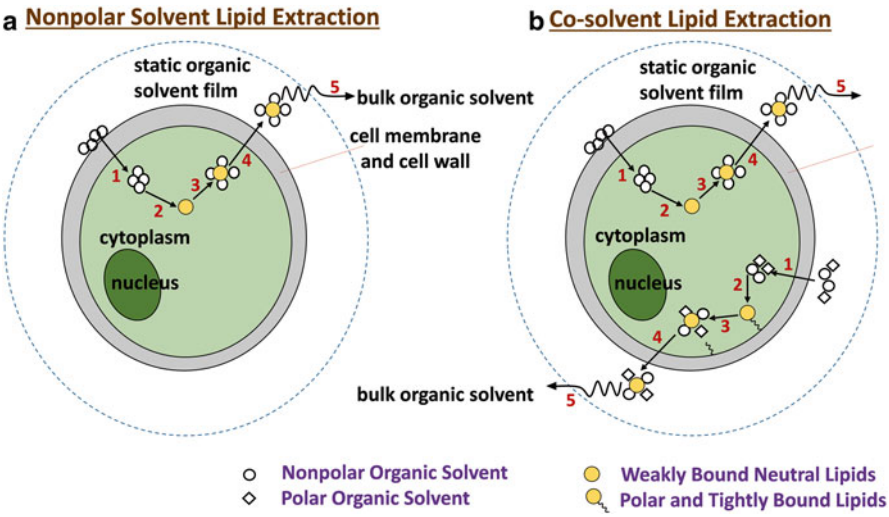

Fig. 4 Conceptual mechanisms of a single non-polar solvent (**a**) and co-solvent (**b**) based lipid extractions. Both mechanisms can be described in five steps. Step 1: penetration of organic solvent through the cell membrane. Step 2: interaction of organic solvent with the lipids. Step 3: formation of organic solvent–lipids complex. Step 4: diffusion of organic solvent–lipids complex across the cell membrane. Step 5: diffusion of organic solvent–lipids complex across the static organic solvent film into the bulk organic solvent (Recreated and modified with permission from Elsevier (Halim et al. 2012a))

strongly linked via hydrogen bonds to proteins in the cell membrane (Halim et al. 2012a). Due to this limitation, only a portion of the neutral lipids are extracted. The polar lipids in biomembranes, on the other hand are in intimate contact with aqueous phase of the electrolytes (Enssani 1990), therefore require the presence of membrane wetting medium such as polar solvents for effective extraction. These limitations led to the development of co-solvent based extraction procedures. Figure 4 depicts the conceptual mechanisms behind the single and co-solvent based extraction techniques.

3.3.2 Co-solvent Based Extraction

The co-solvent extraction method relies on the concept of "like dissolves like" and employs two-solvents for effective extraction. Lipids that are largely hydrophobic (neutral lipids) will favorably interact with relatively non-polar solvents (such as chloroform, ethyl ether, benzene), while membrane-associated polar lipids will require polar solvents (such as ethanol, methanol, isopropanol) to disrupt the hydrogen bonding and electrostatic forces between the lipids and proteins (Kates 1986b; Cooney et al. 2009).

Folch et al. (1957) were the first researchers to report a chloroform/methanol/water phase system for extraction of lipids from biological materials. This method

is still considered as a classic and most reliable method for quantitative extraction of lipids (Iverson et al. 2001). This method uses 1 part of sample to 20 parts of co-solvent (2:1, chloroform/methanol) for the initial extraction into a single phase solution (Folch et al. 1957; Iqbal 2012). After the initial extraction in a single phase liquid, the mixture is subjected to several washings with water, which induces biphasic separation. Neutral and polar lipids will partition to the organic phase containing both the solvents. The non-lipid contaminants (dissolved proteins and carbohydrates) will partition to the aqueous phase.

Bligh and Dyer's co-solvent extraction is the most cited reference method in literature for the extraction of lipids from biological materials (Burja et al. 2007). Although, both Folch and Bligh & Dyer methods are reported comparable (Iverson et al. 2001), the later method uses reduced volumes of solvents. In short, Bligh and Dyer method involves mixing 1 part sample with 3 parts co-solvent (1:2, chloroform/methanol) and conducting the initial extraction in a single phase. The mixture is later converted to biphasic solution by adding metered quantities of chloroform and water. The lipids partition to the heavier chloroform layer, while the non-lipids remain in the upper methanolic layer (Iqbal 2012).

