Science of the Total Environment 977 (2025) 179373

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Semi-hydrophobic eutectic solvents: Sequential extraction of lipids, proteins & carbohydrates, recycling, scalability of microalga *Nannochloropsis oceanica*

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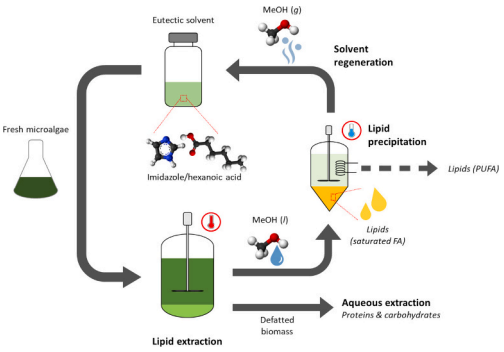
HIGHLIGHTS

- Develop a novel separation concept with semi-hydrophobic eutectic solvents
- Liquid-liquid extraction with hexanoic acid and imidazole as ES system
- Separating saturated fatty acids from mono/poly-unsaturated fatty acids
- Scalability of the biorefinery process
- Reuse of the deep eutectic solvents

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Yifeng Zhang

Keywords:
Eutectic solvents
Lipid extraction
Microalgae
Biorefinery
Semi-hydrophobic
Scalability

ABSTRACT

Semi-hydrophobic eutectic solvents (ES) possess a great potential as lipid extraction solvent from untreated microalgae. However, the low vapor pressure of these solvents and the unknown effects on other biomolecules (e.g., proteins, carbohydrates) limit their application in microalgae biorefinery. In this work, recovery of the extracted lipids was performed by addition of antisolvents and the affecting parameters (i.e., antisolvent type, amount, temperature, ES imidazole content) were studied. The highest recovery was obtained with methanol addition to ES with 15 mol% imidazole at −20 °C, where lipid crystals were formed consisting mainly of saturated fatty acids. The remaining soluble lipids under the same condition were found to be fractions with mono- and poly-unsaturated fatty acids. Furthermore, based on the iterative extractions, the regenerated solvents could create sufficient driving force for lipid extraction despite the lipid accumulation. In addition, a scale-up study of lipid extraction and solvent recycling was performed (2 mL vs 500 mL), whereby the larger scale also showed a good performance. Finally, protein and carbohydrate isolation from the defatted biomass was feasible, but the proposed ES process was not sufficiently mild to maintain native proteins. On the other hand, opportunities are discussed to create new functionalities for proteins and carbohydrates so that a multiproduct biorefinery is feasible for this ES.

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https://doi.org/10.1016/j.scitotenv.2025.179373
Received 20 November 2024; Received in revised form 28 March 2025; Accepted 5 April 2025
Available online 13 April 2025

1. Introduction

Microalgae biorefinery is a set of downstream processing units aiming for the complete valorization of microalgal biomass as these organisms have added value on CO_2 reduction (Frongia et al., 2021). Hence, an ideal biorefinery would translate the various biomolecules into multiple products (Wijffels et al., 2010; Chew et al., 2017). Moreover, the biorefinery typically involves sequential process steps, i.e., harvesting, pre-treatment, extraction, and fractionation (Halim et al., 2012). Besides being complex, these processes are also often energy demanding, i.e., to crack the strong algal cell wall and solvent distillation (Angles et al., 2017a; Günerken et al., 2015). Furthermore, non-renewable, harmful solvents are often used for lipid extraction (Kumar et al., 2017). Therefore, process integrations and improvements are required to simplify and optimize the biorefinery.

Eutectic solvents (including deep eutectic solvents, ES) are a novel class of designer solvents that can be prepared by mixing the constituents with or without heating. The distinctive characteristic of the solvents is that the mixture has a lower melting point than the parental compounds, which allows the solvents to be liquid, although the starting materials are solid (Paiva et al., 2014; Martins et al., 2018). Moreover, their non-flammability, tailorable properties, and cheap preparation attract many researchers from various fields, including microalgae processing. When applied as microalgae pre-treatment, ES have been reported to weaken the cell wall, thus enhancing the extraction of various microalgal components (Lu et al., 2016; Pan et al., 2017). In our previous work, we discovered that the combination of imidazole and hexanoic acid formed an eutectic system with tunable hydrophobicity, depending on the molar composition of the constituents (Lo et al., 2021). Furthermore, we applied this ES on fresh (wet and intact) microalga *Nannochloropsis oceanica* for lipid extraction without cell disruption and drying (Lo et al., 2023). *Nannochloropsis oceanica* is known as omega-3 fatty acid producer with high productivity. Also, *N. oceanica* has a small cell size, making it less susceptible to mechanical cell disruption such as bead milling. Therefore, *N. oceanica* was chosen to be the model species for lipid extraction using ES without cell disruption.

Table 1
The type and levels of the factors investigated through the OD-RSM.

Variable	Type	Low level	Middle level	High level
Imidazole content [mol%]	Categorical	0	–	15
Amount of methanol [mL mL_{ES}^{-1}]	Numerical	0.2	0.5	2
Temperature [°C]	Numerical	−20	3	22

While ES simplify the process, challenges remain in their application for microalgae biorefineries. One of the major challenges is the low vapor pressure of the solvent, which makes solvent-lipid separation and solvent regeneration complex. Previously, we used polar antisolvents, (water, methanol, and ethanol) to induce phase split between the ES and model lipids. Briefly, the polar antisolvents could interact with the ES constituents, making the ES system more polar and thus rejecting the lipid solute. Strongly polar water and methanol caused phase split even at low concentration, whereas less polar ethanol gave low separation yield. Furthermore, the ES was successfully regenerated by evaporating the antisolvents (Lo et al., 2024). However, the model systems were commercial sunflower and culinary algal oil, which were different from the crude lipid extracts in terms of impurities, lipid classes and composition. Additionally, the effect of the ES extraction on the other biomolecules, such as proteins and carbohydrates, from microalgae was not investigated. This issue is essential for the functionality of the multi-products generated by the process. Therefore, this study aims to evaluate the feasibility of microalgae biorefinery based on the semi-hydrophobic ES.

