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Lipid extraction from fresh *Nannochloropsis oceanica* using semi-hydrophobic eutectic solvents

Calvin Lo [a], René H. Wijffels [a,b], Michel H.M. Eppink [a,*]

[a] Bioprocess Engineering, AlgaePARC, Wageningen University, PO Box 16, 6700 AA Wageningen, the Netherlands
[b] Nord University, Faculty of Biosciences and Aquaculture, N-8049 Bodø, Norway

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ABSTRACT

Conventional lipid extraction from microalgae involves energy-intensive pretreatments and the use of non-renewable organic solvents. Eutectic solvents (ES), a new class of designer solvents, hold the potential to improve lipid extraction. Hydrophilic ES have been reported to impair the cell wall of microalgae, bypassing the need for pretreatments. However, other hydrophobic solvents were still required as extraction medium. Recently, ES imidazole and hexanoic acid was discovered to exhibit tuneable hydrophobicity, i.e., dissolving both water and lipids depending on their molar composition. In this work, we evaluated the feasibility of imidazole/hexanoic ES as a single solvent for lipid extraction from intact wet and dried microalga *Nannochloropsis oceanica*. Single-factor multilevel design of experiments is used to evaluate the yield under different conditions (ES composition, temperature, time, solvent/biomass ratio, and water content). Interestingly, the extractions from wet algae paste were higher than dried biomass, reaching a comparable yield to the traditional chloroform/methanol method. From wet biomass, >80 % lipids were extracted by the imidazole/hexanoic acid ES (15:85 mol/mol) at 50 °C within 2 h. Whereas, the extraction yield of dry biomass was lower, reaching only 65 % even after 12 h under the same condition. Supplementation of water during the dry extraction resulted in the same yield as the wet extraction. This research demonstrated that ES can be used to replace non-renewable organic solvents without the need of using mechanical disruption and can be applied directly on wet biomass.

1. Introduction

Microalgae have been considered as a promising feedstock of lipids and biodiesel due to their high productivity and their ability to accumulate high content of lipids [1–3]. However, the extraction of lipids from microalgae typically involves complex sequential process steps. Before having the crude lipid extract, the algae paste (concentrated algae culture) needs to undergo mechanical cell disruption, thermal drying, solvent extraction, and finally solvent removal. Several of these steps, such as cell disruption, drying, and solvent evaporation, are energy-intensive [4–6]. Furthermore, flammable and often dangerous fossil-based organic solvents, such as hexane and chloroform, are often used during the extraction step [5,7,8].

Alternative solvent-based technologies have been proposed to improve lipid extraction from microalgae. Supercritical fluid extraction (SFE) and bio-derived solvents, such as terpenes and dimethyl ether, can replace the fossil-based solvents [5,9,10]. However, SFE operations need a complex setup for the high pressure, and the bio-derived solvents are not yet largely available for this kind of operations. Alternatively, ionic liquids have been applied for their capability of weakening and permeabilising the cell wall [11–14]. Nevertheless, ionic liquids are associated with toxicity and high cost due to the complex synthesis and purification [15–19].

Eutectic solvents (ES) are often considered as alternatives for ionic liquids as they exhibit similar benefits [10,20,21]. However, unlike ionic liquids, which are purely salts made of cation and anion, ES are mixtures of compounds. The characteristic property of these mixtures is that the mixture has a lower melting point than its pure constituents [22]. For example, Abbott et al. [23] discovered that choline chloride and urea (melting point: 302 and 132 °C, respectively) could stay liquid at room temperature when mixed in a 1:2 molar ratio. Furthermore, ES can be prepared from various compounds, including amino acids, sugars and polyols, and carboxylic acids [24–27].

Recently, various ES have been applied on microalgae for pretreatment before the lipid extraction. Hydrophilic ES such as choline chloride/carboxylic acids were used to weaken the cell wall of *Chlorella* sp.,

* Corresponding author.
E-mail address: michel.eppink@wur.nl (M.H.M. Eppink).

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resulting in a 1.5-fold lipid yield compared to untreated biomass [28]. Furthermore, a combination of ES and microwave improved the extraction speed and final yield in *Phaeodactylum tricornutum* [29]. Additionally, a one-pot strategy has been developed to obtain biodiesel from *Chlorella* sp. and *Chlorococcum* sp. by performing ES pretreatment, solvent extraction, and transesterification simultaneously at 90 °C [30]. On the other hand, the use of a switchable ES system as extraction solvent on disrupted *Scenedesmus dimorphus* was also reported [31]. The octanoic acid/dodecanoic acid ES is naturally hydrophobic but can become hydrophilic when mixed with a dilute amine solution. The hydrophobicity can be reversed by exposing the mixture to CO_2 or acid. With this approach, the extraction yield of lipid was comparable to Bligh & Dyer method [31].

