Biomass recovery and lipid extraction processes for microalgae biofuels production: A review

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ABSTRACT

Cell genetics, culture conditions and the recovery efficiency of cells and products are the bottlenecks for industrial bioprocesses from microalgae. Microalgae lipids can be used for different applications, *e.g.*, drugs, food, and biofuels, and their purpose considers the nature and concentration of the lipids that are wished to obtain. For the biotechnological production of lipids, adequate culture conditions are necessary to enable the cell strain to obtain high biomass and lipid yields and productivities. Also, the processes of separating the cells from the culture medium and releasing the lipids from that biomass need to be efficient and economically viable. Considering that cultivation, cell recovery and lipid extraction directly reflect the results obtained, the most appropriate methods for these operations must be applied. Many literature reviews report the lipid and fatty acid contents obtained through the cultivation of different microalgae species and strains. However, few studies relate the contents of these biomolecules, either with the methods of obtaining the biomass or with the extraction of lipids. Even so far there is no review in the literature with such an approach. The aim of this work was to review and discuss the culture conditions for different microalgae strains and their influence on lipid content; the separation of microalgae biomass, including biomass thickening methods, and methods of biomass depletion; the methods of cellular disruption and lipid extraction; the influence on biofuels' production, *e.g.* biodiesel, bioalcohols and biohydrogen; and the influence of green chemistry (solvents and extraction technologies) for a sustainable production of biofuels under the concept of biorefinery.

1. Introduction

Microalgae can be considered as a promising energy source. Among the main reasons is the low use of water in the cultivation; the high biomass yield per area compared to crops; the possibility of using non-agricultural land for cultivation; use of agro-industrial wastes as a source of nutrients, *e.g.*, animal breeding or vinasse effluents [1–3]. Due to its ability to synthesize lipids, it has been studied as raw material for the production of biodiesel and other products with high added value [1,4,5].

The microalgae biomass contains major components, *e.g.*, carbohydrates, proteins, lipids, pigments, among others [5,6]. Each species of microalga is capable of producing different levels of these components and can alter its metabolism according to the changes in the chemical composition of the culture medium and other culture conditions [7].

The use of microalgae biomass presents the potential to be used in several energy processes. However, several technological and economic obstacles must be overcome before starting on an industrial scale

biodiesel production from microalgae. A great challenge is the choice of an effective strategy for biomass recovery and lipid extraction since the scheduling of these processes can be critical, requiring the development of an energetically favorable, environmentally friendly and economically viable process [5,8].

In this work, it is reviewed the main biomass recovery processes and the cellular disruption methods for lipid extraction, relating their influence on the content and yields of lipids and fatty acids to produce biofuels, *e.g.*, biodiesel, bioalcohols, and biohydrogen. Moreover, the utilization of green solvents and green extraction technologies were deeply discussed under the concept of biorefineries.

2. Microalgae

Microalgae are microorganisms that are present in aquatic systems with a great diversity of forms, characteristics and ecological functions. They can be economically exploited under various aspects, *e.g.*, food production for human and animal nutrition (especially in aquaculture),

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pharmaceuticals and biofuels [9–11]. Microalgae can grow in autotrophic, heterotrophic or mixotrophic systems. The latter uses both light and organic substrate as energy sources, as well as CO_2 and organic substrate as carbon sources [12,13].

For the growth of microalgae, it is necessary the interaction between biological, chemical and physical factors. Biological factors refer to their specific metabolic rates of the cultivated species and the influence of other organisms on microalgal development, *e.g.*, fungi, protozoa, and other microorganisms. The main physical and chemical factors that affect microalgae growth are light, temperature, pH, salinity and nutrient availability [9,10,14–16].

There is no single culture medium for all species of microalgae since each has its own specific needs. Thus, the biochemical composition of microalgae, *e.g.*, the total concentration of proteins, lipids, and carbohydrates, varies according to the utilized conditions [15,17–20], *e.g.*, temperature, light incidence, source of nutrients, among others.

3. Culture conditions and their influence on lipid content

Some elements are of great importance for the development of a microalgae culture and should be added in smaller or larger quantities in the culture media to obtain a better development of the species and a higher synthesis of biomolecules of interest. The essential nutrients needed to guarantee a minimum of microalgae growth conditions are carbon, nitrogen, phosphorus, and micronutrients [12,21,22], besides temperature, light, agitation/aeration, availability of nutrients among others [15,23,24].

Carbon is the essential nutrient for cultivation, being its concentration preponderant since it is a basic constituent for the formation of all organic substances synthesized by the cell, *e.g.*, proteins, carbohydrates, nucleic acids, vitamins and lipids [22].

Nitrogen also plays an important role because in the metabolism of microalgae, mainly due to its participation in the formation of proteins. When nitrogen is available in the cultivation, there is an increase in the concentrations of proteins, carotenoids, and chlorophyll. If this element is under limitation, it causes an increase in the lipid content of the microalga [4,12,20,21,25–27].

Temperature affects the metabolic rate of microalgae, being specific for each strain. Constant temperatures provide greater stability in experiments and routine operations, allowing reproducibility. However, in open systems or outdoor photobioreactors, the temperature may oscillate during cultivations [22,23]. The response to growth temperature varies from strain to strain, with no generalized relation [23,24]. Increased microalgal culture temperature may cause an increase in protein content and a decrease in carbohydrate and lipid contents. The percentages of unsaturated fatty acids in the lipid fraction tend to decrease and the saturated ones to increase [28,29].

Microalgae growth also depends on the intensity of light, wavelength, and duration of illumination to which the cells are exposed. The photoperiod is usually 10/14 or 12/12 h of light and dark, respectively. On a laboratory scale, artificial lighting systems with fluorescent lamps are used to simulate natural conditions. The photosynthetic activity rises with increasing irradiation to certain values, and then the inhibition of cell growth begins [22,30]. The formation of polar lipids can be induced by low light intensity. The intensity of light affects the saturation and unsaturation of fatty acids: with high luminous intensity, there is a trend towards the formation of more saturated and monounsaturated fatty acids [29,31].

Aeration and agitation disperse the carbon source in the culture medium, promoting homogenization and avoiding auto-flocculation. In the presence of light, there is the consumption of the CO_2 dissolved in the medium, causing the elevation of the pH. However, the availability of CO_2 can reduce pH and inhibit the growth of some species of microalgae [15,22,30,32].

Some cultivation conditions act directly on the lipid composition and fatty acids profile of microalgae, mainly concerning their nutrient source regarding nitrogen, temperature, and light incidence [33–35]. These stress conditions are strategically used in cultures that have the purpose of producing lipids or other molecules of commercial interest [25,36], being variable for each microalgae strain.

Nutrient deficiency may cause a decrease in growth rates, and under these circumstances, some strains continue to synthesize fatty acids actively [34] and their non-utilization in the formation of membrane lipids would result in the accumulation of triglycerides [37]. The content of the fatty acids and their transformation into triglycerides depend not only on the microalgae strain but also on the culture conditions, including the composition of the medium, aeration, light intensity, temperature and age of the culture [37,38].

In a study with different strains of *Chlorella sorokiniana* cultivated in 10% bovine effluent and Bold Basal medium, cells showed a protein content of up to 24% and fatty acid content of 12% of the biomass, making this cultivation system suitable for the production of biomass for use as animal feed [39]. In another study, the biomass of *Chlorella* sp. obtained from cultures with 150 ppm of $MgSO_4$, 12.5% of salinity and low light intensity showed a high lipid content (32.5%). When cultivated in a lower concentration of $MgSO_4$ and higher salinity and light intensity, it produced lower lipid content (12.5%) [40]. For microalgae cultivation, different combinations of media can be used, combining standard media, *e.g.*, Bold Basal, TAP, Gilliard F/2 among others, and alternative media, *e.g.*, effluents, as well as other parameters, *e.g.*, temperature, pH and photoperiod.

4. Separation of microalgae biomass

Several processes are required after cultivation to exploit the potential of microalgae. One of them is the recovery or harvesting of microalgae biomass from their culture medium [41,42]. The difficulty of separating the biomass is aggravated by the low cell concentration, between 0.1 and 3.0 g L^{-1}; the microscopic size, between 3 and 30 μm [43,44]; the low surface charge, which tends to be negative, preventing or inhibiting cell aggregation; the density similar to water, [45]; the growth phase [46]; the low ionic strength [47], which may hinder their sedimentation. The recovery process of microalgae cultures is an important factor that has a direct influence on the cost and quality of the final products [48].

Thickening methods, *e.g.,* coagulation/flocculation, gravimetric sedimentation, flotation or electroflotation and biomass dewatering methods, *e.g.,* filtration and centrifugation are applied to increase the concentration of microalgae biomass and reduce the volume to be processed, respectively [25,49,50]. Drying methods, *e.g.,* solar drying, greenhouse drying, lyophilization or spray drying are required to obtain dried biomass.

Several authors report that there is no common, simple and low-cost method to be used on a large scale [42,48,51,52]. In this sense, the development of microalgal biomass separation processes to increase recovery efficiencies are fundamental to achieve economic viability of the production of bioproducts.

4.1. Biomass thickening methods

The thickening methods of a microalgae culture lead to the increase of the biomass concentration and the reduction the volume to be processed, contributing to energetic savings [53]. Among the methods of drainage include gravimetric sedimentation, flotation, flocculation. After applying some thickening method, there is still an amount of water that can be drained through drainage methods.

4.1.1. Gravimetric sedimentation and auto flocculation

Sedimentation is a slow, common and rudimentary process, however, with high energy efficiency [54]. The sedimentation of solids is determined by the density and size of the microalgae cells allied to sedimentation velocity. This method is the most common recovery

technique for the microalgae biomass due to the large volumes treated and the low value of the generated biomass. Its applicability is adequate for large microalgae with a size greater than 70 μm [55,56]. The density of microalgae particles plays an important role in the removal of solids by gravity sedimentation, being that microalgae particles with low density do not settle well. Thus they are poorly removed by sedimentation and can re-disperse [57–59].

The recovery of microalgae by sedimentation can be improved with the use of lamella separators and sedimentation ponds [53]. The lamellar separators offer a greater sedimentation area compared to the conventional ones, due to the orientation of the plates and the low energy consumption. The pumping of the microalgae culture to enter the system is continuous and there is the removal of the moistened biomass. Despite reliable and inexpensive, sedimentation ponds are not widely used in industries because it is a slow method to concentrate biomass [50,53].

Auto-flocculation is the spontaneous aggregation of particles, resulting in sedimentation of microalgae [60,61]. Limitation of carbon or certain abiotic factors, *e.g.* reduced or absent aeration may induce auto-flocculation [54,62]. It does not occur for all species of microalgae. It is characterized by a slow process in which redispersion of the flakes can occur [58,59,61]. Increasing the pH using NaOH or another alkaline agent may induce sedimentation. This pH change has the advantage of interfering less in the culture medium compared to the flocculating agents, allowing the medium to be reused [25,57]. This process is also considered auto-flocculation.

Different species of microalgae present distinct recoveries. However, even from the sedimentation and auto-flocculation methods, it is possible to observe the higher recovery of microalgae with the action of a pH-increasing agent and the sedimentation time (Table 1), making the choice of this method highly dependent on the specificity of the microalgae, their interaction between the particles, and the pH adjustment threshold. In studies with auto-flocculation by using NaOH, it was observed the influence of the chemical agent on the lipid content and composition of fatty acids, with lipid reduction and loss of poly-unsaturated fatty acids [63].

Sedimentation and auto-flocculation methods may not be applied to all microalgae species. Microalgae species that form large colonies (50–200 mm), *e.g.*, *Spirulina*, *Actinastrum*, *Micractinium*, *Scenedesmus*, *Coelastrum*, *Pediastrum*, and *Dictyosphaerium*, present a high possibility of sedimentation [54,55]. Auto-flocculation has a greater influence on the recovery of marine than on freshwater microalgae [53,61,64]. However, studies are necessary to determine the pH limit to be used for microalgae sedimentation. Despite their advantages, these methods are not preferred in industrial scale for pre-concentration of microalgae due to the possibility of redispersion of the formed flakes [53,57,59].

4.1.2. Flotation

Flotation is a gravity separation process in which the air or gas bubbles bind to the solid particles and then bring them to the liquid surface, *i.e.*, the insertion of air into the culture in the form of micro-bubbles encompasses the microalgal cells and bring them to the surface [55]. Flotation is often defined as inverted sedimentation or flocculation in reverse. It is commonly applied in wastewater treatment processes because it treats large volumes and requires little space, time and operational equipment [53,54]. Some species of microalgae can float naturally as the lipid content increases [67]. This method is promising for freshwater microalgae. However, for marine microalgae, flotation may be compromised because salinity is a key factor for cell adhesion to the bubble [53,56,68].

Flotation can be classified according to bubble production technology as dissolved air flotation, dispersed flotation or electrolytic flotation. Dissolved air flotation is a process where small bubbles are generated at high pressure, with an average size of 40 μm, which adhere to and load the cells upwards. This system removes microalgae more efficiently than by sedimentation, but large bubbles of dissolved air can break the flakes formed [49,54,56]. The dispersed air flotation bubbles are formed by an air injection system and a high-speed stirrer, or by continuously passing the air through a porous material, forming the bubbles that interact with the negatively charged surfaces of microalgae cells that are then upraised [53,54,69,70]. In electrolytic flotation, the gas bubbles are small and formed by electrolysis. This method is effective on a bench scale, but with intense energetic use [56]. Thus, it is not the best choice for the microalgae recovery [53,57].

In the flotation processes, the chemical coagulation can be used through surfactants followed by flotation of air to increase the yields of the process [53,55,57]. The most commonly used surfactants are aluminum sulfate ($Al_2(SO_4)_3$), iron sulfate ($Fe_2(SO_4)_3$), cetyl-trimethylammonium bromide (CTAB), chitosan and ferric chloride ($FeCl_3$) [71–73].