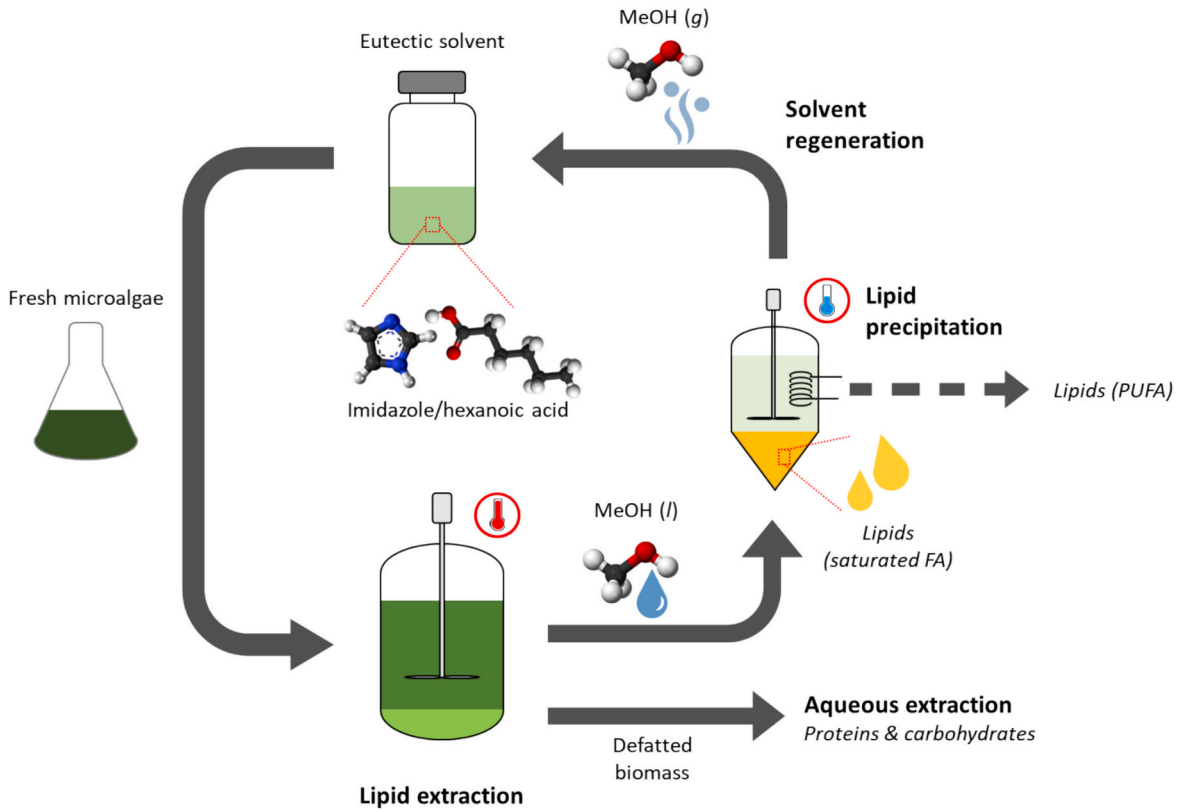

Fig. 1. Operational scheme of the sequential extraction of lipids, proteins and carbohydrates of *Nannochloropsis oceanica* based on the imidazole/hexanoic acid eutectic solvent.

Fig. 2. Precipitation of lipids at the bottom from the ES extract after addition of methanol at −20 °C.

In this study, the recovery of lipids extracted from *N. oceanica* was demonstrated. We investigated several factors affecting the recovery, i.e., antisolvent type, amount, ES polarity (imidazole content) and temperature. The latter was also included since it often influences the solubility of compounds. Also, refrigeration (or winterization) is commonly used in the oleochemical industry to fractionate lipids based on their solubility and melting point (Zaliha et al., 2004). This strategy was also implemented to enhance the separation of lipids from the ES-methanol mixture. Besides that, the recyclability of the solvent and the scalability of the extraction and recovery were discussed. Moreover, the remaining proteins and carbohydrates were isolated from the defatted biomass.

2. Materials and methods

In Fig. 1 a scheme is presented of the process developed with the Eutectic Solvent system which will be further explained in the different sections of materials and methods below.

2.1. Microalgae cultivation

Microalga *Nannochloropsis oceanica* (culture kindly provided by Necton, Portugal) was cultivated in a 12-L stirred tank photobioreactor under constant PAR (photosynthetically active radiation) illumination of 500 $mol_{ph}\ m^{-2}\ s^{-1}$. The growth medium (10*L*) consisted 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), supplemented with 2 g L^{-1} NutriBloom Plus (Necton, Portugal) with modification ($NaNO_3$ 17.65 mM and KH_2PO_4 0.73 mM). The cultivation was performed at room temperature (25 °C) under nitrogen limitation to induce lipid accumulation. Air with 5 % CO_2 was continuously supplied at aeration rate of 0.1 vvm as a carbon source and pH regulator, with 300 rpm agitation. After 7 weeks of cultivation, the culture was centrifuged to obtain biomass paste (32.4 % $g_{DW}\ mL^{-1}$) and stored under refrigeration for no longer than 2 weeks. At the end of cultivation, the fatty acid content of the stressed biomass reached 32.4 % $g_{FA}\ g_{DW}^{-1}$.

2.2. Chemicals & preparation of eutectic solvents

The materials used in this study were hexanoic acid (Sigma-Aldrich, ≥ 99 %), imidazole (Sigma-Aldrich, ≥ 99 %), methanol (Merck Millipore, pure) and water (Milli-Q®, ultrapure). The eutectic solvent imidazole/hexanoic acid (15:85 mol/mol) was prepared by dissolving the pre-weighed imidazole flakes in hexanoic acid at room temperature until a clear homogeneous solution was obtained.

2.3. Lipid extraction, recovery, and solvent regeneration

The lipid extraction from the microalgae was performed following the method described in our previous work (Lo et al., 2024). Algae paste was incubated with hexanoic acid or ES (10 mL g_{DW}^{-1}) for 16 h at 50 °C under constant agitation of 1500 rpm. The extracts were then obtained as the supernatant of the mixtures. The extraction efficiency (EE) was calculated with the equation:

$$EE = \frac{f\ V_S\ \left(c_E - c_L^*\right)}{m_X\ c_X}$$

where f is the factor for water-solvent miscibility (= 1.1 & 1.2, for pure hexanoic acid and 15 mol% imidazole solution), V_S is the amount of solvent or previously regenerated solvent [mL], c_E is the fatty acid concentration in the extract [$g_{FA}\ mL^{-1}$], c_L^* represents the fatty acid concentration in the previously regenerated solvent [$g_{FA}\ mL^{-1}$] (= 0 for the first extraction), m_X is the dry weight of biomass [g_{DW}], and c_X is the fatty acid content in the dried biomass [$g_{FA}\ g_{DW}^{-1}$].

The extracted lipids were recovered by adding a proper amount of water (0.01–0.3 mL mL_{ES}^{-1}) or methanol (0.2–2 mL mL_{ES}^{-1}) to the extract, which induced lipid precipitation. The precipitation was further enhanced by lowering the temperature up to −20 °C. The precipitates were separated from the methanol-rich solvent by centrifugation at 4000 rcf (Allegra X-30 Benchtop Centrifuge (Beckman Coulter)) at specific temperatures up to −20 °C. Furthermore, the liquid phase was vacuum evaporated for 5 h at 8 mbar at 40 °C using a rotary vacuum concentrator (RVC 2–25 CDplus (Christ)), to remove the methanol and thus regenerate the solvent (Chromatograms are added in Supplementary Material (Figure S1)).

The recovered lipids were obtained in the form of solid precipitate. Centrifugation was able to separate the solid phase, but the liquid solvent was still present in the pellet, making the direct quantification complicated. Therefore, the efficiency of recovery (η) was calculated based on the remaining lipid dissolved in the solvent, following equation:

$$\eta = \frac{V_E\ c_E - (V_E + V_M)\ c_L}{V_E\ c_E}$$

where V_E is the amount of extract [mL], c_E is the fatty acid concentration in the extract [g/mL], V_M is the amount of methanol added [mL], and c_L is the concentration of soluble fatty acids in the methanol-rich phase [g/mL].