Here we aim to integrate pretreatment and extraction using a single solvent of semi-hydrophobic ES to further simplify the process. In our recent study, the ES composed of imidazole/hexanoic acid was shown to dissolve both water (hydrophilic) and sunflower oil (hydrophobic), depending on the compositional ratio [32]. Hexanoic acid is a hydrophobic carboxylic acid which can be fermentatively produced and imidazole is a polar aromatic compound that is readily biodegradable [33,34]. The tailorable hydrophobicity is important since the lipids and ES cannot be separated by the solvent evaporation as ES lack of vapor pressure [21]. Thus, in their hydrophobic state, the ES can solubilize the algal lipid and the ES can be separated from the dissolved lipids in their hydrophilic state.

In this study, we performed the direct extraction of lipid from microalgae using the developed ES. *Nannochloropsis oceanica* was used as a model microalga due to its ability to accumulate high amounts of lipid and omega-3 fatty acid [35–37]. Nitrogen limitation was implemented during the cultivation to ensure a high lipid content (25–40 % $g_{FA}\ g_{DW}^{-1}$). Besides that, its small size and strong, multilayered cell wall complicate the conventional cell disruption step [38–40]. Therefore, the avoidance of cell disruption would decrease the energy input for the biomass processing. Furthermore, the effect of solvent hydrophobicity (depending on the imidazole content), temperature, time, solvent loading, and moisture content on the lipid extraction were investigated.

2. Materials & methods

2.1. Microalgae biomass

Two batches of *Nannochloropsis oceanica* (strain provided by Necton, Portugal) were cultivated:

1) Batch 1: Pilot-scale 1500-L tubular photobioreactor (AlgaePARC, Wageningen, The Netherlands); and 2) Batch 2: 10-L stirred tank photobioreactor (Wageningen University, The Netherlands).

The growth medium was made of artificial seawater (NaCl 419.23 mM, Na_2SO_4 22.53 mM, $CaCl_2$ 5.42 mM, K_2SO_4 4.88 mM, and $MgCl_2$ 48.21 mM) enriched with 2 g L^{-1} NutriBloom Plus (Necton, Portugal) with modification ($NaNO_3$ 17.65 mM and KH_2PO_4 0.73 mM). Air with 5 % CO_2 was fed into the reactors as the carbon source and pH regulator. The light source of the pilot reactor was natural light in October – November 2020, while artificial light of 500 $\mu mol_{ph}\ m^{-2}\ s^{-1}$ was continuously supplied to the stirred tank. Both reactors ran for ~2 months to ensure the lipid accumulation due to nitrogen depletion.

The cultures were then harvested by centrifugation (dry weight ~ 30 %) and stored under darkness at 4 °C. The wet paste was stored for up to 10 days before the extraction as a longer storage time could not ensure the biomass freshness. A fraction of the harvested cultures was freeze dried (Zirbus Technology, Germany) for the preparation of dried biomass.

2.2. Materials and ES preparation

The materials used in this study were hexanoic acid (Merck Life Science, ≥99 %), imidazole (Merck Life Science, ≥99 %), and water (Milli-Q®, ultrapure). The eutectic solvent was prepared by dissolving the pre-weighed imidazole flakes in hexanoic acid at room temperature until a clear homogenous solution was obtained. For the conventional lipid extraction and fatty acid analysis, chloroform (BioSolve, min. 99.9 %), methanol (Merck Life Science, ≥99.9 %), and sulfuric acid (Merck Life Science, 95–98 %) were used. Furthermore, two internal standards were used during the Bligh & Dyer lipid extraction, 1) tripentadecanoin (C15:0 TAG, Merck Life Science) and 2) 1,2-didecanoyl-*sn*-glycerol-3-phospho-(1′-*rac*-glycerol) (sodium salt) (C10:0 PG, Avanti Polar Lipids).

2.3. Lipid extraction from microalgae biomass

Fresh or dried microalgal paste was directly subjected to the ES without prior treatment. Unless stated, the default extraction condition was performed for 16 h, with S/B (solvent/biomass ratio) of 10 mL ES/g dry weight at 50 °C under constant agitation (2000 rpm). All experiments were done in duplicate. To study the effect of various parameters on the lipid extraction by ES, single-factor multilevel design was used, which overview is described in Table 1. The factors were imidazole content, temperature, extraction time, S/B ratio, and water content.