The flotation efficiency decreases with high ionic strength. The electrostatic interaction between the collector and the cell surface plays a critical role in the separation processes. Based on bubble generation methods and process ratios, several units were developed to induce flotation [74,75]. The advantages of flotation include substantial recovery efficiency ($\geq 75\%$) of microalgae (Table 2) using various chemical conditions/agents combined with airflow to ensure recovery efficiency. The ease of operation and the ability to process large quantities of microalgae cultures at minimal cost make flotation a promising technology for microalgae harvesting [56,73–75].

The research and applicability of flotation approaches in microalgae recovery are still incipient. Further researches are fundamental to optimizing operational parameters to conduct process scale-up [73–75].

4.1.3. Flocculation

Flocculation is a technology widely used in different industrial processes, *e.g.*, beer production, mining, and water and effluent treatments [25]. It occurs when smaller particles aggregate into larger particles through the interaction of coagulant or flocculating agents and, over time, decanting by sedimentation. It can be accomplished by traditional processes, *e.g.,* chemical flocculation and bioflocculation, and by emerging technologies, *e.g.,* magnetic nanoparticles [51,77,78].

The general mechanisms for flocculation of microalgae are similar to those involved in flocculation for water treatment [45,48,55]. For the choice of the coagulating agent/flocculant, it must be considered its degree of interference in the processing and use for the biomass

Table 1
Harvesting of biomass by sedimentation and auto-flocculation.

Microalga	Method	Condition	Recovery (%)	Ref.
Chaetoceros calcitrans	Auto-flocculation with pH adjustment to 10.2	NaOH: 5 M, ST: 4 h	98	[65]
Chaetoceros calcitrans	Sedimentation	27°C in the light, ST: 8 days	91	[65]
Chaetoceros calcitrans	Sedimentation	4°C in the dark, ST: 8 days	70	[65]
Chlorella vulgaris	Auto-flocculation with pH adjustment to 10.8	NaOH: 9 mg g^{-1} ST: 0.5 h	98	[66]
Chlorella vulgaris	Auto-flocculation with pH adjustment to 10.8	KOH: 12 mg g^{-1} ST: 0.5 h	98	[66]
Chlorella vulgaris	Sedimentation	ST: 3 h	25	[64]
Neochloris oleoabundans	Sedimentation	ST: 3 h	40	[64]

ST: Sedimentation time; Ref.: Reference.

Table 2
Harvesting biomass by flotation.

Microalga	Method	Condition	Recovery (%)	Ref.
Chlorella sp.	Flotation	Air flow: 0114 L min^{-1} Chitosan:10 mg L^{-1} and SDS: 20 mg L^{-1}	90	[74]
Dunaliella salina	Coagulation forward micro flotation	FeCl$_3$: 0,15 g L^{-1}; pH 5; Air flow: 85 L min^{-1}	99	[73]
Dunaliella salina	Coagulation forward micro flotation	Al$_2$(SO$_4$)$_3$: 0,15 g L^{-1}; pH 5; Air flow: 85 L min^{-1}	95	[73]
Microcystis aeruginosa	Electro-coagulation– flotation	Aluminum electrode, pH 4–7	100	[72]
Microcystis aeruginosa	Electro-coagulation– flotation	Iron electrode, pH 4–7	78.9	[72]
Chlorella zofingiensis	Dissolved air flotation	Al$_2$(SO$_4$)$_3$, pH 7–8.2	91.5	[76]
Chlorella sp.	Dissolved air flotation	CTAB - 40 mg L^{-1}, pH 7	92	[74]

SDS: Sodium dodecyl sulfate; CTAB: N-cetyl- N-N-N-trimethylammonium bromide; Ref.: Reference.

obtained, the effectiveness at low concentration, and its cost. The understanding of the flocculation mechanisms is intended for more efficient use of flocculating agents through their interactions between microalgae.

In chemical flocculation, the main agents are metal salts, *e.g.* Al$_2$(SO$_4$)$_3$ and FeCl$_3$ and organic polymers, *e.g.* chitosan and cationic starch [41,58,79]. Although metal salts can be applied for microalga recovery, their use may result in high concentrations of metals in the collected biomass and remain in the biomass residue after extraction of lipids or carotenoids [80]. However, contamination of lipid and fatty acids contents is not observed [42,48]. Organic polymer flocculants, chitosan, and modified starch presented an acceptable recovery of microalgae with a lower dosage and reduced impact on the environment and biomass compared to metal salts [46,81,82].

The use of commercial flocculants, *e.g.* Magnafloc® and CTBA (anionic polyacrylamide) or Flocan® (cationic polyacrylamide) may be alternatives to the recovery of microalgae biomass. To ensure the maximum efficiency of the flocculants, it is often necessary to adjust the pH through alkalinizing agents, *e.g.* NaOH, which plays an important role in the biomass recovery by flocculation similar to the cationic and anionic flocculants, increasing costs [48,68]. Moreover, there is still the influence of the species and the culture parameters on the lipid content. The flocculation carried out only with the pH adjustment, *i.e.*, by the alkalinization to pH 10, becomes slightly more advantageous. After the coagulation, the pH is neutralized by washing cells with an NH$_4$HCO$_2$ solution, returning the pH to 8. This isotonic solution can remove inorganic salts without altering osmotic pressure and preserving the flakes and the lipid content [63].

Bioflocculation is the assistance of some microorganism, *e.g.,* bacteria or microalgae with capacity for flocculation [46] without chemical or polymeric flocculants addition. The bioflocculation of microalgae with bacteria requires an additional substrate for bacterial growth. However, the mechanism involved has not been properly understood up to now [42,64].

Recovery of microalgae biomass by flocculation is reported as a superior method when considered conventional recovery methods

because it allows the treatment of large volumes of microalgae culture for a wide range of species (Table 3). Flocculation when combined with gravimetric sedimentation or filtration increases recovery efficiency [53,54,56,57].

The choice of the flocculating agent must consider its degree of interference in the processing and use of the obtained biomass, the effectiveness at low concentration, and its cost. The microalga specificity directly influences the biomass recovery process [46], which does the search for a recovery system that meets the requirements for the use of biomass with acceptable quality and with lower energy expenditure a great challenge.

4.2. Methods of biomass dewatering

The dewatering of the thickened microalgae biomass can be carried out by using mechanical processes, *e.g.*, centrifugation and filtration. After the dewatering, the recovered biomass is usually dried to improve the efficiency of downstream processes, *e.g.* extraction of lipids or carbohydrates [53,56]. These processes can be performed for thickening, but the efficiency is reduced.

4.2.1. Centrifugation

In this biomass recovery method, the centrifugal acceleration causes the cells to move through the liquid and settle to the bottom or sides of the vessel. Centrifugation is considered an extension of gravity sedimentation where gravitational acceleration (*g*) is replaced by centrifugal acceleration [54,56,88]. Despite its capacity to efficiently recover biomass from most of the microalgae, this process requires high energy consumption and high operational, and capital costs [54,56,88–90], and may limit its use to high-value products, *e.g.*, unsaturated fatty acids, pharmaceuticals and other commodities [53]. Centrifuging is applied as a dewatering method to the microalgae-thickened biomass [44,50,57]. After that, the water is separated by draining the supernatant medium.

Centrifugation can be performed by using two types of equipment: the fixed wall systems, e.g., hydrocyclone, or the rotary wall systems,

Table 3
Harvesting biomass by flocculation.

Microalga	Method	Condition	Recovery (%)	Ref.
Chlorella minutissima	Flocculation forward sedimentation	Al$_2$(SO$_4$)$_3$: 1 g L^{-1}, ST: 1.5 h	60	[58]
Chlorella vulgaris	Flocculation forward sedimentation	NaOH pH: 11–12	95	[83]
Chlorella vulgaris	Flocculation forward sedimentation	Chitosan, 30 mg L^{-1}, ST: 10 min	92	[84]
Chlorella sorokiniana	Flocculation forward sedimentation	Chitosan, 10 mg g^{-1}	99	[85]
Neochloris oleoabundans	Bioflocculation *Tetraselmis suecica*	FT: 3 h, RMM: 0.74	72	[64]
Chlorella vulgaris	Bioflocculation *Ankistrodesmus falcatus*	FT: 3 h, RMM: 1	50	[64]
Nannochloropsis oceanica	Bioflocculation *Solibacillus silvestris*	FT: 10 min, RMM 3:1	88	[86]
Conticribra weissflogi	Flocculation forward sedimentation	FO 4990 SH: 0.001 g L^{-1}, ST: 15 min	75	[87]
Conticribra weissflogi	Flocculation forward sedimentation	FO 4240 SH: 0.001 g L^{-1}, ST: 15 min	93	[87]
Chlorella minutissima	Flocculation forward sedimentation	FeCl$_3$: 0.75 g L^{-1}, ST: 5 h	65	[58]
Chlorella minutissima	Flocculation forward sedimentation	FeCl$_3$: 0.25 g L^{-1}, ST: 5 h	57	[58]
Chlorella minutissima	Flocculation forward sedimentation	Al$_2$(SO$_4$)$_3$: 0.75 g L^{-1}, ST: 5 h	90	[58]
Chlorella minutissima	Flocculation forward sedimentation	Al$_2$(SO$_4$)$_3$: 0.25 g L^{-1}, ST: 5 h	38	[58]

ST: Sedimentation time; FT: Flocculation time; RMM: Ratio microalgae/microalgae; Ref.: Reference.

Table 4

Harvesting biomass by centrifugation.

Microalga	Method	Condition	Recovery (%)	Ref.
Scenedesmus obliquus	Centrifugation	8000 rpm, Time: 10 min	~100	[92]
Nannochloris sp.	Centrifugation	5000g, Time: 10 min	94	[90]
Chlorella sp.	Centrifugation	4000 rpm, Time: 10 min	100	[93]
Pavlova lutheri	Centrifugation – high speed	13,000 g	100	[91]
Pavlova lutheri	Centrifugation – low speed	1300g	66	[91]
Nannochloropsis oculata	Centrifugation – high speed	13,000g	95	[91]
Nannochloropsis oculata	Centrifugation – low speed	1300g	65	[91]

Ref.: Reference.

e.g. centrifugal decanters, disc centrifuges and tubular centrifuges [50,56]. The hydrocyclone consists of a cylinder where the feed with the microalgae culture is upper tangential, thereby forming a downward spiral movement, dragging the larger and heavier particles to the lower outlet of the equipment. The disc centrifuge consists of a shallow cylindrical container containing a stack of closely spaced rotating metal cones (disks). The microalgae culture is fed through the center of the disc stack, and the biomass moves outward, at the bottom of the disks, while the aqueous phase is moved to the center [50,56]. Tubular centrifuges are used to recover microalgae at bench scale for laboratory and performance predictions studies. They do not have a draining system, and the process interruption for the liquid spillage is necessary. The decanter centrifuges are characterized by a horizontal conical container, and their separation is by specific weight difference, with the biomass being separated to the sides and withdrawn by a helical thread [56]. The decanter centrifuges consume more energy than the disc centrifuges [50].

Biomass recovery through centrifugation may be fast and high, depending on the microalgae species/strain, and the type and speed of the centrifuge used [91] (Table 4). However, this method is commonly used as the second treatment for biomass recovery, mainly due to the energy expenditure that it provides. Recovery by centrifugation of 12–25% biomass represents an energy consumption of 50–75 kW [50].

The energy required to operate a centrifuge may be greater than the energy generated in microalgae biodiesel [50], so it is considered an expensive method indicated only for high added value products.

4.2.2. Filtration

Filtration is a solid-liquid separation method that uses a permeable or semipermeable medium, whereby a suspension is passed, where the solids are retained, and the liquid is passed through, concentrating the microalgae biomass [53,94]. Biomass recovery via filtration is one of the most favorable methods due to its ability to collect low-density microalgae [88]. In some filtration systems, cell clogging may occur, increasing operating costs and process time [44,53]. Filtration is generally performed after coagulation, flocculation or flotation to improve biomass recovery efficiency [53].

Filtering methods can be divided into two types: dead-end and tangential flow. The dead-end filtration systems utilize filter cartridge, filter press, and vacuum filter. Tangential flow filtration or cross filtration comprises the ultrafiltration, microfiltration, nanofiltration and reverse osmosis systems.

The filter cartridge system consists of cartridge filters where the operation is continuous, and the cleaning is automatic [56,88]. The filter press is the most common filtration system. It presents low design and maintenance costs and flexibility in the operation. However, it requires manual and labor dismantling. The vacuum filtration system occurs by the application of vacuum, causing the liquid culture to be sucked and the biomass to be retained in the tissue/membrane [53,56,95].

Membrane filtration has been widely used in biotechnology applications. It occurs through the tangential flow that promotes the separation of biomass and liquid. Its high separation efficiency, simple and continuous operation, and no requirement of chemicals in the process brings advantages to this biomass dewatering system [81]. Membranes are permeable, selective barriers and can be made from various organic and inorganic materials. The classification of these membranes occurs according to their porosity and are differentiated by the size of the compounds that they can retain. Non-porous membranes are found for reverse osmosis and nanofiltration while the porous membranes for microfiltration and ultrafiltration [53,56,93]. The size of the particles retained are between 0.01 and 0.001 μm for nanofiltration, between 10 and 0.1 μm for microfiltration and between 0.1 0.01 μm 01 μm for ultrafiltration [49,55,56,88]. However, microfiltration and ultrafiltration have been shown to be more efficient in the recovery of microalgal biomass [93].

The differences between the methods are in the size of the pores and in the size of the particles that will be retained; of the pressures of each method and of the tissue/membrane used [53,56,95]. Dead-end filtrations are effective in the recovery of microalgae cells with a diameter above 70 μm and the tangential flow filtration is more appropriate for the recovery of smaller algae due to reduced fouling problems (Table 5) [50,53,56]. The highest costs of filtration are related to the exchange of membranes, pumping, and energy [44,53].

For the production of microalgae-based biofuels, membrane filtration may facilitate recycling of the culture medium utilized to the microalgae cultivation to retain wasted residual nutrients and to remove protozoa and viruses [96]. The reduction of the filtration flow caused by clogging of the filtering membrane is one of its process restrictions.