Despite very successful and reliable extractions at the lab-scale, oftentimes without any prior cell-disruption, the co-solvent based system is not fully tested for algal lipid extraction at the industrial-scale. The complicated and delicate steps of monophasic extraction, followed by conversion to bi-phasic solutions and water addition/washings is not very conducive to a continuous-flow, industrial-scale lipid extraction systems. Due to these complexities, improved methods of extraction with single solvent were explored (e.g. PLE, discussed below).

3.4 Augmented or Modified Solvent Extraction Methods

3.4.1 Soxhlet Extraction

The solubility of an analyte in solvent (single and co-solvent) is governed by the Gibbs free energy of the dissolution process, which is directly related to the equilibrium constant governing the concentration of the analyte in either phase (Mead et al. 1986). A batch extraction with a limited solvent volume will reach a saturation point as the system will be limited by the lipid mass transfer equilibrium. One way to address this problem is to add continuous fresh solvent, which allows additional solubilization of the analyte in the solvent. However, continuous addition of fresh solvent adds costs and complexity due to recover of lipids from large volumes of solvent. Soxhlet extraction process uses a Soxhlet extraction apparatus, which employs a series of ingenious cycles of solvent evaporation and condensation to provide a continuous supply the fresh solvent to the analyte held in a special thimble. This apparatus overcomes two primary limitations as the solvent is reused multiple times and the extracted lipids are held in a concentrated form within a limited volume of solvent. However, the repeated evaporation and condensation cycles add

an additional economic burden on lipid extraction (Wang and Weller 2006). Halim et al. (2011) found Soxhlet operation of hexane extraction to be significantly more (280 %) efficient than a batch system when used for extracting lipids from *Chlorococcum* sp. However, the elevated temperatures potentially caused lipid degradation (Halim et al. 2012a). Therefore, despite the technical merits, the viability and suitability of an industrial-scale soxhlet extraction system for algal lipids is not clear from the reported literature.

3.4.2 Pressurized Lipid Extraction

In a pressurized lipid extraction (PLE) system, the extractions are carried out at elevated temperatures and pressures. With PLE systems (also known as accelerated solvent extraction or pressurized solvent extraction), the solubility of the analyte is greatly enhanced and the extraction process is completed in a shorter time as the desorption kinetics are greatly accelerated. Higher temperature increases molecular motion of the molecules and thereby decreasing the molecular interactions of hydrogen bonds, van der Waals forces, and dipole interactions (Cooney et al. 2009). Higher pressures increase the penetration power of the solvent through the cell wall and improve the transport of solvent to hard-to-reach areas of the cells (Cooney et al. 2009; Richter et al. 1996). The elevated pressures can reduce the dielectric constant of an otherwise immiscible solvent to values that better match the polarity of the lipids (Cooney et al. 2009; Richter et al. 1996; Herrero et al. 2006). Due to the improved penetration power and lipid extraction efficiency, many researchers are considering PLE systems that employ a single solvent, which is a technically viable alternative to the complicated co-solvent systems.

Accelerated solvent extraction was first reported by Richter et al. (1996) for extraction of chemicals from environmental samples. Numerous researchers have worked on laboratory-scale batch PLE systems with different solvents and different algal strains. Denery et al. (2004) extracted carotenoids and kavalactones from *Haemotococcus pluvialis* and *Dunaliella salina*, respectively. They found the optimum temperature and pressure to be 60 °C and 2000 psi. Presently, this technique is well known for its efficiency, shorter extraction times, and reduced solvent needs (Denery et al. 2004). Apart from the well documented literature on extraction of bioactive compounds from microalgae, the PLE method was not reported for extraction of microalgal lipids for biofuel applications (Iqbal 2012). Due to process advantages of a PLE system (e.g. use of single solvent), this system has potential for adoption at the industrial scale. As demonstrated by our research at LSU, PLE-based systems can be modified to Continuous Flow Lipid Extraction System (CFLES, Fig. 5) (Iqbal and Theegala 2013a). Our results indicated that CLFES achieved significant improvements in total glycerides at moderate temperatures and pressures (100 °C and 50 psi) when compared to Soxhlet extraction (Fig. 5). Despite potential advantages, more information is needed on the economic feasibility of algal lipid extraction using an industrial-scale PLE system.