2.4. Fatty acid analysis

For quantifying the fatty acid extracted in the ES, c_S^L, the lipid-containing ES samples (50 μL) were methylated and analysed using gas chromatography (GC-FID) system following the method described in our previous work [32]. As for the control, the total FA from biomass (C_L^X) was quantified based on Bligh & Dyer method [41] following the protocol described by Remmers et al. [42]. Chloroform/methanol (4:5 v/v) was used to extract the total lipids from microalgae biomass with C15:0 TAG and C10:0 PG as internal standards for neutral and polar lipids, respectively. Then the extract was fractionated into neutral and polar lipids by means of solid phase extraction using a Sep-Pak silica gel cartridge (Waters, USA). Neutral lipids were eluted using nonpolar hexane/diethyl ether mix (7:1 v/v) and polar lipids using methanol/acetone/hexane (2:2:1 v/v). The lipids were further methylated with acid catalyst and analysed using gas chromatography (GC-FID). All analyses were done in triplicate.

2.5. Extraction efficiency

Extraction efficiency (EE) was calculated using this formula:

$$EE = \frac{f\ V_S\ c_L^S}{m_X\ c_X\ c_L^X} \times 100\%$$

where f is a correction factor for water-ES miscibility (for dry extraction, $f = 1$), c_L^S is the concentration of dissolved FA in the solvent phase [g/mL], V_S is the amount of the solvent added [mL], m_X is the amount of added biomass [g], c_X is the dry weight content of the biomass [g/g] ($c_X = 1$ for dried paste), and c_L^X is the FA content in dried biomass [g/g].

For wet extraction, water from the wet biomass (30 % DW) contributed to the final solvent volume since water is soluble in the used ES [32]. The solubility of water in the ES was found to increase with the imidazole content, which is also reported in Table 2. To account the additional volume by water, a correction factor f was used, so that the final extract volume would be $f\ V_S$. The value of f was influenced by imidazole content and S/B ratio (Table 2). The presence of water can be mathematically described as (7/3 g g_{DW}^{-1})/(S/B value). Thus, at low S/B values, more water was introduced in the system and might reach oversaturation. If the presence of water exceeded its solubility, the excess water formed a 3rd bottom phase and did not participate in the extract phase.

Table 1
Single-factor multilevel design of experiment in this work.

Experiment no.	Variable/factor	Imidazole content [mol%]	Temperature [°C]	Time [h]	S/B ratio [mL/gDW]	Water content [mL_{water}/mL_{ES}]	Biomass
1	Imidazole content	0, 15, 25, 35, and 50	35	16	10	Dry: 0 %; Wet: 22 %	Batch 1
2	Temperature	0 and 15	21, 35, 40, 45, 50, and 75	16	10	Dry: 0 %; Wet: 22 %	Batch 1
3	Time	0 and 15	50	0, 0.5, 1, 2, 4, 8, 12, 16, 24, 32, and 48	10	Dry: 0 %; Wet: 22 %	Batch 2
4	S/B ratio	0 and 15	50	16	5, 10, 20, 35, and 50	Dry: 0 %; Wet: 22 %	Batch 2
5	Water content	0 and 15	50	16	10	Dry: 0, 5.5, 11 & 22 %; Wet: 22 %	Batch 2

Table 2
Solubility of water in ES and the correction factor *f* used in this study.

S/B [mL g_{DW}^{-1}]	5	10	20	35	50	Water solubility [g g_{ES}^{-1}] [32]
Imidazole [mol %]						
0	1.1[a]	1.1[a]	1.1	1.1	1.0	0.1
15	1.3[a]	1.2	1.1	1.1	1.0	0.3
25	–	1.2	–	–	–	0.5
35	–	1.2	–	–	–	>1
50	–	1.2	–	–	–	>1

[a] Oversaturation and phase separation occurred.

3. Results & discussion

An outline of the results and discussion is given below. Initially, the fatty acid content of the cultivated biomass was analysed following Bligh & Dyer method, which served as control. Then, the ES extraction was performed on undisrupted wet and dried biomass (Fig. 1). The effect of process parameters, such as ES composition, temperature, time, and solvent loading, were investigated. Finally, we also discussed the effect of moisture presence on extraction performance.

3.1. Fatty acid profile of the cultivated strain

The cultivated biomass underwent nitrogen depletion to induce lipid accumulation. The fatty acid profile of the biomass is shown in Table 3. Batch 1 biomass contained a higher amount of lipid than Batch 2 biomass due to the different stress condition. Since the biomass from Batch 1 received higher irradiance than Batch 2, nitrogen depletion in Batch 1 cultivation began earlier and went longer than Batch 2. Additionally, high light intensity was known to lead to higher lipid accumulation during the stress period. The most obvious difference involved the content of palmitic acid (C16:0), which differed by 2-fold. Furthermore, it was observed that 86 % and 71 % of the total FA belonged to the neutral lipid fraction for the different batches.