4.3. Selection of biomass thickening and drainage method

The microalga specificity directly influences the biomass recovery process [46], driving the search for a recovery system with lower

Table 5

Suspended solids in biomass after filtration.

Microalga	Method	Condition	Suspended solids (%)	Ref.
Coelastrum proboscideum	Vacuum filter	Continuous	18, CF: 180	[97]
Chlorella vulgaris	Microfiltration	Continuous	98	[98]
Phaeodactylum tricornutum	Microfiltration	Continuous	70	[98]
Phaeodactylum tricornutum	High filtration	Batch - 1 h	CF: 10	[99]
Chlorella sp.	Membrane filtration	Continuous	98	[93]

CF: Concentration factor; Ref.: Reference.

Table 6
Harvesting methods.

Harvesting methods	Advantages	Disadvantages	Ref.
Flocculation	Simple and fast Low energy process	Some flocculants could be toxic or expensive	[41,48,51]
Auto-flocculation and bioflocculation	Low cost Non-toxic	Slow process Contamination	[57,64]
Sedimentation	Simple and slow Low costs	Slow process, biomass could be inappropriate for some uses Low efficiency	[56,88]
Flotation	Scale-up Low costs Fast	Chemical flotation agents Low efficiency for seawater microalgae	[68,75]
Filtration	High efficiency Allows the separation of species sensitive to shear	Clogging the pores raising operating costs Membrane change	[22,44,53]
Centrifugation	Fast High costs High-value products	Expensive High energy process Shear some cells	[44,90,100]

Ref.: Reference.

energy expenditure and that meets the requirements for the use of biomass with acceptable quality. Table 6 presents the advantages and disadvantages of different thickening and drainage methods for biomass recovery. The efficiency of these methods depends on the microalgae species, including strain, size, morphology, and composition of the medium utilized for growth. However, there are important aspects for the selection of a microalgae biomass recovery method, including efficiency, economic viability, a subsequent process for biomolecules' extraction, and sustainability. It is important to analyze all available technologies prior to choosing the method to be utilized.

The recovery methods described here are efficient techniques for recovering microalgae biomass from the culture medium. However, there is no universal method that can be applied to harvest all strains of microalgae with the same efficiency [54–56].

To break the bottleneck of microalga recovery some industries (*e.g.*, Algae to Energy, Algaeventure Systems, MBDEnergy, and Scipio Biofuels) are working on the research and development of recovery systems through mechanical (filtration, centrifugation, sedimentation) and biological (bioflocculation) processes, beyond the traditional recovery systems with chemical flocculation followed by filtration [49].

4.4. Effect of biomass recovery methods on lipid content and fatty acids profile

The influence of biomass recovery methods on lipid content is not yet completely understood. Most of the studies that present the lipid content of several microalgae have as their main method of biomass recovery the centrifugation, usually for evaluation of the lipid extraction at bench scale and to evaluate its potential for biofuel production [101–104]. There are few studies that show significant differences when different recovery methods are applied, but there are other studies in which this difference is not significant. It should be noted that the fatty acids profile undergoes small changes (Table 7). Determining the effects of biomass recovery on biomass quality and composition is particularly important when biochemical components (biomolecules) must meet quality standards for further processing of biomass, *e.g.* lipids for biodiesel production [102,105].

Popular coagulant / flocculating agents, *e.g.*, $FeCl_3$ and $Fe_2(SO_4)_3$, widely used in systems for water and effluents' treatment, have a high potential for flocculation and biomass recovery [65,106]. The lipid content extracted from biomass recovered with these salts suffers slight variations, most of them insignificant, with the recovered (lipid content) highly dependent on the species and on the culture parameters [51,89]. About the fatty acids, no studies on this influence were found. The recovery by centrifugation generally presents better lipid results when compared to flocculation or filtration, while the changes in fatty acid profile and composition are insignificant [63,68,89,93], being this

system the most applied for bench studies. The profile and composition of fatty acids undergo interference, such as the increase of PUFA (EPA and ETA) and/or the loss of some fatty acids, when biomass recovery is performed with an alkalinizing agent [48,63].

There is still a lack of studies on the influence of microalgae biomass recovery methods on lipid content and fatty acids profile, showing its potential or adverse effects.

5. Lipid content in microalgae

The lipid content in microalgae can reach up to 75% of their biomass, depending on the cultivation conditions and strain/species chosen (Table 8). Lipids obtained from microalgae can be used as feedstock for biofuels or biomaterials. Lipid production by microalgae can be up to 20 times higher when compared to oilseed plants [5,34,107]. The lipid content of the microalgae can be modified depending on the physiology of the microalgae, their growth phase, and the environmental conditions, *e.g.*, temperature, salinity and nutrients [12,22,25], and the extraction yield depending on the method and solvents used. Microalgae such as *Chlorella* sp., *Nannochloropsis* sp. and *Scenedesmus* sp. are promising candidates for biofuel production because of their high lipid productivity and rapid growth [103,108–110].

The lipids play different roles and can be classified into membrane lipids (polar) and reserve lipids (neutral and nonpolar) [32,37,112–114]. Polar or complex lipids include phospholipids and glycolipids, predominant in most microalgae and in the total lipid composition; the nonpolar and neutral lipids are those who do not contain charged groups, this includes triacylglycerols (TAGs), glycerides, carotenoids, sterols and a limited range of high molecular weight hydrocarbons [29,115–117]. The triacylglycerols are considered as energy storage products, whereas phospholipids and glycolipids are lipid structures present in the cell wall [8,118,119].

There are two types of associations that occur in lipids: hydrogen bonds and electrostatic forces on polar lipids; van der Waals forces in the neutral and nonpolar lipids [115,120,121]. These interactions must be broken for their effective extraction. Polar organic solvents, *e.g.* alcohols (methanol or ethanol) disrupt the hydrogen bonds between polar lipids while nonpolar organic solvents, *e.g.* hexane, are commonly used to break up hydrophobic interactions between neutral and nonpolar lipids. Therefore, the choice of solvent is directly linked to the microalgae strain/species and its lipid arrangements. Cost, toxicity, volatility, polarity, and selectivity should be taken into account when choosing the solvent [89,122,123].

Microalgae lipids are typically composed of glycerol, sugars or bases esterified to fatty acids, containing between 12 and 24 carbons, including medium chain (C10-C14), long chain (C16-18) and long chain fatty acids (C20-C24) [124]. Unsaturated fatty acids are called

Table 7
Lipid recovery under different methods.

Microalga species	Method	Biomass recovery (%)	Lipid yield (%)	Influence on FAME profile	Ref.
Chlorella sp.	Foam flotation followed by centrifugation (air flow of 100 L h^{-1}, 30 min, CTAB 10 mg L^{-1})	30.5	13.4	MUFA increase	[68]
Chlorella sp.	Centrifugation (8700g, 20 min)	n.d.	9.9	PUFA increase	[68]
Chlorella sp.	Centrifugation (4000 rpm, 10 min)	100	27.96	No change	[93]
Chlorella sp.	Membrane filtration (cellulose acetate 47 mm)	90	26.43	No change	[93]
Chlorella sp.	Flocculation (chitosan 10 ppm, 40 min)	95	15.38	No change	[93]
Nannochloropsis oculata	Centrifugation (2276g, 25 min)	n.d.	21.4	Long chain PUFA increase	[63]
Nannochloropsis oculata	Centrifugation followed by washing with water (2276g, 25 min)	n.d.	30.8	Long chain PUFA increase	[63]
Nannochloropsis oculata	Centrifugation followed by washing with NH$_4$HCO$_2$ (2276g, 25 min)	n.d.	45.4	Long chain PUFA increase	[63]
Nannochloropsis oculata	Flocculation with NaOH (pH 10, 1 h)	n.d.	5.3	Higher proportion of SFA; EPA and ETA non-detected	[63]
Nannochloropsis oculata	Flocculation with NaOH followed by washing with water (pH 10, 1 h)	n.d.	3.5	EPA and ETA non-detected	[63]
Nannochloropsis oculata	Flocculation with NaOH followed by washing with NH$_4$HCO$_2$ (pH 10, 1 h)	n.d.	4.4	EPA and ETA non-detected	[63]
Nannochloropsis oculata	Alkalization and flocculation with NaOH (pH 10) and neutralization with HCl (pH 8)	n.d.	11.1	Long chain PUFA increase; higher EPA content	[63]
Nannochloropsis oculata	Alkalization and flocculation with NaOH (pH 10) and neutralization with HCl (pH 8) followed by washing with water	n.d.	10.9	Long chain PUFA increase; higher EPA content	[63]
Nannochloropsis oculata	Alkalization and flocculation with NaOH (pH 10) and neutralization with HCl (pH 8) followed by washing with NH$_4$HCO$_2$	n.d.	21.9	Long chain PUFA increase; higher EPA content	[63]
Nannochloropsis oculata	Alkalization and flocculation with NaOH (pH 10, 1 h)	n.d.	4.3	Balance between SFA, MUFA and PUFA	[48]
Nannochloropsis oculata	Alkalization (NaOH pH 10), flocculation with Magnafloc LT-25 (0.01 dm^3 of solution dm^3 culture) followed by filter paper filtration (1 h)	n.d.	~4.4	Higher C14:0 and lower C20:5	[48]
Nannochloropsis oculata	Alkalization (NaOH pH 10), flocculation with Flopam (0.01 dm^3 of solution dm^3 culture) followed by filter paper filtration (1 h)	n.d.	3.6	Balance between SFA, MUFA, and PUFA	[48]
Thalassiosira weissflogi	Alkalization and flocculation with NaOH (pH 10, 1 h)	n.d.	4.12	C16 0, C18:0 and C18:1n9c increase	[48]
Thalassiosira weissflogi	Alkalization (NaOH pH 10), flocculation with Magnafloc LT-25 (0.01 dm^3 of solution dm^3 culture) followed by filter paper filtration (1 h)	n.d.	~3.1	Balance between SFA, MUFA, and PUFA	[48]
Thalassiosira weissflogi	Alkalization (NaOH pH 10), flocculation with Flopam (0.01 dm^3 of solution dm^3 culture) followed by filter paper filtration (1 h)	n.d.	2.77	Balance between SFA, MUFA, and PUFA	[48]
Choricystis minor	Centrifugation followed by washing with water (8370g, 4 °C, 10 min)	n.d.	30.0	n.d.	[89]
Neochloris sp.	Centrifugation followed by washing with water (8370g, 4 °C, 10 min)	n.d.	16.7	n.d.	[89]
Chlorella vulgaris (FW)	Centrifugation followed by washing with water (8370g, 4 °C, 10 min)	n.d.	35.2	n.d.	[89]
Chlorella vulgaris (SW)	Centrifugation followed by washing with NH$_4$HCO$_2$ (8370g, 4 °C, 10 min)	n.d.	35.7	n.d.	[89]
Nannochloropsis salina	Centrifugation followed by washing with NH$_4$HCO$_2$ (8370g, 4 °C, 10 min)	n.d.	49.1	n.d.	[89]
Cylindrotheca fusiformis	Centrifugation followed by washing with NH$_4$HCO$_2$ (8370g, 4 °C, 10 min)	n.d.	23.6	n.d.	[89]
Choricystis minor	Flocculation followed by centrifugation and washing with water (Al$_2$(SO$_4$)$_3$; 319.0 mg g^{-1}, 1 h)	n.d.	28.3	n.d.	[89]
Neochloris sp.	Flocculation followed by centrifugation and washing with water (Al$_2$(SO$_4$)$_3$; 11.8 mg g^{-1}, 1 h)	n.d.	16.3	n.d.	[89]
Chlorella vulgaris (FW)	Flocculation followed by centrifugation and washing with water (Al$_2$(SO$_4$)$_3$; 503.2 mg g^{-1}, 1 h)	n.d.	36.3	n.d.	[89]
Chlorella vulgaris (SW)	Flocculation followed by centrifugation and washing with NH$_4$HCO$_2$ (Al$_2$(SO$_4$)$_3$; 62.9 mg g^{-1}, 1 h)	n.d.	34.8	n.d.	[89]
Nannochloropsis salina	Flocculation followed by centrifugation and washing with NH$_4$HCO$_2$ (Al$_2$(SO$_4$)$_3$; 229.6 mg g^{-1}, 1 h)	n.d.	48.9	n.d.	[89]
Cylindrotheca fusiformis	Flocculation followed by centrifugation and washing with NH$_4$HCO$_2$ (Al$_2$(SO$_4$)$_3$; 154.6 mg g^{-1}, 1 h)	n.d.	22.1	n.d.	[89]
Choricystis minor	Flocculation followed by centrifugation and washing with water (FeCl$_3$: 257.8 mg g^{-1}, 1 h)	n.d.	29.5	n.d.	[89]
Neochloris sp.	Flocculation followed by centrifugation and washing with water (FeCl$_3$: 32.2 mg g^{-1}, 1 h)	n.d.	15.3	n.d.	[89]
Chlorella vulgaris (FW)	Flocculation followed by centrifugation and washing with water (FeCl$_3$: 265.3 mg g^{-1}, 1 h)	n.d.	36.1	n.d.	[89]
Chlorella vulgaris (SW)	Flocculation followed by centrifugation and washing with NH$_4$HCO$_2$ (FeCl$_3$: 67.7 mg g^{-1}, 1 h)	n.d.	34.9	n.d.	[89]
Nannochloropsis salina	Flocculation followed by centrifugation and washing with NH$_4$HCO$_2$ (FeCl$_3$: 290.1 mg g^{-1},1 h)	n.d.	47.2	n.d.	[89]
Cylindrotheca fusiformis	Flocculation followed by centrifugation and washing with NH$_4$HCO$_2$ (1FeCl$_3$: 32.7 mg g^{-1}, 1 h)	n.d.	21.9	n.d.	[89]

FAME: Fatty acid methyl esters; SFA: Saturated fatty acids (%): fatty acid chains have all or predominantly single bonds; MUFA: Monounsaturated fatty acids (%): fatty acids that have one double bond in the fatty acid chain with all of the remaining carbon atoms being single-bonded; PUFA: Polyunsaturated fatty acids (%): fatty acids that contain more than one double bond in their backbone; ETA: Eicosatetraenoic acid (any straight chain 20:4 fatty acid); EPA: Eicosapentaenoic acid (fatty acid with a 20-carbon chain and 5 cis double bonds); FW: Fresh water; SW: Salt water; ~ Figure data; n.d.: Not determined.

