Applied Microbiology and Biotechnology (2020) 104:9067–9077
https://doi.org/10.1007/s00253-020-10839-x

MINI-REVIEW

Towards green extraction methods from microalgae learning from the classics

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Received: 13 May 2020 / Revised: 29 July 2020 / Accepted: 14 August 2020 / Published online: 22 September 2020

Abstract

Microalgae started receiving attention as producers of third generation of biofuel, but they are rich in many bioactive compounds. Indeed, they produce many molecules endowed with benefic effects on human health which are highly requested in the market. Thus, it would be important to fractionate algal biomass into its several high-value compounds: this represents the basis of the microalgal biorefinery approach. Usually, conventional extraction methods have been used to extract a single class of molecules, with many side effects on the environment and on human health. The development of a green downstream platform could help in obtaining different class of molecules with high purity along with low environmental impact. This review is focused on technical advances that have been performed, from classic methods to the newest and green ones. Indeed, it is fundamental to set up new procedures that do not affect the biological activity of the extracted molecules. A comparative analysis has been performed among the conventional methods and the new extraction techniques, i.e., switchable solvents and microwave-assisted and compressed fluid extractions.

Keywords Microalgae · Green chemistry · Switchable solvents · Microwave-assisted extraction · Compressed fluid extraction · Lipids

Introduction

In the last years, high-value bioproducts extracted from microalgae achieved a foothold in the market (Pulz and Gross 2004). Compared with conventional crops, microalgae are considered a fast and continuous source of polyunsaturated fatty acids, carotenoids, and proteins, which exert beneficial effects on humans (Vega-López et al. 2004; Zhang et al. 2014). Despite microalgae representing a huge alternative to conventional feedstocks, three main drawbacks limit their use at large scale: (i) cultivation, (ii) harvesting, and (iii) downstream costs (Günerken et al. 2015; Zhang et al. 2016; Youn et al. 2017; Gifuni et al. 2019).

The main problems related to cultivation are (i) costs associated to the control of growth parameters, especially the temperature, and (ii) risks related to contaminations (Wang et al. 2013; Molina et al. 2019). To control them, microalgae are generally grown in photobioreactors (PBRs), which allow also obtaining high productivity yields and keeping the cultures axenic (Benedetti et al. 2004; Liu et al. 2019). However, as PBRs are very expensive for industrial applications, microalgae are grown in open pond systems (OPS), which are uncontrolled outdoor systems and do not allow a good productivity. OPS have pros (i–iii) and cons (iv–vi), such as (i) a low initial investment (Narala et al. 2016); (ii) a low power demand (Chen et al. 2013); (iii) low operating and maintenance costs (González-Delgado and Kafarov 2011); (iv) high contamination risk (Banerjee and Ramaswamy 2017); (v) requirement of large areas of land (Norsker et al. 2011); and (vi) high water demand to overcome the poor light utilization (Yin et al. 2020). Moreover, cultivations performed in these systems are strongly influenced by weather and

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environmental conditions. Indeed, controlling the growth parameters, such as temperature, pH, and light intensity is still tricky and may affect the biomass productivity (Carvalho et al. 2006; Slegers et al. 2013; Koley et al. 2019). To face the contaminations, an optimization of highly selective environment is required. Among all the steps involved in the algal biomass production, the harvesting step represents the 20–30% of the overall costs (Rawat et al. 2011; Barros et al. 2015). Thus, the selection of the right technology to harvest the biomass is one of the key issues to make the microalgal exploitation cost-effective at large scale. The high costs are related to several factors, such as the high dilution of the culture that requires an intensive de-watering step; the density of the cells in the medium that is similar to the water density; and the negative charge of algal cells that implies an electrostatic repulsions among them, thus keeping cells in a stable disperse state (Zheng et al. 2012; Hu et al. 2014; Gayen et al. 2019). To date, the most common harvesting industrial procedures are centrifugation, flocculation, coagulation, and immobilization (Drexler and Yeh 2014; Fuad et al. 2018; Hidayah et al. 2019). These techniques present several disadvantages, not only for the elevated energy costs of each operation but also for the low separation efficiency (Danquah et al. 2009; Xu et al. 2010). For these reasons, the optimization of an efficient and economic harvesting procedure is still a challenge.