2.4. Design of experiment

An optimal (custom) quadratic response surface design (OD-RSM) was developed and implemented to predict the region of maximum response for the investigated variables (i.e., imidazole content, antisolvent amount and temperature) under different process constraints (Table 1). The custom fractional factorial design allows the investigation of both numeric and categoric influencing factors under different levels. Moreover, the generated quadratic model provides sufficient resolution

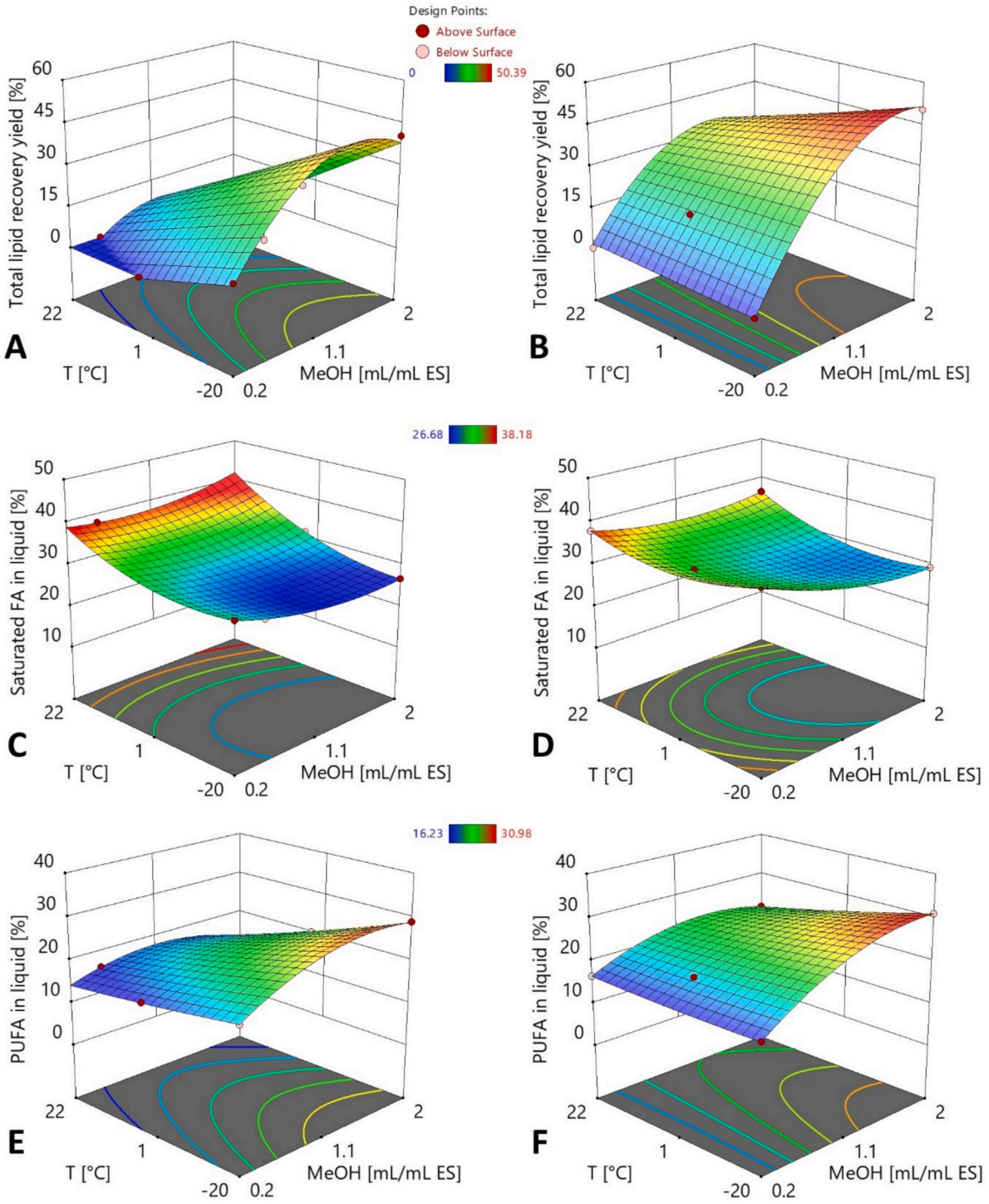

Fig. 3. Results of the OD-RSM lipid extraction experimental design. (A) and (B): Recovery efficiency η of total lipids [%]; (C) and (D): soluble saturated fatty acids (SFA); and (E) and (F): soluble polyunsaturated fatty acids (PUFA); Left panels indicate extraction tests performed using hexanoic acid: (A), (C) and (E), as well as imidazole/hexanoic acid (15:85 mol/mol): (B), (D) and (F). The dots represent some of the measurement data: pink (below the surface model) and red (above the surface model).

to investigate the impact of each of these factors, their two-factor interactions as well as make predictions regarding the regions of maximum response. This is achieved while simultaneously keeping the number of experiments required to a minimum. The experimental design approaches were developed and analyzed using the Design-Expert 13 software suite (StatEase Inc., USA). In this study, the ranges used for the imidazole molar content and the antisolvent amount used was based on the results of our previous studies (Lo et al., 2024) as a starting point for optimal extraction and lipid recovery. The temperature points were selected based on what is feasible using existing scalable equipment.

2.5. Scale-up experiment

In the scale-up experiment (not for cultivation), batch extraction and recovery were performed in a 1-L stirred jacketed glass vessel. Biomass

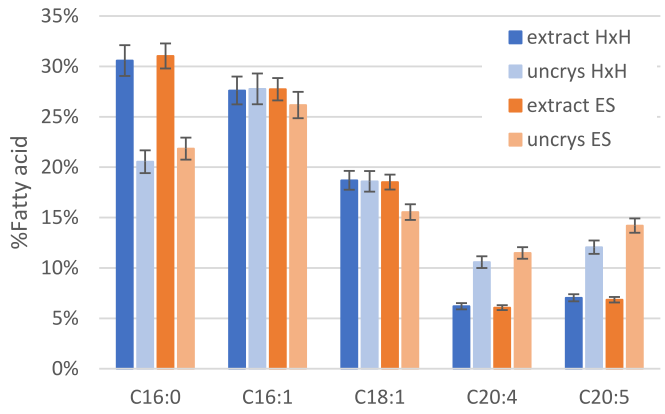

Fig. 4. Relative profile of soluble fatty acids in hexanoic acid HxH (blue) and ES (orange) before (extract) and after (uncrys) the lipid recovery at −20 °C.

Table 2
Composition of fatty acids (FA) in neutral- (NL) and polar lipid (PL) fractions from biomass.

	FA content [g_{FA} / 100 g_{DW}]		
Fatty acids	NL fraction	PL fraction	Sum
C12:0	0.1	0.0	0.1
C13:0	0.0	0.1	0.1
C14:0	1.2	0.6	1.8
C14:1 cis-9	0.0	0.0	0.0
C15:0	–	–	–
C16:0	7.7	2.4	10.1
C16:1	7.2	1.9	9.1
C16:2	0.0	0.0	0.1
C17:0	0.1	0.0	0.1
C16:3	0.1	0.0	0.1
C18:0	0.2	0.0	0.2
C18:1	5.1	0.8	5.9
C18:2	0.3	0.1	0.4
C18:3	0.1	0.0	0.1
C20:4-n3	0.9	0.9	1.9
C20:5-n3	0.8	1.5	2.3
Total FA	23.8	8.7	32.4

was obtained in a similar way as the 5-mL scale. For this experiment, the lipid extraction and recovery was performed using the imidazole/hexanoic acid (15:85 mol/mol) ES. In the scale-up extraction step (300 mL working volume), the temperature (50 °C), solvent ratio (10 mL/g dry weight), and extraction time (16 h) was kept constant, whereas lower agitation was applied due to technical compatibility (150 rpm at 1-L scale and 1500 rpm at the 5-mL scale).

For lipid recovery step, the conditions predicted as optimal after fitting the developed second order polynomial model with the obtained data, were used for the scale-up tests (as detailed in section 3.3 of the manuscript). These are: 2 mL of MeOH per mL extract, extraction temperature of −20 °C and an imidazole/hexanoic acid ratio of 15:85 (mol/mol). These conditions were predicted to maximize both the extraction yield of total lipids, as well as the polyunsaturated fatty acids. Prior to the lipid recovery step, the liquid extract was separated from the defatted biomass by centrifugation (4000 rcf, 15 mins). Methanol (500 mL) was added to the glass container filled with 250 mL ES extract, and the temperature was set at −20 °C. A minimum agitation (~10 rpm) was applied to ensure temperature homogeneity without generating too much shear which could damage the lipid crystals. About 10 mL of suspension was sampled out of the vessel after 16 h of stabilized temperature and analyzed.