Table 8
Lipid content and FAME profile of different species of microalgae.

Microalga species	Total lipids (%)	SFA (%)	MUFA (%)	PUFA (%)	Ref.
Achnanthes sp.	44.5	40.2	45.9	14.3	[101]
Amphora sp. (Persian Gulf)	24	41.28	38.16	8.42	[111]
Ankistrodesmus falcatus	16.49	41.39	28.41	30.20	[103]
Ankistrodesmus fusiformis	20.66	37.33	22.43	40.24	[103]
Ankistrodesmus sp.	17.5	23.43	23.27	37.16	[111]
Botryococcus braunii	44.97	9.85	79.61	10.54	[103]
Botryococcus terribilis	49.00	43.15	44.29	12.56	[103]
Chlamydocapsa bacillus	13.52	35.68	23.58	40.74	[103]
Chlamydomonas reinhardtii	18.9	28.18	22.88	32.07	[111]
Chlamydomonas sp.	15.07	78.61	14.63	6.76	[103]
Chlorella emersonii	18.6	24.55	17.01	38.3	[111]
Chlorella protothecoides	18	22.79	19.23	36.19	[111]
Chlorella salina	11	29.34	18.52	40.63	[111]
Chlorella vulgaris	28.07	52.15	37.51	10.33	[103]
Chlorella vulgaris	17.3	25.06	24.80	45.90	[111]
Coelastrum microporum	20.55	45.87	38.03	16.10	[103]
Desmodesmus brasiliensis	17.99	34.54	44.08	21.38	[103]
Dunaliella salina (Shariati)	18.9	13.93	29.52	42.65	[111]
Dunaliella salina (UTEX)	24	22.77	22.89	34.47	[111]
Dunaliella sp. (Persian Gulf)	22	13.47	24.74	48.1	[111]
Heterosigma sp.	39.9	45.4	31.0	23.7	[101]
Kirchneriella lunaris	17.30	32.06	23.11	44.83	[103]
Nannochloropsis sp.	42.4	35.7	34.8	27.0	[101]
Scenedesmus obliquus	16.73	70.83	21.71	7.46	[103]
Scenedesmus sp.	16	18.59	26.86	30.00	[111]

FAME: Fatty acid methyl esters; SFA: Saturated fatty acids (%); MUFA: Monounsaturated fatty acids (%); PUFA: Polyunsaturated fatty acids (%); Ref.: Reference.

saturated fatty acids (SFA), those with only one unsaturated bond are called monounsaturated fatty acids (MUFAs), those with more than two are polyunsaturated fatty acids (PUFAs) [112]. Saturated fatty acids correspond to the largest fraction of lipids and, in some species, unsaturated fatty acids can represent between 20% and 60% of the total lipids [116,119,121] (Table 8).

Triacylglycerols are preferred to produce biodiesel due to their high content of fatty acids (glycerol structure with three fatty acids) and the absence of other chemical constituents besides glycerol, as occurs in phospholipids or glycolipids [23,119].

5.1. Fatty acids

Fatty acids are components of lipid molecules which denomination is based on two important characteristics: the total number of carbon atoms in the chain and the number of double bonds in the hydrocarbon chain [112,125]. When the carboxyl terminus of the fatty acid molecule is attached to a glycerol group, then a neutral lipid molecule is formed, *e.g.* glyceride; when the association of a fatty acid molecule occurs with a phosphate group, then a polar lipid is formed, *e.g.* phospholipid [11,34].

Fatty acids may be free or esterified and commonly have an even number of carbons disposed in a straight chain. Naturally occurring unsaturated fatty acids usually have a *cis* configuration, since most *trans* fatty acids are not found in nature, but in fats that have gone through artificial processes, especially as a minor product of the hydrogenation of unsaturated fats [11,34,112].

The composition of the fatty acids in microalgae varies according to species/strain and culture parameters, differing in the composition of saturated, monounsaturated and polyunsaturated fatty acids [126,127].

The amount of saturated fatty acids in microalgae usually varies between 13% and 58% [29,37,116]. The dominant fatty acids are C16: 0, C16: 1, C20: 5ω3 and C22: 6ω3 in *Bacillariophyta*, C16: 0, C18: 1, C20: 3 and C20: 4ω3 *Eustigmatophyta*, C16: 0, C18: 1, C18: 2 and C18: 3ω3 in *Chlorophytine*; C16: 0, C20: 1, C18: 3ω3, C18: 4 and C20: 5 *Cryptophyte*, C16: 0, C18: 5ω3 and C22: 6ω3 in *Dinophyte*, and C16: 0, C16: 1, C18: 1, C18: 2 and C18: 3ω3 in *Cyanophyte* [103,116,127,128]. In contrast to the higher plants, there is greater variation in the composition of fatty acids, and some microalgae can synthesize medium chain fatty acids, while others produce very long chain fatty acids [124,127].

The composition of fatty acids is fundamental for the production of biodiesel, as it directly influences the quality of biodiesel. Large amounts of polyunsaturated fatty acids can positively affect viscosity, fog point, cold filter plugging point, but may adversely affect oxidative stability while large amounts of saturated fatty acids have excellent combustion properties [105,129,130].

Fatty acid's metabolism has been scarcely studied in microalgae compared to higher plants. Based on the gene sequence homology and on some shared biochemical characteristics, microalgae and higher plants are involved in the same lipid metabolism [127,131,132].

6. Processes for lipid extraction in microalgae

The extraction and analysis of lipids and fatty acids for microalgae differs from other structures, *e.g.*, vegetable oils and foods, due to the presence of rigid cell wall, and diversity of lipid classes and fatty acids [119,133]. Specific methods must be used to break the cell wall and release the lipids. The conventional methods of lipid extraction established by Bligh & Dyer [134] and Folch [135] use a mixture of chloroform and methanol to release all classes of lipids. However, these methods may not be scaled-up. Table 2 shows the lipids extracted by these conventional methods (Bligh & Dyer and Folch).

When using different methods of cell disruption, allied to solvents, the lipid content tends to be different [119,123,126,133,136]. In the case of an incomplete or selective extraction of lipids, the extraction efficiency of the different lipid classes can vary and consequently influence the composition of fatty acids.

The method for lipid extraction should be fast, efficient and delicate to reduce lipid degradation and be economically viable [5,44,137]. Extraction starts with the microalga cell wall disruption and then the lipids can be extracted in different ways. The cellular disruption process is a prerequisite for efficient extraction of lipids [123]. There are some studies being conducted to improve the process and maximize the extraction at an ever lower cost [81,107,136]. The most widely used methods for lipid extraction from microalgae, separated by sequenced procedures [8,55,123,138,139] up to the removal of the residual solvent, are reviewed in Table 9.

6.1. Methods of cellular disruption

The efficiency of cell disruption methods depends on the microalgae strain/species and on the composition and morphology of the cell membrane. The bursting and extraction costs can be significantly reduced using the appropriate method [81,140]. These methods can use wet biomass or dry biomass, and in this last case, something liquid should be used to promote better dispersion [107,126,141,142].

There are two methods for breaking down the cell membrane: mechanical and non-mechanical. The mechanical methods are ultrasonic, high-pressure homogenization, pressing, ball mill, microwave, while the non-mechanical methods are osmotic shock, chemical breakdown, and enzymes. For industrial scale-up, mechanical methods have the advantage of being fast and monitorable. However, their energy consumption is high [81,139,140]. Conventionally, cell disruption is quantified by the release of metabolites (*e.g.*, lipids or proteins), ultraviolet absorbance, turbidity, particle sizing or cell counting [141].

Table 9
Extraction lipids process.

Process	Method
Disrupt cell methods	Ultrasound
	High-pressure homogenization
	Pressing
	Ball mill
	Microwave
	Osmotic shock
	Enzymatic breakdown
Dry process	Solar drying
	Spray drying
	Freeze drying
Particle size reduction	Milling
	Pressing
Extraction lipids methods	Organic solvents
	Super critic fluid
	Sohxlet
	Disrupt cell methods using solvents
Removal of cellular debris	Filtration
	Centrifugation
Removal of solvents	Distillation
	Vacuum evaporation
	Adsorption column

6.1.1. Ultrasound

The use of ultrasound for cell disruption and lipid extraction of microalgae has been applied in recent years [107,143–147]. The action of the ultrasonic waves propagates in liquid medium and in alternating cycles of high and low pressure, where vacuum microbubbles are produced in the cycles of low pressure and then collapse during the cycle of high pressure, resulting in cavitation, which mechanically breaks the cell structure, allowing the release of lipids [139,148].

Ultrasonic cavitation is significantly more intense at low frequency (18–40 kHz) than at high frequency (400–800 kHz) and is affected by cell wall type, viscosity, reaction time and medium temperature. A low temperature is favorable for an effective sonolysis, to continuously cool the medium and prevent the temperature from increasing due to heat dissipation [81,149]. On the other hand, the energy consumption is increased due to the cooling and the high power of the ultrasound. Moreover, the scaling-up is difficult because the cavitation occurs in regions near the ultrasonic probes [8,141].

Ultrasound performance as a pretreatment method for lipid extraction is satisfactory for some microalgae species. In a study using *Chlorella* sp., *Nostoc* sp. and *Tolypothrix* sp. where different methods of cell disruption were tested for lipid extraction, ultrasound presented the best system for cell disruption and lipid release [149]. For *Chlorella pyrenoidosa* there was no significant difference when using agitation and ultrasound assisted by the 2:1 chloroform: methanol mixture [126]. However, in other studies with *Botryococcus* sp., *Chlorella vulgaris*, and *Schizochytrium* sp. S34, ultrasound showed itself less efficient at breaking down the cell wall to allow the release of the lipid fraction [121,150].

6.1.2. High-pressure homogenization

High-pressure homogenization (HPH) is known as the French press. The cell disruption process uses the hydraulic shear force generated when the biomass at high pressure is sprayed through a narrow tube [81,141]. In this process there is little heat transfer and risk of thermal degradation, low operational cost, average energy consumption when compared to ultrasound, and the possibility of up-scaling [107]. The efficiency of HPH in microalgal cells varies between species and may decrease depending on the rigidity of cell walls [151,152]. Although promising, further evaluation of HPH is required in an industrial scale biofuel production process.

The biomass cell disruption efficiency of *Chlorococcum* sp. with the methods of HPH, ultrasound, ball mill, and sulfuric acid treatment,

indicated by counting cells and measuring colonies' diameter, showed that HPH could destroy 73.8% of the total cells [153]. In a study with *Scenedesmus acutus*, the recovery did not reach 80% in fatty acids, even if the cells were almost completely ruptured [141]. For the extraction of intracellular components of *Nannochloropsis* sp., HPH had the highest efficiency, despite the high energy consumption [154].

6.1.3. Pressing

The cellular disruption of the microalgae wall can be performed by pressing, using mechanical force to break the cells and release the lipid content [139]. The mechanical extraction minimizes the contamination of the microalgae biomass from external sources and maintains the chemical integrity of the substance originally contained [8]. Mechanical pressing is widely used for the industrial extraction of seed oils, *e.g.* soybean and sunflower. This method is considered simple to be applied to the extraction of oil from microalgae. Mechanical technologies for the extraction of microalgae oil include the screw press or piston, extruder and biomass spraying [27].

The extraction of oil from biomass of microalgae using pressing is not easily achieved because part of the biomass can be wasted if flowed in the moisture. In this case, it is necessary to improve the efficiency of the process. Pressing can be applied for both small and large scales to obtain microalgae lipids for the production of biodiesel [27].

The efficiency of this extraction method can reach 75%. However, it is slow and requires a large amount of biomass of filamentous microalgae [138,139]. Although the application of this method is not exhaustively treated in the literature for the extraction of lipids from microalgae, it should be considered as a viable option for the industrial applications in view that some industries already operate in this segment.

6.1.4. Ball mill

The ball mill consists of a rotating cylinder with metallic or quartz beads, which act as grinding frame. This system causes direct damage to the cell wall, by collision or friction, through the speed of rotation of the beads. This method is commonly used to extract DNA from biological samples [81,139]. Damage caused by beads can break a cell within minutes without applying any preparation to biomass (wet or dry). The ball mill system has been used concomitantly with solvent at laboratories and industries [107].

Several factors affect the rupture efficiency and the energy consumption of the method, *e.g.*, the shape of the container; the stirring speed; the size, type, and quantity of spheres. The simplicity of the equipment and the speed of the process bring advantages to this method. However, its scheduling requires an intensive cooling system to avoid thermal degradation of the lipids [81,139,149,153].

The performance of the ball mill as a pretreatment method for lipid extraction is advantageous for some species of microalgae. Literature reports an extraction of 28% of lipids from *Botryococcus* sp. when using the ball mill system, which was 20% superior if compared to the ultrasound followed by the solvent extraction. However, when used for *Scenedesmus* sp., the ball mill was, *e.g.* less efficient than the microwave for lipid extraction [107]. However, for *Tolypothrix* sp. and *Chlorella* sp. there was no difference in lipid extraction when compared to ultrasound [149]. In other studies, the ball mill was not as efficient as the other methods for cell disruption [155].

6.1.5. Microwave oven

Microwaves are electromagnetic radiations of frequency from 0.3 to 300 GHz, which are lower than the infrared and higher than the radio waves [81,139,141]. However, only small scale microwaves of approximately 2450 MHz are used in microwave ovens for cell disruption [81,146].