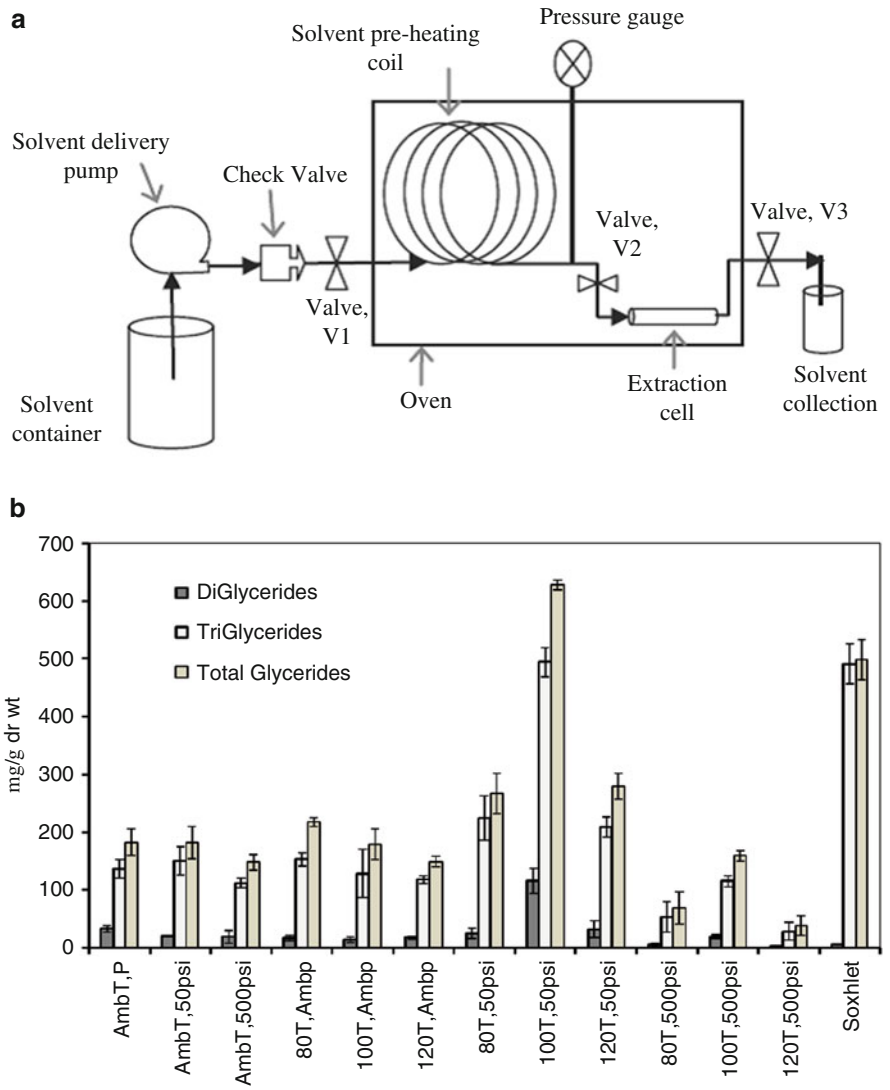

Fig. 5 (**a**) A schematic of the developed and tested Continuous Flow Lipid Extraction System (CFLES), and (**b**) comparison of total bound glycerides (mono-, di-, and triglycerides) extracted from *Nannochloropsis* sp. under different temperature and pressure combinations in CFLES and Soxhlet extraction (Reprinted with permission from Wiley (Iqbal and Theegala 2013a))

3.4.3 Microwave Assisted Solvent Lipid Extraction

Microwave radiation can be employed for assisting solvent extraction. When cells receive this radiation, localized superheating occurs which leads to instantaneous increases in temperatures and pressure within the cell matrices (Halim et al. 2012a).

