Calvin Lo

Eutectic Solvents as a Novel Extraction System for Microalgae Biorefinery

Propositions

1. Green solvents are not always green.
   (this thesis)

2. Non-volatile solvents are like a double-edged sword, inflammable but difficult to regenerate.
   (this thesis)

3. Protocols are not rituals.

4. Curiosity should be the only prerequisite of doing science.

5. Visa limit globalization.

6. The COVID-19 pandemic enhances the integration of introverts into society.

Propositions belonging to the thesis, entitled

Eutectic solvents as a novel extraction system for microalgae biorefinery

Calvin Lo
Wageningen, 3 December 2021

Eutectic solvents as a novel extraction system for microalgae biorefinery

Calvin Lo

Thesis committee

Promotors
Prof. Dr R.H. Wijffels
Professor of Bioprocess Engineering
Wageningen University & Research

Prof. Dr M.H.M. Eppink
Special professor, Biorefinery with Focus on Mild Separation Technologies of Complex Biomolecules
Wageningen University & Research

Other members
Prof. Dr J.H. Bitter, Wageningen University & Research
Prof. Dr B. Schuur, University of Twente, Enschede
Prof. Dr J.A.P. Coutinho, University of Aveiro, Portugal
Dr J.P. Cadoret, Algama, Malakoff, France

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

Eutectic solvents as a novel extraction system for microalgae biorefinery

Calvin Lo

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 3 December 2021
at 1.30 p.m. in the Aula.

Calvin Lo
Eutectic solvents as a novel extraction system for microalgae biorefinery
146 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands, (2021)
With references, with summary in English

ISBN: 978-94-6395-965-0
DOI: 10.18174/553274

Table of Contents


0.2um_PTFE
WHATMAN
SPIRULINA

Chapter 1

Introduction and thesis outline

1. Introduction

The current practice of lipid production poses serious threats to the environment. It drives major deforestation, accelerating climate change and causing the loss of habitat for biodiversity.[1,2] Microalgae, photosynthetic microorganisms, are gaining popularity as the next generation of lipid feedstock. Not only they can accumulate lipids with high productivity, but also they can be cultivated in seawater on non-arable land.[3–5] Even more, certain microalgal species could synthesize essential fatty acids, such as omega-3 fatty acids. These fatty acids hold huge health benefits and cannot be produced by the human body.[6]

Commonly, the extraction of lipids from microalgae involves several processing steps (Fig. 1).[5] Prior to the solvent extraction, cell disruption is necessary to liberate lipids, which are located intracellularly. Typically, cell disruption is performed by bead milling or homogenization.[7–10] Besides that, the presence of moisture could negatively affect the extraction yield. Thus, dry biomass is normally desired for lipid extraction. The drying can be done by spray drying or freeze-drying.[11,12] The latter is advantageous to keep the lipids from thermal degradation. During the solvent extraction, the lipids migrate to the organic solvents due to their affinity, while the hydrophilic compounds are left in the biomass matrix. After the extraction, the solvent is separated from the crude lipids by distillation and further reused.

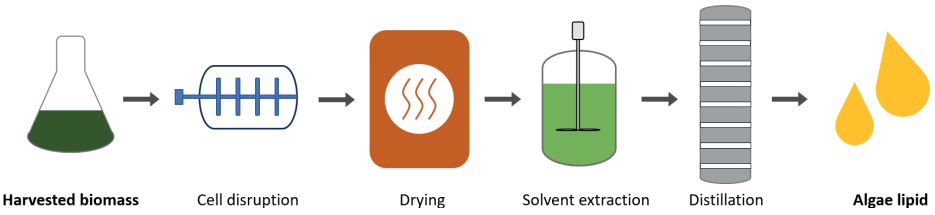


Fig. 1. Schematic overview of conventional lipid extraction from microalgae

The sequential processes require different unit operations, which directly contribute to the capital cost. Furthermore, the rigid cell wall of microalgae and the high latent heat for water evaporation necessitate high energy inputs

for cell disruption and drying.[13] The high energy requirement would affect not only the operational costs but also the process sustainability. Furthermore, the typical organic solvents for lipid extraction, such as hexane and alcohols, are still fossil-derived and flammable.[14,15] Therefore, process development and simplification are required to improve the process economy and sustainability.

Recently, the energy-intensive mechanical cell disruption was bypassed with the use of neoteric solvents. Ionic liquids and hydrophilic eutectic solvents were reported to interact with the cell wall component, creating pores and cracks in the cell wall of microalgae.[16,17] Hydrophobic organic solvent, like hexane, could then penetrate the permeabilized cell wall and solubilize the lipids. Based on these facts, we hypothesized that the pretreatment steps, especially the cell disruption step, could be avoided entirely if the hydrophobic version of those solvents is used. Additionally, despite having some similarities, eutectic solvents are argued to be better than ionic liquids in terms of production cost and potential toxicity.[18–21]

Another advantage of the use of eutectic solvents is that they could stabilize biomolecules.[22,23] This creates the possibility to improve the economy and sustainability of the microalgae industries to employ the biorefinery concept. This concept is a set of downstream processing that valorizes all the valuable biomolecules into multiple products. For instance, pigments, proteins, and carbohydrates could be used as ingredients for cosmetics and food/feed, biostimulants, or substrates for biofuels fermentation.[24–26] With this approach, biomass underutilization would be avoided, and multiple products could generate more added values.

2. Eutectic solvents

A eutectic system is a mixture with lower melting point than its parental compounds. The depression of melting point can allow some mixtures that started with solid or liquid material to be liquid at room temperature. Those liquids could then be used as solvents, hence the term of eutectic solvents (ES).

Thermodynamically, the depression of melting point of a eutectic system is often described by this equation:

$$\ln(x_i\gamma_i) = \frac{\Delta_m h}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)$$

where x_i is the molar fraction of compound i in the mixture, γ_i is the activity coefficient of compound i in the mixture (γ = 1 for ideal mixture), $\Delta_m h$ is the fusion enthalpy of pure compound i in J mol^{-1}, R is the ideal gas constant (8.314 J mol^{-1} K^{-1}), T_m and T are the melting point of pure compound i and the mixture in K, respectively.

A sub-class of eutectic solvents, deep eutectic solvents (DES), has even lower melting point than the ideal eutectic mixture.[27] Fig. 2 illustrates solid-liquid phase diagram of a (deep) eutectic solvent made of compound A and B. The non-ideal eutectic behavior (γ < 1) is often associated with the strong interaction between the constituents, such as hydrogen bonding or other van der Waals interactions. However, in the literature, the distinction between ES and DES is unclear since the phase diagrams of the mixtures were not available. Thus, both terms are used to refer the mixtures of Brønsted or Lewis acids and bases that exhibit depression of melting point.[27–29] Since it becomes the matter of semantics, we use the term of eutectic solvents (ES) to also refer the sub-class in this thesis.

Eutectic solvents are often considered analogous to ionic liquids. They both are thermally stable, nonflammable, and have tailorable properties.[18,28,30,31] The properties of ionic liquids are determined by the cation and anion pairs, whereas that of ES are determined by the composition of their acids and bases constituents. However, in contrast to ionic liquids which are pure molten salts, the ES are mixtures that can be simply prepared from the starting materials without the need of complex purification steps.[32] Furthermore, the ES are often boasted to be greener than ionic liquids since they can be prepared from various bio-derived and biodegradable compounds, such as amino acids, sugars, polyols, terpenes, and organic acids.[19,33,34]

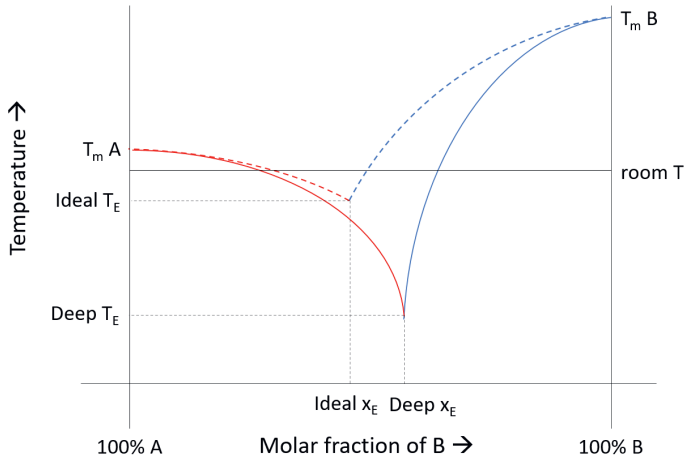


Fig. 2. Phase diagram (solid-liquid equilibria) of eutectic mixture of compound A and B. Red, blue, green lines represent ideal eutectic (γ = 1), deep eutectic (γ < 1), and room temperature, respectively. Area below the room temperature indicate liquid phase at room temperature. T_m is melting point of the pure component, T_E is eutectic temperature, and x_E is the eutectic composition of A and B.

Due to their advantages, ES have been actively researched in various fields, including extraction of biomolecules and pre-treatment of lignocellulosic material.[20,28,30,31,35] In the field of lipid extraction from microalgae, ES have also been applied to weaken the cell wall, which enhanced the lipid extraction from undisrupted biomass.[17,36,37] However, since the used ES were hydrophilic, other organic solvents were still used to extract the lipids. That said, we hypothesized that the integration of cell disruption and lipid extraction would be possible if hydrophobic ES were used.

One of the biggest weaknesses of using ES as an extraction solvent is the lack of vapor pressure. The low volatility suggests that the solvent regeneration after extraction would be challenging since the solvent cannot be separated from lipids *via* the normal solvent evaporation. Furthermore, hydrophobic ES would have a strong affinity towards the lipids. On the one hand, the strong affinity ensures the good solubilization of lipids and the extraction yield. On the other hand, it also means the strong bonding between the lipids and the ES,

which is not desired for solvent removal. Therefore, we proposed to use ES with tuneable hydrophobicity, to facilitate both extraction and solvent regeneration.

3. Aim and thesis outline

The goal of this thesis is to develop eutectic solvents with tuneable hydrophobicity for lipid extraction as the basis of microalgae biorefinery. Moreover, the research questions of the thesis were formulated as follow:

- What combinations make eutectic solvents with tuneable hydrophobicity?
- How to regenerate the eutectic solvent?
- Can such eutectic solvent be used to extract lipid from intact microalgae? What are the influencing parameters?
- Is the lipid extraction using such eutectic solvent mild for biorefinery?