2.6. Analysis of fatty acids and solvent components

The lipids (fatty acids) were quantified with gas chromatography coupled with a flame ionization detector (GC-FID). The lipid analysis prepared from microalgal biomass was performed based on the method reported by Remmers et al. (Remmers et al., 2017). Briefly, the total lipids were extracted using chloroform/methanol (4:5 *v*/v) containing C15:0 TAG and C10:0 PG as internal standards. Then the separation of polar and neutral lipids was performed on the lipid extract by solid phase extraction using a Sep-Pak silica gel cartridge (Waters, USA). The neutral lipids were eluted using hexane/diethyl ether mix and the polar lipids were eluted with methanol/acetone/hexane mix. The separated lipids were then methylated in excess of methanol and acid catalyst (H_2SO_4 5 %). Then, the fatty acid methyl esters (FAME) were extracted in hexane before injection to the GC-FID. The lipid analysis of the hexanoic acid and ES extracts was performed based on method described in our previous work (Lo et al., 2023). The extracts were directly methylated with acid catalyst and transferred to hexane phase containing C15:0 FAME before injected to GC-FID.

The solvent components (i.e., methanol, hexanoic acid, and imidazole) were also analyzed using GC-FID system with the previously developed protocol (Lo et al., 2024). The solvent phases were diluted 10× in (ethanol-stabilized) chloroform prior to the injection into a DB-FFAP column. Additionally, the water content present in the samples were determined with Karl-Fischer titration (831 KF Coulometer, Metrohm).

2.7. Isolation and analysis of carbohydrates & protein

After centrifugation of the extraction mixtures, the defatted biomass was obtained as the heavy phase of each mixture. These suspensions were thoroughly mixed before resuspended in the aqueous solution of NaOH 1 M to reach pH 4, 7, and 13. Afterwards, the mixtures were diluted with water to reach 5 % biomass (*w/v*). For the disruption of biomass, these mixtures were subjected to bead-beating (Bertin Instruments, France) at 3 × 6000 rpm. For protein determination, a *DC* protein assay kit (Bio-Rad, US) based on the Lowry method (Lowry et al., 1951) was used with bovine serum albumin as standard. The carbohydrate content was measured with the phenol-sulfuric acid method (Dubois et al., 1956) using glucose as standard.

3. Results and discussion

3.1. Lipid recovery by methanol-induced precipitation

Separation of the extracted lipid from the solvent is necessary for recovery of the lipids, based on the previous study (Lo et al., 2023), the lipid extraction was only suitable with ES with low imidazole content. Therefore, in this study, lipid was extracted using pure hexanoic acid (0 % imidazole) and imidazole/hexanoic acid (15:85 mol/mol) ES, which gave the highest extraction yields in the previous study (Lo et al., 2023). Moreover, the temperature is known to influence the lipid solubility and induce phase transition of lipids (Zaliha et al., 2004). Therefore, we designed and performed an experiment to investigate the effect of the mentioned factors.

Despite showing the most potent antisolvent activity in our previous study (Lo et al., 2024), the addition of water, unfortunately, did not induce lipid recovery (data not shown). The hexanoic acid extract was already saturated with water during the extraction step (water content = 10 % *w*/w); thus, the extra addition of water formed another liquid phase. For the imidazole/hexanoic acid (15:85 mol/mol) extract, the water content was ~20 % w/w, while the saturation point was previously found to be ~30 % w/w (Lo et al., 2021). Thus, a small amount of water could still be dissolved in the extract. However, the formation of the lipid-rich phase was not observed. On the other hand, the addition of the larger amount of water (i.e., beyond the saturation point) caused a

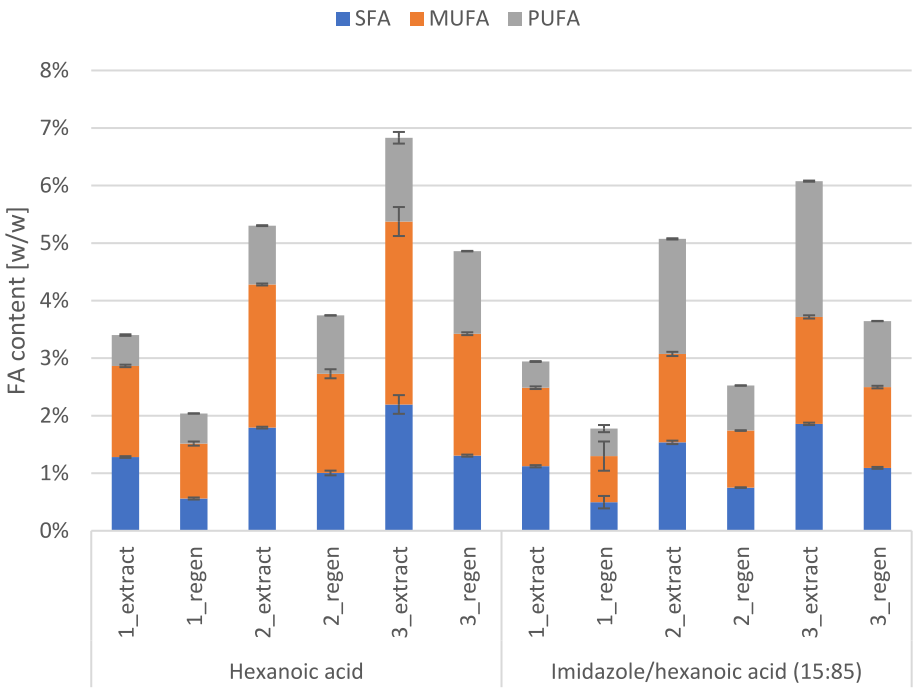

Fig. 5. The fatty acid content in the solvent extracts and the regenerated solvents throughout the different cycles. SFA = Saturated Fatty Acid; MUFA = Mono Unsaturated Fatty Acid; PUFA = Poly Unsaturated Fatty Acid.

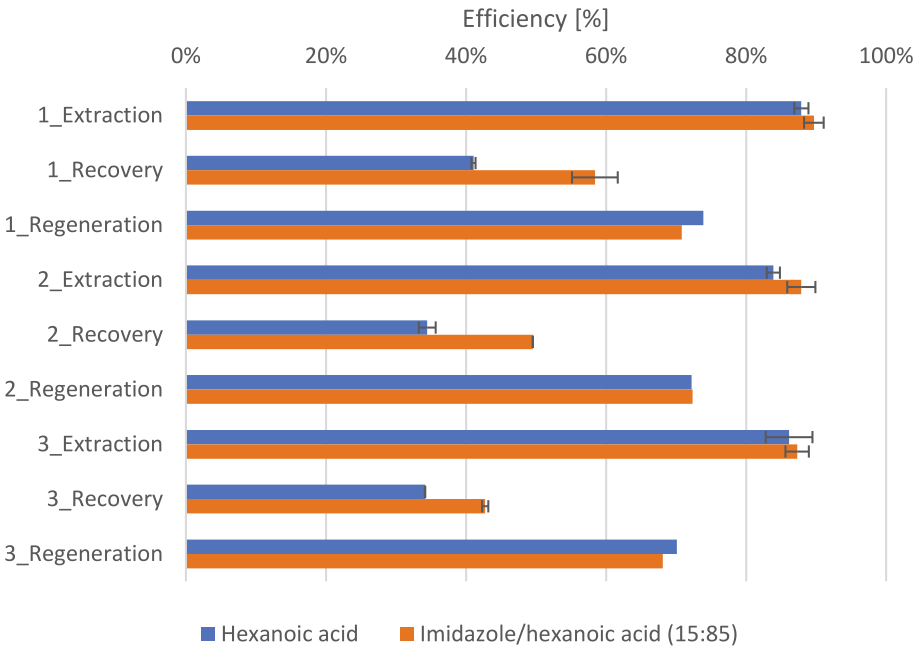

Fig. 6. The efficiency of lipid extraction, recovery, and solvent regeneration in three cycles.

phase split between the hydrophobic organic layer and the aqueous phase. The lipids remained in the organic layer, whereas imidazole leached to the aqueous phase, reducing the ES polarity and enriching the dissolved lipids in the organic layer. Moreover, the unsuccessful lipid recovery occurred regardless of the temperature, indicating that the lipids could not be recovered by water addition.