The weak hydrogen bonds are disrupted by promoting the rotation of molecular dipoles, an effect opposed by the viscosity of the medium and strongly dependent upon the solvent and matrix (Cravotto et al. 2008). Our research at LSU has indicated that microwave assisted solvent extraction can have better yield than soxhlet extraction at 120 °C (Fig. 6a) (Iqbal and Theegala 2013b). As indicated earlier, microwaves have been effectively used for cell-disruption. Lee et al. (2010) have indicated that microwaves and bead-beating were the best cell-disruptors on their experiments with 3 different species of microalgae. Our experiments on extracting lipids from microwave assisted solvent extraction system indicated that cells are heavily disrupted (Fig. 6b). However, it is not clear if the microwave radiation merely causes cell-disruption or it directly aids in the solvent extraction process apart from cell disruption. This question can be answered by conducting controlled microwave assisted solvent extraction experiments on pre-disrupted algal cells.

The energy consumption for the microwave assistance is another important parameter that needs to be quantified. If one were to look at the well-established

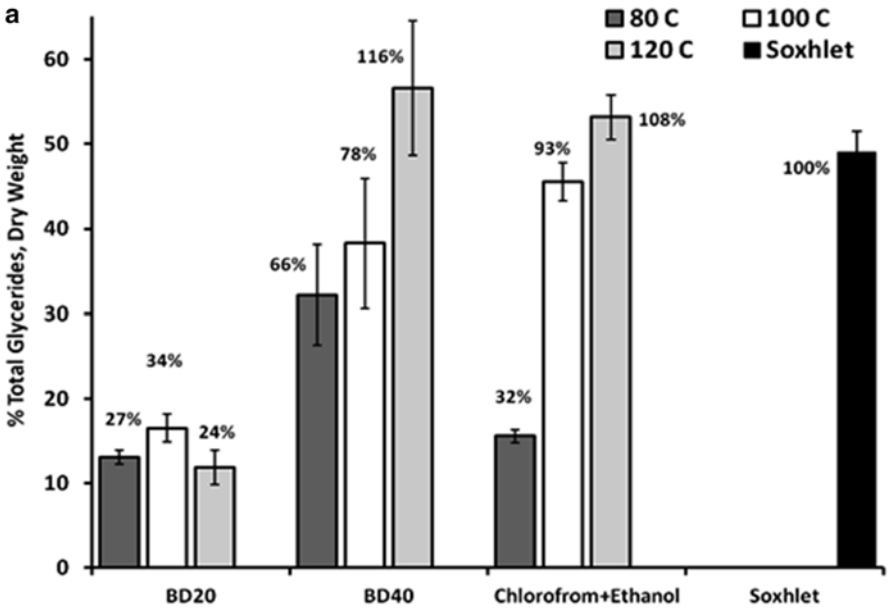

Fig. 6 (**a**) Total saturated fatty acid methyl esters (FAMEs) produced from oil extracted with microwave assisted extraction using BD20 (20 % ethanol in biodiesel), BD40 (40 % ethanol), and chloroform with ethanol as compared to conventional Soxhlet extraction. (**b**) Scanning electron microscope (SEM) images of *Nannochloropsis* sp. showing that microwave energy efficiently disrupted the microalgal cell structures. The top image shows intact cells, while the bottom image shows the cells exposed to BD40 in microwave assisted extraction at 100 °C (Reprinted with permission from Elsevier (Iqbal and Theegala 2013b))