Table 3
Fatty acid (FA) profile of polar (PL) and neutral lipid (NL) fraction of the cultivated biomass when extracted using Bligh & Dyer method.

Fatty acid (FA)	FA content [g_{FA} g_{DW}^{-1}]			
	Batch 1		Batch 2	
	PL fraction	NL fraction	PL fraction	NL fraction
C12:0	0.0 %	0.1 %	0.0 %	0.1 %
C13:0	0.1 %	0.0 %	0.1 %	0.0 %
C14:0	0.4 %	1.4 %	0.7 %	1.1 %
C14:1 cis-9	0.0 %	0.0 %	0.0 %	0.0 %
C15:0	n.d.	n.d.	n.d.	n.d.
C16:0	1.6 %	14.1 %	2.2 %	5.6 %
C16:1	0.9 %	9.0 %	1.7 %	5.7 %
C17:0	0.0 %	0.1 %	0.0 %	0.1 %
C16:3	0.0 %	0.1 %	0.0 %	0.1 %
C18:0	0.0 %	0.3 %	0.0 %	0.2 %
C18:1	0.2 %	6.1 %	0.7 %	4.7 %
C18:2	0.0 %	0.2 %	0.1 %	0.2 %
C18:3	0.1 %	0.0 %	0.0 %	0.1 %
C20:4	0.4 %	0.6 %	0.7 %	0.5 %
C20:5	1.6 %	1.1 %	1.4 %	0.6 %
Total FA	5.2 %	33.1 %	7.7 %	19.1 %

3.2. Effect of ES composition: imidazole content & hydrophobicity

According to the principle of '*like dissolves like*', hydrophobic solvent is required for lipid extraction. In our previous work, we showed that the

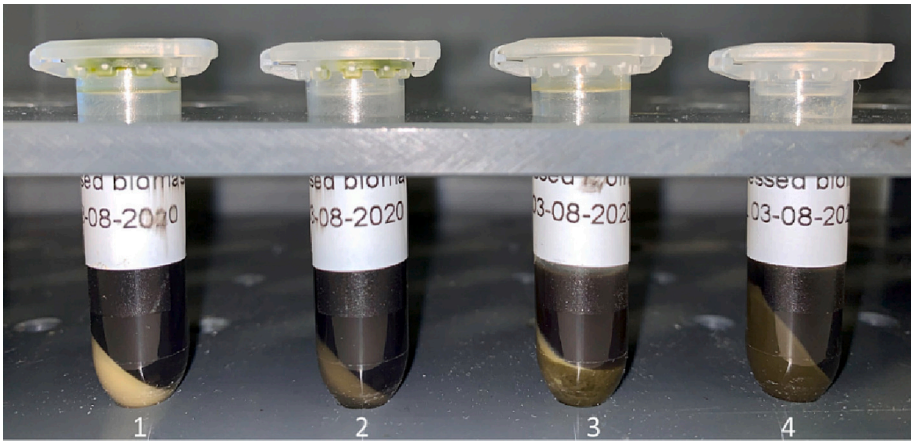

Fig. 1. Direct extraction of lipid from intact microalga *Nannochloropsis oceanica* with different conditions: hexanoic acid on wet (1) and dried biomass (3), and imidazole/hexanoic acid (15:85 mol/mol) on wet (2) and dried biomass (4). The extraction was performed at 50 °C.

hydrophobicity of imidazole/hexanoic acid ES depends on the ES composition [32]. At low imidazole content, the solubility of sunflower oil in ES is particularly high and decreases at higher imidazole content. Hence, it can also be expected that the lipid extraction yield from microalgae would decrease at higher imidazole content.

Fig. 2a shows the extraction efficiency (EE) of Batch 1 biomass at 35 °C with ES with different imidazole content. The temperature was selected to minimise the unspecific release of intracellular components due to heat, but also not too low to avoid the low extraction yield. For wet extraction, the highest EE was achieved with pure hexanoic acid (the lowest imidazole content) and decreased with imidazole content. On the other hand, the dry route reached a maximum at 15 mol% imidazole, indicating a small amount of imidazole was beneficial for the dry extraction. This benefit may be associated with the amphiprotic property of imidazole, which contributed to the overall solvent basicity. The basicity is essential to intercept the intramolecular hydrogen bonding in cellulose, effectively destabilising the cell wall [43]. Furthermore, Medronho and coworkers suggested that the presence of amphiphilic compounds could solvate cellulose, increasing the aqueous solubility and permeability of cellulose [44].

Moreover, wet extraction reached higher yields than dry extraction, except at ≥50 mol% imidazole. At the highest imidazole content, the ES became too hydrophilic and had a stronger affinity towards water than to lipid. From this result, it was concluded to continue with ES with imidazole molar content of 0 % (i.e., hexanoic acid) and 15 %.