Another important issue to consider is the selection of the right extraction procedure to be employed. In fact, when extracting molecules from the biomass, one should choose a fully biocompatible buffer which will not alter the bioactivity of the extracted molecule. Currently, conventional extraction techniques involve the use of organic solvents, such as chloroform, acetone, methanol, and diethyl ether to be used in large amounts, for a long time and also the use of dry biomass as a starting material (Ghasemi Naghdi et al. 2016; Saini and Keum 2018; Zhang et al. 2019)

Recently, a new generation of extracting techniques, which do not require the involvement of toxic solvents, is being developed. Much effort has been done to set up green extraction procedures without using toxic solvents, thus minimizing environmental impact (Chemat et al. 2012; Armenta et al. 2019). Moreover, the new techniques allow to reduce the extraction time and to improve the extraction yields, without affecting the biological activity (Esquivel-Hernández et al. 2017; Dixon and Wilken 2018). To date, only few algae strains are considered suitable for the large-scale production (Brennan and Owende 2010; Kothari et al. 2017; De-Luca et al. 2019), such as *Spirulina*, *Chlorella*, *Dunaliella salina*, *Aphanizomenon flosaquae*, *Haematococcus pluvialis*, *Crypthecodinium cohnii*, and *Shizochytrium* (García et al. 2017).

One way to reduce the overall costs of microalgae cultivation on a large-scale production is the valorization of different microalgal biomass components (Lam et al. 2018; Chandra et al. 2019). In the last decades, the linear economy has given way to the circular economy, to promote a better use of resources by utilizing wastes and natural products as starting material, and to develop an integrated platform able to produce different bioproducts from biomasses (Bhalamurugan et al. 2018; Mathimani and Pugazhendhi 2019). In this context, microalgal biorefinery could be considered the most efficient and cost-effective approach to obtain different molecules, starting from the one endowed with the highest market value. This requires an appropriate selection of the extraction procedure to be employed. In this review, we will try to provide an overview on the different extraction techniques used for microalgae with a special focus on the improvements obtained.

Organic-solvent extraction techniques

The extraction step represents one of the main drawbacks in algae-based industries (Gifuni et al. 2019). Lipids and carotenoids are commonly recovered by using organic solvents, such as hexane, chloroform, acetone, methanol, and diethyl ether (Saini and Keum 2018). Conventional extractions usually require large amounts of organic solvent and long extraction times and they generally need dry biomass (Mansour et al. 2019; Sati et al. 2019). Moreover, a pretreatment step is often required before the extraction, thus increasing the overall costs (Alzate et al. 2012; Kadir et al. 2018). A brief description of the conventional methods, generally used to extract lipids, is reported, and the results obtained are summarized in Table 1.

The Folch method

The Folch method (Folch 1957) is one of the oldest methods employed for the extraction of lipids from microalgae and cyanobacteria. This procedure is fast and easy; however, it is less sensitive compared with the most recent procedures (Kumar et al. 2015). It requires chloroform and methanol as solvents, and it still represents one of the most used methods to estimate, spectrophotometrically, algal lipids. Banskota et al. (2019) extracted lipids from several microalgal strains. Extractions were performed starting from freeze-dried biomass using chloroform to methanol (2:1). They found that the method was able to extract from 30 to 40% (w/w of dry biomass) of lipids, with the exception of *Nannochloropsis granulata* in which the lipid content was 49.3 ± 4.0% (w/w of dry biomass). Authors demonstrated also that the lipid content was directly related to ORAC values (Banskota et al. 2019). Schipper et al. (2019) studied novel microalgal strains isolated from extreme desert environments: *Tetraselmis* sp.