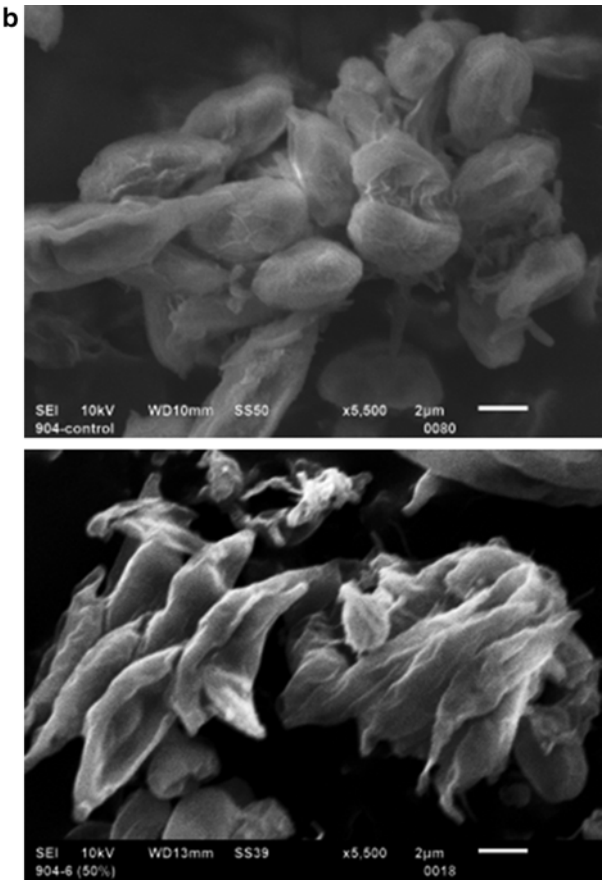

Fig. 6 (continued)

chemical industry, it can be clearly seen that electricity is the least preferred source for heating. If the efficiency factor for conversion of electricity to microwave energy is incorporated into the computations, the economic viability of microwave technology may be further impeded. Apart from these possible limitations, microwave assistance has several undisputed benefits for algal lipid extraction. When compared to traditional heating, microwaves can impart the energy in a very short time period. Secondly, as microwave heating is done without any direct liquid contact, development of an industrial-scale, continuous-flow microwave assisted solvent extraction system appears to be technically feasible. Thirdly, microwave assistance improves the lipid yields significantly (better than Soxhlet extraction). However, as indicated earlier, the improvements from microwave based methods have to be compared with other methods and standardized against energy consumption (e.g. energy consumed per unit increase in lipid yield).

3.4.4 Ultrasound Assisted Solvent Lipid Extraction

Apart from the use of ultrasound for cell-disruptions (discussed earlier), ultrasound has been employed during solvent extraction or along with a solvent. Wiltshire et al. (2000) have reported more than 90 % extraction of fatty acids and pigments from *Scenedesmus obliquus* with ultrasound (Wiltshire et al. 2000). Complete extraction of lipids from *Chaetoceros gracilis* by using ultrasound was subsequently evaluated by Pernet and Tremblay (2003). It was concluded that ultrasonic method increased the extraction rate, which directly influences the overall lipid recovery. However, it is not clear if ultrasound employed during/along-side the solvent extraction mainly assists in the cell-disruption or if it enhances extraction kinetics due to other mechanisms. Experimental results that quantify the improvements in lipid yield (per unit input energy) from employing ultrasound techniques, either at cell-disruption stage or during solvent extraction, are critically needed. In contrast to positive results, ultrasound was also reported as ineffective by researchers focusing on cell-disruption. Halim et al. (2012b) indicated that ultrasonication for 25 min of both low-density and high-density cultures, even at the highest power level (130 W), failed to effectively rupture the cells (*Chlorococcum* with thick cell walls), indicating that cell walls play a critical role. Apart from several unanswered technical questions, sufficient information on the feasibility or economics for a commercial-scale algal ultrasonication system is still not reported in literature (Singh and Gu 2010). Despite the drawback and limited/conflicting results, the ultrasound technology lends itself perfectly to a continuous-flow, industrial-scale process (be it cell-disruption or lipid extraction), therefore, merits further exploration and viability assessment.

3.5 Other Extraction Methods

3.5.1 Supercritical Fluids Extraction

Supercritical fluid extraction (SFE) is an emerging green technology that has the potential to replace the traditional organic solvent extraction (Halim et al. 2012a). In simple terms, when the pressure and temperature of a fluid are raised above their critical values (Tc and Pc), the fluid enters a supercritical region. In supercritical state, the fluid attains gas-like mass transfer properties and liquid-like solvating properties with diffusion coefficients greater than those of a liquid (Luque de Castro et al. 1994; Romanik et al. 2007; Leonard et al. 2008). While most of the applications employed CO_2 and water, several other fluids such as methanol, ethanol, and pentane have been reported in literature. Carbon dioxide has gained utmost importance as it has moderate critical properties (Fig. 7, 31.1 °C and 73.8 bar), low toxicity, low flammability, and chemical inertness (Cooney et al. 2009; Halim et al. 2012a). Several researchers have reported the use of SFE for effective algal lipid, fatty acid, and pigment extraction (Mendes et al. 1995, 2003, 2006; Taylor 1996).