Furthermore, imidazole content was found to influence the selectivity of extracted fatty acids based on their saturation degree. Fig. 2b shows the mass distribution of different FA profile in different extracts. Polyunsaturated FA (PUFA) content increased with the imidazole content, reaching up to 2.5- and 1.5-fold for wet and dry extraction, respectively. On the contrary, the saturated and monounsaturated FA (SFA and MUFA, respectively) decreased at higher imidazole content, explaining the reduced EE as SFA & MUFA composed most of the total FA. This higher affinity towards double bonds might be explained by the favourable noncovalent π-π interaction between imidazole's aromatic ring and the unsaturated bonds [45]. This work agrees with our previous finding that the solubility of sunflower oil (rich in C18:2) in this ES was higher than culinary algae oil (mainly C18:1) [32]. This selectivity can be applied to produce lipid fraction with an enriched content of certain FA, such as omega-3 FA (C20:5), which is desired for its nutritional and biological value.

3.3. Effect of temperature

Moreover, the effect of temperature was also investigated. It is generally known that molecules move faster at higher temperatures. This is favourable for the extraction since high temperature enhances both mass transfer and lipid-solvent (hydrophobic) interaction while lessening lipid-biomass interaction (e.g., hydrogen bond and polar interaction). In this experiment, hexanoic acid and ES with 15 % imidazole (S/B = 10 mL/g) were used to extract biomass from Batch 1 at different temperatures for 16 h (Fig. 3a). The ES with lower imidazole content (0 and 15 %) were chosen for their highest extraction yields from wet and dry biomass, respectively. For the wet extraction, the yield increased with higher temperature (21–75 °C) for both solvents, reaching EE ≅ 100 %. For the dry extraction, the presence of imidazole (15 mol%) in the ES gave higher extraction yield than only hexanoic acid, which is similar to the previous result. The effect of temperature in dry extraction was less extensive than in wet extraction. Furthermore, extracted EPA content slightly decreased with increasing temperature (data not shown), indicating lipid degradation.

It is worth noting that despite the higher FA yield, elevated extraction temperature is not optimal for energy consumption and is associated with the risk of product degradation. Degradation of lipid, such as lipid oxidation, is accelerated at higher temperature [46]. Besides that, protein from the biomass may lose its three-dimensional structure and eventually its functionality, which is not desired in multi-product biorefinery [47,48].

3.4. Effect of extraction time (kinetics)

Besides temperature, extraction time is also known to increase the extraction yield until the maximum is achieved positively. With disrupted biomass, the extraction rate is usually high as the solvent can easily access the lipid solute. However, with undisrupted biomass, the rate can be slow as the solvent needs to penetrate the cell matrix before reaching the lipid. The low extraction rate is undesired since it means the overall process takes longer, and a larger solvent/biomass range is required. Therefore, in this study, we also investigated the extraction rate.

The effect of the extraction period on the extraction efficiency is shown in Fig. 3b. The extractions were performed at 50 °C with solvent/biomass ratio of 10 mL/g. The highest extraction rate was achieved in wet extraction of ES with 15 mol% imidazole, followed by dry extraction using the same solvent (the steady state was achieved after approx. 2 and 4 h, respectively). In comparison, extraction using hexanoic acid

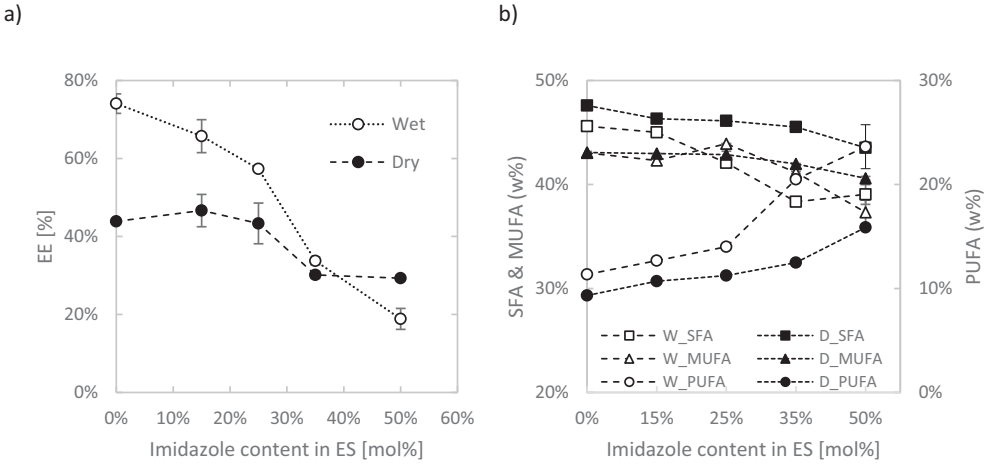

Fig. 2. a) EE from wet (white circle) and dry (black circle) stressed *N. oceanica* Batch 1 using ES with different imidazole content at 35 °C for 16 h (S/B – 10 mL/g); b) Saturated FA (SFA, square), monounsaturated FA (MUFA, triangle) and polyunsaturated FA (PUFA, circle) weight fraction of total FA in different extracts from wet (white) and dry (black) biomass with the same condition as above.

