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Review

In-situ lipid and fatty acid extraction methods to recover viable products from *Nannochloropsis* sp.

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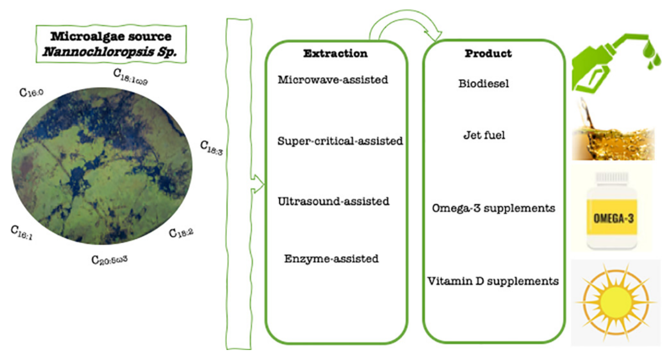
HIGHLIGHTS

- A critical review of FA and lipid extraction methods for application to microalgae.
- Combined extraction methods (*e.g.* microwave and ultrasound) show improved extraction performance
- Enzyme-assisted extraction offers green production, but scale up is a challenge.
- A summary of the FA composition obtained using extraction methods is provided.
- Viable products from *Nannochloropsis* Sp. are given, highlighting biodiesel and Omega-based products.

GRAPHICAL ABSTRACT



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ABSTRACT

Nannochloropsis sp. has received increased attention by researchers in recent years due to its complexity and abundance of lipid structures. The lipids of this microalgae species have been identified to contain large quantities of neutral lipids which are capable of producing raw materials for nutraceuticals, food additives and biofuels. The production of biodiesel has received the greatest attention as there is an increase in global demand for both more fuel and more environmentally sustainable methods to produce such resources. The greatest challenges facing industries to mass produce viable products from microalgae involve the degradation of the cell wall and extracting the fatty acid of interest due to high costs. Various studies have shown that the extraction lipids from the microalgae can greatly influence the overall fatty acid composition. Different extraction methods can result in recovering higher quantities of either saturated fatty acids, monounsaturated fatty acids or polyunsaturated fatty acids. Biodiesel production requires higher quantities of saturated fatty acids and monosaturated fatty acids as increased quantities of polyunsaturated fatty acids result in oxidation which decreases the performance of the biodiesel. Whereas, polyunsaturated fatty acids are required in order to produce pharmaceuticals and food additives such as omega 3.

This review will focus on how different *in-situ* extraction methods for lipid and fatty acid recovery, influence the fatty acid composition of various *Nannochloropsis* species (*oculate, gaditana, salina* and *oceanica*). The mechanical methods (microwave, ultrasonic and supercritical-carbon dioxide) of extraction for *Nannochloropsis* sp. will be critically evaluated. The use of enzymes will also be addressed, for their ability to extract fatty acids in a more environmentally friendly manner. This paper will report on the viable by-products which can be produced using different extraction methods.

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Contents

1. Introduction

Microalgae are naturally occurring green unicellular organisms which can be found in either freshwater or marine systems which produce high levels of lipids (Wu et al., 2017). The environmental impact of microalgae on fresh and marine waterways has received much interest. They are often preferred to other crops for biomass production due to their ability to grow on water and not compete for arable land for food supplies (Bermúdez Menéndez et al., 2014). Their high lipid content can be 200 times greater than other plants producing lipid volumes of 32,374.9 L/ha year^{-1} (Ali and Watson, 2015; Qadariyah et al., 2018). Microalgae is capable of adsorbing CO_2 making it an attractive green process with reports showing 100 tons of microalgal biomass fixing 183 tons of CO_2 (Ali and Watson, 2015; Ranjan et al., 2010). It has been reported by various authors that microalgae can be used to deplete nitrogen levels up to 100% removal. However, no studies have focused on the use of *Nannochloropsis* sp. to reduce nitrogen levels (Babatsouli et al., 2015; Church et al., 2017; Shen et al., 2015). The use of microalgae to produce viable products such as biodiesel is favoured. (Huerlimann et al., 2010; Zuorro et al., 2016a). This is due to reduced environmental impacts compared with conventional fossil oil recovery. Additionally, recovering biodiesel from microalgae over plants which can be used for human consumption is favoured in order to prevent a depletion of food sources and competing for arable land.

Nannochloropsis sp. has been reported to be a favoured microalgae species for a number of reasons (Ljubic et al., 2020). It is capable of accumulating in large amounts, it can grow on brackish and other wastewaters under adverse conditions and accumulate large amounts of polar lipids (up to 50% of total biomass being lipid) (Qiu et al., 2019; Zuorro et al., 2016b). The lipids present in microalgae are made up of mainly polar (phospholipids, glycolipids and betaine) and non-polar (acylglycerols, sterols and free fatty acid (FFA)) lipids (Natarajan et al., 2014). Studies looking at *Nannochloropsis* sp. have reported high concentrations of C_{16}, $C_{16.1}$, C_{18} and $C_{18.1}$ which are suitable for biodiesel production. *Nannochloropsis* sp. *also* contain high concentrations of $C_{20:5\omega3}$ which contribute to the production of omega-3 products (Adam et al., 2012; Qiu et al., 2019). Recovering these FA from the microalgae species would allow for the production of viable products. However, it is a difficult process due to the complex structure of the organism. The cell structure (composed of cellulose, polysaccharides, proteins and lipids) has a strong resistance to mechanical and chemical treatments (Chua and Schenk, 2017; Zuorro et al., 2016a). Species of *Nannochloropsis* used in fatty acid recovery, include *oculata, gaditana, salina and oceanica.*