To answer the research questions, the thesis was outlined as follows. In **Chapter 2**, we screened the ES with semi-hydrophobic properties, which made of imidazole and hexanoic acid. At low imidazole content, the mixture dissolved lipids, whereas the mixture rejected lipids at high imidazole content. In **Chapter 3**, the solvent regeneration step was emphasized. Since imidazole could not easily be removed from the ES, alternative auxiliaries were used to shift the hydrophobicity instead of imidazole. The ES was then regenerated by evaporating the volatile auxiliaries. In **Chapter 4**, we studied the lipid extraction from fresh microalgae *Nannochloropsis oceanica* with the imidazole/hexanoic acid ES. In **Chapter 5**, the separation of the algae lipid from the solvents was studied. The process recyclability and scalability were also evaluated. Furthermore, we made a step further for the biorefining, i.e., by isolating the proteins and carbohydrates from the defatted biomass. Finally, **Chapter 6** reflects on the major findings and challenges in this thesis. An outlook for further studies based on this work is also given.

References

1. Carlson, K. M. *et al.* Effect of oil palm sustainability certification on deforestation and fire in Indonesia. *Proc. Natl. Acad. Sci.* **115**, 121–126 (2018).
2. Vijay, V., Pimm, S. L., Jenkins, C. N. & Smith, S. J. The Impacts of Oil Palm on Recent Deforestation and Biodiversity Loss. *PLoS One* **11**, e0159668 (2016).
3. Wijffels, R. H., Barbosa, M. J. & Eppink, M. H. M. Microalgae for the production of bulk chemicals and biofuels. *Biofuels, Bioprod. Bioref.* **4**, 287–295 (2010).
4. Ruiz Gonzalez, J. *et al.* Towards industrial products from microalgae. *Energy Environ. Sci.* (2016). doi:10.1039/C6EE01493C
5. Halim, R., Danquah, M. K. & Webley, P. A. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnol. Adv.* **30**, 709–732 (2012).
6. Chua, E. T. & Schenk, P. M. A biorefinery for Nannochloropsis: Induction, harvesting, and extraction of EPA-rich oil and high-value protein. *Bioresour. Technol.* **244**, 1416–1424 (2017).
7. Günerken, E. *et al.* Cell disruption for microalgae biorefineries. *Biotechnology Advances* (2015). doi:10.1016/j.biotechadv.2015.01.008
8. Postma, P. R. *et al.* Energy efficient bead milling of microalgae: Effect of bead size on disintegration and release of proteins and carbohydrates. *Bioresour. Technol.* **224**, 670–679 (2017).
9. Zinkoné, T. R., Gifuni, I., Lavenant, L., Pruvost, J. & Marchal, L. Bead milling disruption kinetics of microalgae: Process modeling, optimization and application to biomolecules recovery from Chlorella sorokiniana. *Bioresour. Technol.* **267**, 458–465 (2018).
10. Suarez Garcia, E., Lo, C., Eppink, M. H. M., Wijffels, R. H. & van den Berg, C. Understanding mild cell disintegration of microalgae in bead mills for the release of biomolecules. *Chem. Eng. Sci.* **203**, 380–390 (2019).
11. Show, K. Y., Lee, D. J., Tay, J. H., Lee, T. M. & Chang, J. S. Microalgal drying and cell disruption – Recent advances. *Bioresour. Technol.* **184**, 258–266 (2015).
12. Stirk, W. A. *et al.* Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass. *J. Biotechnol.* **307**, 35–43 (2020).
13. Xu, L., Wim Brilman, D. W. F., Withag, J. A. M., Brem, G. & Kersten, S.

Assessment of a dry and a wet route for the production of biofuels from microalgae: Energy balance analysis. *Bioresour. Technol.* **102**, 5113–5122 (2011).

14. Halim, R., Rupasinghe, T. W. T., Tull, D. L. & Webley, P. A. Modelling the kinetics of lipid extraction from wet microalgal concentrate: A novel perspective on a classical process. *Chem. Eng. J.* **242**, 234–253 (2014).
15. Kumar, J., Garlapati, V. K., Dash, A., Scholz, P. & Banerjee, R. Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Res.* **21**, 138–147 (2017).
16. Orr, V. C. A., Plechkova, N. V., Seddon, K. R. & Rehmann, L. Disruption and Wet Extraction of the Microalgae Chlorella vulgaris Using Room-Temperature Ionic Liquids. *ACS Sustain. Chem. Eng.* **4**, 591–600 (2016).
17. Lu, W. *et al.* A new approach of microalgal biomass pretreatment using deep eutectic solvents for enhanced lipid recovery for biodiesel production. *Bioresour. Technol.* **218**, 123–128 (2016).
18. Paiva, A. *et al.* Natural Deep Eutectic Solvents − Solvents for the 21st Century. *ACS Sustain. Chem. Eng.* **2**, 1063–1071 (2014).
19. Florindo, C., Romero, L., Rintoul, I., Branco, L. C. & Marrucho, I. M. From Phase Change Materials to Green Solvents: Hydrophobic Low Viscous Fatty Acid-Based Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **6**, 3888–3895 (2018).
20. Vanda, H., Dai, Y., Wilson, E. G., Verpoorte, R. & Choi, Y. H. Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents. *Comptes Rendus Chim.* **21**, 628–638 (2018).
21. Eppink, M. H. M., Ventura, S. P. M., Coutinho, J. A. P. & Wijffels, R. H. Multiproduct Microalgae Biorefineries Mediated by Ionic Liquids. *Trends Biotechnol.* (2021). doi:10.1016/j.tibtech.2021.02.009
22. Gertrudes, A. *et al.* How Do Animals Survive Extreme Temperature Amplitudes? The Role of Natural Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **5**, 9542–9553 (2017).
23. Sanchez-Fernandez, A. & Jackson, A. J. Proteins in deep eutectic solvents: Structure, dynamics and interactions with the solvent. *Adv. Bot. Res.* **97**, 69–94 (2021).
24. ’t Lam, G. P., Vermuë, M. H., Eppink, M. H. M., Wijffels, R. H. & van den Berg, C. Multi-Product Microalgae Biorefineries: From Concept Towards

Reality. *Trends Biotechnol.* **36**, 216–227 (2017).

25. Vanthoor-Koopmans, M., Wijffels, R. H., Barbosa, M. J. & Eppink, M. H. M. Biorefinery of microalgae for food and fuel. *Bioresour. Technol.* (2013). doi:10.1016/j.biortech.2012.10.135
26. Lee, S. Y., Cho, J. M., Chang, Y. K. & Oh, Y.-K. K. Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresour. Technol.* **244**, 1317–1328 (2017).
27. Martins, M. A. R., Pinho, S. P. & Coutinho, J. A. P. Insights into the Nature of Eutectic and Deep Eutectic Mixtures. *J. Solution Chem.* **48**, 962–982 (2018).
28. Smith, E. L., Abbott, A. P. & Ryder, K. S. Deep Eutectic Solvents (DESs) and Their Applications. *Chem. Rev.* **114**, 11060–11082 (2014).
29. Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K. & Tambyrajah, V. Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* 70–71 (2003). doi:10.1039/b210714g
30. Zhang, Q., De, K., Vigier, O., Royer, S. & Jeoe, F. Deep eutectic solvents: syntheses, properties and applications. *Chem. Soc. Rev.* **41**, 7108–7146 (2012).
31. Van Osch, D. J. G. P. *et al.* Ionic liquids and deep eutectic solvents for lignocellulosic biomass fractionation. *Phys. Chem. Chem. Phys.* **19**, 2636–2665 (2016).
32. Florindo, C., Branco, L. C. & Marrucho, I. M. Quest for Green-Solvent Design: From Hydrophilic to Hydrophobic (Deep) Eutectic Solvents. *ChemSusChem* **12**, 1549–1559 (2019).
33. Francisco, M., Van Den Bruinhorst, A. & Kroon, M. C. Low-transition-temperature mixtures (LTTMs): A new generation of designer solvents. *Angew. Chemie - Int. Ed.* **52**, 3074–3085 (2013).
34. Choi, Y. H. *et al.* Are Natural Deep Eutectic Solvents the Missing Link in Understanding Cellular Metabolism and Physiology? *PLANT Physiol.* **156**, 1701–1705 (2011).
35. Smink, D., Kersten, S. R. A. & Schuur, B. Recovery of lignin from deep eutectic solvents by liquid-liquid extraction. *Sep. Purif. Technol.* **235**, 116127 (2020).
36. Tommasi, E. *et al.* Enhanced and Selective Lipid Extraction from the Microalga P. tricornutum by Dimethyl Carbonate and Supercritical CO 2

Using Deep Eutectic Solvents and Microwaves as Pretreatment. *ACS Sustain. Chem. Eng.* **5**, 8316–8322 (2017).

37. Pan, Y. et al. One-step production of biodiesel from wet and unbroken microalgae biomass using deep eutectic solvent. Bioresour. Technol. 238, 157–163 (2017).

Chapter 2

Eutectic solvents with tuneable hydrophobicity: lipid dissolution and recovery

This chapter has been published as:

Lo, C., Semerel, J., van den Berg, C., Wijffels, R. H. & Eppink, M. H. M. Eutectic solvents with tuneable hydrophobicity: lipid dissolution and recovery. *RSC Adv.* **11**, 8142–8149 (2021).

Abstract

Despite the promising advantages of eutectic solvents, the application of these solvents as an extraction solvent is still limited due to the challenging product recovery. Previously, it was reported that lipid could be recovered from a hydrophobic eutectic solvent with the principle of switchable hydrophobicity. However, this method still involves additional chemicals, such as polymeric amine, water, and CO_2, which need to be removed later. In this study, we proposed a different approach by shifting the hydrophobicity spectrum of semi-hydrophobic solvent. Made of hydrophilic imidazole and hydrophobic hexanoic acid, this combination showed tuneable hydrophobicity when the composition was changed, shown by the change of dipolarity (π*) scale from solvatochromic analysis. At low imidazole content, the solvent was able to dissolve sunflower oil and algae oil, whereas, at high imidazole content, the solvent showed high affinity towards water. By adding imidazole to the solution of oil and the solvent, a phase split was induced between the oil-rich upper phase and the solvent-rich lower phase. With this approach, ~75% of recovery efficiency was achieved for the two oils, with the purity of ~100% for sunflower oil and 86% for algae oil.

1. Introduction

Eutectic solvents, including "deep eutectic solvents" (DES), have been gaining interest as alternative green solvents in the field of extraction of natural compounds. This is due to the attractive advantages of these solvents, such as low vapor pressure, high thermal stability, and high carrying capacity.[1–3] Moreover, a eutectic solvent can be cheaply and easily prepared by mixing Brønsted or Lewis acid and base (or "hydrogen bond donor" and "hydrogen bond acceptor").[1,2,4,5] Furthermore, the properties of these solvents are claimed to be designable.[6–8] For example, if the mixture is made of bio-derived, safe, and biodegradable compounds, then the solvent might also be benign and sustainable.[5,9,10]

These mixtures melt at lower temperatures than the parental constituents, such that allows the mixtures to be liquid at room temperature, even if the parental compounds are solids.[11–15] The depression of melting point can be found in not only "deep eutectic solvents", but also normal, ideal mixtures. Group of Coutinho[13] proposed to specifically classify DES as mixtures which exhibit further reduction of melting point when compared to ideal eutectic mixture. In this study, we use the term of eutectic solvents to have wider selection of mixtures, regardless the deviation of the melting point reduction.