Table 1 Different conventional extraction methods and their relative yields

Microalgal species	Extraction method	Solvent	Biomass	Lipid yield (%)	Reference
N. oleoabundans	Folch	Chloroform/methanol	Dry	31.8 ± 6.7	(Banskota et al. 2019)
B. braunii				41.1 ± 5.5	(Banskota et al. 2019)
P. tricornutum				44.8 ± 3.6	(Banskota et al. 2019)
N. granulata				49.3 ± 4.0	(Banskota et al. 2019)
C. sorokiniana				32.3 ± 2.4	(Banskota et al. 2019)
P. aerugineum				30.9 ± 6.1	(Banskota et al. 2019)
S. obliquus				40.5 ± 7.8	(Banskota et al. 2019)
Scenedesmus sp.				36.3 ± 12.5	(Banskota et al. 2019)
T. chui				32.1 ± 5.5	(Banskota et al. 2019)
T. subcordiformis QUCCCM51				25.6 ± 0.9	(Banskota et al. 2019)
P. maculatum QUCCCM127				28.0 ± 2.0	(Banskota et al. 2019)
N. oculata				24.4	(Wei et al. 2014)
T.subcordiformis				22.2	(Wei et al. 2014)
C. acidophila LAFIC-004	Bligh and Dyer	Chloroform/methanol/water	Dry	54.6	(Souza et al. 2017)
I. galbana				25.3 ± 0.2	(Bonfanti et al. 2018)
C. sorokiniana				29.9	(Rasouli et al. 2018)
M. capsulatus				21.8	(Rasouli et al. 2018)
C. vulgaris		Chloroform/methanol		10.4	(Zullaikah et al. 2019)
G. phlegrea	Soxhlet	Chloroform/methanol	Dry	79 ± 26	(Imbimbo et al. 2019)
S. obliquus				17.4 ± 0.4	(Wang et al. 2019)
Chlorophyta sp.		*n*-Hexane/ether		18.3 ± 0.4	(Yusuff 2019)
C. gracilis				12.3	(Kanda et al. 2020)
P. carterae		*n*-Hexane		7.5	(Kanda et al. 2020)
C. vulgaris		Heptane		57.5 ± 0.5	(Minyak et al. 2017)

and *Picochlorum* sp., characterized by their tolerance to high temperature and to high CO_2 concentrations. The species were isolated, and lipid extraction showed that the two novel strains contained significant amounts of lipids, up to 25.6 ± 0.9% and 28.0 ± 2.0% (w/w of dry biomass), for *Tetraselmis* sp. and *Picochlorum* sp., respectively. The method is very reliable as different authors reported the same extraction yield for the same strain (Danquah et al. 2009; Wei et al. 2014).

Bligh and Dyer

The Bligh and Dyer method (Bligh and Dyer 1959) is similar to Folch method. It allows for the extraction of lipids from homogenized cells, generally using a mixture of chloroform/methanol. It is a rapid and effective procedure, thus becoming a standard method for the lipid content determination in biological tissues (Iverson et al. 2001).

Souza and co-workers (Souza et al. 2017) studied the acidophilic microalga *Chlamydomonas acidophila* LAFIC-004 performing a lipid extraction by the Bligh and Dyer method and obtained 54.6% (w/w of dry biomass) of lipids. Bonfanti et al. (2018) performed lipid extraction starting from *Isochrysis galbana*, with a 25.3 ± 0.2% (w/w of dry biomass) yield. Rasouli and co-workers (Rasouli et al. 2018) extracted about 30% of lipids from *Chlorella sorokiniana*, a value similar to that reported using the Folch method (Schipper et al. 2019), thus suggesting that all the methodologies are able to extract the same amount of lipids when the same strain and the same experimental procedure are followed.

Soxhlet extraction

Soxhlet extraction is a conventional procedure employed for the extraction of lipids and carotenoids. It is performed by using solvents at boiling temperature and ambient pressure, and even if it requires high amount of solvents and a long extraction time, it provides high yields and does not affect the bioactivity of the extracted molecules. We recently reported a Soxhlet extraction with chloroform to methanol (2:1) to obtain lipids from *Galdieria phlegrea* (Imbimbo et al. 2019).

This procedure allowed obtaining a recovery of 79 ± 26% (w/w of dry biomass) of lipids starting from the dried biomass (Imbimbo et al. 2019). Yusuff reported an oil extraction performed by Soxhlet from the green microalga *Chlorophyta* sp. The extraction was performed by using *n*-hexane to ether (4:1) mixture and allowed a yield of 18.3 ± 0.4% (w/w of dry biomass) (Yusuff 2019). Kanda and colleagues used two different microalgae strains to extract lipids: *Chaetoceros gracilis* and *Pleurochrysis carterae*. Extractions were performed by Soxhlet using pure *n*-hexane. This technique allowed achieving yields of 12.3% (w/w of dry biomass) for *C. gracilis* and 7.5% (w/w of dry biomass) for *P. carterae* (Kanda et al. 2020).