For years scientists have attempted to disrupt the strong cell structures of *Nannochloropsis* sp. using conventional methods such as soxhlet extraction and chemical degradation (Zghaibi et al., 2019). After the lipids are extracted, they undergo a transesterification into fatty acid methyl esters (FAME) using an alkali or acid catalyst at high temperatures in the presence of methanol (Santana et al., 2012). However, this conventional two step method requires high temperatures which may deteriorate the polyunsaturated fatty acids (PUFA) (Aliev and Abdulagatov, 2017; Taher et al., 2020). These disadvantages have called for *in-situ* extraction methods which use less harmful chemicals and can disrupt the microalgae cell efficiently (Goh et al., 2019).

Due to negative aspects of the conventional methods, novel *in-situ* methods for extraction of lipids and FA from *Nannochloropsis* sp. are needed to produce viable products. Many methods have gained attention in the last 5 years. (Ali and Watson, 2015; Qiu et al., 2019; Wiyarno et al., 2011). From this period, microwave-, ultrasound-, supercritical CO_2 (SC-CO_2) and enzyme-assisted extractions are the most studied methods on *Nannochloropsis* sp. and therefore they are the focus of this paper. The data presented in this review also suggests that these are the most promising methods in achieving lipid and FA recovery.

The energy requirement to dry, extract and hydrolyse the FA, accounts for 10.7, 14.5 and 14.8% of the total energy consumption, respectively (Wang et al., 2017). In order to reduce these energy inputs, the use of *in-situ* methods must be integrated (Qadariyah et al., 2012). Wet biomass extraction can be difficult and result in the recovery of fewer lipids. This is due to the algal cells gathering a hydrated shell which acts as a barrier to energy and mass transfer (Martinez-Guerra et al., 2018). The choice of a particular technique for lipid extraction depends on algal species, initial lipid content and desired FA (Ranjan et al., 2010). SC-CO_2 have been reported as a suitable method for PUFA recovery but high pressures result in high energy costs (Zghaibi et al., 2019).

In-situ extraction and derivatisation methods have received increasing interest in recent years for the recovery of lipids from *Nannochloropsis* sp.

Many papers have assessed the methods used in terms of the lipids and proteins recovered. However, to the authors knowledge there has been no review on the most common methods to critically review their performance in terms of FA composition and the possible viable products. A large number of studies have focused on the lipid recovery from *Nannochloropsis* sp. using various methods. This paper reviews the most common *in-situ* lipid and FA extraction methods for *Nannochloropsis*. The review covers 10 years of studies involving *Nannochloropsis* sp. as a source of lipids and FAs for bio-based products. Search terms such as *Nannochloropsis* sp. lipid extraction, microwave-, ultrasound-, supercritical- or enzyme-assisted extraction of lipids from *Nannochloropsis* sp. and recovery of viable products from *Nannochloropsis* sp. were used. Over 140 references were gathered and subsequently >70 references have been included in this review. It addresses how the methods influence the FA composition of the end product. The viable products from the microalgae are also discussed.

2. Extraction-assisted methods

2.1. Microwave-assisted extraction

The use of microwave (MW) to aid in FAME recovery from microalgae has increased recently. MW allows for a controlled and accurate heating with uniform thermal transfer to ensure all the samples are heated up equally (Martinez-Guerra et al., 2018; Mubarak et al., 2015). Microwave heating leads to localized high temperature and pressure gradient which results in cellular wall degradation and enhanced mass transfer (Martinez-Guerra et al., 2018). This unique heating mechanism leads to a reduced extraction time (15 min) compared to conventional heating (>60 min) (Brennan et al., 2020).

2.1.1. Principles of MW assisted extraction

The principle of MW assisted lipid extraction is described in detail by (Mandal and Hemalatha, 2007) who used the method for medicinal plants. However, the same principle is used for microalgae.

2.1.2. Factors which effect lipid recovery using MW

The use of MW for lipid and FA recovery extracts up to 51.8% of the dry biomass as lipids with 13.2% SFA, 3.5% MUFA and 35% PUFA as reported by (Brennan et al., 2020). The drying and extraction processes are the most energy demanding steps in the recovery of viable products from *Nannochloropsis sp.* (Mubarak et al., 2015). However, some authors have described the process of having much lower retention times. Therefore this decreases the cost, also making it a greener process (Zghaibi et al., 2019). A study by (Ali and Watson, 2015) investigating *Nannochloropsis oculata* showed that the energy and cost from the MW contribution of the process for lipid recovery was very low at 0.160 mJ/kg of lipid recovered. The same study showed that the use of ethanol contributed more to the energy consumption at 90.9 mJ/kg of lipid recovered, suggesting that the solvent choice is important. A study by (McKennedy et al., 2016) using *Nannochloropsis oculate*, extracted lipids with methanol leading to a lower yield. However, as seen in Fig. 1 and Table 1, ethanol is reported to achieve the optimal extraction of lipids using MW radiation (Mandal and Hemalatha, 2007).