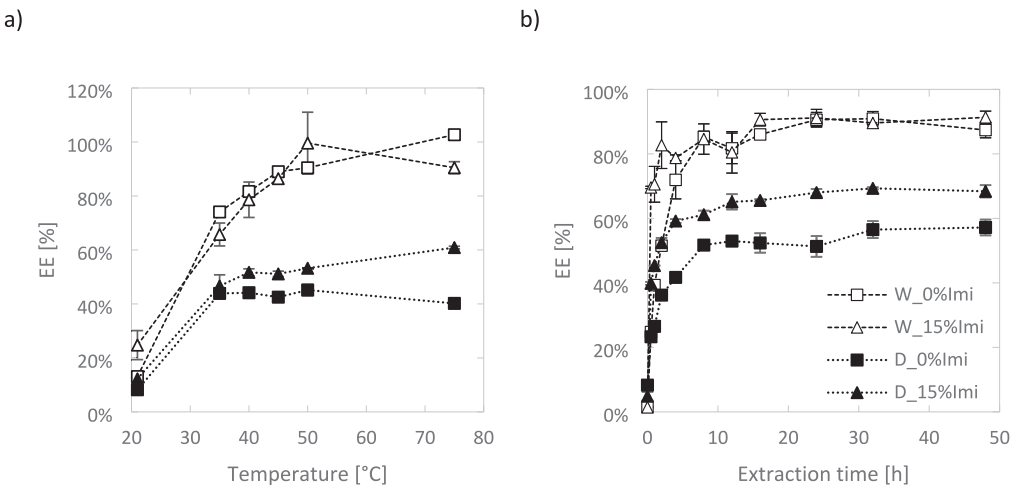

Fig. 3. EE of hexanoic acid (square) and 15 mol% imidazole ES (triangle) from wet (white) and dried (black) biomass under different conditions: a) different temperature (Batch 1, 16 h), and b) different extraction time (Batch 2, 50 °C). The beginning of steady state was approximately at 8 h. All experiments were performed at S/B = 10 mL/g.

reached a steady-state after approx. 8 h for both wet and dry extraction. This result agrees with our hypothesis that the basicity of imidazole enhanced the solvent penetration. Despite the absence of imidazole, hexanoic acid was still able to penetrate the cell wall even at a lower extraction rate. In general, acids have long been used to hydrolyse cellulose and chemically disintegrate the cell wall of microalgae [39]. Furthermore, cellulose tends to be more water-soluble and permeable when ionized at low or high pH [44].

3.5. Effect of solvent to biomass ratio (S/B)

Effect of solvent loading on dry weight basis was investigated on biomass Batch 2 and shown in Fig. 4. At higher solvent loading, the overall system is more diluted and thus creating a larger driving force, indicated by lower FA concentration but higher yield. For wet extraction, hexanoic acid reached maximum efficiency at S/B = 20, whereas for the ES at S/B = 35. Meanwhile, at a lower S/B ratio (5–10), the obtained EE was already >80 %, with extract concentration > 8-fold larger than that of the highest S/B (50). The dry extraction using the ES behaved similarly to the wet extraction. Unexpectedly, for hexanoic acid extraction on dry biomass, the extraction yield did not increase with the S/B ratio.

The opposing trends between the yield and the extract concentration is a classical trade-off in the field of extraction. On the one hand, a high S/B ratio would be suitable for efficient extraction. On the other hand, the lower S/B ratio would be desired for the lower solvent consumption, the smaller extractor size, and the higher extract concentration. The last is particularly important for further downstream processing — product-solvent separation.

3.6. Wet vs dry extraction

Moisture content is a topic of importance in the field of eutectic solvent. This is because the solvent physicochemical properties are highly influenced by the presence of water, even at a low concentration. For instance, the presence of water in ES is known to reduce the solvent viscosity, increases solvent polarity, decreases the mixture melting point, and even disrupts the interaction between ES parental compounds [49,50]. In the current case, water may influence the lipid extraction by not only reducing viscosity on the one hand but also increasing solvent polarity on the other hand.