Green extraction techniques

Recently, the demand for greener, safer, and more natural products that do not require the involvement of toxic solvent increased. The development of green extraction procedures to recover valuable compounds from natural sources represents a significant advance. These eco-friendly techniques allow obtaining bioactive products by reducing or completely replacing toxic solvents, thus minimizing the environmental impact, in agreement with several Green Chemistry principles (Capello et al. 2007; Anastas and Eghbali 2010; Jeevan Kumar et al. 2017). Moreover, a reduction in the extraction time and an improvement in the extraction yields have been obtained. Nevertheless, only few innovative techniques succeeded so far.

Ionic liquids and switchable solvents

Ionic liquids (ILs) are organic solution of salts that can melt at mild temperature (< 100 °C). They are typically composed of a large number of inorganic or organic cations and are characterized by synthetic flexibility and thermal stability. Moreover, they are non-volatile and non-flammable (Vekariya 2017; Harris et al. 2018), being a good alternative to conventional solvents. They are generally employed for lipid extraction; however, to date, only limited papers are available in literature (Motlagh et al. 2019). One of the main drawbacks of ILs is the unrealistic application at industrial scale, due to their costs and the environmental impact (Zhang et al. 2008). Indeed, many ILs have been proved to be not harmful for humans, but their synthesis involves many steps that require expensive, toxic, and volatile reagents (Domínguez de María 2017; Harris et al. 2018; Singh and Savoy 2020). In recent years, a second generation of ILs has been developed: switchable solvents (SSs). First reported by Philipp Jessop et al. (2005), SSs are non-volatile liquids able to switch from hydrophobic to hydrophilic state and vice versa in response to external stimuli, such as temperature or pH variation and/or the addition or removal of a gas (i.e., CO_2) (Al-Ameri and Al-Zuhair 2019; Do Yook et al. 2019). Theoretically, these SSs have many pros: (i) they allow performing cascade extractions of high-value molecules, (ii) it is possible to recover and reuse the solvent; thus, they are considered economically competitive and they require low energy consumption (iii); (iv) they are eco-friendly; (v) they are highly selective; and (vi) they enable extractions in a short time (Pollet et al. 2011; Jeevan Kumar et al. 2017; Clarke et al. 2018). For all these reasons, the second generation of SSs is considered green (Vanderveen et al. 2014; Jeevan Kumar et al. 2017). However, many cons have emerged with respect to solvent loss. This is mainly due to (i) the use of CO_2 in the switching process; (ii) the impossibility to completely remove the solvent from the residual biomass after the process; and (iii) the release of the solvent in water. To improve SS properties, functional groups may be incorporated into the structure during the chemical synthesis, with increase in production costs (Clarke et al. 2018). Nowadays, primary, secondary, and tertiary amines are among the most SSs (Schuur et al. 2019). Table 2 reports a comparison between the lipid yield extraction by using SSs and conventional methods.

Research on SSs is quite recent. In 2018, Cicci et al. (2018) used N,N-dimethyl-cyclohexylamine (DMCHA) on the wet biomass of *Scenedesmus dimorphus* to extract lipids. The lipid yield was 35.6% (w/w of dry biomass), about 1.2-fold more than the yield obtained by Gour and colleagues with a conventional method (Bligh and Dyer) (Gour et al. 2020). Nevertheless, the experimental procedure seemed to be able to extract similar amount of lipids independently from the strain. Indeed, the lipid yields obtained by Cicci et al. (2018) on *Scenedesmus dimorphus* can be compared with the lipid yield obtained by Samorì et al. (2013) on *Tetraselmis suecica* (31.9%). Instead, Du et al. (2013) showed that N-eyhylbutylamine (EBA) was able to extract lipids from *Desmodesmus* sp. with a yield of 16.8% (w/w of dry biomass), a value lower than that obtained by Samorì and colleagues who used DMCHA (29.2%) (Samorì et al. 2013). Indeed, the yield was higher than that obtained by the Bligh and Dyer method (Du et al. 2013; Samorì et al. 2013). Probably, the tertiary amine DMCHA allows a better extraction of the lipid fraction from the biomass, as it is more hydrophobic than the secondary amine EBA. Afterwards, Du et al. (2018) showed that, starting from a stressed culture of *Neochloris oleoabundans*, EBA allowed to obtain an increase in the lipid yield, from 47.0 to 61.3% (w/w of dry biomass), only by increasing the number of extractions. In this case, authors found that EBA extracted about 4 times more lipids than the Bligh and Dyer method. It has to be noticed, however, that the switch back has not been reported in literature yet, so that the use of SSs is still far from being used in a biorefinery approach.