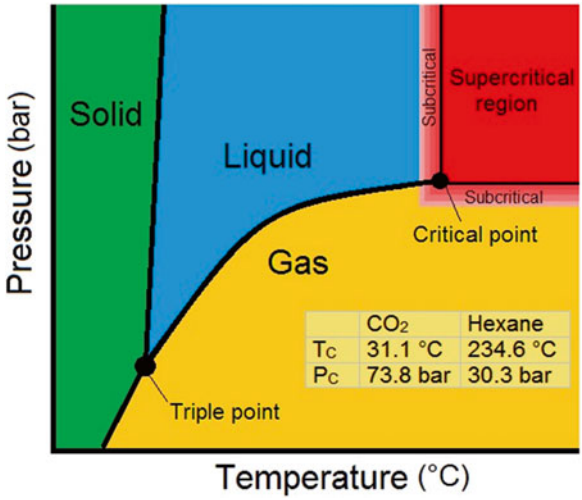

Fig. 7 A typical P-T phase diagram showing the solid, liquid, and gas phases and supercritical point. The supercritical temperature (Tc) and Supercritical pressure (Pc) for CO_2 and hexane are shown

Canela et al. (2002) looked into supercritical extraction of fatty acids and carotenoids from *Spirulina maxima*. Their experiments indicated that the temperature and pressure had little effect (above Tc and Pc), but the extraction rates were significantly different. Andrich et al. (2005) explored extraction of bioactive lipids from *Nannochloropsis* sp. using supercritical fluid extraction. They reported SFE to have comparable extraction efficiency to that of solvent extraction using hexane. Researchers have also looked into the variations to the traditional CO_2-based SFE. Subcritical solvent extraction was explored and found to have certain advantages over supercritical solvent extraction. The less intensive subcritical conditions retain certain features of the supercritical solvent extraction at a lower operational cost (Herrero et al. 2005). Chen et al. (2011) used ethanol at subcritical conditions and extracted lipids from wet past of *Nannochloropsis sp*. with a maximum efficiency of 90.21 % of total lipids.

Despite the lack of demonstrated deployment of the SLE technologies at an industrial-scale for algal lipids, the SLE process warrants further consideration as it has several potential merits, some of which include: (1) tunable solvating power, (2) improved lipid yield due to higher penetration power of supercritical fluids, (3) shorter extraction time as the fluids have liquid–gas properties, (4) production of solvent-free crude lipids, (5) inherent safety of an industrial-scale SFE system, and (6) process suitability for a continuous-flow, industrial-scale SFE unit (Halim et al. 2012a; Halim et al. 2011). Presently, the biggest drawback is its intensive energy needs (Cooney et al. 2009). Future research may perhaps offer a lower energy demanding process (or sub-critical process) with improved energy reuse.

3.5.2 Direct Transesterification

Historically, biodiesel oils are first extracted and purified and later subjected to transesterification to produce biodiesels. Direct transesterification is a "single-step" process that converts saponifiable lipids directly to fatty acid methyl esters (FAME) using an in-situ extraction/transesterification process. This method is gaining a lot of attention, at least at the laboratory-scale for analytical applications. In short, the process involves adding alcohol (such as methanol) and catalyst (base or acid catalyst) to algal paste (or dried algal biomass) and produce fatty acid methyl ester at elevated temperatures. Several researchers have employed reaction enhancing techniques such as microwaves and ultrasonication to achieve better extraction efficiencies. Positive results were reported from both assisted and un-assisted direct transesterification techniques. Griffiths et al. (2010) indicate that the most commonly used method of Bligh and Dyer was the least effective method for fatty acid production from three different microalgae, when compared to Smedes and Askland, Folch, and direct transesterification. They employed wet algal paste (19.6–27.4 % dry weight) and indicated that up to 10 % of water of the total reaction volume had no detrimental effects on transesterification. Koberg et al. (2011) demonstrated that both microwave and sonification- assisted direct transesterification process had better yields than the 2-stage processes. In another study, wet algal biomass (*Nannochloropsis* sp.) with 90 % water was subjected to a simultaneous lipid extraction/transesterification process using supercritical methanol (Patil et al. 2011). They indicated the single-step process has favorable energy balance as the drying and extraction needs are eliminated. Despite promising results at the laboratory scale, it remains to be seen whether direct transesterification can be proven to be viable and cost-effective for industrial-scale conversion of algal lipids to biodiesel.