Despite the advantages, the use of eutectic solvents still faces several challenges, such as difficult product recovery and solvent regeneration.[16] This is mainly because these solvents have low volatility. In the typical organic solvent process, these two processes were done via distillation and solvent condensation. However, applying distillation to eutectic solvents would require a tremendous amount of energy. Therefore, other strategies need to be developed to tackle this bottleneck in the near future.

One of the most promising techniques is switching the solvent hydrophobicity, which approach was inspired by switchable solvents.[17] Bravi and coworkers[18] have reported the use of a switchable-hydrophilicity eutectic solvent system, based on the previously reported hydrophobic eutectic solvent octanoic acid/dodecanoic acid (3:1).[19] When this solvent is mixed with an aqueous

solution of Jeffamine D-230, they form hydrophilic ionic liquid made of the protonated amine and deprotonated acid (forward switching). Then, CO_2 or acid could be used to protonate back the acid, thus, obtaining the hydrophobic eutectic solvent (backward switching).[18] With this system, both hydrophilic and hydrophobic biomolecules can be extracted and separated.[18] However, this approach involved two additional compounds (the amine and carbon dioxide or acid) which later need to be removed further downstream.

In this study, we propose another approach to tune the hydrophobicity of a semi-hydrophobic solvent, consisting of hydrophilic and hydrophobic compounds. With this approach, the eutectic solvent can have a spectrum of hydrophobicity, depending on the composition of the parental compounds. Hence, at the hydrophobic state, the solvent can be used to dissolve hydrophobic solutes, such as lipids. Afterwards, the lipid can be recovered by adding an excess of the hydrophilic parental compound, making the overall solvent hydrophilic. Here in this study, we use sunflower oil and culinary algae oil as model lipids to demonstrate the principle.

To obtain the semi-hydrophobic solvent, a list of compounds was made based on the reported parental compounds for hydrophilic and hydrophobic eutectic solvents, such as C2 – C10 carboxylic acids, quaternary ammonium salts (Aliquat® 336 – methyltrioctylammonium chloride and tetrabutylammonium bromide), terpene DL-menthol, and heteroaromatics (imidazole and pyrazole). These non-green compounds are used as a proof-of-principle of this current strategy, which later can be applied on greener compounds with similar or better performance. The hydrophobicity of each individual compound was indicated by the partition in octanol/water system (log K_{ow}) and the molar solubility in water (log S).

Several combinations were made by pairing hydrophobic and hydrophilic compounds, which were screened based on the miscibility with water and dodecane. The combinations which dissolve both water and dodecane were further characterized with polarity estimation and lipids solubility. The polarity of eutectic solvents with different acid/base ratios was estimated by

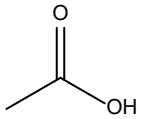
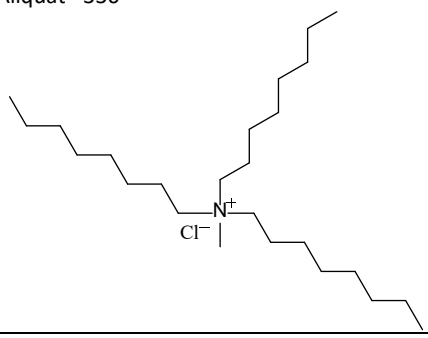
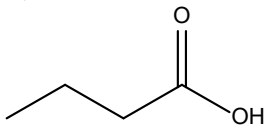
solvatochromic analysis.[14,20–24] Imidazole/hexanoic acid combination was further used to demonstrate the dissolution and recovery of the model oils.

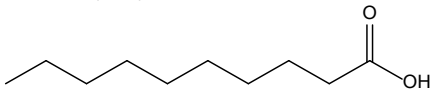
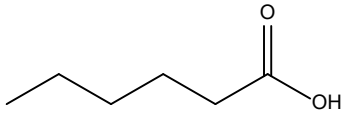
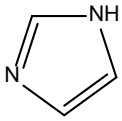
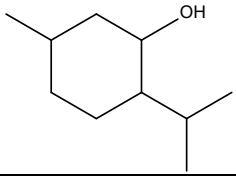
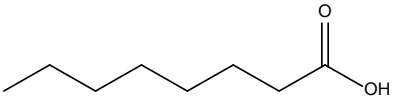
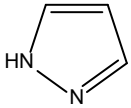
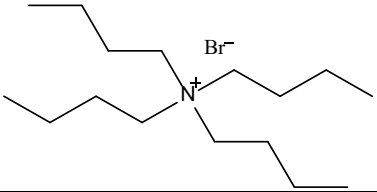
2. Materials & Methods

2.1. Materials

The starting compounds of the eutectic solvents used in this study are listed in Table 1. The logarithmic values of the octanol/water partition coefficient, log K_{ow}, and the logarithmic values of solubility in water (in mol L^{-1}), log S, indicate the hydrophobicity of the pure compounds. Model oils used in this study were commercial sunflower oil (Jumbo Supermarkten B.V., The Netherlands) and culinary algae oil (Corbion Biotech, Inc., United States). For solvatochromic analysis, *N,N*-dimethyl-4-nitroaniline (98+%) was purchased from Alfa Aesar.

Table 1. List of chemicals used to prepare eutectic solvents and their properties (data taken from PubChem[25])

Compounds	Supplier, purity	Brønsted-	Log K_{ow}	Log S
Acetic (C2) acid 	Sigma-Aldrich, 100%	acid	-0.17	1.22
Aliquat® 336 	Alfa Aesar, n.a.	base	6.74*	-2.70†
Butyric (C4) acid 	Sigma-Aldrich, ≥99%	acid	0.79	-0.17

Decanoic (C10) acid 	Sigma-Aldrich, ≥98%	acid	4.09	-3.45
Hexanoic (C6) acid 	Sigma-Aldrich, ≥99%	acid	1.92	-1.05
Imidazole 	Sigma-Aldrich, ≥99%	acid or base	-0.08	0.99
DL-Menthol 	Sigma-Aldrich, ≥98%	acid or base	3.20	-2.57
Octanoic (C8) acid 	Acros Organics, 99%	acid	3.05	-2.26
Pyrazole 	Sigma-Aldrich, ≥99%	base	0.26	-0.55
Tetrabutylammonium bromide (TBAB) 	Sigma-Aldrich, ≥99%	base	1.71*	0.27

* Simulated by VCCLAB[26], http://www.vcclab.org

† Dissolved in 2M HCl [27]

2.2. Preparation of the eutectic solvent

The eutectic solvents were made by mixing the proper combination of acids and bases at 60 °C until clear homogeneous solutions were formed. No prior purification step was performed. The mixtures which did not stay

homogeneous for at least 24 hours were categorized as unstable. The stable mixtures were then stored for no longer than one week for further use.

2.3. Dissolution of dodecane and water

The miscibility of dodecane and water in the eutectic solvents were used as indicators of the solvent hydrophobicity. This fast analysis was done by adding dodecane or water into the eutectic mixtures equivolume. Based on the final volume and visual observation of each phase after mixing, the miscibility was evaluated. The combinations which dissolve both dodecane and water were further analyzed with solvatochromic analysis.

2.4. Solubility of model oils and water

The solubility of sunflower oil, algae oil, and water were determined in imidazole/hexanoic acid mixture with different molar fraction of imidazole by adding them dropwise until the solution became turbid.

2.5. Recovery of model oils

The recovery of sunflower oil and algae oil from imidazole/hexanoic acid was done by adding a proper amount of imidazole. The addition of imidazole induced a phase split, which was accelerated through centrifugation. The recovered oil formed a top phase, while the hydrophilic mixture together with the remaining oil formed the bottom phase.

The partition coefficient (K) and separation efficiency (η) of the lipid recovery were calculated by using these equations, respectively:

$$K = \frac{c_O}{c_S}$$

$$\text{Eff} = \frac{V_O\, c_O}{V_O\, c_O + V_S\, c_S}$$

where V is the total amount of the phase in gram, c is the total fatty acids concentration in g/g, subscript O and S denote the oil-rich and solvent-rich phase, respectively.

2.6. Solvatochromic analysis

The solvatochromic analysis was performed to quantify the polarity of the eutectic solvents that dissolve both dodecane and water. The quantitation was based on Kamlet-Taft dipolarity scale (π^*), which is normally based on the red-shift of the absorption spectrum of *N,N*-diethyl-4-nitroaniline dissolved in the solvent.[14,20,21] However, due to the difficulty in finding this dye commercially, the analogue *N.N*-dimethyl-4-nitroaniline was used in this study instead.[28,29] A known amount of the dye was dissolved in the eutectic solvent with a concentration of ~0.1 mg/mL before the spectrum measurement using a quartz cuvette with 10 mm light path. The dipolarity scale (π^*) was calculated with the formula:

$$\pi^* = \frac{\lambda_{solvent}^{-1} - \lambda_{cyclohexane}^{-1}}{\lambda_{DMSO}^{-1} - \lambda_{cyclohexane}^{-1}}$$

where λ is the UV/Vis wavelength [nm] at which maximum absorbance of the dye occurred. The scale uses cyclohexane and dimethyl sulfoxide (DMSO) as references with a value of 0 and 1, respectively.

2.7. Gas chromatography

The analysis of fatty acids was performed using GC-FID system (Agilent Technologies) with H_2 as a carrier gas. Methylation was performed on the samples (~5 mg) in advance by adding 3 mL of methanol & 5% H_2SO_4 at 100 °C for 1 hour. Then, the methylated products were extracted using hexane that contained C15:0 methyl ester as an internal standard. The samples were then run through Nukol™ column (30m x 0.53mm x 1.0µm, Supelco) with a split ratio of 0.1:1 and split flow of 3.55 mL/min. The oven temperature profile was 90 °C to 200 °C at 44.08 °C/min and held for 7.5 minutes. In this study, the total amount of fatty acids was assumed as the amount of lipid.