The effect of water content on lipid extraction was also investigated.

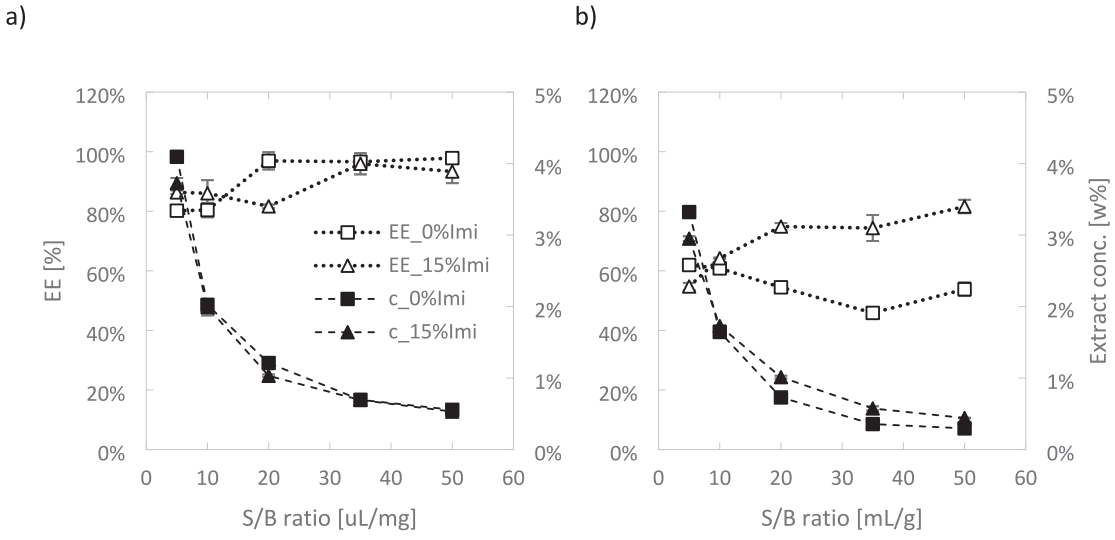

Fig. 4. EE (white, left y-axis) and the FA concentration (black, right y-axis) from wet (a) and dry biomass (b) at different solvent-to-biomass ratio (S/B, v/w). The extraction was performed at 50 °C on biomass Batch 2 using hexanoic acid (square) and ES with 15 mol% imidazole (triangle) for 16 h.

In this experiment, the extraction at 50 °C was performed on dried biomass (S/B = 10 mL/g) with water supplementation for 16 h. It was observed that water addition increased the extraction yield, even reached the same EE as the extraction with wet biomass (Fig. 5). It implied that regardless of its origin (i.e., intracellular or external addition), water positively contributed to the lipid extraction. Furthermore, EE remained relatively constant regardless of the water content. This result showed that even a small amount of water could influence the accessibility of lipid by the solvent.

Throughout the results in this study, the wet extraction consistently outperformed the dry extraction if the imidazole content in the solvent was kept low. This is a peculiar case since moisture is generally antagonistic in lipid extraction. Interestingly, the presence of water improved the final equilibrium at the steady-state instead of the extraction rate in the initial hours (Fig. 3b). It implies that water did not significantly increase the mass transfer, which is associated with the extraction rate. Moreover, the contradicting nature of water and lipid could not explain the ameliorated equilibrium. These rationales might signal that a different mechanism occurred where water could participate and improve the overall yield.

Different from the disrupted biomass, lipids in the intact cell are not readily accessible by the solvent. First, the solvent needs to penetrate the cell wall, which has a bilayer structure of the inner cellulosic wall and the outer hydrophobic algaenan shell [38,51,52]. Furthermore, prior to dissolution, the lipids (especially the polar ones) must be disentangled from the neighbouring biomolecules. The lipids, particularly the polar lipids, are bound to other biomolecules (i.e., proteins and carbohydrates) via electrostatic forces [5,52]. Since water would oppose the lipid dissolution and interaction with algaenan, it is suspected that water played a role in the solvent-cellulose interaction and disruption of lipid-biomolecules bonds.

Acid-base and electrostatic interactions, including hydrogen bonding, highly depend on the solvent's nature [53,54]. The high polarity of water facilitates ion dissociation and solvation, whereas hexanoic acid promotes ion association [55]; the dielectric constants of water and hexanoic acid are 80 and 2.6, respectively [56]. This fact implies that water enhances the deprotonation of hexanoic acid and promotes its acidity. Acidic environment (solvent) destabilises electrostatic forces, including the hydrogen bond within cellulose structure or lipid-protein interactions, which led to higher extraction yield. In the absence of water, the weak acidity of hexanoic acid alone might not be sufficient to weaken the cell wall and liberate the lipid.