3.5.3 Cell Milking

Cell milking for microalgal lipids is a relatively new concept. In concept, just like "milking cows", a portion of the lipids inside the cells are extracted without affecting the cell viability. The "milked cells" are returned for continued growth and repeatedly milking. Hejazi et al. (2002) reported successful extraction of β-carotene from *Dunaliella salina*. According to their observations, solvents with higher hydrophobicity (decane and deodecane) are gentle on the cell walls and can extract triglycerides from microalgal cells without loss of cell viability. However, Cooney et al. (2009) stated that the effectiveness of cell milking is limited to cells that are "porous" or have "open pores" such as *Dunaliella*. They also indicated that long term testing of cell viability remains to be done.

3.5.4 Genetic Engineering

Although not fully documented or demonstrated, one emerging concept of lipid extraction involves genetic modification of photosynthetic organisms to secrete lipids through their cell membrane into the culture media. REG Life Sciences is one company that claims to have produced bacteria-based biological catalysts, which have been engineered to selectively convert various sugars using a single-step fermentation process to drop-in and differentiated products (REG Life Sciences 2015). Although conceptually appealing, such processes are reliant on a sugar source supporting fermentation (Cooney et al. 2009). Therefore, genetic engineering on autotrophic microalgae is perhaps the most logical direction from a sustainability point-of-view.

3.5.5 Hydrothermal Liquefaction

Hydrothermal liquefaction (HTL) is one technology that has caught the attention of numerous researchers and industries. However, the HTL process does not produce saponifiable lipids that can be converted to biodiesels. The HTL process produces bio-oils (that resemble fossil crude) from wet biomass slurries at elevated temperatures and pressures. Our prior research has demonstrated that oil production from the HTL process using pine sawdust and switchgrass is particularly promising (Midgett et al. 2012). However using oily biomass as feedstock for the HTL process does not appear to be justifiable. Producing a concoction of oxygenated products from an oily feedstock that is rich in triglycerides does not sound like a logical approach (Fig. 8). Our research has indicated that oily feedstocks (such as tallow seeds, peanuts, and pure vegetable oil), although may result in higher oil production and energy density (MJ/kg), they do not offer major advantages (energy content or compositional) over low value cellulosic or waste feedstocks (such as dairy manure, poultry litter, pine sawdust, and switchgrass) (Midgett et al. 2012). Looking from a practicality and economic perspective, it makes sense to use a slightly larger volume of low-value cellulosis feedstock as opposed to oily feedstocks. For example, approximately 1.4 tons of switchgrass produces the same energy (in MJ) as 1 ton of crushed peanuts in a HTL system. Therefore, using algal biomass in a HTL system may not be justifiable due to difficulties in production and drying of large quantities of algal biomass.

4 Potential Industrial Scale Algal Lipid Extraction Technologies

Despite decades of results from laboratory-based experiments, there is no settlement on the most effective algal lipid extraction method (Lee et al. 2010). Identification of a universally effective and economically viable algal lipid extraction technology is difficult given the diversity of algal lipids, variations in cell wall composition, end use of the extracted lipids, and limitations of various cell