Table 2 Yields obtained with switchable solvent method from different microalgae

Microalgal species	Extraction method	Solvent	Biomass	Lipid yield (%)	Fold increase	Reference
S. dimorphus (UTEX 1237)	Switchable solvent	DMCHA	Wet	35.6 ± 1.9	1.2	(Cicci et al. 2018)
S. dimorphus (Sd12)	Bligh and Dyer	Chloroform/methanol/water	Dry	30.7		(Gour et al. 2020)
N. gaditana	Switchable solvent	DMCHA	Wet	57.9 ± 1.3	1.3	(Samorì et al. 2013)
	Bligh and Dyer	Chloroform/methanol/water	Dry	45.1 ± 0.9		(Samorì et al. 2013)
T. suecica	Switchable solvent	DMCHA	Wet	31.9 ± 1.5	1.3	(Samorì et al. 2013)
	Bligh and Dyer	Chloroform/methanol/water	Dry	25.4 ± 2.6		(Samorì et al. 2013)
D. communis	Switchable solvent	DMCHA	Wet	29.2 ± 0.9	1.6	(Samorì et al. 2013)
	Bligh and Dyer	Chloroform/methanol/water	Dry	17.8 ± 0.1		(Samorì et al. 2013)
Desmodesmus sp.	Switchable solvent	EBA	Wet	16.8 ± 0.5	1.3	(Du et al. 2013)
	Bligh and Dyer	Chloroform/methanol/water	Dry	12.8 ± 0.6		(Du et al. 2013)
N. oleoabundans	Switchable solvent	EBA	Wet	47.0	1	(Du et al. 2017; Du et al. 2018)
	Bligh and Dyer	Chloroform/methanol/water	Dry	13.1		(Du et al. 2017; Du et al. 2018)
Chlorella sp.	Switchable solvent	EBA	Wet	12.3 ± 3.2	3.2* 1.3**	(Al-Ameri and Al-Zuhair 2019)
		DMCHA		13.3 ± 0.4	3.5* 1.4**	(Al-Ameri and Al-Zuhair 2019)
		Dipropylamine		7.0 ± 1.3		(Al-Ameri and Al-Zuhair 2019)
	Conventional	[Bmim][PF6]	Dry	3.8 ± 1.1		(Al-Ameri and Al-Zuhair 2019)
		n-Hexane		9.4 ± 0.7		(Al-Ameri and Al-Zuhair 2019)

*With respect to [Bmim][PF6]

**With respect to *n*-hexane

Microwave-assisted extraction

Microwave-assisted extraction (MAE) involves the use of microwaves to heat up the solvent in contact with the cell thus allowing to extract pigments, lipids, and other bioactive molecules (Juin et al. 2015). The heating is caused by two phenomena: dipole rotation and ionic conduction, which may happen individually or simultaneously (Tatke and Jaiswal 2011). MAE is generally performed in closed systems to avoid heating dissipation. By this way, the heating mechanism is targeted and selective, thus reducing the extraction time and improving the final yield. However, the main limitation of this method is the high temperature required that might affect the bioactivity of the extracted molecules. A study from Mahfud's group indicated that MAE was able to increase by almost 10 times the lipid extraction yield in *Spirulina platensis*, with respect to Soxhlet (Kalsum et al. 2019), when *n*-hexane was used as solvent. These and other results are reported in Table 3, with a comparison with conventional methods.