Factors which effect MW lipid extraction include temperature, time, matrix characteristics, solvent type and dielectric properties (Mandal and Hemalatha, 2007; Zghaibi et al., 2019). Solvents which have a lower dielectric loss, allow for less microwave adsorption and thus greater heating efficiency. Polar solvents such as methanol and ethanol achieve better extraction than hexane and chloroform. It was demonstrated by (Bermúdez Menéndez et al., 2014) that using a non-polar solvent such as hexane with *Nannochloropsis gaditana* recovered only 14.8% lipids from the dry biomass. (Zghaibi et al., 2019) used NaCl on *Nannochloropsis oceanica* to recover lipids (Table 1). This represents a greener process however, the recovery was low due to its low dielectric constant (Hoekstra and Cappillino, 1971). NaCl is reported to recover less lipid and FA volume, but it is more cost efficient. The cost to extract 1 kg of lipid with the Bligh Dyer method and Soxhlet extraction is $1380 and $10,683, respectively. The costs of the MW method with NaCl was reported to be in the region of $774 to recover the same volume of FA (Zghaibi et al., 2019).

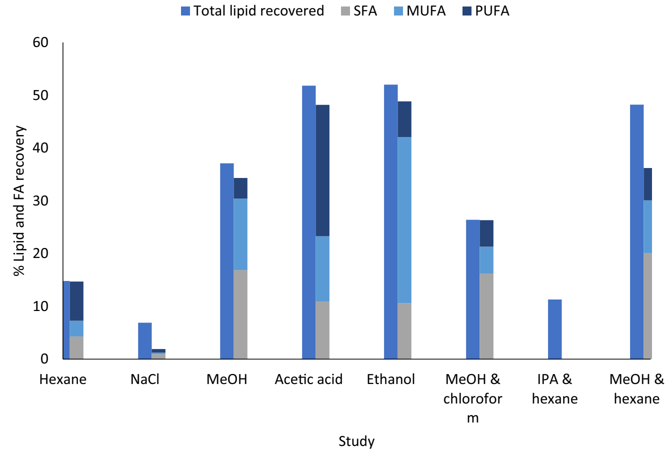

Fig. 1. MW-assisted extraction of lipids and FA from Nannochloropsis sp. using different solvent(s). (Hexane (Bermúdez Menéndez et al., 2014); NaCl (Abugrara et al., 2019); MeOH (McKennedy et al., 2016); Acetic acid (Brennan et al., 2020); Ethanol (Ali and Watson, 2015); MeOH&chloroform and IPA&hexane (Teo and Idris, 2014); MeOH&hexane (Martinez-Guerra et al., 2018)).

2.1.3. MW in-situ methods

The mechanical effect of ultrasonication (US) can allow for the release of soluble compounds from the microalgae (by disrupting the cell wall by cavitation and bubble collapse). This enhances mass transfer and facilitates solvent access to the cell components (Bermúdez Menéndez et al., 2014). By combining MW and US enhances mass transfer by MW with US improving rapid heating (Martinez-Guerra et al., 2018). A study by (Martinez-Guerra et al., 2018) showed that using MW with methanol and hexane, obtained a lipid recovery of 22.8%. By adding US in the process, more than doubled the recovery to 48.2% and lowered the energy required. The results showed that the US method did not affect the FA composition. The recovered fraction was composed of $C_{16:0}$ and other SFA with very low PUFA, allowing for good quality biodiesel production (Martinez-Guerra et al., 2018).

2.1.4. Efficiency of lipid and FA extraction using MW

Studies to determine the efficiency of MW for extracting lipids and FA from *Nannochloropsis* sp. indicate a huge variation in lipid recovery based on the solvents (Fig. 1 and Table 2). Studies have shown recovery of similar FA compositions (Fig. 1), high SFA and MUFA, with low PUFA

Table 1
Lipid and FA recovery from Nannochloropsis Sp. using different solvents with the aid of MW-assisted extraction (Ali and Watson, 2015; Bermúdez Menéndez et al., 2014; Brennan et al., 2020; Martinez-Guerra et al., 2018; McKennedy et al., 2016; Teo and Idris, 2014; Zghaibi et al., 2019).

Solvent composition	Lipid recovery (%)	SFA (%)	MUFA	PUFA
Hexane	14.8	29.1	20.1	50.2
Sodium chloride	6.9	13.5	4.4	9.9
Methanol	37.1	45.5	36.5	10.5
Acetic acid	51.8	21.0	23.9	48.1
Ethanol	52.0	20.4	60.5	13.0
Methanol and chloroform	26.4	61.2	19.5	19.0
Propanol and hexane	11.3	NR	NR	NR
Methanol and hexane	48.2	41.7	20.7	12.7

Table 2
Lipid and FA composition of Nannochloropsis Sp. investigated using MW with different solvent(s) ((Abugrara et al., 2019; Ali and Watson, 2015; Bermúdez Menéndez et al., 2014; Brennan et al., 2020; Martinez-Guerra et al., 2018; McKennedy et al., 2016; Teo and Idris, 2014).