The contrast findings of the antisolvent activity of water in the different studies can be explained by the different imidazole content. The presence of imidazole was known to dramatically increase the solvent affinity towards water (Lo et al., 2021). In the previous work, the model lipids were recovered from ES with 25 mol% imidazole (Herrera and Hartel, 2000), whereas in this study, the imidazole content was ≤15 mol%. Therefore, it could be concluded that hexanoic acid and the ES used in this study were too hydrophobic to interact with water.

On the other hand, methanol is fully miscible with both hexanoic acid and ES regardless of the imidazole content, eliminating the risk of methanol forming another phase. The addition of methanol was found to induce lipid precipitation, especially at lower temperatures. The precipitates might contain not only glycerides but also sterols and other unsaponifiables as those fractions have been found in *Nannochloropsis* sp. and *N. oceanica* (Yao et al., *2015*; Lu et al., *2014*). Furthermore, from the visual observation, the precipitate seemed to be less colored,

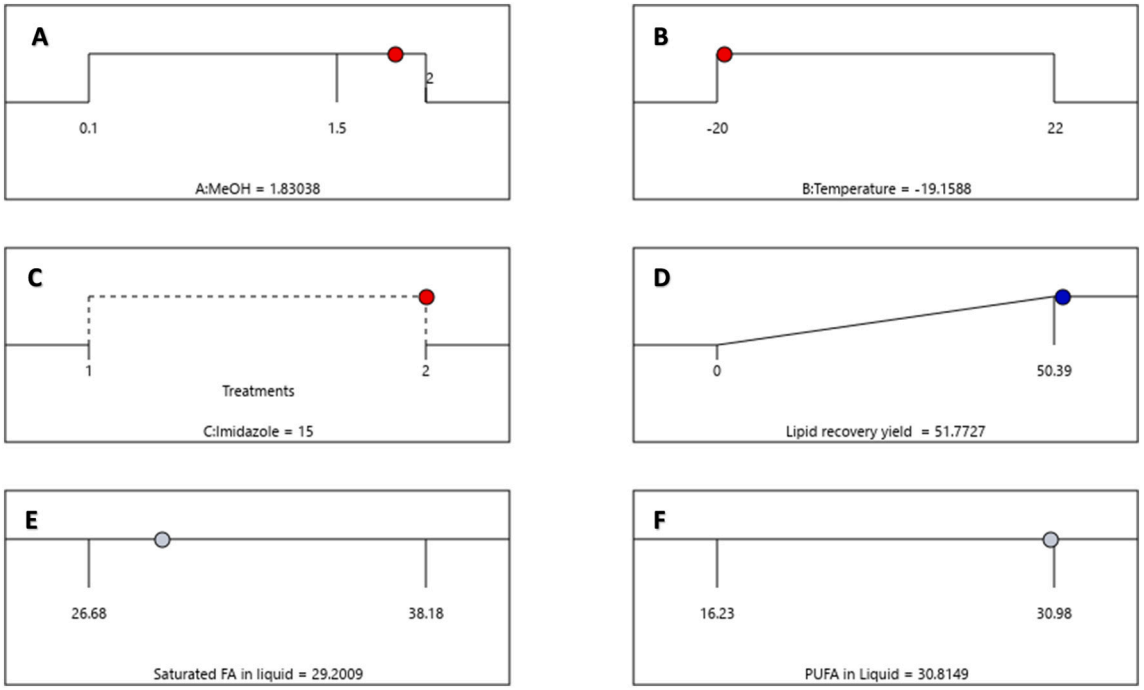

Fig. 7. Optimization scenario in which total lipid recovery is maximized while the other variables and responses are kept in range. Using 1.8 mL mL_{ES}^{-1} of MeOH (A), an extraction temperature of −19 °C (B) and 15 mol% imidazole (C), predicts the recovery of 51.7 % total lipids (D), and FA distribution in the liquid: 29.2 % SFA (E) and 30.8 % PUFA (F). Red dots represent predicted values of investigated variables, blue dots predicted values of targeted responses, and gray dots, predicted values of responses untargeted by the optimization.

Table 3
Scale-up of eutectic solvent extraction and recovery under optimized conditions.

Parameters	Smaller scale	Larger scale
Extraction		
Solvent volume [mL]	1.5	300
Biomass (dry weight) [g]	0.15	31.55
Temperature [°C]	50	50
Agitation [rpm]	1500	150
Extraction period [h]	16	16
FA concentration in extract [w%]	2.70	2.64
Saturated FA [%FA]	38	41
PUFA [%FA]	16	9
Extraction efficiency	1	0.92
Recovery		
Extract volume [mL]	1	250
Methanol volume [mL]	2	500
Temperature [°C]	-20	-20
Agitation [rpm]	–	10 (low)
Recovery duration [h]	16	16
Concentration of remaining FA [w%]	0.48	0.55
Saturated FA [%FA]	29	28
PUFA [%FA]	29	28
Recovery efficiency	0.58	0.46

indicating the lower content of chlorophyll (Fig. 2). The recovery efficiency was found to be dependent on the methanol amount and temperature (Fig. 3a). The highest recovery (40 % and 50 % for hexanoic acid and ES extracts, respectively) was achieved at the lowest temperature with the highest amount of methanol (i.e., −20 °C and 2 mL/mL extract). The precipitation yield was somewhat unsatisfactory compared to our previous work using model lipids which yielded >90 %. It is worth noting that the initial lipid concentration in this study was 10-fold lower compared with the previous study (Lo et al., 2024). Less lipid content in the extract would imply a lower lipid recovery since the oversaturation, the driving force for phase separation, would also be lower. More methanol might improve the lipid recovery (i.e., further reducing the lipid solubility) but would also require more energy to evaporate and an extra cost of the process.

The effect of temperature was more significant for the hexanoic acid extract than for the ES extract. Typically, lower temperature benefits the recovery by reducing the lipid solubility in the solvents and by allowing lipid crystallization (Fig. 2). Furthermore, the strength of hydrogen bonds increases at lower temperatures (Ross et al., 1996). It is then hypothesized that hexanoic acid could establish stronger hydrogen bonds with methanol at low temperatures, weakening the hydrophobic interaction between lipids and hexanoic acid. For the ES extract, however, the presence of polar imidazole already decreased the hydrophobic interaction. Therefore, higher recovery of lipids might be achieved at even lower temperatures or with higher imidazole content in the ES. The first hypothesis is rather impractical for large-scale production, while the latter hypothesis gave a trade-off with lower lipid extraction efficiency (Lo et al., 2023).

A clear effect of temperature and methanol was observed on the recovery of SFA and PUFA. The analysis of variance (ANOVA) tests suggest the obtained model is statistically significant for all of the investigated responses, while the lack of fit is not significant (Supplementary Material Table S1 and Table S2). Overall, increasing the methanol concentration has a clear effect on the total lipid recovery (Fig. 3, Panels A and B). The soluble saturated fatty acids increases with temperature but at decreasing methanol content (Fig. 3, Panels C and D), whereas for the polyunsaturated fatty acids the opposite was observed (Fig. 3, Panels E and F). Due to the difference in the degree of saturation and thus melting point, lipids rich in saturated fatty acids (SFA) solidified easier than lipids rich in unsaturated fatty acids. Thus, lower temperatures could provide selectivity of lipid recovery. At low temperatures, the concentration of dissolved SFA decreased, whereas the concentration of PUFA (polyunsaturated fatty acids) increased as compensation for crystallized SFA-rich lipid.