3. Results & Discussion

An outline of the results and discussion section is given below. First, the potential parental compounds, as listed in Table 1, were combined, and screened to obtain semi-hydrophobic eutectic solvents via miscibility with

water and dodecane. Then, the polarity of selected mixtures with different composition was quantified by solvatochromic analysis. One mixture with the widest spectrum of dipolarity, imidazole/hexanoic acid, was further selected to demonstrate the lipid recovery via changing the solvent composition. Hence, the solubility of model oils was measured in the eutectic solvent with varying imidazole concentration. Furthermore, the actual lipid recovery experiment was performed by inducing phase split. In this part, the partition coefficient, the separation efficiency, and the purity of recovered oils were evaluated. Finally, we compared the basic energy requirement of this process to that of the switchable eutectic solvent approach to evaluate the process feasibility.

3.1. Screening of semi-hydrophobic eutectic solvents

The potential semi-hydrophobic eutectic solvents were screened based on the miscibility with water and dodecane. The miscibility was observed based on the phase volume after being mixed with water or dodecane at 1:1 v/v. Each combination was categorized into four groups: 1) dissolve only water, 2) dissolve only dodecane, 3) dissolve both water and dodecane, and 4) dissolve neither water nor dodecane. Additionally, in this study, we used the sum of log S as a crude indicator of the mixture hydrophobicity, regardless of the composition. This indicator may not properly function for other combinations outside this study. It is also worth to mention that this indicator does not imply any thermodynamic properties of the mixtures.

Table 2 shows the combinations of each group. As expected, combinations which belong to Group 1 are made from hydrophilic acids and bases (sum log $S > 0.8$), whereas Group 2 are generally combinations of two hydrophobic compounds (sum log $S < -2.8$). Furthermore, all combinations of Aliquat® 336 always belong to Group 2 due to the long alkyl chains of the cation. Semi-hydrophobic eutectic solvents, Group 3, are combinations of hydrophilic acids and hydrophobic bases, or vice versa ($-2.8 <$ sum log $S < 0.8$). Moreover, despite the similar values of sum log S to Group 3, Group 4 showed little to no miscibility with water and dodecane.

Besides that, there were also unstable combinations, such as TBAB/decanoic acid and DL-menthol/imidazole, which formed solids when mixed with water. The solid formation indicated the alteration of solid-liquid equilibrium of the eutectic combination. This alteration may be due to either the incorporation of water in the overall supramolecular interaction or the change in the eutectic mixture composition. Based on reported previous studies, the presence of water in choline chloride/urea caused a further depression of melting point.[30,31] Therefore, it is more likely that the latter took place. Due to the large difference in water affinity of the constituents, the hydrophilic TBAB and imidazole leached to water. Hence, the effective molar ratio of TBAB or imidazole in the non-aqueous phase decreased, resulting in less reduction of the melting point and the solid formation. This leaching phenomenon may also occur in other combinations, but with less obvious visual effect. In fact, several cases of leaching have been reported in the mixtures based on tetrabutylammonium chloride, and DL-menthol when combined with short carboxylic acid.[32]

Table 2. Combinations of characterized deep eutectic solvents in this study. The group categories are 1) dissolve only water, 2) dissolve only dodecane, 3) dissolve both water and dodecane, and 4) dissolve neither water nor dodecane

Group	Base	Acid	sum log S	Base:Acid ratio
2	Aliquat® 336	Decanoic acid	-6.15	1:2
2	DL-Menthol	Decanoic acid	-6.02	1:2
2	Aliquat® 336	DL-Menthol	-5.27	1:2
2	Aliquat® 336	Octanoic acid	-4.96	1:2
2	DL-Menthol	Octanoic acid	-4.83	1:2
2	Pyrazole	Decanoic acid	-4.00	1:2
2	Aliquat® 336	Hexanoic acid	-3.75	1:2
2	DL-Menthol	Hexanoic acid	-3.62	1:2
unstable	TBAB	Decanoic acid	-3.18	1:2
2	DL-Menthol	Pyrazole	-3.12	3:1
2	Aliquat® 336	Butyric acid	-2.87	1:2
2	Pyrazole	Octanoic acid	-2.81	1:2
2	DL-Menthol	Butyric acid	-2.74	1:2

solid	Imidazole	Decanoic acid	-2.46	1:2
3	TBAB	DL-Menthol	-2.30	1:3
4	TBAB	Octanoic acid	-1.99	1:2
4	Aliquat® 336	Imidazole	-1.71	1:1
3	Pyrazole	Hexanoic acid	-1.60	1:2
unstable	DL-Menthol	Imidazole	-1.58	3:1
2	Aliquat® 336	Acetic acid	-1.48	1:2
3	DL-Menthol	Acetic acid	-1.35	1:2
3	Imidazole	Octanoic acid	-1.27	1:2
4	TBAB	Hexanoic acid	-0.78	1:2
3	Pyrazole	Butyric acid	-0.72	1:2
3	Imidazole	Hexanoic acid	-0.06	1:2
1	TBAB	Butyric acid	0.10	1:2
solid	Imidazole	Pyrazole	0.44	1:1
3	Pyrazole	Acetic acid	0.67	1:2
1	Imidazole	Butyric acid	0.82	1:2
1	TBAB	Imidazole	1.26	1:2
1	TBAB	Acetic acid	1.49	1:2
1	Imidazole	Acetic acid	2.21	1:2

3.2. Solvatochromic analysis of semi-hydrophobic eutectic solvent

The solvatochromic analysis was performed on the eutectic solvents in Group 3 to measure the solvent polarity with various compositions. Fig. 1 shows the polarity scale of the eutectic solvents with various compositions. In general, the polarity is heavily influenced by the nature of the parental compounds, with stronger emphasis from the bases. The permanent charge of TBAB caused a high dipolarity value, followed by the aromatic imidazole and pyrazole, and lastly by DL-menthol, which is neutral. Besides that, the acids also influence the mixture polarity to a lesser degree. For example, pyrazole/acetic acid has higher dipolarity than pyrazole/butyric acid. Similarly, it can also be observed between imidazole/hexanoic acid pair and imidazole/octanoic acid to a lower extent. These findings agree with the previously reported results.[14,20,21]

More importantly, this result confirms our hypothesis that the polarity of these solvents is influenced by the composition of parental compounds. For example, in the case of imidazole/hexanoic acid and pyrazole/hexanoic acid, the solvent

polarity increases at lower concentrations of the hydrophobic acid. Hence, the solvent hydrophobicity can be tuned by changing the constituent molar ratio, which is the basic principle of polarity shifting proposed in this study. Among the combinations, imidazole/hexanoic acid showed the largest change in dipolarity value (Fig. 1), indicating the widest range for tuning the hydrophobicity. Hence, this combination was used to demonstrate the lipid recovery by this hydrophobicity shifting approach.

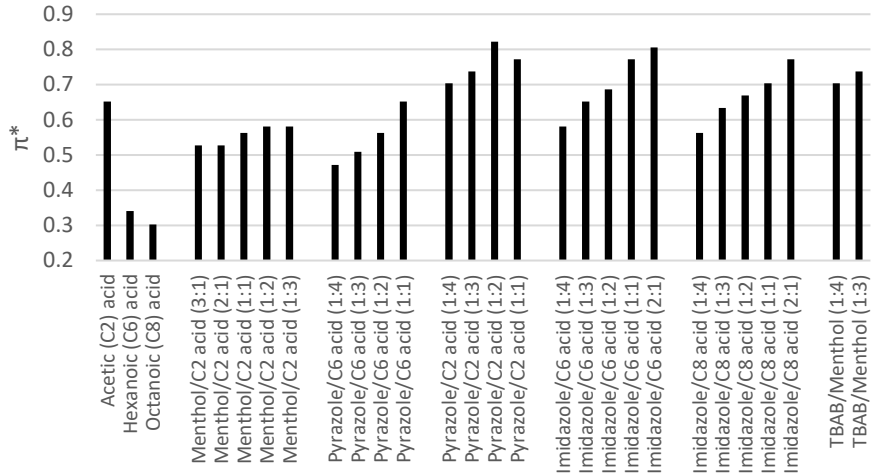


Fig. 1. Dipolarity π* values of different compounds and semi-hydrophobic eutectic solvents. Imidazole/hexanoic acid showed the largest change of dipolarity.

3.3. Solubility of model oils and water in imidazole/hexanoic acid eutectic solvent

The eutectic solvent imidazole/hexanoic acid was further characterized by measuring the solubility of sunflower oil, algae oil, and water in different imidazole/hexanoic acid molar ratio (Fig. 2). Since imidazole is a polar and hydrophilic compound, the increased presence of imidazole results in the higher degree of solvent hydrophilicity. Thus, the model oils dissolved well in the solvent with low imidazole content and became less soluble at higher imidazole molar ratio. Sunflower oil generally dissolves better than algae oil in the eutectic solvent. This solubility difference may be associated with the different chemical composition of the oils (Table S1). On the other hand, the

solubility of water is positively correlated to imidazole content and reaches high miscibility (≥ 50%-weight) at 30%.

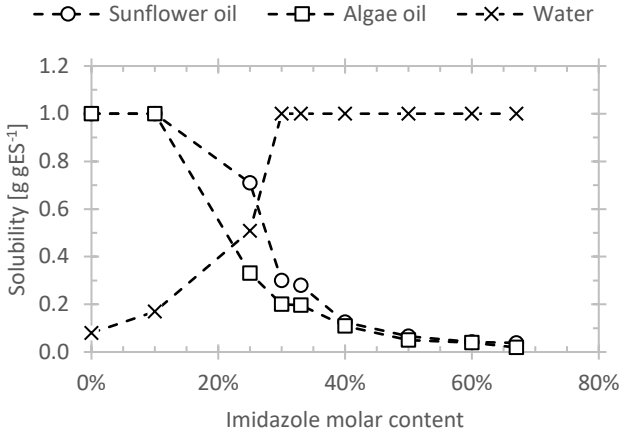


Fig. 2. Solubility of sunflower oil (circle), algae oil (square), and water (cross) in imidazole/hexanoic acid with different molar content of imidazole.

Despite hexanoic acid being the dominant constituent, the solvent still has a high affinity towards water at > 30% imidazole molar content. This might be explained by the result of acid-base interaction between hexanoic acid and imidazole (pKa = 4.88 and pKb = 7.05, for respective compounds). Unlike the normally reported eutectic solvents that formed due to hydrogen bonding,[2,11,33,34] this interaction produces the ionic liquid imidazolium hexanoate.[35] The formed ions could interact strongly with water, causing the high affinity towards water. Furthermore, the presence of the permanent charge of imidazolium hexanoate can also explain the rapid decline of lipid solubility in the range of 10 – 30% imidazole molar content. Assuming the spontaneous formation of imidazolium hexanoate at equimolar (1:1) ratio, the mixture might very well be composed of imidazolium hexanoate and the unreacted species. For example, at imidazole/hexanoic acid (1:3), which equivalent to 25% imidazole content, all imidazole reacted with hexanoic acid to form imidazolium hexanoate and unreacted hexanoic acid with 1:2 ratio. The melting point of the imidazole and hexanoic acid combination at different

ratios showed eutectic behavior, i.e., depressed melting point (phase diagram: Fig. S1).