Fatty acid composition (%)	Hexane	Sodium chloride	Methanol	Acetic acid	Ethanol	Methanol and chloroform	Methanol and hexane
$C_{14:0}$	4.1	4.0	6.1	0.4	–	11.9	10.8
$C_{16:0}$	24.9	9.2	38.4	17.8	18.0	16.5	11.3
$C_{16:1}$	15.8	0.5	27.7	19.7	4.5	–	6.2
$C_{18:0}$	–	0.3	1.0	2.5	2.4	32.9	8.7
$C_{18:1\omega9}$	4.3	1.1	8.8	2.5	56.0	10.6	4.7
$C_{18:1\omega9t}$	–	1.6	–	1.7	–	9.0	–
$C_{18:2}$	14.6	0.1	1.2	9.3	–	19.0	6.1
$C_{18:3}$	12.0	0.3	0.7	–	13.0	–	6.6
$C_{20:0}$	–	0.1	–	–	–	–	10.9
$C_{20:4\omega6}$	1.9	1.2	–	2.7	–	–	–
$C_{20:5\omega3}$	7.4	8.2	6.5	36.1	–	–	–
$C_{22:1}$	–	0.7	–	–	–	–	9.9

levels suggesting suitability for biodiesel recovery. A study by (Brennan et al., 2020) showed that PUFA have a greater affinity to acetic acid. The high PUFA levels are suitable for omega 3 supplementary products. However, this study was based on microalgae cultivated from brine wastewater, unsuitable for pharmaceutical (Brennan et al., 2020; Gadipelly et al., 2014). The use of multiple solvents, (Qadariyah et al., 2012) is reported to aid lipid recovery, with different solvents allowing for the extraction of different FAs (Brennan et al., 2020; Martinez-Guerra et al., 2018; Teo and Idris, 2014). Table 2 and Fig. 1 show the FA composition obtained for *Nannochloropsis* sp. from different solvent (s); the average SFA is 32%, 17% MUFA and 12.2% PUFA from all of the methods investigated. According to (Martinez-Guerra et al., 2018), these compositions make MW the ideal method to produce biodiesel from *Nannochloropsis.* The ideal cetane number, viscosity, oxidation potential and heating capacity discussed further in Section 3.1, are achieved.

2.2. Supercritical fluid-assisted extraction

Supercritical fluid extraction (SFE) is the process of using CO_2 or other solvents under high pressure to extract oils/lipids from the matrix (Pushpangadan and George, 2012; Obeid et al., 2018). SFE has been recognised as an efficient alternative to organic solvents and conventional methods (Mouahid et al., 2013). Supercritical CO_2 (SC-CO_2) has a lipid solubility similar to organic solvents with higher diffusivity and lower viscosity (Taher et al., 2020). The use of SC-CO_2 over conventional methods has been reported to extract up to 2.5 times more lipid and use less energy (Aliev and Abdulagatov, 2017). In addition, the SC-CO_2 method is efficient at recovering non-polar lipids. Polar solvents can be incorporated in order to influence the recovery of polar lipids (Patil et al., 2018). However, SC-CO_2 is a complex process with costly apparatus compared to conventional methods (Brennan et al., 2020). The SC-CO_2 method is affected by water content in the sample therefore impacting the biomass recovery and reducing the lipid recovery (Taher et al., 2020).

2.2.1. SC-CO_2 in-situ methods

Nannochloropsis salina (Patil et al., 2018) was used with a MW step to rupture the cell walls, prior to extraction by SC-CO_2. The results showed that a lipid recovery of 12% of the total biomass, could be obtained. This was double that obtained using the conventional method. (Taher et al., 2020) who investigated *Nannochloropsis gaditana* incorporated an enzymatic process prior to the SC-CO_2 extraction to further derivatize the lipids into FAMEs. Fig. 2 shows that lipase had increases of yield up to 80%. However, the cost of enzymes can greatly increase the cost of biodiesel production. A study by (McKennedy et al., 2016) (using *Nannochloropsis* oculata) showed that by incorporating co-solvents can allow for reduced temperature and pressure. Therefore operation costs were reduced, while producing FAME suitable for biodiesel. Ethanol has a low solubility to lipids however, when under supercritical conditions it can dissolve more lipids (Chen et al., 2012). By mixing ethanol with hexane as a co-solvent for SC-CO_2, improved lipid recovery is achieved (in comparison with conventional SC-CO_2) and FAME production. (Patil et al., 2018). Fig. 2 shows the lipid recovery obtained as a % of the total biomass for SC-CO_2 and SC-CO_2 assisted methods (Aliev and Abdulagatov, 2017; Andrich et al., 2005; Chen et al., 2012; Patil et al., 2018; Taher et al., 2020).

2.2.2. FA composition obtained by SC-CO_2

Results obtained from SC-CO_2 studies for FAME recovery (Table 3) show that the composition of the lipid is dependent on the extraction technique. SC-CO_2 does not recover polar lipids but shows higher concentrations of less polar lipids useful for omega 3 and omega 6 medicinal production (Andrich et al., 2005; Solana et al., 2014). Studies by (Mouahid et al., 2013) and (Taher et al., 2020) using conventional SC-CO_2 on *Nannochloropsis oculata* and *gaditana*, respectively showed that more PUFA FA were recovered rather than the more polar SFA (Table 3). (McKennedy et al., 2016) stated that using different co-solvents demonstrates the tunability of SC-CO_2 to produce a desired product. Solvents such as hexane and ethanol allow for the more oxidative FA which are suitable for biodiesel production. Table 3 shows the

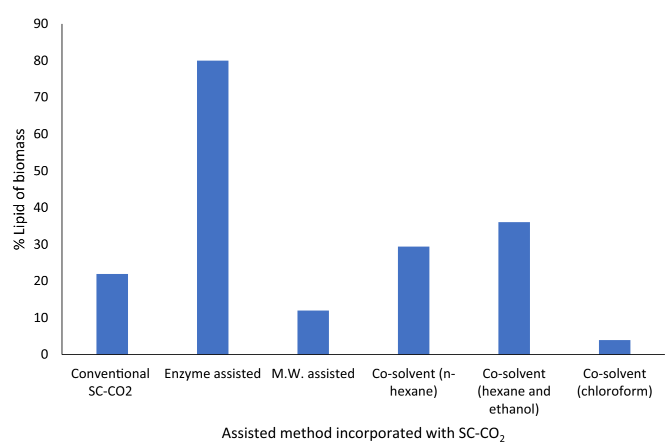

Fig. 2. Lipid recovery with SC-CO_2 using different assisting methods (enzyme- (Taher et al., 2020), MW- (Patil et al., 2018) and co-solvent- (n-hexane with *Nannochloropsis* sp. *salina*) (Aliev and Abdulagatov, 2017); (hexane & ethanol) (Chen et al., 2012); (chloroform with *Nannochloropsis* sp. *salina*) (Aliev and Abdulagatov, 2017) assisted methods) compared with conventional SC-CO_2 (Andrich et al., 2005).