Besides temperature, imidazole concentration was also expected to influence the selectivity of unsaturated fatty acids. It was hypothesized that imidazole could establish π-π interactions with unsaturated bonds

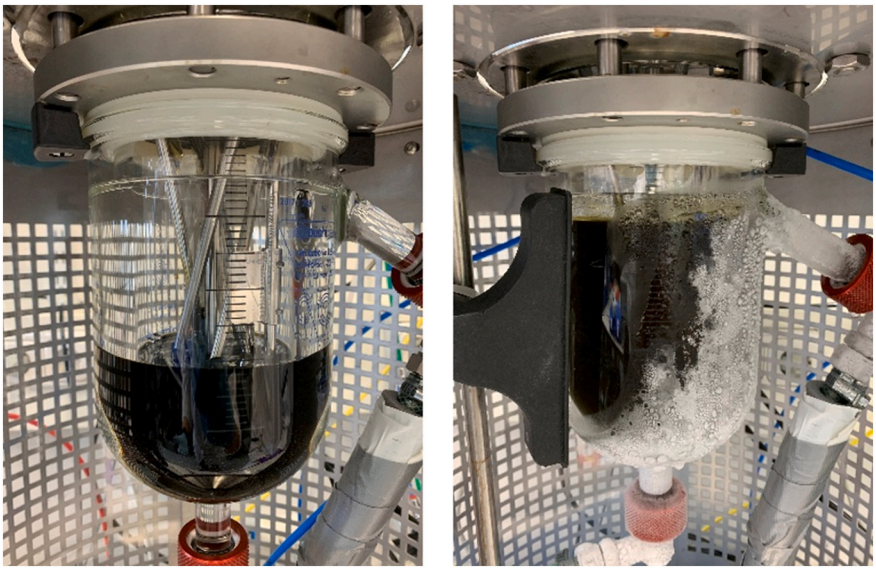

Fig. 8. Scaled-Up extraction at 50 °C using imidazole/hexanoic acid ES (left), and during lipid recovery at −20 °C with methanol addition.

Table 4
Biochemical compositions of the obtained fractions.

	FA	Protein	Carbohydrate
Dried intact *N. oceanica* [mg g_{DW}^{-1}]	324.2 ± 3.6	248.6 ± 13.7	116.4 ± 30.9
Hexanoic acid extract [mg mL^{-1}]	32.4 ± 0.6	8.4 ± 1.2	n.a.[1]
ES extract [mg mL^{-1}]	27.7 ± 0.5	12.2 ± 2.1	n.a.[1]
C6[2] acid-defatted biomass [mg $g_{suspension}^{-1}$]	n.a.[1]	68.7 ± 2.9	46.8 ± 13.7
ES-defatted biomass [mg $g_{suspension}^{-1}$]	n.a.[1]	84.8 ± 4.2	57.5 ± 10.1

[1] n.a. = not analyzed.
[2] hexanoic acid.

(Lo et al., 2021). However, the fatty acid profile of the hexanoic acid extract and ES extract was the same regardless the imidazole concentration (Fig. 4). Furthermore, only a slight difference in PUFA content was observed in the uncrystallized extracts after the recovery step.

A deeper look at the fatty acid distribution and lipid classes gave a further hint on the selectivity. The majority of PUFA in the biomass was detected in the polar lipid (PL) fraction, whereas >75 % of SFA and MUFA (monounsaturated fatty acids) were present in the neutral lipid (NL) fraction (Table 2). Polar solvents such as methanol are known to dissolve polar lipids. Thus, it is likely that polar lipids were not solidified even at low temperatures. In this study, the fatty acid in the PL fraction was ~25 %, which was much higher than in the model lipids (> 10 %, Lo et al., 2024 (Lo et al., 2023)). The high PL content may lead to the formation of micellar structures, which could facilitate the dispersion of other lipid fractions in the methanol-rich phases, making it more difficult to recover the lipids.

Besides the recovery conditions, several factors during the extraction influenced the lipid-solvent separation, such as the lipid concentration in the extract. In the typical precipitation or crystallization method, the solute concentration must be higher than the saturation or solubility limit to cause the phase separation. Similarly, the higher initial lipid concentration would cause the larger difference from the saturation, resulting in a higher recovery.

3.2. Iterative extraction

Solvent recyclability is an important issue since the process would be neither economically feasible nor sustainable if the solvent is not reusable. In our previous study, we successfully recycled the ES after the dissolution and recovery of model lipids. The ES regeneration was performed by vacuum evaporating the antisolvents (Lo et al., 2024). However, as observed above, the lipid profile of the model lipids differs from the extracted lipids from *N. oceanica*. Furthermore, the lipid dissolution did not reflect the actual extraction from *N. oceanica*, which also includes solvent penetration and lipids partitioning. Thus, in this study, we performed three repeated extraction cycles. The extraction step was with 10 solvent-to-biomass ratio at 50 °C, the recovery step was by addition of methanol 2 mL/mL extract, and the regeneration was performed by vacuum evaporating methanol. The cycle was then repeated by applying the regenerated solvents onto the fresh biomass.

Throughout the cycles, the lipids accumulated due to the incomplete lipid removal during recovery (Fig. 5). In accordance with the above

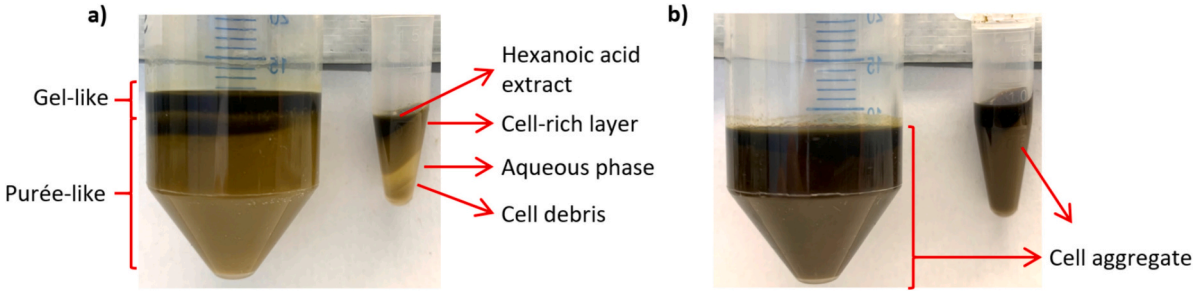

Fig. 9. The suspension of residual biomass which were defatted by hexanoic acid (a) and imidazole/hexanoic acid ES (b). The suspensions were centrifuged at 4000 rcf (in larger tubes) and at 20000 rcf (in smaller tubes).