3.4. Recovery of model oils by phase split

The proposed strategy to shift the solvent hydrophobicity by adding imidazole was then applied for the recovery of the model oils. This experiment began with the model oils dissolved in imidazole/hexanoic acid (1:3) – 25% imidazole molar content, resulting in a homogeneous solution (Fig. 3). The initial concentration of sunflower oil and algae oil in the eutectic solvent is 0.55 and 0.28 g oil/g solvent, respectively. Then, excess of imidazole was added to the system until reaching 40%, 50%, 60%, and 75% imidazole content. The high imidazole concentrations induced phase split between the light lipid-rich phase and the heavy solvent-rich phase (the interface is shown by the arrows). At 75% imidazole molar content (3:1), not all imidazole dissolved and remained as white solids in the bottom part of the tube. This indicated that this system was beyond saturation (phase diagram: Fig. S1). All formed phases were then analyzed by gas chromatography to measure the total fatty acid content, which further assumed to be the lipid content. Based on this analysis, partition coefficient, recovery efficiency, and purity of the recovered product were evaluated. The overview can be seen in Table 3.

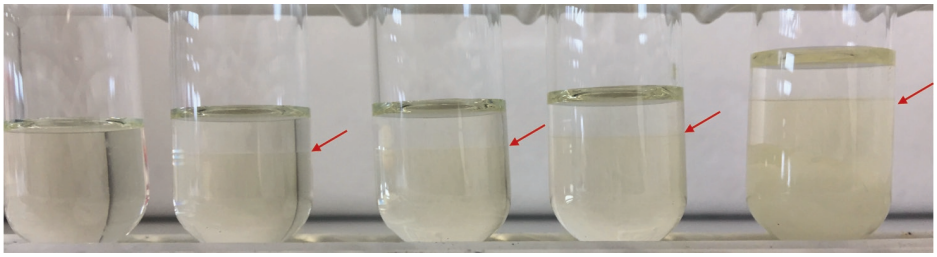


Fig. 3. Recovery of sunflower oil from eutectic solvent via induced phase split by adding imidazole. The arrows show the interface between the recovered oil and modified eutectic solvent. From left to right, the eutectic solvent contained 25%, 40%, 50%, 60%, and 75% imidazole molar content. The initial concentration of sunflower oil is 0.55 g/g solvent.

Table 3. Results overview of the lipid recovery experiment. c_O and c_S are the lipid concentration in oil- and solvent-rich phase, respectively; V_O and V_S are the total mass of oil- and solvent-rich phase, respectively, K is the lipid partition coefficient (c_O/c_S), and Eff is the separation efficiency.

Sunflower oil

Imidazole [mol%]	c_O [g/g]	V_O [g]	c_S [g/g]	V_S [g]	K	Eff [%]
40%	0.98 ± 0.03	0.301 ± 0.017	0.13 ± 0.00	1.406 ± 0.001	7.3 ± 0.1	61
50%	0.90 ± 0.12	0.375 ± 0.012	0.08 ± 0.00	1.495 ± 0.027	11.0 ± 1.4	73
60%	0.95 ± 0.00	0.384 ± 0.000	0.14 ± 0.00	1.737 ± 0.000	6.8 ± 0.0	60
75%	1.01 ± 0.00	0.369 ± 0.013	0.13 ± 0.07	2.495 ± 0.043	9.5 ± 5.3	56

Algae oil

Imidazole [mol%]	c_O [g/g]	V_O [g]	c_S [g/g]	V_S [g]	K	Eff [%]
40%	0.75 ± 0.11	0.110 ± 0.011	0.11 ± 0.05	1.296 ± 0.009	7.9 ± 4.5	38
50%	0.82 ± 0.07	0.176 ± 0.005	0.05 ± 0.01	1.413 ± 0.012	17.0 ± 4.4	67
60%	0.85 ± 0.01	0.183 ± 0.042	0.03 ± 0.01	1.627 ± 0.022	27.7 ± 6.6	75
75%	0.86 ± 0.02	0.206 ± 0.017	0.03 ± 0.01	2.352 ± 0.001	27.9 ± 6.3	71

Partition coefficient

The partition coefficient is important to understand how the lipid distributed in the light and heavy phase. With higher partition coefficients, the lipids are less distributed and more likely to be found in the light phase. Fig. 4 shows the partition of the oils between the light and heavy phase, based on the concentration ratio. The partition of algae oil is positively correlated to imidazole content, whereas that of sunflower oil remains relatively constant. Moreover, generally, algae oil reached higher partitions than sunflower oil. The highest obtained partition coefficient for sunflower and algae oil is 11 and 28, respectively.

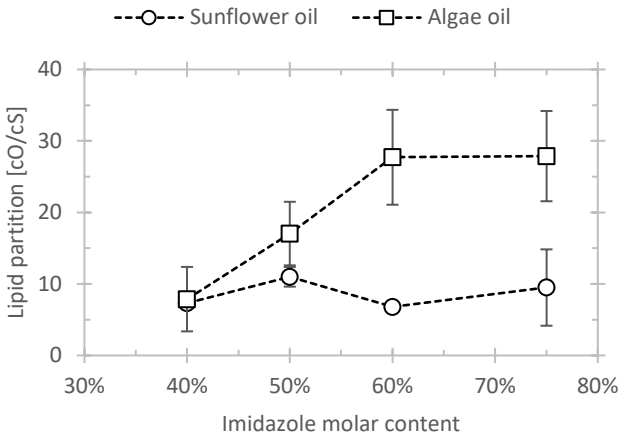


Fig. 4. The partition coefficient of sunflower oil (circle) and algae oil (square) between the oil-rich and solvent-rich phase.

The different behavior may be owed to the different nature of the two oils, e.g., the difference in fatty acid profile (Table S1). The algae oil contains > 93% monounsaturated fatty acid (MUFA) C18:1, whereas the most abundant fatty acid in sunflower oil is polyunsaturated fatty acid (PUFA) C18:2 (58%). It is known that the unsaturated bonds in fatty acids can interact with cations or other aromatic compounds.[36–38] Hence, the polar solvent may have a relatively higher affinity towards PUFA than towards MUFA. This causes the relatively better dissolution of sunflower oil than algae oil in the polar state of eutectic solvent, which leads to a lower partition coefficient of sunflower oil.

Recovery efficiency

Besides the partition behavior, we also evaluated the recovery efficiency of the model oils, which is based on the oil mass. Hence, the recovery is not only determined by the partition equilibrium, but also by the amount of the formed phases. Fig. 5 shows the recovery efficiency of the model oils, which is influenced by the imidazole content. For sunflower oil, the maximum recovery of 73% was achieved at 50% imidazole content, and it tends to decline at higher imidazole content despite being not statistically significant. Whereas for algae oil, the recovery resembles the trend of partitioning, which rises with higher

amount of imidazole. The highest achieved efficiency for algae oil was 75% at 60% imidazole molar content.

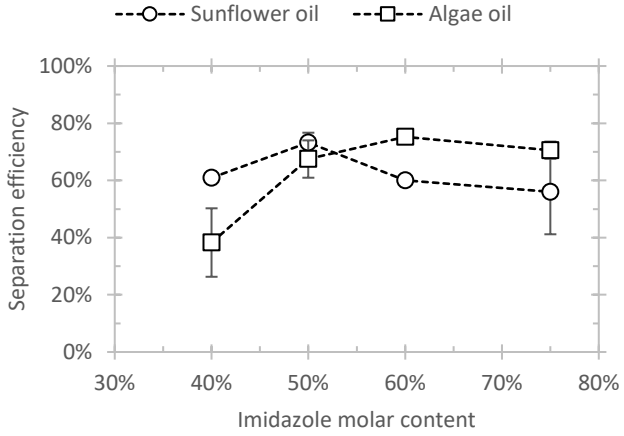


Fig. 5. The recovery efficiency of sunflower oil (circle) and algae (square) oil from imidazole/hexanoic acid with the increasing amount of imidazole.

As mentioned before, the recovery is determined by the amount of the phase formed. The added imidazole, which induced the phase split, in fact, created a dilution effect on the heavy solvent phase. This dilution then reduces the recovery efficiency since, with the increased amount of bottom phase, the amount of unrecovered lipid is also increased. This may explain the declining trend of sunflower oil at high imidazole content. The slightly declining trend can also be observed in the case of algae oil. However, since less algae oil is dissolved in the solvent-rich phase when compared to sunflower oil (> 2.5-fold in partition coefficient), the dilution effect is not as obvious.

Purity of recovered oil

Furthermore, the concentration of total fatty acid in the oil-rich phase was further assumed to represent the purity of recovered oil (Fig. 6). The overall purity of the recovered oils was relatively high, with sunflower oil reached > 90%, and algae oil reached 75 – 86%. The possible impurities in the oil-rich phase include eutectic solvent constituents, hexanoic acid, imidazole, and their derivatives. Based on the gas chromatography analysis, the presence of

hexanoic acid is in the range of 2 – 5%, which decreases with higher imidazole content. Besides that, the remaining mass would be associated with several possible compounds, such as imidazole, glycerol, the polar functional groups of polar lipids, and vitamins.

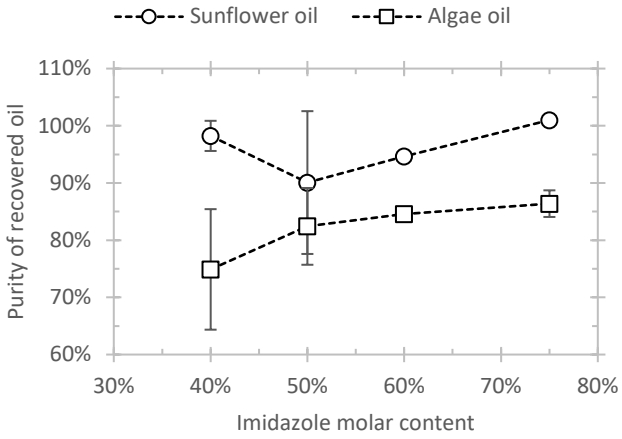


Fig. 6. The purity of the recovered sunflower oil (circle) and algae oil (square) based on total fatty acids.

3.5. Eutectic solvent regeneration

Despite the little energy requirement for the lipid recovery, the regeneration of the eutectic solvent to its initial ratio is not yet considered. One can simply add hexanoic acid to readjust the molar composition, but that approach is not sustainable since the eutectic solvent would accumulate along with the process cycles, while fresh imidazole and hexanoic acid need to be continuously supplied as makeup. Therefore, other techniques for the eutectic solvent regeneration are still being explored in the future studies.