Table 3
FA composition for Nannochloropsis Sp. lipids extracted by SC-CO_2.

SC-CO_2 condition	Conventional SC-CO_2	Conventional SC-CO_2	Co-solvent (hexane and ethanol)	Co-solvent (n-hexane)	Co-solvent (chloroform)
Lipid recovery (% total biomass)	33	21.9	36	29.4	3.92
SFA	25.3	21	42.37	23.44	23.35
MUFA	14	32	52.8	46.19	46.31
PUFA	43.2	43	0.62	11.9	12.22
$C_{12:0}$	0	0	0	0.39	0.27
$C_{14:0}$	5.7	3	2.88	3.2	3.06
$C_{16:0}$	17.8	12	35.67	18.62	18.91
$C_{16:1}$	11.4	12.5	25.96	26.61	27.11
$C_{18:0}$	1.8	6	3.82	1.23	1.11
$C_{18:1\omega9}$	2.6	19.5	26.84	19.58	19.2
$C_{18:2}$	5.2	8	0	5.92	6.34
$C_{20:4\omega6}$	5	5	0	5.98	5.88
$C_{20:5\omega3}$	33	30	0.62	0	0

FAME composition obtained for different solvents and it shows that hexane-ethanol produce high levels of SFA and MUFA which are suitable for biodiesel production (Aliev and Abdulagatov, 2017; Andrich et al., 2005; Chen et al., 2012; Mouahid et al., 2013; Patil et al., 2018; Taher et al., 2020).

2.3. Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) refers to an extraction process of lipids from solid samples using ultrasound waves to disrupt the cell walls (Bendicho and Lavilla, 2018). This allows for a greater solvent penetration into the microalgae, increasing contact between the sample and solvent thereby improving mass transfer (Duarte et al., 2014; Mubarak et al., 2015). An improved extraction yield, reduction in temperature, solvent consumption and extraction time have been reported (Parniakov et al., 2015). UAE has been reported to give a higher purity product (Wiyarno et al., 2011) where optimisation of temperature (Adam et al., 2012) and retention time making it a greener process (Adam et al., 2012). A drawback of UAE is the difficulty of recovering solvents to be reused (Mandal et al., 2015). (Duarte et al., 2014) reported that it is difficult to incorporate UAE with other methods – although (Martinez-Guerra et al., 2018) and (Bermúdez Menéndez et al., 2014) showed that UAE and MW assisted extraction can be incorporated and increase the lipid yield more efficiently. The UAE method is outlined extensively by (Ferreira et al., 2016; Mubarak et al., 2015; Natarajan et al., 2014).

The FAME composition for *Nannochloropsis* sp. obtained for different UAE studies and their conventional methods is presented in Table 4. A high SFA and MUFA content is reported which suggests that the method may be suitable for biodiesel production. However, comparisons against conventional methods suggest that there is no significance in the composition of the FA prepared by different methods. Overall, *Nannochloropsis* sp. has received little attention with the use of UAE for lipid recovery. UAE represents a quick method which successfully produces FA of industrial use and it can be easily scaled up. However, it does not allow for variation in FA composition.

Table 4
FAME composition for Nannochloropsis Sp. investigated by UAE and conventional Soxhlet method (Adam et al., 2012; Bermúdez Menéndez et al., 2014; Natarajan et al., 2014; Wiyarno et al., 2011) (NR = Not recorded) (Lipid recovery reported as % of total biomass and FA reported as % of the lipid).

Fatty acid composition (%)	UAE	UAE	UAE	Conventional	UAE
Lipid recovery	14.76	NR	55	21	6.8
SFA	28.8	34.0	23.4	24.6	29.2
MUFA	20.0	10.0	37.6	31.6	43.5
PUFA	43.7	39.5	38.1	41.7	5.9
$C_{14:0}$	4.0	9	7.4	7.4	11.1
$C_{16:0}$	24.7	18	14.5	16.8	12.5
$C_{16:1}$	15.8	–	33.6	27.1	–
$C_{16:2}$	6.3	–	1.7	1.2	–
$C_{18:0}$	–	7	1.5	0.4	5.6
$C_{18:1\omega9}$	4.2	10	4	4.5	43.5
$C_{18:2}$	15.3	10.5	1.5	2.6	5.9
$C_{18:3}$	12.2	20	–	0.7	–
$C_{20:4\omega6}$	1.9	–	5.3	3.4	–
$C_{20:5\omega3}$	8.0	9	29.6	33.8	–