The use of microwaves for lipid extraction is performed by waves that break the cell wall by induction of heat and interact with molecules thus releasing lipids [54,139]. Microwave-assisted heating is faster than

conventional heating as heat is transmitted via radiation rather than convection or conduction. This method is very selective for polar solvents, *e.g.* water, which generates steam and breaks the cell wall, releasing intracellular contents [141] and leads to an efficient lipid extraction procedure [27]. Due to the high temperature, some products, *e.g.* lipids and fatty acids can be degraded during the process. In this case, it is required a cooling system or a reduced process time to prevent some bioproducts from being degraded during the process. The use of microwave can be a method with reduced extraction time and less demand for solvents, but it has a high energy cost considering its scaling-up [8,156].

In a study using the hexane-assisted microwave, it was identified a higher recovery of lipids and a higher proportion of fatty acids compared to the sole solvent extraction [146]. In another study using a pool of microalgae, it was tested different methods for extraction of lipids. The microwave method was the fastest and most efficient (33.7%) followed by electroflotation and autoclave methods [136]. In experiments with different solvent-assisted cell disruption methods, using *Botryococcus* sp., *Scenedesmus* sp., and *Chlorella vulgaris*, the best results for all strains were obtained with the use of microwave [107].

6.1.6. Osmotic shock

Osmotic shock occurs through a sudden rise or fall in the salt concentration of the medium, which disturbs the equilibrium of the osmotic pressure between the inside and the outside of the cells, causing the cells in solution to rupture and to release the lipids [81,141]. There are two technologies of osmotic stresses capable of damaging the cells: hyperosmotic stress and hypo-osmotic stress. When the salt concentration is higher on the outside: the cells suffer from hyperosmotic stress, and as the cells contract, the fluids within the cells diffuse outward, causing damage to the cell wall. Hypo-osmotic stress occurs when the salt concentration is lower on the outside: water flows into the cells to balance the osmotic pressure, swelling up to burst, releasing the lipids. However, this process requires a large amount of water for dilution of the liquid medium, which makes process scale-up impracticable [81,149].

Osmotic shock uses low-cost chemical agents, *e.g.* sorbitol and NaCl through a simple process, but its performance is often not as efficient as that obtained by other techniques, as it results in effluents with high salinity. Moreover, this method is specific for microalgae with a cell wall permeable to this solution [142,150,157]. In order to obtain better extraction of lipids, it is necessary to include a later step with solvent use [141,158].

In a study with wet biomass of *Chlamydomonas reinhardtii* and osmotic shock with NaCl, followed by solvent extraction, showed yields of 23.81% and 34.50% during the stationary and post-stationary phases [150]. Using trituration, ultrasound, microwave and osmotic shock with NaCl, followed by a solvent to extract lipids from *Schizochytrium* sp. S35 and *Thraustochytrium* sp. the results showed that osmotic shock had the highest efficiency compared to ultrasound and microwave [142]. However, in a study with *Scenedesmus* sp., *Chlorella vulgaris* and *Botryococcus* sp., the efficiency of osmotic shock was lower compared to that obtained with microwaves and ball mill methods [107]. It means that the use of osmotic shock should be directed to species that tend to break their cell wall from saline solutions.

6.1.7. Chemical breakdown

Microalgae cells can be disrupted by using acids, alkalis, or surfactants, which can degrade chemical bonds in the cell wall and induce the release of intracellular biomolecules. The permeability of the cell wall can be enhanced by chemicals, *e.g.*, polymyxin, lysine polymers, protamine, polycationic peptides, and cationic detergents. In these cases, if the permeability exceeds a certain limit, the cells will break [81]. The energy consumption is lower compared to mechanical methods since it does not require a lot of heat or electricity to break. However, it is still necessary to use solvents to release the lipids and

carry them to the micelle [54,55,107,159]. Chemical agents should be consumed continuously. Otherwise acids and alkalis can corrode/encrust the surface of the reactors. The neutralization of acids and alkalis doubles the cost.

The chemical breakdown method may be a promising technique to facilitate the lipid extraction of microalgae since it breaks the cell walls and the bonds between the lipids and the biomass matrix making them accessible to the solvent [141]. In an extraction using acids and alkalis to break the moist biomass of a mixture of *Chlorella* sp. and *Scenedesmus* sp., the cell disruption was performed with 1 M H_2SO_4 1 M and 5 M NaOH at 90 °C for 30 min. After chlorophyll is dissolved, the free fatty acids were converted by a 0.5 M H_2SO_4 1 M solution [160].

The direct transesterification process of microalgae biomass can reduce the cost of biodiesel production of microalgae and increase the yield of fatty acids by the extraction and transesterification that occur simultaneously in a single stage [126,161] with acid catalysis and lipid conversion in fatty acids. This process can be considered as a method of breaking cell by chemical breakdown.

6.1.8. Enzyme breakdown

The breakdown of the cell wall of microalgae through enzymes is considered a biological method of cellular disruption. Enzymes are preferred because of their commercial availability, and the process is more easily controlled than in autolysis or phagocytosis. However, it is more expensive than the mechanical or chemical methods [81,162]. Enzymes may selectively degrade a specific chemical bond, and this does not occur in mechanical methods where they destroy almost all particles in the solution, and the chemicals may induce secondary reactions of the bioproducts [81,155,163]. The combination of different enzymes does not always give better results because the inhibition of the reaction can occur if they are of competitive absorption in substrates [155].

Enzymes must be chosen cautiously for effective cell disruption. However, the high cost of enzymes is still a limiting factor for the scale-up of this process. There are two ways to reduce the cost of an enzymatic process: the immobilization of enzymes or the combination of this process with other methods [81].

In a study with *Chlorella salina*, the yeast *Rhodotorula mucilaginosa* was immobilized in sugar cane bagasse and utilized to break down the microalgae cell wall and release the lipids to produce biodiesel [164]. The enzymes lysozyme and cellulase were used to breakdown the biomass cells of *Scenedesmus sp.* for extraction of lipids using solvents, reaching yields of 16.6% and 16.0% respectively. Immobilized enzymes can efficiently degrade the cell walls of *Chlorella pyrenoidosa* and increase the lipid extraction yield by 75% [81].

6.2. Selection of cellular disruption methods

The main function of biomass pretreatment is to expose lipids to improve their extraction. The pretreatment methods of biomass for cell disruption are summarized in Table 10. The high energy consumption is due to the combination of several factors, including the temperature and pressure conditions of the extraction process, the cost of distillation associated with the separation of lipids from organic solvents, and the cost of drying biomass. However, these factors are linked directly to the cell wall of the microalgae, which is often composed of a thick and rigid layer, with mechanical and chemical resistance [8,81,139,140]. Table 10 classifies some of the requirements regarding high, medium and low difficulty for scale-up [81,139,141,153].

The search for less energetic and high-efficiency processes to break the cell wall and release the lipids should be specific to the purpose of the biomolecules and the specificities of the microalga, requiring a synergistic approach combining different methods. It obligatory must be considered (a) the energy consumption of the process, (b) the cost, use and impact of solvents in bioproducts, (c) the processing time, and (d) the scale-up procedures.

Table 10

Comparison between disrupt cell, scale up process for biodiesel production.

Method for cell disruption	Scale up	Energy consumption	Operational cost	Increase capacity	Solvent utilization
High pressure homogenization	H	L-H	A-H	L	L
Bead mill	H	H	L-A	L	A-H
Ultrasound	L-A	H	A-H	L	A-H
Microwave	A-H	A-H	A-H	L	A-H
Enzymatic breakdown	H	L-A	H	A-H	L
Chemical breakdown	H	L	A-H	A-H	A-H
Osmotic shock	L-A	L-A	H	L-A	A-H
Pressing	H	L	L-A	L	L

H: High; A: Average, L: Low. Adapted from literature [55,81,139].

Table 11

Influence of disrupt cell methods on lipid extraction.

Microalga species	Method for cell disruption	Drying	Lipid yield (%)	Ref.
Chlorella vulgaris	Auto-flocculation	Solar	3*	[155]
Chlorella vulgaris	Wet biomass pressing with silica powder	Solar	4.7*	[155]
Chlorella vulgaris	Dry biomass pressing with silica powder	Solar	6*	[155]
Chlorella vulgaris	Pressing with liquid nitrogen	Solar	29*	[155]
Chlorella vulgaris	Ultrasound (600 W, 20 min)	Solar	14*	[155]
Chlorella vulgaris	Ball mill (1500 rpm, 20 min)	Solar	9.6*	[155]
Chlorella vulgaris	Snailase (37 °C, 2 h)	–	6.8*	[155]
Chlorella vulgaris	Lysozyme (55 °C, 10 h)	–	24*	[155]
Chlorella vulgaris	Cellulase (55 °C, 10 h)	–	22*	[155]
Chlorella vulgaris	Microwave (2450 MHz, 100 °C, 5 min)	Solar	17*	[155]
Spirulina sp.	Auto-flocculation	Yes, n.e.	5.86	[156]
Spirulina sp.	Ball mill (600 rpm, 2 h)	Yes, n.e.	5.82	[156]
Spirulina sp.	Microwave (2450 MHz, 2 min)	Yes, n.e.	5.7	[156]
Spirulina sp.	Autoclaving (0.2 MPa, 30 min)	Yes, n.e.	5.85	[156]
Chlorella sp.	Auto-flocculation	Yes, n.e.	15*	[149]
Chlorella sp.	Autoclaving (15 lbs, 121 °C, 5 min)	Yes, n.e.	24*	[149]
Chlorella sp.	Ball mill (3500 rpm, 5 min)	Yes, n.e.	30*	[149]
Chlorella sp.	Microwave (2450 MHz, 100 °C, 5 min)	Yes, n.e.	36*	[149]
Chlorella sp.	Ultrasound (50 kHz, 15 min)	Yes, n.e.	38*	[149]
Chlorella sp.	Osmotic shock (10% NaCl) with vortexing (1 min) and maintained for 48 h	Yes, n.e.	34*	[149]
Nostoc sp.	Auto-flocculation	Yes, n.e.	14.8*	[149]
Nostoc sp.	Autoclaving (15 lbs, 121 °C, 5 min)	Yes, n.e.	20*	[149]
Nostoc sp.	Ball mill (3500 rpm, 5 min)	Yes, n.e.	26*	[149]
Nostoc sp.	Microwave (2450 MHz, 100 °C, 5 min)	Yes, n.e.	32*	[149]
Nostoc sp.	Ultrasound (50 kHz, 15 min)	Yes, n.e.	35*	[149]
Nostoc sp.	Osmotic shock (10% NaCl) with vortexing (1 min) and maintained for 48 h	Yes, n.e.	24*	[149]
Tolypothrix sp.	Auto-flocculation	Yes, n.e.	6*	[149]
Tolypothrix sp.	Autoclaving (15 lbs, 121 °C, 5 min)	Yes, n.e.	18*	[149]
Tolypothrix sp.	Ball mill (3500 rpm, 5 min)	Yes, n.e.	28*	[149]
Tolypothrix sp.	Microwave (2450 MHz, 100 °C, 5 min)	Yes, n.e.	32*	[149]
Tolypothrix sp.	Ultrasound (50 kHz, 15 min)	Yes, n.e.	28*	[149]
Tolypothrix sp.	Osmotic shock (10% NaCl) with vortexing (1 min) and maintained for 48 h	Yes, n.e.	26*	[149]
Botryococcus sp.	Auto-flocculation	Yes, n.e.	7*	[107]
Botryococcus sp.	Autoclaving (1.5 MPa, 125 °C, 5 min)	Yes, n.e.	11*	[107]
Botryococcus sp.	Ball mill (2800 rpm, 5 min)	Yes, n.e.	28*	[107]
Botryococcus sp.	Microwave (2450 MHz, 100 °C, 5 min)	Yes, n.e.	28.5*	[107]
Botryococcus sp.	Ultrasound (10 kHz, 5 min)	Yes, n.e.	8*	[107]
Botryococcus sp.	Osmotic shock (10% NaCl) with vortexing (1 min) and maintained for 48 h	Yes, n.e.	10*	[107]
Chlorella vulgaris	Auto-flocculation	Yes, n.e.	5*	[107]
Chlorella vulgaris	Autoclaving (1.5 MPa, 125 °C, 5 min)	Yes, n.e.	10*	[107]
Chlorella vulgaris	Ball mill (2800 rpm, 5 min)	Yes, n.e.	8*	[107]
Chlorella vulgaris	Microwave (2450 MHz, 100 °C, 5 min)	Yes, n.e.	10*	[107]
Chlorella vulgaris	Ultrasound (10 kHz, 5 min)	Yes, n.e.	5.5*	[107]
Chlorella vulgaris	Osmotic shock (10% NaCl) with vortexing (1 min) and maintained for 48 h	Yes, n.e.	8*	[107]
Scenedesmus sp.	Auto-flocculation	Yes, n.e.	2*	[107]
Scenedesmus sp.	Autoclaving (1.5 MPa, 125 °C, 5 min)	Yes, n.e.	5*	[107]
Scenedesmus sp.	Ball mill (2800 rpm, 5 min)	Yes, n.e.	9*	[107]
Scenedesmus sp.	Microwave (2450 MHz, 100 °C, 5 min)	Yes, n.e.	10*	[107]
Scenedesmus sp.	Ultrasound (10 kHz, 5 min)	Yes, n.e.	7*	[107]
Scenedesmus sp.	Osmotic shock (10% NaCl) with vortexing (1 min) and maintained for 48 h	Yes, n.e.	7*	[107]

n.e.: Not specified; * Values obtained from graphic; Ref.: Reference.

Table 11 shows the lipid yield of several species of microalgae, which after cell disruption, underwent a drying process, and then a solvent extraction (Bligh & Dyer method). This influence shows that no single method can be beneficial for all microalgae species, except for *Spirulina* sp. [165]. However, it is observed that the ultrasound, microwave and osmotic shock methods presented the highest lipid yield efficiencies.

7. Methods of drying biomass and reducing the particle size

After the pre-treatment of cell disruption, the biomass, which is often moist due to the cell wall breaking process, must be dry and in microparticles for the next processing steps, depending on the type of process that is desired, *e.g.*, extraction of lipids, carbohydrates, pigments, among others. Several studies use biomass drying methods after their recovery [89,107,136,146]. Drying can be done by oven drying, spray-drying, freeze-drying, solar drying, and other forms of drying, *e.g.* microwave and infrared [4,27,44].