4. Conclusion

In this study, a new approach of lipid recovery from a semi-hydrophobic eutectic solvent was developed by shifting the hydrophobicity spectrum. The tuneable hydrophobicity of the eutectic solvent was achieved by changing the compositional ratio. A solvent mixture which is composed of imidazole and

hexanoic acid was shown to have this tuneable hydrophobicity, which hydrophobicity reduced with higher imidazole. Therefore, this combination was used to demonstrate the dissolution and recovery of sunflower and algae oil. By adding imidazole, the solubility of the model oil decreased, which induced a phase split between the upper oil-rich phase and the lower solvent-rich phase. With this approach, about 75% of the oils can be recovered with relatively high purity (which can reach > 90%). The recovery was also affected by the natural composition of the model lipids.

References

1. Zhang, Q., De, K., Vigier, O., Royer, S. & Jeoe, F. Deep eutectic solvents: syntheses, properties and applications. *Chem. Soc. Rev.* **41**, 7108–7146 (2012).
2. Smith, E. L., Abbott, A. P. & Ryder, K. S. Deep Eutectic Solvents (DESs) and Their Applications. *Chem. Rev.* **114**, 11060–11082 (2014).
3. Lee, J., Jung, D. & Park, K. Hydrophobic deep eutectic solvents for the extraction of organic and inorganic analytes from aqueous environments. *TrAC - Trends in Analytical Chemistry* **118**, 853–868 (2019).
4. Fernandez, L. *et al.* Indirect assessment of the fusion properties of choline chloride from solid-liquid equilibria data. *Fluid Phase Equilib.* **448**, 9–14 (2017).
5. Francisco, M., Van Den Bruinhorst, A. & Kroon, M. C. Low-transition-temperature mixtures (LTTMs): A new generation of designer solvents. *Angew. Chemie - Int. Ed.* **52**, 3074–3085 (2013).
6. Cao, L. *et al.* Imidazole tailored deep eutectic solvents for CO2 capture enhanced by hydrogen bonds. *Phys. Chem. Chem. Phys* **17**, 27306–27316 (2015).
7. van den Bruinhorst, A. *et al.* Hydrophobic eutectic mixtures as volatile fatty acid extractants. *Sep. Purif. Technol.* **216**, 147–157 (2019).
8. Jeong, K. M. *et al.* Tailoring and recycling of deep eutectic solvents as sustainable and efficient extraction media. *J. Chromatogr. A* **1424**, 10–17 (2015).
9. Macário, I. P. E. *et al.* Unraveling the ecotoxicity of deep eutectic solvents using the mixture toxicity theory. *Chemosphere* **212**, 890–897 (2018).
10. Radošević, K. *et al.* Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. *Ecotoxicol. Environ. Saf.* **112**, 46–53 (2014).
11. Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K. & Tambyrajah, V. Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* 70–71 (2003). doi:10.1039/b210714g
12. Silva, L. P. *et al.* Design and Characterization of Sugar-Based Deep Eutectic Solvents Using Conductor-like Screening Model for Real Solvents. *ACS Sustain. Chem. Eng.* **6**, 10724–10734 (2018).
13. Martins, M. A. R., Pinho, S. P. & Coutinho, J. A. P. Insights into the Nature

of Eutectic and Deep Eutectic Mixtures. *J. Solution Chem.* **48**, 962–982 (2018).

14. Martins, M. A. R. *et al.* Tunable Hydrophobic Eutectic Solvents Based on Terpenes and Monocarboxylic Acids. *ACS Sustain. Chem. Eng.* **6**, 8836–8846 (2018).
15. Kollau, L. J. B. M. *et al.* Quantification of the liquid window of deep eutectic solvents †. *Chem. Commun* **54**, 13351 (2018).
16. Smink, D., Kersten, S. R. A. & Schuur, B. Recovery of lignin from deep eutectic solvents by liquid-liquid extraction. *Sep. Purif. Technol.* **235**, 116127 (2020).
17. Jessop, P. G., Mercer, S. M. & Heldebrant, D. J. CO2-triggered switchable solvents, surfactants, and other materials. *Energy Environ. Sci.* **5**, 7240–7253 (2012).
18. Sed, G., Cicci, A., Jessop, P. G. & Bravi, M. A novel switchable-hydrophilicity, natural deep eutectic solvent (NaDES)-based system for bio-safe biorefinery. *RSC Adv.* **8**, 37092–37097 (2018).
19. Florindo, C., Romero, L., Rintoul, I., Branco, L. C. & Marrucho, I. M. From Phase Change Materials to Green Solvents: Hydrophobic Low Viscous Fatty Acid-Based Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **6**, 3888–3895 (2018).
20. Florindo, C., McIntosh, A. J. S., Welton, T., Branco, L. C. & Marrucho, I. M. A closer look into deep eutectic solvents: Exploring intermolecular interactions using solvatochromic probes. *Phys. Chem. Chem. Phys.* **20**, 206–213 (2017).
21. Teles, A. R. R. *et al.* Solvatochromic parameters of deep eutectic solvents formed by ammonium-based salts and carboxylic acids. *Fluid Phase Equilib.* **448**, 15–21 (2017).
22. Abbott, A. P. *et al.* Glycerol eutectics as sustainable solvent systems. *Green Chem.* **13**, 82–90 (2011).
23. Li, X. & Row, K. H. Development of deep eutectic solvents applied in extraction and separation. *J. Sep. Sci.* **39**, 3505–3520 (2016).
24. Pandey, A. & Pandey, S. Solvatochromic Probe Behavior within Choline Chloride-Based Deep Eutectic Solvents: Effect of Temperature and Water. *J. Phys. Chem. B* **118**, 14652–14661 (2014).
25. Kim, S. *et al.* PubChem substance and compound databases. *Nucleic Acids Res.* **44**, D1202–D1213 (2016).

26. Tetko, I. V. *et al.* Virtual computational chemistry laboratory - Design and description. *J. Comput. Aided. Mol. Des.* **19**, 453–463 (2005).
27. Xu, J., Paimin, R., Shen, W. & Wang, X. An investigation of solubility of Aliquat 336 in different extracted solutions. *Fibers Polym.* **4**, 27–31 (2003).
28. Oka, H. & Kajimoto, O. UV absorption solvatochromic shift of 4-nitroaniline in supercritical water. *Phys. Chem. Chem. Phys.* **5**, 2535–2540 (2003).
29. Minami, K., Mizuta, M., Suzuki, M., Aizawa, T. & Arai, K. Determination of Kamlet-Taft solvent parameters p* of high pressure and supercritical water by the UV-Vis absorption spectral shift of 4-nitroanisole. *Phys. Chem. Chem. Phys.* **8**, 2257–2264 (2006).
30. Meng, X., Ballerat-Busserolles, K., Husson, P. & Andanson, J.-M. Impact of water on the melting temperature of urea + choline chloride deep eutectic solvent. *New J. Chem.* **40**, 4492–4499 (2016).
31. Smith, P. J., Arroyo, C. B., Lopez Hernandez, F. & Goeltz, J. C. Ternary Deep Eutectic Solvent Behavior of Water and Urea Choline Chloride Mixtures. *J. Phys. Chem. B* **123**, 5302–5306 (2019).
32. Florindo, C., Branco, L. C. & Marrucho, I. M. Development of hydrophobic deep eutectic solvents for extraction of pesticides from aqueous environments. *Fluid Phase Equilib.* **448**, 135–142 (2017).
33. Dai, Y., Witkamp, G.-J., Verpoorte, R. & Choi, Y. H. Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. *Food Chem.* **187**, 14–19 (2015).
34. Stefanovic, R., Ludwig, M., Webber, G. B., Atkin, R. & Page, A. J. Nanostructure, hydrogen bonding and rheology in choline chloride deep eutectic solvents as a function of the hydrogen bond donor. *Phys. Chem. Chem. Phys.* **19**, 3297–3306 (2017).
35. Anouti, M. *et al.* Aggregation behavior in water of new imidazolium and pyrrolidinium alkycarboxylates protic ionic liquids. *J. Colloid Interface Sci.* **340**, 104–111 (2009).
36. Winstein, S. & Lucas, H. J. The Coördination of Silver Ion with Unsaturated Compounds. *J. Am. Chem. Soc.* **60**, 836–847 (1938).
37. Wydro, P., Krajewska, B. & Hąc-Wydro, K. Chitosan as a lipid binder: A langmuir monolayer study of chitosan-lipid interactions. *Biomacromolecules* **8**, 2611–2617 (2007).
38. Aleem, A. M. *et al.* Probing the Electrostatic and Steric Requirements for Substrate Binding in Human Platelet-Type 12-Lipoxygenase. *Biochemistry*

58, 848–857 (2019).

39. Almeida, A. R. R. P. & Monte, M. J. S. Thermodynamic study of phase transitions of imidazoles and 1-methylimidazoles. *J. Chem. Thermodyn.* **44**, 163–168 (2012).
40. Ceondó. Chemical Properties of Hexanoic acid. Available at: https://www.chemeo.com/cid/49-496-8/Hexanoic acid. (Accessed: 8th November 2020)

Supplementary Information

Phase diagram of imidazole/hexanoic acid

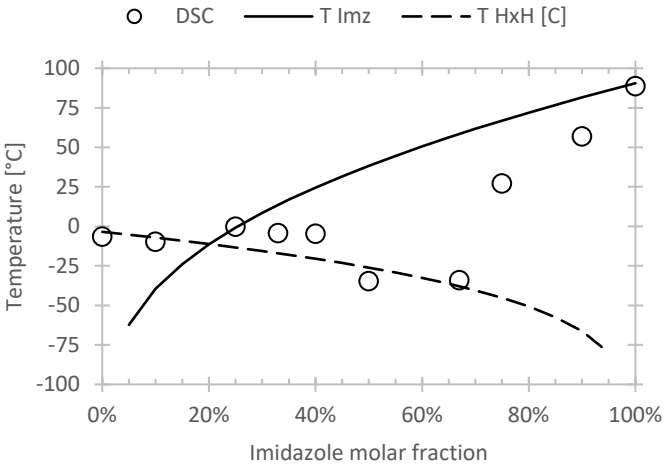


Fig. S1. Phase diagram of imidazole/hexanoic acid eutectic solvent. The circles represent the DSC onset temperatures; the continued and dashed line represent the model of ideal eutectic behavior of imidazole (Imz, continuous line) and hexanoic acid (HxH, dashed line), respectively.