Furthermore, it is known that the microalgal cell surface is negatively charged at physiological pH, which preventing the cell cohesion [57]. This surface charge (zeta potential) is a net charge of ionized functional groups of proteins and carbohydrates present on the cell surface [58], which is heavily hampered in the absence of water. The diminishing zeta potential could promote cell aggregation, resulting in the reduced cell/solvent contact and ultimately the minimal accessibility of lipid in the inner aggregate by the solvent. The clumping of freeze-dried biomass was indeed observed after the extraction.

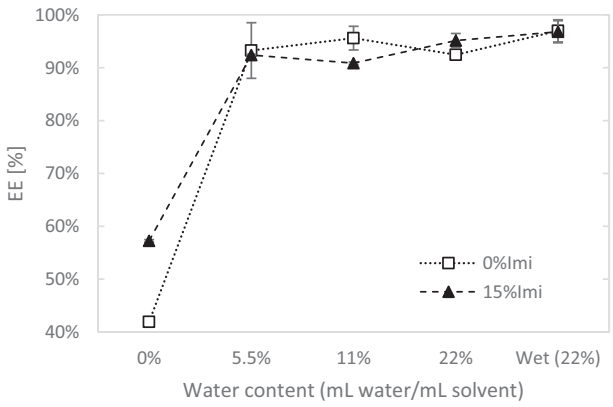

Fig. 5. Effect of water supplementation on lipid extraction from dried biomass (Batch 2, S/B = 10 mL/g) using ES with 0 (white) and 15 mol% (black) imidazole at 50 °C for 16 h. Fresh (22 %) indicates the extraction from wet algae paste.

Additionally, the presence of water may also affect the extraction by inducing lipolysis. In the presence of water, lipolytic enzymes and an acidic environment, the lipids can be hydrolysed and release free fatty acids. The fatty acids are smaller molecules than the glycerides, which can diffuse and dissolve better in the solvent. However, with the analytical method used in this study, it is impossible to detect the hydrolysis. The product quality can be dramatically hampered if the hydrolysis indeed took place. Free FA is generally undesired since it is associated with health risk for consumption and saponification issue for biodiesel production [59–61].

Besides the chemical mechanism, water influenced the cellular structure. For instance, during the freeze-drying, the cellular components underwent physicochemical changes, such as the alteration of protein conformation and the collapse of cytoplasm. The latter ultimately led to cell size reduction and cell wall compression. Freeze-drying has been reported to induce damage to the cell wall [62], improving the extraction from dried biomass. On the contrary, the opposite was observed in this study. Thus, we instead proposed that the cell wall compaction increased the cell wall strength. Günther et al. reported the increase of required energy to mechanically disrupt microalga *Chlorella vulgaris* when the cell was exposed to medium with high osmolality. Medium with high osmolality forced intracellular water to escape from the cytoplasm, causing the decline of turgor pressure and enhanced cell wall flexibility [63]. In addition, nitrogen starved *Nannochloropsis* sp. was found to have higher resistance towards mechanical disruption than the nitrogen replete cultures, indicating stronger cell wall and might further hamper the solvent penetration [64–68].

Finally, to dry or not to dry the biomass has been a long debate among microalgae experts. On the one hand, drying is essential for significant volume reduction, stable product quality, longer shelf-life, higher lipid extraction yield, and lower solvent consumption. On the other hand, dehydration by evaporation is energy-intensive, and simplifying unit operation reduces the capital cost. Furthermore, if the drying is performed at a higher temperature, there is always an increased risk of product degradation. Based on the obtained results, we propose not to dry the biomass before the ES extraction. However, when the starting biomass is already dried, water can be added externally to facilitate high lipid extraction.

4. Conclusion

In this study, we performed lipid extraction from untreated microalgae *Nannochloropsis oceanica* using eutectic solvent (ES) imidazole/hexanoic acid. In addition, the effects of physicochemical parameters (i.e., ES composition, temperature, extraction time, solvent/biomass ratio, and water content) on the extraction yield were investigated. The extraction from wet algae paste resulted in a higher yield than freeze-dried biomass, reaching a very high extraction efficiency to the benchmark Bligh & Dyer method. The imidazole content influenced ES hydrophobicity and thus solvent affinity towards lipid. The temperature, extraction time, and S/B ratio positively influenced the extraction yield. It was also found that the external water could be supplemented during the dry extraction facilitating the same yield as the wet extraction.

CRediT authorship contribution statement

Calvin Lo: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **René H. Wijffels:** Supervision, Funding acquisition, Writing – review & editing. **Michel H.M. Eppink:** Supervision, Project administration, Writing – review &

editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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