2.4. Enzyme-assisted extraction

2.4.1. Principles of enzyme-assisted extraction

The process of enzyme recovery is based on the selected enzyme (s) breaking the cell wall of the microalgae, isolating the lipid and derivatizing the lipid to FAME (Maffei et al., 2018; Zuorro et al., 2016b). The use of enzymes for FAME recovery offers a reduction in energy, due to decreased temperatures required. (Wang et al., 2017) who studied *Nannochloropsis oceanica* demonstrated that enzymatic recovery of FAME could be carried out at room temperature allowing for FAME recovery of up to 99%. Disadvantages of enzymatic-assisted extraction include the inability to upscale due to the high cost of enzymes (Lopez et al., 2015; Wu et al., 2017). However, various studies by (Castillo López et al., 2015) on *Nannochloropsis gaditana* showed that novozym has a high reuse rate of up to 6 times. A study by (Wang et al., 2017) also using novozym enzymes, showed that they could be reused up to 110 times. The increased life time may be due to the addition of t-butanol and methanol when compared to the other study, which used methanol. When using enzymes to recover FAMEs, the cost of the enzymes represent 22% of the total costs which can be reduced in half by recycling the enzymes as long as the enzymes working efficiency remained at 90% (Wang et al., 2017).

2.4.2. FA composition obtained by enzyme-assisted extraction

The most common enzymes which have been tested include novozym, cellulose, hemicellulose and lysozyme (Onumaegbu et al., 2018; Zhang et al., 2018). Fig. 3 shows the lipid recovery for various enzyme(s) all with a concentration of 10 mg/g which was determined to recover the greatest volume of lipids by various studies by (Zuorro et al., 2016b, 2016a). An investigation by (Qiu et al., 2019) showed that *Nannochloropsis* sp. lipids contain up to 60% polar lipids which can be converted to FAMEs using enzyme (lipids were used for food applications in this study). Cellulose which was the most common enzyme to be used alone, gave lipid recovery of between 11.73 and 53.2% in different studies ((He et al., 2020; Liang et al., 2012; Maffei et al., 2018; Qiu et al., 2019). The composition of the cellulose lipid reported by multiple authors showed an average of 48.9% SFA, 35.4% MUFA and 15.5% PUFA as seen in Fig. 3 (He et al., 2020; Qiu et al., 2019). This composition of FA in the microalgae represents an ideal biodiesel raw material, with a very small percentage of PUFA (Martinez-Guerra et al., 2018). The FAMEs which are at highest

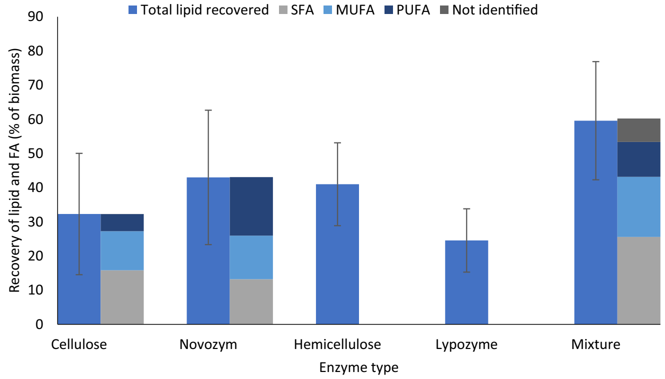

Fig. 3. Summary of data gathered on the average of % lipid recovery and FA composition from *Nannochloropsis* sp. using different enzymesbased on a number of studies (Cellulose: Zuorro et al., 2016a, 2016b, Qiu et al., 2019, Liang et al., 2012 and Castillo López et al., 2015; Novozym: Zuorro et al., 2016a, Wang et al., 2017 and Ali and Watson, 2015; Hemicellulose: Zuorro et al., 2016a, 2016b and Maffei et al., 2018; Lypozyme: Zuorro et al., 2016a and Lopez et al., 2015; Mixture: Zuorro et al., 2016a, 2016b, Wang et al., 2017 and Maffei et al., 2018.).

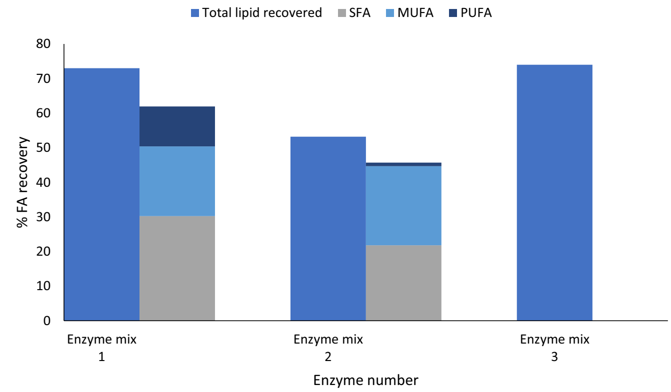

Fig. 4. Summary lipid recovery and FA composition from Nannochloropsis sp. using enzyme mixtures from 3 different studies; enzyme mix 1 = mixture of cellulose/protease/lysozyme/pectinose (Wu et al., 2017), enzyme mix 2 = cellulose/hemicellulose by (Zuorro et al., 2016a) and enzyme mix 3 = cellulose/hemicellulose by (Maffei et al., 2018).

concentrations include $C_{16:0}$ and $C_{16:1}$ which are characteristic biodiesel carbons outlined in Table 5 (Teo et al., 2014).