The melting temperatures were the onset temperatures measured by differential scanning calorimeter (Perkin-Elmer DSC 8000 series). The temperature profile was as follow: 1) fast heating from 20 °C to 90 °C (100 °C /min), 2) hold at 90 °C for 10 min, 3) fast cooling from 90 °C to -90 °C (100 °C /min), 4) scanning from -90 °C to 90 °C (10 °C /min), 5) fast cooling from 90 °C to -90 °C (100 °C /min), 6) scanning from -90 °C to 90 °C (10 °C /min), 7) fast cooling to 20 °C.

The ideal eutectic behavior was calculated with equation:

$$\ln(x_i) = \frac{\Delta_m H}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)$$

where x_i is the liquid molar fraction of compound i, T is the apparent temperature [K], T_m and $\Delta_m H$ are the melting temperature and enthalpy of

the pure compound i, respectively, and R is the universal gas constant. The values of T_m and $\Delta_m H$ for imidazole are 363.7 K and 12.5 kJ/mol, respectively[39]; and those for hexanoic acid are 269.7 K and 16.98 kJ/mol, respectively[40].

Fatty acid composition of sunflower oil and algae oil

Table S1. The fatty acid profile of the model oils used in this study

Fatty Acid [%]	Sunflower oil	Algae oil
C12:0	0.00%	0.14%
C13:0	0.00%	0.00%
C14:0	0.10%	0.47%
C14:1 cis-9	0.00%	0.00%
C16:0	6.85%	1.77%
C16:1	0.17%	0.30%
C16:2	0.00%	0.00%
C16:3	0.00%	0.00%
C17:0	0.00%	0.00%
C18:0	3.16%	0.99%
C18:1	30.70%	93.35%
C18:2	57.86%	1.69%
C18:3	0.00%	0.00%
C19:0	0.09%	0.19%
C20:0	0.24%	0.04%
C20:1	0.17%	1.07%
C20:2	0.00%	0.00%
C20:3	0.00%	0.00%
C20:4	0.00%	0.00%
C20:5	0.00%	0.00%
C22:0	0.65%	0.00%
C22:1	0.00%	0.00%
C24:0	0.00%	0.00%
C22:6	0.00%	0.00%

300
200

Chapter 3

Lipid recovery from semi-hydrophobic eutectic solvents by polar antisolvents

This chapter has been submitted as:

Lo, C., Wijffels, R. H. & Eppink, M. H. M. Lipid recovery from semi-hydrophobic eutectic solvents by polar antisolvents.

Abstract

Due to the low volatility of eutectic solvents (ES), the recovery of lipids is difficult. We developed a novel strategy to separate the dissolved sunflower and algae oil from imidazole/hexanoic acid ES by using polar antisolvents (water, methanol, and ethanol). The polarity and the amount of antisolvent influenced the lipid solubility in ES. While water was the strongest antisolvent, the alcohols were easier to evaporate, ensuring easy ES regeneration. By adding small amounts of water and methanol, more than 90% of the lipids were recovered in the form of high purity oils (>90%). In the case of ethanol, a large amount of methanol was required, which diluted the solvent-rich phase and solubilized more lipids in it. Based on three repeated cycles, > 90% of the eutectic solvent could be regenerated.

1. Introduction

For the common extraction of lipids from microalgae, energy-intensive cell disruption methods and unsustainable solvents are used.[1–3] Recently, a greener alternative of ionic liquid termed as eutectic solvents (ES, including "deep eutectic solvents") have been applied. ES have the capability to induce pores or cracks in the algae cell wall, allowing the lipid extraction without mechanical pre-treatment.[4] However, the used ES were hydrophilic, which are not suitable for lipid extraction. In theory, with hydrophobic ES, it might be possible to crack the cell wall and extract the lipids simultaneously. This would simplify the extraction method and benefit the process economy. However, unlike the organic molecular solvents, ES suffer from low volatility, suggesting the challenging separation of lipids from ES.[5] Therefore, to avoid the complicated separation further downstream, the ES should not be completely hydrophobic.

In our previous work, we screened semi-hydrophobic ES and developed a lipid recovery strategy by tuning the solvent polarity.[6] The ES with different ratios imidazole and hexanoic acid could dissolve water (hydrophilic) or lipids (hydrophobic). At high imidazole content, the solvent became hydrophilic and rejected the dissolved lipids. However, this strategy is unsuitable for ES regeneration since imidazole is strongly bound to hexanoic acid.[7] Therefore, another strategy is needed for regeneration.

Antisolvents have been widely used to separate the target product from polar ES. For example, the addition of water, alcohols, or acetone induced the precipitation of phenolic compounds, lignin, cellulose, and insoluble protein from ES.[8–15] Typically, the target solute has a low, if not negligible, solubility in the antisolvents, whereas the ES has a strong affinity towards the antisolvent. Furthermore, the antisolvent can then be evaporated to regenerate the ES. In general, the product recovery is directly proportional to the amount of antisolvent. However, it is worth noting that the antisolvent amount directly correlates to the energy required for antisolvent removal.

It is then hypothesized that polar antisolvents could promote the hydrophilicity of imidazole/hexanoic acid ES, consequently lowering the lipid solubility. Furthermore, if the antisolvents have relatively high vapor pressure, ES could also be easily regenerated. To the best of our knowledge, the use of ES and antisolvents in the lipid extraction process is still limited.

This study aims to assess the feasibility of using antisolvents for recovery of dissolved lipids and solvent regeneration. Water, methanol, and ethanol were used to separate sunflower and algae oil from imidazole/hexanoic acid ES. Afterwards, the ES was regenerated by evaporating the antisolvents before re-used in three consecutive cycles. Based on the compositional analysis, the antisolvent performance and the loss throughout the process were evaluated.

2. Materials and Methods

2.1. Materials

The materials which were used in this study were algae oil (Corbion Biotech, Inc., United States), ethanol (Merck Millipore, absolute), hexanoic acid (Sigma-Aldrich, ≥ 99%), imidazole (Sigma-Aldrich, ≥ 99%), methanol (Merck Millipore, pure), water (Milli-Q®, ultrapure), and sunflower oil (Jumbo Supermarkten BV, The Netherlands).

2.2. Preparation of the eutectic solvent

In this study, the combination imidazole/hexanoic acid (molar ratio 1:3) was used to represent the semi-hydrophobic ES. The ES 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 dissolution in eutectic solvent and recovery using antisolvents

Sunflower and algae oil were used as model lipids in this study. To measure the effect of antisolvents, the lipid solubility was measured *via* cloud point determination. This was performed by a continuous addition of the oils to the ES which contained various concentration of water, methanol, and ethanol. The experiment was performed at room temperature and stopped as soon as the solution turned cloudy.

Besides that, the lipid recovery by addition of antisolvent was also performed. For this experiment, the initial ES solution contained sunflower oil (38 wt%) or algae oil (31 wt%). Then, various amount of antisolvents were added to the solutions at room temperature, which induced a phase split between the recovered lipid and the solvent-rich phase. The mixtures were centrifuged at 4000 rcf before separated and stored for further analysis.

2.4. Evaporation of antisolvents and eutectic solvent regeneration

The antisolvents were evaporated from the solvent-rich phases using a rotational vacuum concentrator at 8 mbar, 35 °C. The low evaporation temperature was chosen to minimize the risk of undesired side reactions, such as esterification. Unless stated, the evaporation occurred ~16 hours for the alcohols-containing mixtures and 24 hours for water mixtures.

2.5. Gas chromatography analysis

All the analyses were performed using gas chromatography-flame ionization detector (GC-FID) system (Agilent Technologies) with H_2 as a carrier gas. The fatty acid analysis was performed based on the method described in our previous work.[6] For analyses of methanol, ethanol, imidazole, and hexanoic acid, the solvent-rich phases were diluted 10x in chloroform and run with DB-FFAP column (part #122-3232, Agilent Technologies) with a split-ratio of 20:1 and a split-flow of 45.27 mL/min. The oven temperature profile was 45 °C to 80 °C with 6.30 °C/min, and to 220 °C with 26.24 °C/min and held for 3 minutes.

2.6. Partition coefficient and separation efficiency

The partition coefficient K and separation efficiency of the lipid recovery were calculated by using these equations, respectively:

$$K = c_O / c_S$$

$$\text{Efficiency} = \frac{V_O\, c_O}{V_O\, c_O + V_S\, c_S}$$

where V is the total amount of the phase in gram, c is the total fatty acids concentration in mg/mg, subscript O and S denote the oil-rich and solvent-rich phase, respectively.

3. Results and Discussions

Initially, we evaluated sunflower and algae oil solubility in the eutectic solvent containing different concentrations of water, methanol, and ethanol. Next, the dissolved lipids were recovered from ES mixtures with varying amount of antisolvents. Furthermore, the antisolvents were evaporated from the solvent mixtures to mimic the ES regeneration step. Finally, three repeated cycles of dissolution of sunflower oil, recovery and solvent regeneration were performed to analyze the mass composition and to identify potential losses.

3.1. Reduced lipids solubility in the eutectic solvent containing antisolvents

To enable the lipid separation from the solvent, the addition of antisolvents should decrease the lipid solubility in the imidazole/hexanoic acid (1:3) ES. Both the concentration and the polarity of antisolvents influenced the solubility of sunflower and algae oil (Fig. 1). In general, lipids were less soluble at higher antisolvent concentrations regardless the antisolvent. Water caused the most substantial solubility reduction, followed by methanol and ethanol. It is worth to note that water has limited solubility in the eutectic solvent (~0.4 g/g eutectic solvent), whereas the alcohols are completely miscible with the solvent. Furthermore, it was known before that the algae oil has a lower solubility in ES than sunflower oil. It is due to the different degree of fatty acid saturation in the lipids, where algae oil contains > 90% C18:1 fatty acid and sunflower oil is rich in C18:2 (> 56%).[6]

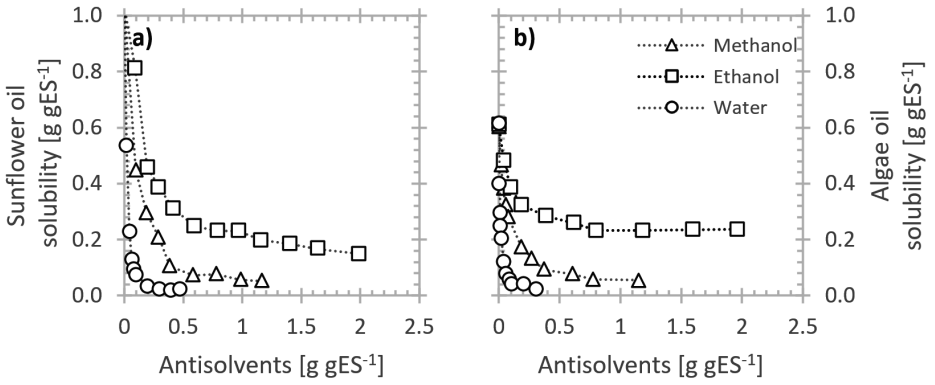


Fig. 1. Solubility of sunflower oil (a) and algae oil (b) in the eutectic solvent with various concentrations of water (circle), methanol (triangle), and ethanol (square).