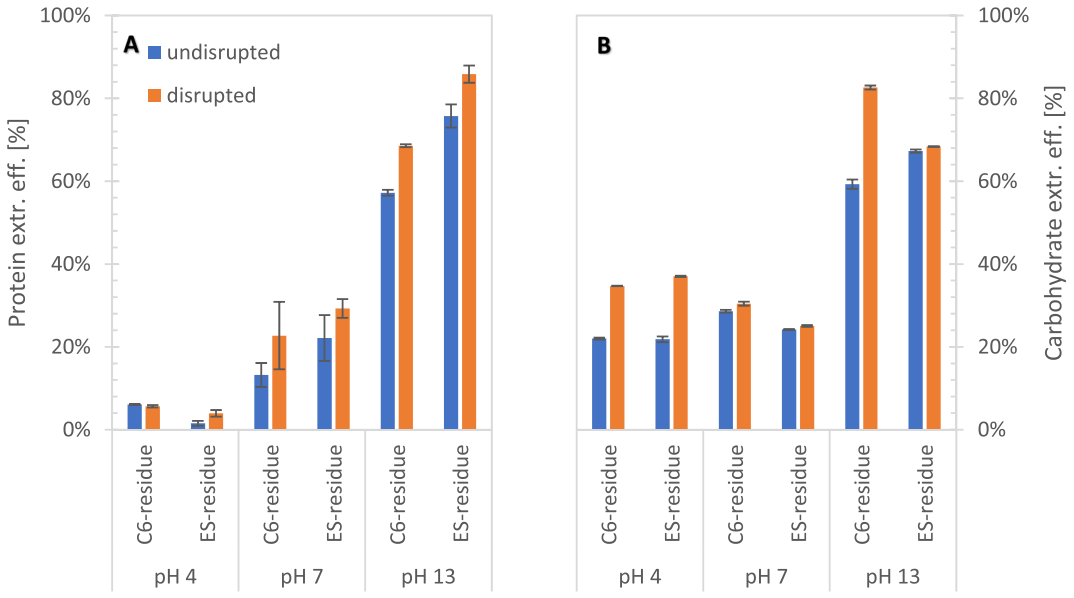

Fig. 10. The extraction efficiency of protein (A) and carbohydrate (B) from the defatted biomass.

finding, the fatty acid profile also changed throughout the cycles with different solvents. PUFA content increased more significantly in the ES than in hexanoic acid. Interestingly, the increase was not only observed during the recovery (between the extract and the regenerated solvent), but also during the second and third extraction. Again, this could be associated with the ES affinity for unsaturated FA. The unrecovered lipids might induce a positive feedback loop for the extraction of PUFA. By the third iteration, the soluble PUFA content reached 2-fold of the initial extract.

Despite the accumulation of fatty acid, the extraction efficiencies remained relatively constant, about 86 % and 88 % for hexanoic acid and 15 mol%-imidazole ES, respectively (Fig. 6). The lipid accumulation in hexanoic acid and ES phase should reduce the solvents' carrying capacity and would eventually saturate the solvents. However, the negligible reduction of lipid yield after three cycles indicated that the carrying capacity of hexanoic acid and the ES was still far from saturated. This is plausible since the solubility of sunflower oil and culinary algae oil (Lo et al., 2021) were $\geq$ 10-fold larger than the lipid concentration in the algae extract. However, it is expected that the extraction efficiency would eventually diminish due to the accumulated lipids, although the maximum cycle number is still unknown.

Nevertheless, since the yield was not much affected, one could propose to reuse the solvent for several extractions before proceeding to the lipid crystallization and methanol evaporation step. This strategy would obtain a higher lipid concentration and reduce the energy consumption per amount of lipid produced. However, it also leads to the accumulation of water due to the moisture from the wet biomass. The high content of water would limit the solubility of lipid, hence decreasing the extraction yield (Lo et al., 2024). In this work, water did not accumulate as 97 % of water was removed during the vacuum evaporation of methanol.

On the other hand, despite the higher lipid concentration, recovery efficiency already started to decline during the three cycles (Fig. 6). Typically for crystallization or precipitation, a higher concentration (thus farther from the saturation point) would cause higher recovery. However, this was not observed as the unrecovered lipid are most likely to be PL. This might be due to the higher solubility of PL in the methanol-rich phase, and the concentration after three iterations were not above the solubility limit. The mass distribution of the phases during three consecutive cycles is available in Supplementary Table S4.

In addition, about 70 % of the solvents were regenerated within a single iteration (Fig. 6). While a negligible amount of the solvents was intended for analytics, the remaining losses mainly occurred during solid-liquid separation. For instance, fractions of solvents were entrapped within the defatted biomass and filled the porous space among the lipid solids. For the latter, instead of centrifugation, vacuum filtration could be used to salvage more liquid solvent. As for the one within the cell matrix, an additional extraction using another solvent, such as alcohols, may be necessary to retrieve the ES or hexanoic acid back.

3.3. Scale-up experiment

Scalability is an important issue for process design. Therefore, we performed a scale-up experiment of lipid extraction and recovery. The solvent regeneration part was not scaled-up since methanol evaporation is a widely implemented technology, i.e., via distillation. The data generated through the implementation of the lab-scale solvent extraction OD-RSM was used to fit a quadratic model (Supplementary Table S1). The ANOVA test showed the model to be statistically significant (below a *p*-value of 0.05) while the lack of fit statistically not significant (above a p-value of 0.05), for all the investigated responses (total lipids, saturated as well as polyunsaturated fatty acids) (Supplementary Table S2). This allows one to navigate the design space not only to determine the direct impact and interactions among the investigated variables on the selected responses, but also to predict the regions of minimum and maximum response. Thus, multiple optimization scenarios have been predicted, with the most interesting one being used for scale-up and confirmation experiments.

The initial optimization scenario focused on maximizing the extraction yield of total lipids, while keeping the investigated variables in range (Fig. 7). This resulted in predicted total lipids extraction yields slightly above the highest measured value of 50 %. These same conditions also predict PUFA extraction yields very close to the maximum obtained ones during the implementation of the lab-scale OD-RSM. Thus, a second and third optimization scenario was performed in order to also identify the optimal conditions to maximize not only the total lipid extraction efficiency, but also the extraction of either the saturated or the unsaturated fatty acids (Supplementary Table S3). Interestingly, the predicted extraction conditions for maximizing PUFA extraction are very similar to the ones predicted to maximize total lipids extraction yields, resulting also in similar recovery yields. This is very promising for a future biorefinery concept, as an oil fraction rich in PUFA is highly

valuable due to the health benefits. On the other hand, the conditions predicted to maximize the recovery yields of the saturated fatty acids differ, especially with regards to the extraction temperature, from -20 °C as in the case of the PUFA, to +20 °C for the saturated ones. This suggests that within the developed process, tuning the temperature alone will allow for the selection of desired fatty acids and thus a tailor-made approach. This provides relative simplicity and flexibility, factors crucial to the scaling-up process. Based on these results, the optimization scenario targeting maximizing both the total lipid as well as PUFA extraction yields was scaled up. Thus, the solvent used was the ES imidazole/hexanoic acid (15:85), at -20 °C and 2 mL mL_{ES}^{-1} MeOH. The working volume was increased by >100-fold. The agitation in the extraction vessel was slightly different than the small-scale tests due to the technical limitation of the available facility.

For the extraction, the efficiency in the larger scale was slightly lower than in the smaller scale (Table 3). Fig. 8 shows the reactor that was used for the lipid extraction and recovery. This was expected since the lower mixing rate reduced the cell-solvent contact and increased the risk of non-homogeneous concentration gradient for the extraction (Halim et al., 2014); homogeneity is essential to ensure a reliable scale-up process. Besides the agitation speed, the container geometry could also heavily influence the mixing profile of the solvent. Therefore, these two parameters are essential for scaling-up the lipid extraction using the eutectic solvent.

The lipid recovery of the larger scale was also lower than that of the smaller scale (Table 4). Despite the same temperature and methanol content, the concentration of remaining lipid dissolved was higher in the large scale. Stirring, which was necessary to avoid uneven temperature profiles, might influence the precipitation or crystallization process. Too harsh agitation would damage the formed crystals, but too gentle agitation would induce less contact between the lipid particles, making it difficult to form crystals (Herrera and Hartel, 2000). Besides the agitation, the cooling rate in the larger volume was completely different from the smaller scale. In contrast to the instantaneous cooling in the smaller scale, the cooling in the larger scale was achieved gradually. Such difference would, of course, cause the variation in the crystallization process since the temperature profile determines the system saturation and thus the lipid recovery (Djagny et al., 2001).