In the extraction processes using microwave, the use of a mixture of solvents may result in an increase of the yield. As an example, the mixture *n*-hexane/methanol is a non-polar solvent able to solve oils from the matrix cells of microalgae. On the other hand, methanol allows microalgae to absorb more microwave energy with a consequent increase in microalgal disruption (Kalsum et al. 2019).

Krishnan and colleagues studied the importance of different ILs in the MAE extraction system on *Chlorella vulgaris*. Interestingly, they found that the extraction yield increased from 10.9 (Bligh and Dyer method) to 19.2% (w/w of dry biomass) when the 1-octyl-3-methylimidazolium acetate ([Omim][OAc]) was used (Krishnan et al. 2020). In general, they found that the polarity of the ILs and the electronegativity of the anions used played an important role in the type of lipids extracted: the higher the hydrophobicity of the anion used, the higher the extraction of non-polar compounds.

Recently, Zghaibi et al. (2019) found that it was possible to use MAE to extract lipids from *Nannochloropsis* sp. by using only 10% NaCl (6.9% yield). In particular, the lipid extraction yield was similar with respect to Soxhlet extraction (4.5%), and lower with respect to the Bligh and Dyer (18%). However, MAE fully replaced the use of organic and harmful solvents, and, noteworthy, a better quality of lipids was obtained (polyunsaturated fatty acids and omega-3) (Zghaibi et al. 2019). It has to be considered that water, which as a highly polar solvent, can absorb microwave energy, and NaCl can improve the dielectric loss responsible for converting microwave energy into heat. The result is a higher efficiency in PUFA recovery.

Table 3 Yields obtained with MAE method from different microalgae

Microalgal species	Extraction method	Solvent	Biomass	Operative parameters	Lipid yield (%)	Fold increase	Reference
S. platensis	MAE	Methanol/*n*-hexane	Dry	600 W; 40 min	12.5	9.6	(Kalsum et al. 2019)
	Soxhlet	*n*-Hexane			1.3		(Kalsum et al. 2019)
C. vulgaris	MAE	Chloroform/methanol/water [Omim][OAc] 2.5%		700 W; 10 min	19.2	1.8	(Krishnan et al. 2020)
	Bligh and Dyer	Chloroform/methanol/water			10.9		(Krishnan et al. 2020)
Nannochloropsis sp.	MAE	Water/sodium chloride 10%		800 W; 30 min	6.9	1.5* 0.38**	(Zghaibi et al. 2019)
	Soxhlet	*n*-hexane			4.5		(Zghaibi et al. 2019)
	Bligh and Dyer	Chloroform/methanol/water			18		(Zghaibi et al. 2019)

*With respect to Soxhlet

**With respect to Bligh and Dyer

Compressed fluid extractions

Compressed fluid extractions are considered valuable green alternatives to conventional extractions. They include subcritical water extraction (SWE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE). Solvents involved in PLE and SWE are maintained at a temperature above the boiling point and at a pressure high enough to keep fluids in their liquid states (Ramos et al. 2002). On the other hand, SFE operates at temperature and pressure above the critical point of the solvent selected (Herrero et al. 2013). These conditions allow for the increase of diffusivity of the solvent, thus improving the penetration of the solvent into the matrix (Phelps et al. 1996). Besides the differences between these techniques, they all require minimum amount of GRAS solvents to perform selective extraction of bioactive compounds, without affecting the bioactivity or the chemical structure (Herrero and Ibañez 2018). Unfortunately, they are still not diffused due to the high investment costs (Herrero and Ibañez 2018). So far, CO_2 is the most used solvent, especially in supercritical extractions (Goto et al. 2015). CO_2 is an economic, non-harmful, non-flammable, and recyclable solvent. Due to its thermodynamic properties, at supercritical conditions, CO_2 shows a high diffusivity and a high density that allow a better penetration into the matrix (Goto et al. 2015; Molino et al. 2020). However, supercritical CO_2 ($ScCO_2$) is limited to the extraction of non-polar or low polar compounds (Gilbert-López et al. 2015; Gallego et al. 2019). To overcome this problem, a low amount of co-solvent (e.g., ethanol) can be used to increase the CO_2 polarity.