Drying in greenhouses is the most commonly used method for removing water by heating. It is a slow method that can take from 3 to 24 h at temperatures of 60 or 105 °C, depending on the process. It is a cheap and simple process, but the accuracy to measure the removal of water or other liquid depends on several factors, *e.g.* drying temperature, particle size and crust formation on the sample surface [4,44]. Solar drying consists of the outdoor exposing of the biomass to take advantage of the light incidence and heat to evaporate the water. This method is inexpensive but dependent on the weather conditions.

Spray drying is a process which consists of spraying the biomass into a chamber subjected to a controlled hot airflow, thereby achieving evaporation of the water, resulting in an ultrafast separation of the biomass with the degradation of the product, resulting in the recovery of the powder product. This method is widely used in the food industry [166].

Freeze-drying, also known as lyophilization, is a drying process where water is removed by sublimation. It consists of freeze the biomass before applying vacuum sublimation; then the temperature is gradually increased, reducing the pressure, allowing the frozen water passes from the solid to the gas, without altering or degrading the properties of the biomass.

Drying by infrared is a very effective process and involves heat action inside the sample, reducing the drying time by up to 1/3 of the total. The method consists of dehydration using an infrared radiation lamp with 250–500 W, whose filament develops a temperature close to 700 °C. Microwave drying is a non-standardized method, but it is quick and simple. The heat in the sample is evenly distributed both on the surface and internally in the sample, facilitating evaporation of the water/liquid and preventing the formation of crust on the surface [166].

The drying temperature affects both the lipid composition and the lipid yield of any matrix [167–169]. Drying temperatures below 60 °C still maintain a high TAG concentration in the lipids and only slightly decreases lipid yield, while with higher temperatures decreases both the TAG concentration and the lipid yield [167]. In a study with *Scenedesmus* sp., three different biomass drying methods and two cell disruption methods for lipid extraction (ultrasound and microwave) were tested, where no significant differences were observed between the drying methods, but a significant difference between the cell disruption methods for lipid extraction [170]. However, the energy consumption and the time were crucial in the process. In a study with *Spirulina* sp., the drying method associated with different cell disruption methods did not have a significant difference in cold lipid extraction while that in the hot method, lipid yields decreased by 60% [156]. In a study comparing three methods of drying (freeze-drying, greenhouse, and solar drying), no effect was observed for lipid extraction, although there was an increase in free fatty acids when solar drying was conducted [146].

The methods for making biomass dried in tiny particles are similar to those used in the food industry. The larger the contact area, the better the solvent's possibilities in carrying the lipids to the micelle. In laboratory scale the dry biomass can be crushed using mortar and pistil or by grinding with sieves. The spray-drying and freeze-drying processes also act to promote the microparticles.

8. Methods of lipid extraction

The yield of lipid content may exhibit variations depending on the method for lipid extraction chosen. The content of lipids extracted is influenced by the solubility of the fatty acids, and by the ability of the solvent to permeate the cell membrane (already ruptured by the cellular disruption methods) in releasing the lipid content. These methods should be rapid, scalable and do not damage bioproducts [54,55,139,141]. Depending on the pre-treatment chosen, the biomass may be moist or dry. There are several studies addressing lipid extraction using humid biomass [89,171–174]. However, most of the studies report the use of biomass dried in a greenhouse or lyophilized [103,107,136,144].

During extraction, the lipids are removed from the cell matrices by an extraction solvent. The lipids must be separated from the cellular debris, isolated from the extraction solvent and any residual water, and finally converted to biodiesel by transesterification or fractionated according to the desired lipid class.

The disruption of the cells generates a distribution of cellular debris, of various particle sizes, that need to be removed. Such removal can be accomplished by separation techniques, which are commonly filtration and centrifugation [8], processes similar to those mentioned for recovering the biomass to separate biomass from the culture medium. The miscible lipids in the solvent should be separated by distillation, vacuum evaporation or solid phase absorption techniques and it is often possible to recover the solvent and to use the process again. The remaining biomass may be inappropriate for animal consumption in cases of excessive contamination by solvents used during the lipid extraction process; then a distillation process is necessary to remove this solvent from the biomass [27].

The main technologies for the extraction of lipids utilize organic solvents or supercritical fluid [8,122,174,175]. Methods that employ solvents are commonly used concomitantly with other methods of cell disruption. Direct transesterification has emerged as a method of lipid extraction and transesterification for the production of fatty acids in a single process [176].

8.1. Supercritical fluid

Supercritical fluid extraction which is an emerging green technology that is gaining considerable attention and acceptance in recent years due to its high selectivity and the use of substances that have properties of liquids and gases when exposed to high temperatures and pressures [55,136,177,178]. When the temperature and pressure of fluid reach its critical values, the fluid enters the supercritical region. This property allows them to act as solvent extraction, leaving no residue when the system is brought back to atmospheric pressure and room temperature.

Extraction of lipids using supercritical carbon dioxide (SC-CO_2) has the potential to supplant traditional methods of lipid extraction by organic solvents [179]. The process consists of a system for compressing and transporting liquid CO_2 to the extraction vessel, installed inside an oven, and a heating valve to depressurize the input SC-CO_2. Once the furnace is heated, the compressed CO_2 enters a supercritical state and extracts the lipid from the microalgae, and then CO_2 evaporates like a gas into the environment, forcing the extracted lipid to precipitate [54,139,174,180]. SC-CO_2 has high solvation power and low toxicity. The intermediate diffusion and viscosity properties of the fluid lead to a favorable mass transfer equilibrium and this process produces solvent-free lipids. However, high infrastructure and operating costs associated with this process are its main disadvantages [139].

The performance of lipid extraction from microalgae *Chlorococcum* sp. with SC-CO_2 when compared to Soxhlet and hexane extraction had higher lipid yields and exhibited an adequate profile of fatty acids for biofuel production [152]. Extraction of lipids from *Chlorella vulgaris* using SC-CO_2 had the lipid extraction yields increased with increasing

Table 12
Types of lipid extract using solvents.

Solvent	Extracted compound
Hexane	Hydrocarbons and triacylglycerols
Chloroform	Hydrocarbons, carotenoids, chlorophyll, sterols, triacylglycerols, waxes and aldehydes
Acetone	Diacylglycerols, cerebrosides, and sulfolipids
Ethanol	Phospholipids and glycolipids
Methanol	Phospholipids and glycolipids

Adapted from literature [181].

pressure [179]. This method may not be suitable for all microalgae species.

8.2. Solvents

The solvent extraction of microalgae biomass is widely used to extract metabolites, *e.g.* astaxanthin, β-carotene, and lipids. Organic solvents may extract different lipid classes, according to their polarity, within the "similar dissolve similar" premise. Most common nonpolar solvents are hexane, benzene, toluene, diethyl ether, chloroform, and polar solvents are methanol, acetone, ethyl acetate and ethanol. Table 12 shows the polarity of the solvents and the types of lipid classes they can release. With increased polarity of the solvents, lipid extraction was increased in several studies, and the use of mixtures of nonpolar and polar solvents may increase lipid yields [126,136,142].

In the process for extraction of lipids by non-polar solvent, six steps were identified: (a) exposing a microalga to a solvent; (b) penetration of the solvent through the already ruptured cell membrane; (c) interaction of the solvent with the neutral/non-polar lipid, canceling out the weak Van der Walls force; (d) formation of a solvent-lipid complex; (e) passage of the complex through the cell membrane by concentration gradient; and (f) formation of a micelle out of the cell. The extracted lipids remain dissolved in the solvent [139].

Some lipids are found in the cytoplasm through complex polar lipids. This complex is strongly bound via hydrogen bonds and proteins in the cell membrane. The Van der Waals interactions formed between the nonpolar solvent and the neutral lipids in the complex are inadequate to disrupt these lipid associations-proteins of the membranes, and then a polar solvent can disrupt the associations, releasing the polar lipids. The mechanism of action is similar to that mentioned above, but the use of solvent mixtures would have the advantage of extracting all lipid classes. This practice is commonly used for quantification of total lipids, *e.g.* by the traditional methods Bligh & Dyer [134], using a mixture of chloroform, methanol and water, and Folch et al. [135] with a mixture of chloroform and methanol.

Solvent extraction using Soxhlet [182] equipment is a very ancient technique (1879) and bears the name of its inventor. It is based on the solid-liquid extraction, where the solvent extracts, evaporates and condenses, making it always in contact with the biomass [8,139]. In some species of microalgae, this method has high efficiency when coupled with methods of cellular disruption [145,164]. Because it is a hot method, degradation of the lipids and fatty acids present in the microalga can occur. Its scale-up is difficult due to the complexity of the equipment [81,182].

Solvents must be inexpensive, volatile (to be removed and reused later), of low toxicity, pure, water-immiscible and selective, *i.e.*, they should not extract undesirable compounds. Organic solvents are widely used in lipid extraction, but because of the antioxidant potential and polarity of the compounds, the yield depends on the type of solvents used [8,123,183]. The methods of extraction of lipids by solvents are usually assisted by ultrasound and microwave, making the process faster and with higher yields [17,138,139,174].

In several studies with polar, nonpolar and mixtures of solvents for lipid extraction of *Chlorella pyrenoidosa* [126], *Chlorella vulgaris* [144],

Botrycoccus braunii [107] and *Schizochytrium* sp. S31 [142], it was observed that the highest lipid yields were obtained for the systems using blends of polar and non-polar solvents. For the genus *Chlorella* and the *Botryoccus braunii* species, higher yields were obtained with polar solvents. However, for *Schizochytrium* sp. the nonpolar solvents showed better yields than the polar ones. Different methods of cellular disruption assisted by solvent mixtures for lipid extraction have a significant effect on yield [107,142,149,156]. It means that the search for the most appropriate method to extract the desired lipids (polar, nonpolar or both) is specific for each species of microalgae, culture conditions, biomolecule purpose, as well as factors that influence the process up-scale.

8.3. Effect of cell disruption and lipid extraction methods on lipid content and fatty acids profile

The production of biodiesel from microalgae involves four main stages that are cultivation, recovery of biomass, extraction of lipids and conversion of lipids into biodiesel. Suitable cell disruption and lipid extraction methods are prerequisites for ensuring efficiency and cost-effectiveness for process scale-up. Except for the use of supercritical fluid for lipid extraction, the other methods of cell disruption are assisted by solvents; this means that the efficiency of the lipid extraction depends on the polarity of the solvent and the combination of the solvent mixture, together with the specificity of each microalga strain.

To select the most efficient method to maintain the lipid content and fatty acid's profile suitable for the purpose, it is necessary to know the behavior of the microalgae biomass against the methods, through bench experiments [142] and then analyze their effects for the scale-up process. Some factors must be considered, *e.g.*, energy consumption, operating costs, productivity in lipid extraction (inputs and outputs in the process), solvent toxicity, process time, among others.

The effect of the cellular disruption method, assisted by polar and/or non-polar solvent, and/or a combination of solvents is significant, clear and specific on the lipid extracted yield (Table 13). The use of mixtures of polar and nonpolar solvents, *e.g.*, chloroform and methanol, hexane and ethanol, dichloromethane and methanol, usually results in superior results when compared to the extraction without solvent mixtures. Even with the use of these mixtures (polar + nonpolar), there will still be an impact on the lipid content, depending on the method of cellular disruption chosen. It means that to know the lipid content of a certain species of microalgae, the ideal is to use different methods combined with the polar and nonpolar solvents, *i.e.*, through the conventional Bligh & Dyer method. However, if the aim is the scale-up of the lipid extraction process, it is necessary to evaluate the best system of cellular disruption and solvents, and the technological process as a whole.

Regarding the profile and composition of fatty acids, the variation is minimal, often non-significant [126,142,174,183]. However, Soares et al. [19] observed both differences when comparing different microalgae species and methods with combinations of polar and nonpolar solvents, *e.g.* Folch, Bligh & Dyer, Rose Gottlieb, direct transesterification, hexane/ethanol mixture, dichloromethane/methanol, and hexane.

9. Biodiesel

Biodiesel is an alternative to diesel that can be obtained from vegetable oils, animal fats, frying oil or other materials containing lipids or TAG, through a transesterification reaction with an alcohol, usually methanol or ethanol to form esters methyl or ethyl fatty acids (FAME or FAEE) [5,129,184]. Biodiesel was developed to be used in diesel engines with compression ignition, mixed with diesel fuel in various proportions or pure [1,5,184,185].

Standards and regulations for biodiesel have been established in several countries, including the American Society for Testing and Materials (ASTM) D6751, the European standard EN 14214 and the

Table 13
Lipid yields by different methods of disrupt cell.