Novozym is the most widely used commercially available enzyme for assisting in the production of biodiesel as it allows for high lipid recovery (Castillo López et al., 2015). As seen in Fig. 3, the lipids recovered (average of 3 studies by (Castillo López et al., 2015; Lopez et al., 2015; Wang et al., 2017)) by novozym showed to have 30.7, 29.7 and 39.8% for SFA, MUFA and PUFA respectively. The compositions of these FA appear to have equal amounts of SFA, PUFA and MUFA which would mean they are not suitable for biodiesel production (Wang et al., 2017; Wu et al., 2017; Zuorro et al., 2016b). Hemicellulose and lypozyme were also investigated demonstrating recovery of favourable levels of lipids at 41 and 24.5%, respectively (Ali and Watson, 2015; Lopez et al., 2015; Maffei et al., 2018; Wang et al., 2017; Zuorro et al., 2016b, 2016a).

2.4.3. Factors effecting enzyme-assisted recovery

Fig. 3 shows that a mixture of enzymes can recover the highest volume of lipids (Maffei et al., 2018; Wu et al., 2017; Zuorro et al., 2016b). Fig. 4 shows the lipid and FA composition obtained by different enzyme mixtures (enzyme mix 1; mixture of cellulose/protease/lysozyme/pectinose (Wu et al., 2017), enzyme mix 2; cellulose/hemicellulose by (Zuorro et al., 2016a) and enzyme mix 3; cellulose/hemicellulose by (Maffei et al., 2018). A study by (Zuorro et al., 2016b) showed that lipid recovery with no enzymes gave 14% lipids from biomass. By introducing enzymes to assist with lipid extraction showed improvements with hemicellulose, cellulose and lysozyme at 34, 24 and 18%, respectively. Hemicellulose is reported to have optimal impact due to its composition of mannanase, galactosidase and gluconate. Zuorro's results showed (Zuorro et al., 2016b) that a combination of enzymes (cellulose and hemicellulose) led to greater lipid recovery at 53.2%. These lipids were further characterized to contain 41% SFA and 43% PUFA, which is an ideal ratio for the production of biodiesel. Another enzyme mixture consisting of cellulose and hemicellulose (Maffei et al., 2018) found that the mixture allowed for a recovery of 74%. A study carried out by (Wu et al., 2017) showed that an enzyme cocktail of cellulose, protease, lysozyme and pectinase extracted 73.1% of the lipid from microalgae. The results in Table 5 indicate that enzyme-assisted extraction enhances the recovery of SFA and MUFA.

Table 5
FA composition obtained by enzymatic-assisted extraction.

Fatty acid composition (%)	Enzyme mixture	Cellulose	Nonozyme
SFA	42.91	48.99	30.6
MUFA	28.63	35.44	28.7
PUFA	16.87	15	40.4
$C_{14:0}$	6.5	7.48	7.3
$C_{16:0}$	35.4	35.26	23.3
$C_{16:1}$	28.46	28.15	24.8
$C_{18:0}$	0.32	6.25	–
$C_{18:1\omega9}$	–	7.29	3.9
$C_{18:2}$	2.95	1.61	3.8
$C_{18:3}$	0.26	0.53	–
$C_{20:4\omega6}$	3.61	1.13	6.8
$C_{20:5\omega3}$	10.31	12.26	29.8

3. Viable products from *Nannochloropsis* sp.

3.1. Biodiesel

Nannochloropsis sp. microalgae has received increased interest as sources of biodiesel. This is due to advantages such as carbon neutrality, reduced emissions and availability of biomass feedstock due to fast growth (Cehn et al., 2018; Lee et al., 2014; Bwapwa et al., 2020). Biodiesel can be either used in pure form or in combination with diesel for engines which do not need to be modified to facilitate them (Mubarak et al., 2015). Biodiesel is synthesised by transesterification in which the triglyceride in the matrix reacts with a catalyst to yield esters of FA and glycerol (Ranjan et al., 2010). There is a growing need for biodiesel due to the impact of fossil fuel resources on climate change (Castillo López et al., 2015).

3.1.1. Biodiesel FA composition

FA compositions of lipid extracted from *Nannochloropsis* sp. determines if the species is suitable for biodiesel production. Studies by (Ali and Watson, 2015) and (Ma et al., 2014) showed that $C_{16:0}$, $C_{16.1}$, C_{18} and $C_{18.1}$ were found in *Nannochloropsis* sp. The presence of SFA such as $C_{16:0}$ and $C_{18:0}$ determines the cetane number, which improves the ignition quality and the heat of combustion of the fuel. UFA such as $C_{16.1}$ and $C_{18.1}$ are oxidatively unstable due to the double bonds which are susceptible to reaction with oxygen (Ali and Watson, 2015). It has been suggested that MUFA act as a balance between oxidative stability and low temperature properties (Ma et al., 2016). The recommended FA ratio for optimal biodiesel is 5:4:1 for $C_{16.1}$:$C_{18.1}$:C_{14} (Huerlimann et al., 2010). The equations used to calculate the most important properties of biodiesel (including cetane number, saponification value, iodine number, viscosity, density and higher heating value) were reported by (Martinez-Guerra et al., 2018). Of these properties, the most important include the cetane number which indicates the fuel ignition quality, oxidation stability which should be high to avoid oxidation and to avoid longer storage times, viscosity values which are higher to alter injection spray characteristics damaging the chamber and more viscous fuel can damage the fuel pump (Ali and Watson, 2015; Ma et al., 2016). The required properties set out by the American and European fuel agencies is outlined in Table 6 (Martinez-Guerra et al., 2018). A study by (Ma et al., 2014) showed that biodiesel could be successfully produced to consist of the suitable properties cetane number 54.61, iodine number 104.85 and low cloud point of 3.45 °C. This suggests a suitable product (Table 6) for engines can be produced using *Nannochloropsis* sp.