3.4. Outlook for multi-product biorefinery

Preliminary multiproduct microalgae biorefinery investigations have been subsequently performed in order to valorize the proteins and carbohydrates from *N. oceanica*. These were possibly distributed in the solvent extracts and in the residual biomass, which was separated via centrifugation at 4000 rcf. Fig. 9 provides a visual observation of the heavy phases of each extraction mixture. While the ES heavy phase was a suspension of cell aggregate in ES liquid extract, the heavy phase of hexanoic acid was composed of a dark-colored gel-like structure and a denser purée-like layer. The formed structures indicated the presence of polymers or molecules with high molecular weight. When centrifuged at higher acceleration (20,000 rcf), the ES heavy phase remained the same, but the hexanoic acid one was split into three major phases. The lightest phase was liquid hexanoic acid extract, the middle phase being the cell-rich layer, and the densest phase was an aqueous phase with an extra layer of cell debris. The heavy phases from the slower centrifugation (4000 rcf) were homogenized before further treatments.

The composition of biomolecules from the obtained fractions in the process is shown in Table 4. Due to the nitrogen limitation, the major content of microalgae was fatty acids (32 %), followed by protein (25 %), and carbohydrate (12 %). Interestingly, some proteins (~1 % of suspension) were also detected in the hexanoic acid and ES extract. As hexanoic acid and the ES were both relatively hydrophobic, the dissolved proteins might also be hydrophobic. From the total proteins in the original microalgae, only 20 % were found to be water-soluble in the supernatant after mechanical cell disruption, which was similarly reported in the literature (Schwenzfeier et al., 2011; Suarez Garcia et al., 2018). Hence, about 80 % of the proteins were hydrophobic, membrane- or cell wall-associated proteins. Due to the low economic value, little attention has been given to these proteins. Till recently, Dai and co-workers (Dai et al., 2020) valorized the insoluble proteins from *Chlorella protothecoides* as foam stabilizer.

Moreover, without the residual solvent, the major content of the defatted biomass should be proteins and carbohydrates (Table 4). The latter were most likely cell wall components and storage polysaccharides called laminarin (Vogler et al., 2018; Scholz et al., 2014). The proteins could be hydrophilic (insoluble in hydrophobic solvent) or large proteins, as well as denatured and aggregated proteins, which could not pass the cell wall pores. The protein content of the defatted biomass (6.9–8.5 %) was rather lower than the reported protein content from isopropanol-defatted *Nannochloropsis* spp., which contained about 12 % (Gerde et al., 2013). The difference could be associated with the presence of residual hexanoic acid and the ES (together with water), whereas in the reported literature study, the biomass was thoroughly dried. Drying the residual biomass would be difficult due to the low vapor pressure of hexanoic acid and imidazole. Alternatively, the residual biomass can be washed using methanol to remove the ES molecules.

Additionally, proteins and carbohydrates were isolated from the defatted biomass. The extraction was performed by resuspending the defatted biomass in aqueous solutions with different pH values using 1 M NaOH to reach pH 4, 7, and 13, with and without bead beating for cell disruption. For protein extraction, both alkalinity and cell disruption improved the extraction efficiency (Fig. 10a). It is widely known that alkaline conditions drive proteins to be negatively charged, enhanced their solubility. Additionally, at an extremely high pH value of 13, hydrolysis of protein might occur, producing smaller peptides that tend to dissolve easily. Furthermore, the cell disruption opened the entire cell matrix, liberating the large proteins or protein aggregates. However, for carbohydrates, the pH effect was only distinctive at the highest pH (Fig. 10b). Similar to proteins, carbohydrates could also be ionized and hydrolyzed at such pH.

The process mildness has been one of the central issues in multi-product biorefinery as it guarantees low energy consumption and the quality of the obtained biomolecules. In mild conditions, biomolecules, especially proteins, do not lose their three-dimensional structure and thus the functionality (Desai et al., 2018; Suarez Ruiz et al., 2018). Therefore, we also evaluated the quality of the solubilized proteins from the defatted biomass and in the solvent extracts using SDS-PAGE (results not shown). Based on the molecular size, protein aggregation and hydrolysis could be evaluated. The analysis results suggested that proteins strongly aggregated during the lipid extraction. Also, protein hydrolysis indeed took place when extracted at pH 13, including the hydrophobic proteins in the lipid extracts.

Despite being denatured, the obtained proteins could still be valorized. For instance, gelatin, which is one of the most common food ingredients, is obtained through the denaturation of collagen of vertebrates (Djagny et al., 2001). In this case, the peptides from protein hydrolysis might be valorized as amino acid precursors or possess certain bioactivities, such as antimicrobial, antioxidative, health-beneficial, etc. (Montone et al., 2018; Ejike et al., 2017) Moreover, the aggregation suggests that the proteins could have a thickening or gelling property. Meanwhile, the obtained carbohydrates might be used as filler in food/feed products or as a carbon source for fermentation.

The protein denaturation indicated that the lipid extraction using the ES or hexanoic acid was not sufficiently mild for protein valorization. The loss of protein structure might be due to the solvent property, which was acidic and amphiphilic. The latter enabled the solvents to behave as a surfactant, exposing the hydrophobic regions of proteins and causing them to aggregate (Jelinska et al., 2017). Acidity would influence the net charge of the proteins, inducing coagulation and irreversible precipitation. Even further, hexanoic acid and imidazole might establish hydrogen bonds with the proteins and somehow facilitate the

aggregation. Alternatively, the elevated temperature during the extraction might be responsible for the denaturation as the temperature destabilized the intramolecular hydrogen bonding of the proteins. A further investigation of what caused the denaturation is required to develop a mild process based on the used solvents.

Furthermore, a short overview is given below on the (dis)advantages of ES compared to organic solvents and ionic liquids for extracting lipid components from *Nannochloropsis* sp.:

Extraction method	Advantage	Disadvantage	Reference
Organic Solvent	- Extraction of lipids - Scalable process - Cost of compounds	- Use of organic solvents - ATEX implementation for scale-up - Energy intensive process - Protein denaturation	(Angles et al., 2017b; Brennan and Regan, 2020)
Ionic Liquids	- Extraction of lipids - New technology	- Expensive/complex compounds - Energy intensive process - Protein denaturation - Scalability (to be investigated)	(Rezaei Motlagh et al., 2024)
Eutectic Solvents	- Extraction of lipids - Available components - New technology	- Scalability (to be investigated) - Protein denaturation	This study

4. Conclusion

This study was a preliminary feasibility study of microalgae multiproduct biorefinery based on semi-hydrophobic eutectic solvents (Fig. 1). The lipids extracted in the semi-hydrophobic solvents were recovered via precipitation after methanol addition. The efficiency of recovery was proportional to imidazole and methanol content and enhanced at lower temperatures, with a maximum recovery of ~60 %. The solvents were then regenerated and reused in three extraction cycles. During the iterations, no significant reduction in the extraction efficiency was observed regardless of the lipid accumulation. Besides that, the scalability of the proposed process was feasible with a lower efficiency and recovery. Unfortunately, the lipid extraction using the solvents was not sufficiently mild for the other fractions, the suspensions of defatted biomass contained proteins and carbohydrates, as the proteins lost their native structure based on the protein analysis. Further investigations on the critical points discussed in this study are required to develop a viable multiproduct biorefinery process.

CRediT authorship contribution statement

Calvin Lo: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Iulian Boboescu:** Visualization, Resources, Formal analysis. **Sebastiaan Haemers:** Resources, Investigation. **René H. Wijffels:** Writing – review & editing, Supervision, Funding acquisition. **Michel H. M. Eppink:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Prof. Dr. R.H. Wijffels reports financial support was provided by Bio-based Industries Joint Undertaking. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research is part of MAGNIFICENT project, funded by the Bio-Based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation program (grant agreement no. 745754).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179373.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

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