Microalgae species	Method for lipid extraction	Solvent	Lipid yield (%)	Ref.
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Chloroform	9.7	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Methanol	7.5	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Dichloromethane	9	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Diethyl ether	7	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Hexane	12.5	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Toluene	3	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Isopropyl alcohol	3	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Ethanol	7	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Heptane	11	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Chloroform: methanol (2:1)	22	[142]
Schizochytrium sp. S31	Vortex and centrifugation (4000 rpm) solvent-assisted	Chloroform: hexane (2:1)	13.4	[142]
Schizochytrium sp. S31	Shaking solvent-assisted	Bligh & Dyer	22.1	[142]
Tetraselmis sp.	Soxhlet (7 h)	Hexane	2.4	[122]
Tetraselmis sp.	Soxhlet (7 h)	Hexane: Ethanol (3:1)	6.6	[122]
Tetraselmis sp.	Supercritical CO_2 (15 MPa, 40 °C, 12 h)	–	10.88	[122]
Chlorella pyrenoidosa	Shaking (700 rpm) solvent-assisted	Chloroform: Methanol (2:1)	20.2	[126]
Chlorella pyrenoidosa	Shaking (700 rpm) solvent-assisted	Methanol	8.3	[126]
Chlorella pyrenoidosa	Shaking (700 rpm) solvent-assisted	Ethanol	7.2	[126]
Chlorella pyrenoidosa	Shaking (700 rpm) solvent-assisted	Chloroform	9.1	[126]
Chlorella pyrenoidosa	Shaking (700 rpm) solvent-assisted	Hexane	1.7	[126]
Chlorella vulgaris	Ultrasound (40 kHz) solvent-assisted	Bligh & Dyer	52.5	[144]
Chlorella vulgaris	Ultrasound (40 kHz) solvent-assisted	Methanol: Dichlorometane (2:1)	10.9	[144]
Chlorella vulgaris	Ultrasound (40 kHz) solvent-assisted	Folch	16.1	[144]
Chlorella vulgaris	Ultrasound (40 kHz) solvent-assisted	Isopropanol: Hexane (2:3)	2.2	[144]
Chlorella vulgaris	Soxhlet (8 h)	Acetone	1.8	[144]
Nannochloropsis sp.	Ultrasound (30 W, 50 Hz) solvent-assisted	Chloroform: Methanol (2:1)	34.3	[146]
Nannochloropsis sp.	Rapid solvent extraction (100 °C, 200 kPa)	Chloroform: Methanol (2:1)	32.6	[146]
Nannochloropsis sp.	Homogenizing (810 W, 12,000 rpm)	Chloroform: Methanol (2:1)	37.7	[146]
Nannochloropsis sp.	Soxhlet (6 h)	Chloroform: Methanol (2:1)	33.4	[146]
Nannochloropsis sp.	Ultrasound (30 W, 50 Hz) solvent-assisted	Hexane: Methanol (3:2)	22.3	[146]
Nannochloropsis sp.	Rapid solvent extraction (100 °C, 200 kPa)	Hexane: Methanol (3:2)	30	[146]
Nannochloropsis sp.	Homogenizing (810 W, 12,000 rpm)	Hexane: Methanol (3:2)	29.1	[146]
Nannochloropsis sp.	Soxhlet (6 h)	Hexane: Methanol (3:2)	30.8	[146]
Schizochytrium sp. S31	Liquid nitrogen pressing solvent-assisted	Chloroform: Methanol (2:1)	45.8	[142]
Schizochytrium sp. S31	Vortexing with glass balls solvent-assisted	Chloroform: Methanol (2:1)	22.8	[142]
Schizochytrium sp. S31	Ultrasound (20 kHz) solvent-assisted	Chloroform: Methanol (2:1)	31.4	[142]
Schizochytrium sp. S31	Osmotic shock (NaCl 10%) solvent-assisted	Chloroform: Methanol (2:1)	48.7	[142]
Schizochytrium sp. S31	Thermolysis (90 °C) solvent-assisted	Chloroform: Methanol (2:1)	20	[142]
Schizochytrium sp. S31	Shaking (1060 cycles min^{-1}) solvent-assisted	Chloroform: Methanol (2:1)	31.4	[142]
Thraustochytrium sp.	Solvent	Chloroform: Methanol (2:1)	10.7	[142]
Thraustochytrium sp.	Liquid nitrogen pressing solvent-assisted	Chloroform: Methanol (2:1)	9.3	[142]
Thraustochytrium sp.	Vortexing with glass balls solvent-assisted	Chloroform: Methanol (2:1)	25.7	[142]
Thraustochytrium sp.	Ultrasound (20 kHz) solvent-assisted	Chloroform: Methanol (2:1)	10	[142]
Thraustochytrium sp.	Osmotic shock (NaCl 10%) solvent-assisted	Chloroform: Methanol (2:1)	29.1	[142]
Thraustochytrium sp.	Thermolysis (90 °C) solvent-assisted	Chloroform: Methanol (2:1)	8.5	[142]
Thraustochytrium sp.	Shaking (1060 cycles min^{-1}) solvent-assisted	Chloroform: Methanol (2:1)	10	[142]

Brazilian ANP 45/2014. They serve as guidelines for the development of standards and assurance of the quality and characteristics of biodiesel.

The production of biodiesel in Brazil is still dependent on a single raw material, soybean and frying oil, contrarily to the National Biodiesel Production and Use Program (PNPB which aims to sustain a diversity of greasy materials chains available in the various regions of the country [186]. Several options that can be used as raw material for biodiesel or be used as blends to soybean biodiesel, including palm, bovine tallow, and the most promising source: microalgae [1,187,188].

9.1. Microalgae as a potential for biodiesel production

The raw materials most utilized around the world for biodiesel production are rapeseed oil (59%), soybean oil (25%), palm oil (10%), sunflower oil (5%), and other sources (1%) which include: coconut, jatropha *sp.*, camelina, peanut, safflower, mustard, hemp, corn (maize), waste frying oil, animal fat, and algae [189].

Some species of microalgae cultured in optimized growth conditions have the potential to yield 47,000–141,000 L of algal oil per hectare per year, which represents a yield of oil over 200 times the yield from the best-performing plant vegetable oils [190]. The problem is that the energy requirement for increasing biomass concentration exceeds the energy which could be potentially obtained from algal biomass [191]. As a result, most existing large-scale microalgal plants are currently aimed at producing high-value products such as nutrition supplement and cosmetics instead of biofuels. Therefore, for algal biodiesel to be economically attractive, this bottleneck should be removed [192] so that cost of producing microalgae biofuel decrease to 10 times to compete with the price of crude oil in the international market [193].

Despite the economic aspects that still must be solved, the biodiesel obtained from microalgae has several potentialities, *e.g.* selective accumulation of lipids depending on the cultivation conditions; selective or genetic manipulation of microalgae species; ease of cultivation, with use of non-arable areas or sources of low-cost nutrients, even industrial effluents; and high growth rates and lipid productivities [4,5,43,139].

The most studied microalgae for the production of biodiesel are those belonging to the cyanophyte, chlorophyceae (green algae) and diatomaceous groups. The genera reported as those with the highest lipid contents are *Chlorella* sp., from 20% to 30%; *Dunaliella* sp., from 17.5% to 67%, and *Scenedesmus* sp., from 11% to 55% [31,103,104]. After extraction, the lipid transesterification is performed, and for microalgae, acid catalysis is generally used [27,174]. Some authors verified the production of biodiesel from microalgae using conventional

Table 14
Biodiesel properties.

Characteristics	EN 14214	CS [103]	CV [111]	CP [111]	NO [205]	SD [205]	SB [200]	PB [130]	BT [200]
SFA	–	52.15	25.0	22.79	39.4	18.1	15	44.7	25.2
MUFA	–	37.51	24.80	19.23	49.6	17.4	24.7	46.4	33.4
PUFA	–	10.33	45.90	36.19	9.6	64.5	60.3	8.9	41.1
ID	–	58.17	116.59	91.60	–	–	–	64.2	–
CN	Min 51	61.83	44.0	54.57	57.9	37.1	51.7	61	49.2
SV	–	199.37	194.00	163.37	203	195.7	–	–	–
IV	Max 120	52.63	135.26	111.75	80.6	183.7	–	57	–
LCSF	–	1.57	6.71	4.93	3.7	3.8	–	7.7	–
CFPP*	Variable	− 10.81	4.60	− 0.99	− 4.8	− 4.6	–	10	–
CP	–	–	2.66	3.51	–	–	0	–	3
HHV	–	–	–	–	39.8	40.2	39.79	–	39.77
V	3.5–5.0	–	–	–	4.2	3.6	4.10	4.5	4.29
D	0.86–0.9	–	–	–	0.9	0.9	0.881	–	0.877

CS: *Chlorella sorokiniana*, CV: *Chlorella vulgaris*, CP: *Chlorella protothecoides*, NO: *Nannochlopsis oculata*, SD: *Scenedesmus dimorphos* SB: soybean biodiesel, PB: palm biodiesel, BT: beef tallow, SFA: saturated fatty acids (%), MUFA: monounsaturated fatty acids (%), PUFA: polyunsaturated fatty acids (%), ID: degree of unsaturation, SV: saponification value (mg g^{-1}), IV: iodine value (g I$_2$ 100 g^{-1}), CN: cetane number, LCSF: long chain saturated factor, CFPP: cold filter plugging point (°C), CP: Cloud point (°C), HHV: higher heating value, (MJ kg^{-1}), V: viscosity (mm^2 s^{-1}), D: density (g cm^{-3}). CFPP*: seasonal variable.

alkaline catalysts, and these were not suitable for the transesterification of microalgae lipids due to the parallel saponification reaction that occurs due to the high levels of free fatty acids [194,195].

In a study with *Botryococcus braunii*, it was observed that the higher rate of esterifiable lipids was achieved through extraction using polar and nonpolar solvents than when using a sole solvent, impacting in the conversion to biodiesel [183]. However, in another study with *Botryococcus* sp., a higher lipid extraction was observed when using a ball mill and microwave, exhibiting a high percentage of oleic acid [107]. Similar results were found for *Nostoc* sp. and *Tolypothrix* sp., which presented higher content of oleic acid, extracted by ultrasound and microwave, respectively, making them suitable for the production of good quality biodiesel.

Several studies have verified that there is a carbon chain predominance between C14 and C24 in the profile of fatty acids in microalgae, but in different compositions, which determines different characteristics to the biodiesel [19,101,103,104]. This suggests that each microalga provides a unique biodiesel composition, influenced by the characteristics of their fatty acid profile. However, some species are more suitable than others for both high lipid content and their fatty acid profile and composition.

9.2. Direct transesterification

Direct transesterification or *in situ* transesterification has been studied as a form of biodiesel production without the extraction and purification step of the oil [161,177,178]. It occurs when the microalgae biomass, an alcohol and a catalyst, usually acid, are mixed and heated to high temperature. Lipid extraction and transesterification occur simultaneously, and biodiesel is produced [139,170,196,197]. This process can be carried out with wet [161,198] or dry biomass [87,140], and besides reducing the steps for biodiesel production and energy consumption of the process, it also reduces process cost and final cost [126,197,198]. The remaining biomass is separated from biodiesel and cell debris, glycerol and excess alcohol using filtration or centrifugation methods [199]. Direct transesterification can be used to know the profile and composition of fatty acids in a small sample of microalgae [161]. The limitation of this method is that when converting lipids to fatty acids, lipids can no longer be separated and evaluated in different classes, *e.g.*, phospholipids, glycolipids, and triacylglycerols. If the study requires the differentiation of the lipid classes, solvent extraction is required [177].

Comparative yields of biodiesel from *in situ* transesterification are often higher than the production of conventional route biodiesel [176], but in some cases, similar results were achieved when using direct

transesterification or extraction followed by transesterification in biodiesel production [126]. Regarding the profile and composition of fatty acids, there is a change between the conventional method, extraction using several solvents (polar and nonpolar) and the direct transesterification for *Nannochlorophisis oculata*, *Chaetoceros muelleri* and *Chlorella* sp. similar to that occurs when comparing the extraction with several solvents [19]. However, the quantification of fatty acids by direct transesterification did not show a significant difference when compared to the conventional Folch method, although it was significant for the Bligh & Dyer and Smedes & Askland methods in all analyzed microalgae (*Chlorella vulgaris*, *Scenedesmus* sp. *Nannochloropsis* sp.) [177].

9.3. Properties of biodiesel from microalgae

In addition to the lipid content, the profile and composition of fatty acids should be considered to produce biodiesel. The most common fatty acids contained in biodiesel are palmitic, stearic, oleic and linolenic acids [105]. The carbon chain length and the number of double bonds, the amount and detailed composition of the fatty acids are some of the main properties that influence the biodiesel quality [5,105].

The biodiesel quality characteristics are directly related to the configuration of the fatty acid chain [130,200–204]. Saturated chains have higher oxidation stability, making oxidation stability parameters easier to handle than in the case of the presence of unsaturated chains. Unsaturated chains ease the possibility of gelation of biodiesel, so the cold clogging point of the filter will be a minor problem in cold climates [105,200,203].

To verify the quality and characteristics of biodiesel, and comply with the regulations (ANP 45 for Brazil and EN 14214 for the European Union), several analyzes are necessary, often expensive, and time-consuming, requiring large quantities of samples. However, in some cases, it may be impossible to obtain a large sample of biodiesel from an emerging raw material oil for detailed analyzes, *e.g.* microalgae. Several studies have shown that some relevant biodiesel properties can be predicted using information on the lipid profile and composition of the lipid matrix, especially for microalgae [31,129,200,202,205]. The fuel properties mentioned can be determined by empirical equations, with accuracy and reliability showed elsewhere [40,130,200,202].

Table 14 presents the characteristics of several microalgal biodiesels and from other origins, as soybean, palm, and bovine tallow. It shows a clear influence of the profile and composition (verified by SFA, MUFA, PUFA) on the biodiesel characteristics. The properties based on the profile and composition of fatty acids are the degree of instauration, cetane number, iodine number, saponification index, cloud point, cold filter clogging point, calorific value, kinematic viscosity, density, and

oxidative stability.

(M)Ethyl esters of fatty acids with higher chain length and lower degree of unsaturation, measured by MUFA and PUFA percentages in the carbon chains are more suitable for high-quality biodiesel [105,130]. The high concentration of saturated fatty acids contributes to incompatible viscosities, being this parameter closely related to the number of unsaturations and poor cold flow properties of the fuel, *e.g.*, cloud point and cold clogging point, related to chain length and saturation. The molecular mass and percentage of each fatty acid coupled with a number of double bands affect the iodine and saponification indices, which simultaneously impact the cetane number, *i.e.*, the fuel's ability to burn rapidly after being injected [206]. Oxidative stability expresses the susceptibility to oxidation, related to the number of unsaturations and iodine number [130,184,200]. Heat power increases with saturated long chain fatty acids [205].

In a work with 12 species of microalgae, the ones that presented the most adequate lipid content and fatty acid profile for the production of high biodiesel were selected. Among them, *Chlorella* sp., *Botryococcus braunii*, and *Botryococcus terribilis* had the highest lipid contents, while *Chlamydomonas* sp. and *Scenedesmus obliquus* presented fatty acids with greater stability to oxidation and higher cetane number [103]. In another study with three different species of microalgae and several solvents for lipid extraction, it was evidenced that the best extractions occurred using a mixture of nonpolar and polar solvents. While the species *Chaetoceros muelleri* presented within the standards specified by the norm EN 14214, the other microalgae were favorable for the extraction of essential fatty acids [19].