3.1.2. Biodiesel case studies

Nannochloropsis sp. for biodiesel production is reported to be an attractive alternative to fossil fuels as it is compatible with current engines. (Carrero et al., 2015). A study by (Teo et al., 2016) showed that the retention time to produce biodiesel from *Nannochloropsis* sp. can be performed in 20–30 min using a MW noncatalytic method. However, problems associated with the production of biodiesel from microalgae include the cost of production. This is due mostly to cultivation and extraction (Goh et al., 2019; Lee et al., 2014; Onumaegbu et al., 2018). A study by (Kim et al., 2015) found that *Nannochloropsis* sp. lipids and FA were capable of producing a biodiesel with 100% yield at lab scale analysis. However, biodiesel production from *Nannochloropsis* sp. requires improvement in the cultivation and drying methods in order to achieve the scale required (Ma et al., 2016). Some authors have predicted microalgae can make a viable biodiesel product (Santos-Sánchez et al., 2016) in five years from 2016.

3.2. Omega-3

Omega-3 FA are recovered from $C_{20:5\omega3}$ and $C_{22:6\omega3}$ which are PUFA. They can be used to produce food, nutraceuticals and pharmaceuticals. Omega-3 is reported to provide health benefits such as preventing cardio-vascular diseases, cancer, asthma, arthritis and high blood pressure (Aliev and Abdulagatov, 2016; Craggs et al., 2011). Furthermore, the intake of omega-3 containing FAs are known to play a role in controlling depression and promote animal growth (Li et al., 2019; Ma et al., 2016). It was reported (Figueiredo et al., 2019) that omega-3 consumption by humans worldwide is below the recommended. Therefore, the use of microalgae is of interest to produce FA for enriching foods or production of nutraceutical supplements. PUFA such as EPA and DHA have been reported to provide benefits for health conditions with high concentrations from *Nannochloropsis* sp. lipids (containing up to 30% of the biomass) (Aliev and Abdulagatov, 2016; McKennedy et al., 2016; Santos-Sánchez et al., 2016). (Ma et al., 2016) reported that the recommended daily intake of EPA and DHA is 250 mg to 2 g in order to have a healthy lifestyle. A study by (Brennan et al., 2020) reported 0.36 mg EPA in a 2 mg *Nannochloropsis* sp. sample therefore in order to meet the recommended levels, >690 mg of Nannochloropsis sp. would be required. Nannochloropsis sp. are a suitable species to supply these FAs as they've been reported to contain up to 50% lipids in their biomass which is capable of doubling daily and producing high levels of $C_{20:5\omega3}$ when compared to other microalgae species (McKennedy et al., 2016; Ranjan et al., 2010).

Table 6
Regulation set out by US and EU authorities for biodiesel properties for to be used in engines.

Property	Unit	ASTM 6751–02	EN 14214
Cetane number	–	≥47	≥51
Saponification value	(mg KOH/g)	–	–
Iodine value	(g I_2 100/g)	–	≤120
Degree of unsaturation	–	–	–
Cold filter plugging point	(°C)	NA	≤5/≤ − 20
Cloud point		Report	Report
Viscosity	(mm^2/s)	1.9–6.0	3.5–5.0
Density	(g/cm^3)	NA	0.96–90
Oxidative stability	(h)	–	≥6

4. Conclusion

Extensive research has previously been carried out characterising FA from *Nannochloropsis* sp. using different extraction methods. However, of the studies investigated, no study has looked at how different methods impact the FA composition of the recovered lipid. This is a comprehensive review of the *in-situ* mechanical and enzymatic methods of recovering FAMEs from *Nannochloropsis* sp. The methods discussed include microwave-, ultrasound-, SC-CO_2- and enzyme assisted extraction. The viable products which can be produced from the FA recovered are discussed. The comparison of methods suggest that the MW method produces high levels of SFA and MUFA which makes it suitable for biodiesel production. However, MW is a difficult and costly method to scale up which presents challenges to using it at industrial scale. SC-CO_2-assisted extraction shows some merits which allows for the conditions to be altered in order to produce desired FAs. The UAE method showed to have little research focusing on *Nannochloropsis* sp. However, from the reviewed literature it could be concluded that the simple and low costing method produces highest levels of SFA and MUFA. Enzyme-assisted extraction was reported to offer a green alternative with lower temperatures and chemical consumption. A mixture of enzymes allowed for the greatest recovery. Using a mixture resulted in a maximum disruption of the cell wall. For the recovery of nutraceuticals, SC-CO_2-assisted extraction appears to be the most efficient as the system parameters can be modified to obtain the highest concentrations of EPA and DHA. In terms of biodiesel, enzymatic-assisted extraction shows great potential with its high lipid recovery, ability to reuse enzyme substrates and green approach to help lower CO_2 emissions. Published literature has focused on the recovery of biodiesel and omega-3 lipids extensively. Biodiesel recovery shows great potential but there are still further developments to make it a viable product. EPA and DHA have become very popular for producing omega-3 containing nutraceuticals.

The review shows that different extraction methods can impact the FA composition recovered from the microalgae species. The MW method shows high recovery of biodiesel suitable FA while SC-CO_2 shows the greatest recovery for nutraceutical suitable FA. The review also shows strong potential to produce viable products from the FA recovered from *Nannochloropsis* sp. Further research is required to address the challenge of scaling up these operations.

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Declaration of competing interest

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