According to this, Nobre and co-workers performed a lipid extraction starting from the dried biomass of *Nannochloropsis* sp. (NANNO-2) by using $ScCO_2$ in the presence and in the absence of a co-solvent (20% ethanol). Authors found that $ScCO_2$ combined with ethanol was able to increase lipid yield, as 45% (w/w of dry biomass) of lipid yield was obtained with respect to 32% yield (w/w of dry biomass), in the absence of co-entrainer (Nobre et al. 2013). Moreover, Zullaikah et al. (2019) performed a lipid extraction from the wet raw biomass of *Chlorella vulgaris* by SWE. Besides the co-solvent, time and temperature may also affect the extraction yields. For this reason, experiments were performed in the presence or absence of co-solvents, at different temperatures and different time. In particular, chloroform, methanol, ethanol, ethyl acetate, and *n*-hexane were tested as co-solvents. Extractions were performed at 160 °C, 80 bar, at 160 °C, 180 °C and 200 °C, for 15 min, 30 min, 1 h, 3 h, and 5 h. All the results were then compared with conventional Bligh and Dyer extraction. The study revealed that SWE performed at 200 °C, 80 bar for 30 min, using ethyl acetate as co-solvent, gave the highest lipid yield (65.94%, w/w of dry biomass), while conventional chloroform/methanol extraction allowed for the obtaining of a lipid yield of 10.43% (w/w of dry biomass). Altenhofen da Silva and co-workers evaluated the effect of supercritical carbon dioxide ($ScCO_2$) on freeze-dried biomass of *Desmodesmus subspicatus*. Extractions were performed comparing two different pressures (20 and 30 MPa, that correspond to 200 and 300 bar, respectively) at 60 °C. Authors found out that a direct correlation was observed with pressure and lipid yield, as, at 30 MPa, 45% of lipids were recovered, a value which is almost the double of that obtained at 20 MPa or with Soxhlet (23% at and 20%, respectively) (Altenhofen et al. 2016).

Zimmerer et al. (2019) employed *Phaeodactylum tricornutum* dry biomass for lipid extraction. Cells were disrupted by ultrasonication prior the extraction with $ScCO_2$. Among the different pressures and temperatures tested, 90 °C and 621 bar were found to be the best conditions, as a 25% yield was obtained (w/w of dry biomass). As a reference, lipids were extracted by Folch method, using a mixture of

water, methanol, and chloroform. The lipid yield obtained by the conventional method was 28% (w/w of dry biomass). He and co-workers set up a process to obtain lipids from *Isochrysis* sp. dried biomass. Lipids were extracted by 3 cycles of 5 min each of PLE, at 103 bar, 80 °C, using two different solvents: *n*-hexane and ethanol. Soxhlet extraction (with hexane) and Folch method (with a mixture of chloroform/methanol/water) were performed to compare the extraction yields. PLE with *n*-hexane gave a higher yield (34.42 %) when compared with Soxhlet extraction performed with the same solvent (about 19% yield). However, PLE performed by using ethanol improved the process, as the lipid yield was 38.94% (w/w of dry biomass) (He et al. 2019).

We recently reported a process intensification to obtain three different high-value molecules in a biorefinery approach (Imbimbo et al. 2019). In particular, we improved lipid extraction as the third step of the cascade process. The extraction was performed using pure CO_2 as solvent, at 350 bar, 60 °C for 100 min starting from the wet biomass of *Galdieria phlegrea*. The yield was then compared with the one obtained by a conventional extraction performed with 0.37% NaCl in chloroform/methanol (2:1) on dry biomass. SFE allowed for the obtaining of 18.4% yield (w/w of dry biomass), in comparison with 11% yield (w/w of dry biomass) obtained by the conventional extraction method (Imbimbo et al. 2020). All the extraction yields are reported in Table 4.