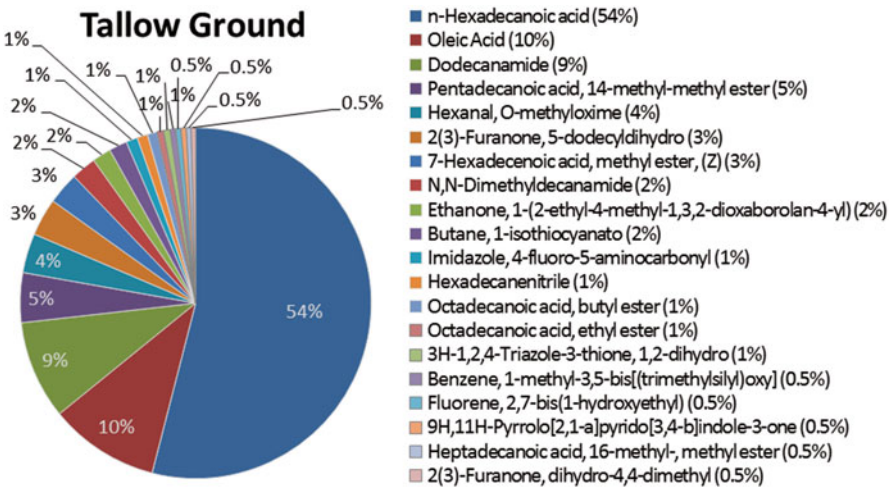

Fig. 8 HTL process converts homogenous triglycerides in oily seeds (including microalgae) to a concoction of low-value oxygenated compounds. The top 20 most abundant compounds in the acetone soluble fraction produced from tallow ground accounted for 100 % of the area of the feedstock. The top 20 selection was based on the largest peak areas in the GC-MS chromatogram. All values are reported as weight percent (Reprinted with permission from Springer (Midgett et al. 2012))

disruption and extraction methods. There is clearly a dearth of information on pilot or commercial-scale algal cell disruption and lipid extraction (Mercer and Armenta 2011; Halim et al. 2012b). Therefore any recommendations and suggestions for potential industrial-scale lipid extraction processes are based on the reported (at laboratory scale) process advantages, limitations, economics, logical reasoning, and perceived process viability at an industrial-scale.

Irrespective of the solvent or solvents used for lipid extraction, cell disruption appears to be a logical choice due to relatively lower energy needs. Bead beating or sonication process, although not proven for industrial algal applications, appear to be the most suitable options for continuous flow industrial operations. As drying algal paste beyond the 20 % solids content may introduce negative energy balance, lowering the moisture below 80 % does not make economic sense. However, availability of reject heat or alternative-energy-based drying options (e.g. solar or geothermal energy) may justify further drying. If cells are subjected to further drying (below 80 % moisture) it is important to disrupt the algal cells first before drying as drying is known to irreversibly close the pores in the cell wall and retard solvent access (Roder and Sixta 2004).

Disrupted cells can then be subjected to lipid extraction. Selection of the best lipid extraction method is more complicated than cell disruption as several technically viable options exist. The final choice of the process will have to be based on process efficiency, amenability to continuous flow mode of operation, commercial scalability, operational costs, solvent cost, chemical stability of the desired end product (which is affected by the choice of extraction method), environmental

impact of the method, and suitability to a biorefinery model. Direct transesterification or use of biodiesel as a co-solvent showed promise at the laboratory level and may be an option if biodiesel is the primary target product. However, this method seriously hampers the biorefinery approach as exposure to toxic chemicals limit the usability of the leftover proteins, carbohydrates, and other value-added products for human or animal uses. Cell milking is likely to have very limited applicability as only selective species with weak cell walls can be milked. Out of the remaining methods, pressurized solvent extraction, ultrasound assisted solvent extraction, microwave assisted solvent extraction, and supercritical fluid extraction processes appear to be conducive to continuous-flow, industrial-scale algal lipid extraction systems. Microwave technology, despite its merits, may be limited by the high operational expenses. Microwave technology starts with electricity (which is an expensive option for industrial heat) and adds an additional efficiency factor (conversion of electricity to microwave energy), which further adds to the economic burden and therefore may be limited to high-value applications (other than biodiesel). Exclusion of the MW technology points to pressurized solvent extraction, ultrasound solvent extraction, and supercritical fluid (CO_2) extraction as the three finalist algal lipid extraction technologies. Each of the finalist processes have their own merits and limitations and need validation at a pilot-plant or industrial-scale. From an environmental perspective, supercritical CO_2 is perhaps the most benign technology as it uses naturally abundant and non-toxic CO_2. The residual biomass and byproducts from supercritical CO_2 extraction process will likely have the widest applications and therefore is the most suitable option for algal biorefineries.

List of Abbreviations

BD20	Solvent mixture with 20 % ethanol and 80 % biodiesel
BD40	Solvent mixture with 40 % ethanol and 60 % biodiesel
CFLES	Continuous flow lipid extraction system
DAG	Diacylglycerols
FAME	Fatty acid methyl esters
FFA	Free fatty acids
GL	Glycolipids
HTL	Hydrothermal Liquefaction
MAG	Monoacylglycerols
NL	Neutral lipids
PL	Phospholipids
PLE	Pressurized lipid extraction
TAG	Triacylglycerols
SEM	Scanning electron microscope
SFE	Supercritical fluid extraction

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