The influence of each fatty acid on biodiesel properties has a significant impact on the choice of the whole process for the effective production of the same. This shows that not only the strain/species must be taken into account, but the conditions under which it is cultivated, the method of recovery of this biomass and especially the choice of the most effective, efficient and sustainable method for extraction of the lipids and their conversion into biodiesel [207].

10. Bioalcohols and biohydrogen

Similarly to the biodiesel production, where the selective accumulation of lipids, depending on the cultivation conditions, is an important parameter to be observed, the production of bioalcohols and biohydrogen from microalgae is strictly dependent on the carbohydrate content in biomass [208,209]. In other words, cells must be driven to produce large amounts of carbohydrates instead of proteins and lipids, which will be briefly discussed here in terms of results obtained with different separation methods for biomass from some microalgae species.

Different pretreatments could be applied to disrupt the cell wall, liberate the polysaccharides, and hydrolyze them to simple sugars. The efficiency of these processes highly depends on biomass type and composition [208]. Thus, the pretreatment strategy depends on the microalgae biomass composition [210].

As the physical/mechanical methods are not specific, making no distinction among different biomass fractions, proteins, lipids, and carbohydrates are equally liberated, which may reduce the economic feasibility and decrease the quality of derived bioproducts [208].

Recent reviews on bioethanol production from algae were published elsewhere [208,211–213]. Some references regarding bioethanol production include the study with an aqueous suspension of *Scenedesmus obliquus* biomass in which was obtained a sugar release yield lower than $0.03\ g_{eq\ glucose}\ g_{algae}^{-1}$ using physical methods (homogenization, sonication, bead beating) and $0.04\ g_{eq\ glucose}\ g_{algae}^{-1}$ using thermal/pressure pretreatment (120 °C and 1.2 bar for 30 min) [214]. In another work using the bead milling for *Neochloris oleoabundans* biomass, sugars were released in the supernatant up to $0.12\ g_{sugar}\ g_{algae}^{-1}$ [215]. Regarding chemical pretreatments, it was reported elsewhere a sugar yield of $0.350\ g_{sugar}\ g_{algae}^{-1}$ using 0.75% w/v NaOH at 120 °C for 30 min of

Chlorococcum infusionum biomass [216], a glucose equivalent yield of $0.025\ g_{eq\ glucose}\ g_{algae}^{-1}$ after alkali pretreatment with 3 N NaOH at 120 °C and 1.2 bar for 30 min of *S. obliquus* and of 0.082 and 0.081 $g_{eq\ glucose}\ g_{algae}^{-1}$ after acid hydrolysis with 3 N H_2SO_4, and 3 N HCl, respectively, at identical conditions [214]. Markou et al. (2013) [217] applied different chemical reagents at four concentrations and four temperatures to pretreat *Spirulina platensis* for bioethanol production, obtaining a sugar yield of $0.522\ g_{sugar}\ g_{algae}^{-1}$ using 0.5 N HNO_3 at 100 °C for 180 min. Some other authors, *e.g.* report the obtaining of $0.472\ g_{sugar}\ g_{algae}^{-1}$ using 1% (v/v) H_2SO_4 at 121 °C for 120 min of *Chlorella vulgaris* [218] and $0.580\ g_{sugar}\ g_{algae}^{-1}$ using 3% (v/v) H_2SO_4 at 110 °C for 30 min of *Chlamydomonas reindhardtii* [219].

Fermentation can be performed with separated hydrolysis and fermentation (SHF) and simultaneous hydrolysis and fermentation in the same vessel (SSF). Literature reports, *e.g.* ethanol yields of $0.214\ g_{ethanol}\ g_{algae}^{-1}$ with *Zymomonas mobilis* from sugar obtained of *C. vulgaris* during SSF [218], and of $0.235\ g_{ethanol}\ g_{algae}^{-1}$ with *Saccharomyces cerevisiae* from sugar synthesized by *C. reindhardtii* during SHF [220].

Regarding hydrogen production, few photosynthetic microorganisms has evolved the ability to use light energy to drive hydrogen gas production from water, and among microalgae *C. reinhardtii* is considered one of the most promising H_2 producing species [209].

Recent reviews on biohydrogen production from algae were published elsewhere [209,221–223]. Some of the results reported include the H_2 production at different levels, depending on the pretreatment conditions utilized, *e.g.*, 36.5 mL H_2 gTS (total solids)$^{-1}$ using 1.6% HCl for 30 min of *C. vulgaris* [224], 37.9 mL H_2 gTS^{-1} using ultrasonication (100 KJ gTS^{-1}) of *C. vulgaris* [224], 81.0 mL H_2 gTS^{-1} using 1.5% HCl at 121 °C for 20 min of *C. vulgaris* [225], 90.3 mL H_2 gTS^{-1} at 121 °C for 15 min of *S. oblicuus* [226], 96.6 mL H_2 gTS^{-1} using 1% H_2SO_4 at 140 °C microwave for 15 min of *Arthrospira platensis* [227], and 43.1 mL H_2 gTS^{-1} using hydrolytic extracellular enzyme solution of *C. vulgaris* [228].

At present, both alcohol and hydrogen energies from microalgae are economically less feasible due to the huge capital cost of facilities and high production cost. Nevertheless, commercialization of biofuels from microalgae may be accelerated by using technological advancements and integrative processes of production [211,223].

11. Environmental green chemistry aspects for microalgae extraction and biofuel production under the concept of biorefinery

Although a variety of methods have been reported, the appropriate selection of the extraction solvent remains a key challenge to enable developing sustainable biofuel production processes [229]. Conventional petroleum-derived organic solvents used in the lipid extraction process are inexpensive and easy to execute. However, they are toxic, consume a large volume of solvent and time and comprise more downstream processing (DSP) steps, and may further aggravate the quality of the product by dissolving other compounds [230]. Moreover, most of them may represent risks of fire and explosion [231]. Their utilization is strictly regulated by European Directives such as REACH (2006/1907/EC) [229].

To circumvent the problem, green solvents and process intensification methods/techniques (green extraction technologies) potentially improve the characteristics of energy reduction, eco-friendliness, non-toxicity and efficient lipid extraction [230]. These renewable bio-based solvents produced from biomass feedstock have emerged as a new generation of highly sought-after chemicals for microalgal lipid extraction [229].

Terpenes, *e.g.* are natural green solvents extracted from citrus fruits and many other plants, and possess excellent chemical and technical properties. In *Chlorella vulgaris*, extraction with some terpene solvents gave better yield when compared to extraction with hexane, without change in FAME composition [232]. Recently, commercialized bio-based solvents include ethyl lactate and methyl soyate, produced from

renewable resources such as corn, citrus, and soybeans [233].

In very recent research, lipid extraction studies conducted on *C. vulgaris* and *Nannochloropsis* sp., via Soxhlet method using various bio-based solvents (*i.e.*, ethyl acetate, ethyl lactate, cyclopentyl methyl ether, and 2-methyltetrahydrofuran were compared to the conventional solvent hexane. All bio-based solvents provided up to three-fold lipid extraction yield in comparison with hexane, with FAME profiles suitable to extract target lipids for biodiesel production [229]. The green solvents are recyclable and work as a single solvent system avoiding the solvent recovery that usually takes place in the conventional process. Thus, these solvents could eventually replace the conventional and other green solvents in different industrial extraction processes. The high cost of green solvents can be easily compensated in terms of DSP steps minimization, energy reduction and saving time with less or no by-products [230].

Algal-based bioenergy products have faced multiple economic and environmental problems [234]. Designing an efficient, sustainable (both economic and ecological) and safe method of lipid extraction and obtaining maximum FAME recovery is still a challenge in algal fuel industry [231]. Moreover, sole production of biofuel on the analyzed scale would not cover all the production costs. Thus, multiple-products production would enhance the overall viability of the plant [235].

For example, to effectively reduce greenhouse gas emissions, biofuel's production must be sustainable in order that sustainable feedstocks must be chosen to provide CO_2 emission savings in comparison with fossil fuels; should lead to some benefits for local communities; and should be based on clean and safe manufacturing processes featuring minimal production of wastes [236]. The valorization of multiple co-products could improve the economic viability of microalgae-based biofuels [234]. It was reported elsewhere that *Chlorella* sp. and *Tetraselmis suecica* present a huge potential to be used for future clean and sustainable mechanisms for biofixation of CO_2 and production of biofuels (ethanol, butanol, and acetone) and as feedstock for organic acid (acetic and butyric acid) production after anaerobic fermentation of both the microalgal biomasses using the bacteria *Clostridium saccharoperbutylaticum* [237].

Thus, the concept of biorefinery has been at the center of attention with an aim to address these challenges by promoting an integral use of biomass to allow the production of multiple products in order to obtain economic, environmental, and social advantages over individual processes. Each biorefinery scheme must be individually evaluated to ensure achieving the highest level of sustainability from both economic viability and environmental friendliness based mainly on the selection of technologies, raw materials, and products since the additional extraction/separation processes could impose excessive costs on the biorefinery [235].

Some recent studies on algal biorefinery include a circular biorefinery proposed for biogenic H_2 formation from a microalgae consortium, where intermediate products such as volatile fatty acids could be converted to biopolymers or undergo anaerobic digestion for the production of additional energy source, *i.e.*, methane [238].

Applying the concepts of biorefinery, two combined methods of biofuel production using *Scenedesmus* sp. were evaluated: (a). The biomass was pretreated with H_2SO_4 and autoclaved for sugar extraction; then the supernatant was used for ethanol fermentation and the biomass used for the direct transesterification (without oil extraction process); (b). The biomass was utilized first for the direct transesterification and then the residues used for sugar extraction and ethanol fermentation. The pretreatment of *Scenedesmus* sp. resolved the limitations of energetic and economic issues and allowed the production of methyl ester by direct transesterification. However, the second combined method was identified as the best biorefinery approach to obtain maximum product yield [239].

Recent advances and challenges of waste biorefineries associated with the integration of anaerobic waste treatment and microalgal cultivation for bioenergy production were reviewed elsewhere [240]. C/

N/P contents from the anaerobic digestate were indicated to produce microalgal biomass that serves as feedstock for biofuels, while biogas upgrading simultaneously fixed phototrophic CO_2 during microalgal growth. According to these authors, several bottlenecks need to be addressed prior to implementation on a large scale, including the identification of a robust microalgae; the optimization of the biotic and abiotic factors associated with anaerobic digestion and microalgal cultivation; the application of low-cost and effective harvesting methods; and the employment of genetically modified strains to meet the process requirements [240].

Multiple environmental and economic assessments have analyzed the concept of biorefinery [234]. Despite the variety of proposed strategies, most studies use too many assumptions, incorporate inadequate data and thus result in erroneous analyses of assessment of sustainability [231]. To avoid that, engineering methods and indicators are being developed and applied for decision making regarding biofuel production and utilization routes. They are intended to help determine the most resource-efficient, cost-effective, and ecologically benign synthesis pathways and consumption patterns. These methods offer great promise for the quantitative and qualitative assessment of biofuel production and consumption systems and thus should help the biofuel industry advance [241]. Some indicators are considered essential for biofuels, *e.g.*, Life Cycle Energy Efficiency (LCEE); Fossil Energy Ratio (FER); Contribution to Global Warming (GW) or Carbon Footprint (CF); Land Use Intensity (LUI): Carbon Stock Change Emissions (CSCE) [242]. A combination of one or more of the available assessment strategies may be employed to analyze the sustainability and feasibility of the pathway. While immediate commercialization of the algal fuel is not possible, refining these production pathways based on their sustainability assessment data could very well allow for its commercialization in the future [231].

12. Concluding remarks and prospects

The lipid yield of microalgae depends on the choice of the strain/species to be cultivated, the culture parameters, *e.g.*, light intensity and nitrogen stress, the biomass recovery system, and especially the lipid extraction systems (cell disruption and extraction itself). The effects of lipid content and fatty acid profile and composition on the characteristics and quality of biodiesel have been identified and related to all the processes presented here.

In relation to biomass recovery, centrifugation is the most commonly used method, without or with minimal influence on lipids. However, it is a highly energetic method, and difficult for the process scale-up. On the other hand, flocculation shows advantages regarding its low influence in the lipid content, and relatively low operating and energy costs, beyond being considered a process of easy scale-up. These two processes are possibly the most beneficial for biodiesel production.

The efficiency of the extraction method depends on several factors that affect for the choice of the most appropriate method for cell rupture, including the species/strain and the nature of their cell wall, the type of lipid present (polar or non-polar), beyond the operating and energy costs. Solvent-assisted ultrasound and microwave are reported as efficient for cell disruption and lipid extraction but are highly energy-consuming and difficult to scale-up.

The main way to develop a process of biofuel production from microalgae lipids is to obtain it at a competitive cost, through the selection of the best strain/species and cultivation conditions that allow maximum lipid productivity with a fatty acids profile that grant biodiesel quality.

For obtaining sustainable and cleaner algae-based fuels of commercial viability, more emphasis has to be given to green solvents and extraction techniques based on eco-friendliness, with less usage of solvent, enhancing the quality of the biofuel and reducing energy/time consumption and downstream processing steps. These solvents should be assessed in terms of effective lipid extraction to make the biorefinery

processes viable, building a bio-economy based on renewable sources. For example, biodiesel and bioethanol production can be integrated. In this case, biodiesel is produced from microalgae fatty acids and bioethanol from the de-fatted biomass while the methyl solvent is replaced by the ethyl solvent in transesterification, instead of using petroleum-derived solvents as presented in several examples of algal biorefinery found in the literature. Thus, the use of green solvents for microalgae extraction and biofuel production is decisive for a successful economic, environmental, and social production of multiple products over individual processes, under the concept of a biorefinery.

Acknowledgments

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