Conclusions

Microalgae represent a natural source of bioactive compounds to be used in pharmaceutical, nutraceutical, cosmetic, and food sectors. In particular, many hydrophobic molecules endowed with special biological activity can be extracted from microalgae and used. Of course, during extraction, many parameters have to be considered. An ideal extraction method should allow to operate at low costs and to preserve both the original characteristics of the isolated molecule and of the residual biomass. Green extraction techniques seem to combine environmentally friendly and cost-effective extractions. Most of them are economically and environmentally sustainable and non-toxic and can increase the selectivity and

Table 4 Yields obtained with compressed fluid extraction methods from different microalgae

Microalgal species	Extraction method	Solvent	Biomass	Lipid yield (%)	Fold increase	Reference
Nannochloropsis sp. (NANNO-2)	SFE	CO_2	Dry	34	1.3*	(Nobre et al. 2013)
		CO_2 + 20% ethanol		45		(Nobre et al. 2013)
	Soxhlet	*n*-Hexane		40.7		(Nobre et al. 2013)
		Ethanol		50.6		(Nobre et al. 2013)
	Bligh and Dyer	Chloroform/methanol/water		25.3		(Nobre et al. 2013)
G. phlegrea	SFE	CO_2	Wet	18.4 ± 0.5	1.7	(Imbimbo et al. 2020)
	Conventional	Chloroform/methanol/sodium chloride	Dry	11 ± 0.3		(Imbimbo et al. 2020)
C. vulgaris	SWE	Water/ethyl acetate	Wet	65.9	6.3	(Zullaikah et al. 2019)
	Bligh and Dyer	Chloroform/methanol	Dry	10.4		(Zullaikah et al. 2019)
D. subspicatus	SFE	*n*-Hexane	Dry	45	2.3	(Altenhofen et al. 2016)
	Soxhlet	Chloroform/methanol/water		20		(Altenhofen et al. 2016)
P. tricornutum	SFE	CO_2	Dry	25	0.9	(Zimmerer et al. 2019)
	Folch	Chloroform/methanol/water		28		(Zimmerer et al. 2019)
Isochrysis sp.	PLE	*n*-Hexane	Dry	34.41	1.4** 1.8***	(He et al. 2019)
	PLE	Ethanol		38.94	1.5** 2***	(He et al. 2019)
	Folch	Chloroform/methanol/water		25.36		(He et al. 2019)
	Soxhlet	*n*-Hexane		19		(He et al. 2019)

*With respect to SFE in absence of co-solvent

**With respect to Folch

***With respect to Soxhlet

extraction efficiency (Domínguez de María 2017; Häckl and Kunz 2018; Singh and Savoy 2020). However, all of them have pros and cons with respect to the industrial benchmark of extraction with organic solvent.

In particular, MAE is a green technique used to recover different thermo-stable molecules. Of course, this can be a problem in the case of thermolabile molecules as the experimental conditions used may affect the physical-chemical properties of the isolated molecules. Furthermore, the efficiency of the extraction is often lower with respect to organic solvents.

Compressed fluid extractions can represent an excellent alternative to recover thermolabile molecules. The major advantage resides in the possibility to recover and recycle the solvent. Furthermore, the solvent polarity can be tuned by combining the neutral CO_2 with polar co-solvents, such as ethanol or isopropanol. The major limitation of compressed fluid extractions is represented by its initial investment costs. Lab-scale tests seem efficient when achieving > 300-bar pressure, which is often economically unfeasible at industrial scale. In case of switchable solvents, solvent separation and recycle are the main advantage. Furthermore, the tunability of the polarity allows these solvents to extract both hydrophobic and hydrophilic molecules only by switching a chemical-physical factor. What is still a pending point for SSs is their effect on the residual biomass after the extraction. Unfortunately, there is still much effort to be done to use these solvents, as no evidence of their extraction abilities have been reported after the switch.

Generally speaking, one should keep in mind that microalgae can be used as an excellent source of bioactive molecules provided that a biorefinery approach has to be used. Thus, microalgae costs have to be paid by obtaining more than a class of molecules, starting from the one with the highest market value. So, if the biorefinery approach includes downstream processes able to fulfill the requirements of Green Chemistry, it will end up with a new and sustainable process. With respect to green extraction techniques of lipids and pigments, less is known on two important aspects: the residual amount of solvent in the biomass and, mainly, the effect of the extraction on the other molecules in the leftover spent biomass. This review provides a step further in the extraction knowledge that can help to valorize microalgae biomass by using innovative extraction techniques, which comply with the Green Chemistry principles.

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