3.2. Lipid recovery from eutectic solvent extract using antisolvent

The pre-dissolved sunflower and algae oil (initial concentration: 38 and 34 w%, respectively) were separated from the ES by adding various amounts of antisolvents. This led to oversaturation and eventually induced phase split between the oil-rich phase and the solvent mixture. The formation of phases was further accelerated by centrifugation. Fig. 2 provides the visual observation of mixtures treated with water and methanol. The increasing amount of water and methanol are ordered from left to right in the pictures. The location of oil-rich phase depends on the density of the formed phases (ρ of ES, algae and sunflower oil are 0.98, 0.90 and 0.92 g mL^{-1}, respectively). The water/ES mixtures were constantly denser than the oil-rich phase, whereas density of alcohol/ES mixture depends on the alcohols' concentration. Since the alcohols (ρ ≅ 0.79 g mL^{-1}) are much lighter than the lipids, the alcohol-rich mixtures are lighter than the oils.

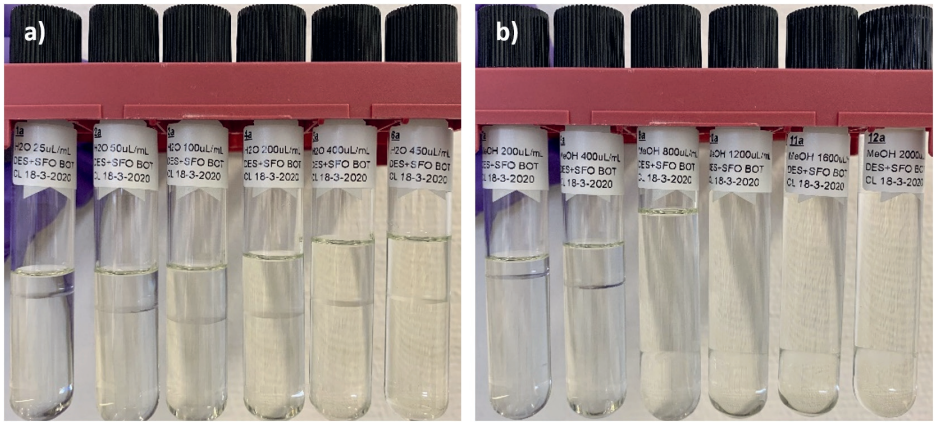


Fig. 2. Phase separation between the recovered oil and solvent mixtures by adding (a) water and (b) methanol, increasing concentrations ordered left to right. While water/ES mixtures were consistently denser than the lipids, the concentration of methanol determined the density of the solvent mixtures. Methanol-rich mixtures were lighter than the lipids, whereas the mixtures with low methanol concentrations were heavier.

Each phase was then analyzed to quantify the partition of the model oils at equilibrium, K (Fig. 3a). A high partition coefficient means that the lipid is less likely to be dissolved in the ES-rich phase, leading to a better separation. The

partition coefficients linearly correlate to the amount of antisolvent added. Moreover, the polarity of antisolvent also determines the sensitivity of K towards the antisolvent amount (shown by the slope). For both model lipids, water achieved the highest K with the steepest slope, closely followed by methanol. As mentioned before, methanol is more soluble than water in ES. This might imply that higher K values could be achieved at higher methanol concentrations, whereas that from water reached the maximum considering the limited solubility of water in ES. Moreover, ethanol, as expected, did not give good partition since it did not induce enough polarity to reduce the lipid solubility in the solvent phase. Besides that, K values of algae oil are higher than that of sunflower oil. This implies that at the same antisolvent concentration, the solvent phase dissolved more sunflower oil than algae oil. This result is consistent with the antisolvent effect which was discussed above.

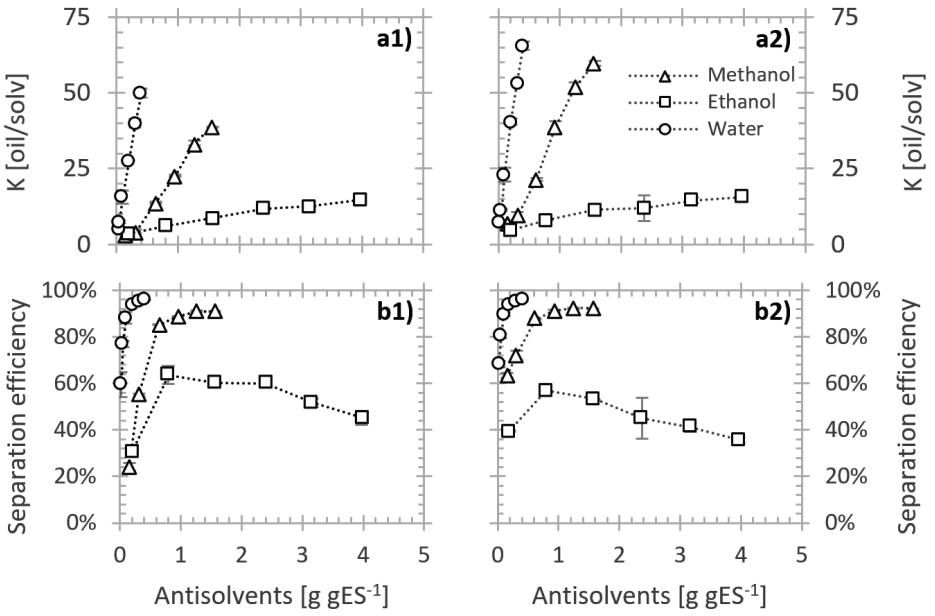


Fig. 3. a) Partition coefficient K and b) separation efficiency of sunflower oil (1) and algae oil (2) recovered by addition of water (circle), methanol (triangle), and ethanol (square).

Furthermore, from the fatty acid analysis, the separation efficiencies in the lipid recovery step were calculated (Fig. 3b). The separation efficiency steeply increases with amount of water and methanol until reaching about ~90% recovery, then slowly increases until maximum was reached. As expected, water gave the highest recovery for both model oils (efficiency of 96%), while methanol reached the efficiency of ≥ 90%. Ethanol, however, performed poorly at higher concentrations with the highest obtained efficiency of ~60%. Unlike water and methanol, a much larger amount of ethanol was added to reduce the lipid solubility, thus creating a dilution effect. The addition of ethanol significantly increased the volume of solvent-rich phase, which consequently increased the absolute amount of lipids dissolved in that phase.

Besides that, the purity of recovered oil was also evaluated (Fig. 4). Overall, the purity the recovered oil was high, with the recovered algae oil being slightly higher than the sunflower oil. While methanol reached the highest purity, ~100%, water and ethanol yielded a lower purity, 80 – 90%. The contradicting nature of polar antisolvents and hydrophobic hexanoic acid influenced the distribution of hexanoic acid. Water, being more polar than methanol, caused extra leaching of hexanoic acid to the oil phase and ultimately lower the oil purity. However, in the case of ethanol, ethanol itself is also a contaminant in the oil phase. This might be due to the lower polarity of ethanol and its large presence in the overall system, causing distribution of ethanol over the phases. A quantitative analysis on the oil-rich phase can be found in Section 3.4. Recycle of the eutectic solvent.

The presence of the contaminants may decrease the quality of the recovered oil. The alcohols can easily be removed by evaporation. The presence of hexanoic acid in the oil is less desired since it would give rancid, unpleasant odor and increase the free fatty acid (FFA) content of the oil. High FFA lipids are associated with lower food quality[16], health issues when consumed[17] and lead to saponification problem in the biodiesel synthesis.[18,19] Therefore, lipid deodorization and deacidification should be performed in the further downstream steps.

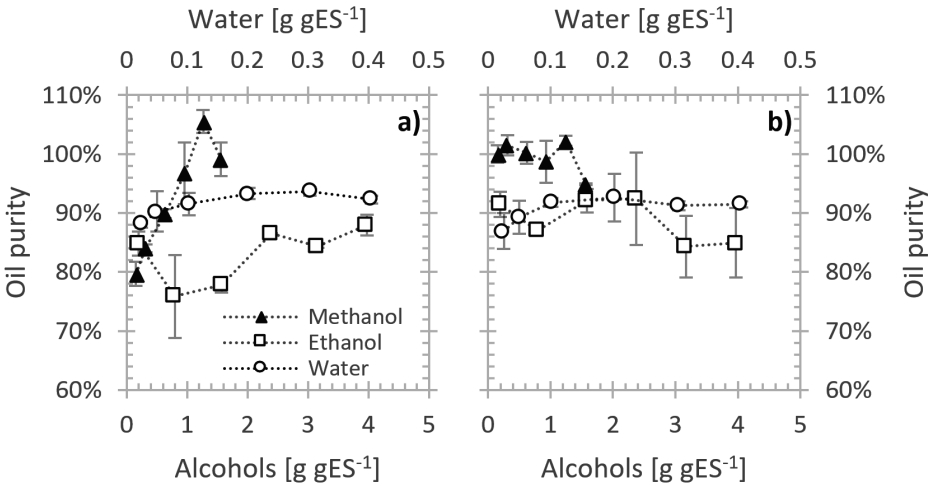


Fig. 4. Purity of recovered sunflower oil (a) and algae oil (b) with different antisolvents. Mind the different scale of water and alcohols concentrations.

3.3. Eutectic solvent regeneration via antisolvent evaporation

The ease of eutectic solvent regeneration after the lipid separation step was evaluated based on the evaporation kinetics. In this experiment, we performed the evaporation of antisolvents at two different concentrations to mimic the possible mixtures after the recovery step at 8 mbar and 35 °C. This low temperature was chosen to avoid not only the evaporation of imidazole and hexanoic acid, but the undesired esterification of both hexanoic acid and the alcohols as well. This was also confirmed by gas chromatography analysis.

Fig. 5 shows the evaporation rate of water, methanol, and ethanol in the ES solutions. The evaporation rate of the antisolvents follow the volatility of the pure compounds, i.e., methanol and ethanol have much higher vapor pressures than water (276, 135, and 42 mbar at 35 °C, respectively).[20] Additionally, the low water concentration in the solution and the possibility of strong interactions between water molecules and eutectic solvent components could further hamper the volatility of water, resulting in problematic regeneration. Therefore, to ensure the easy regeneration step, the use of methanol or ethanol is favorable. Furthermore, unlike the case with water, complete removal of the alcohols is not necessary since the lipid

solubility is less sensitive towards the presence of the alcohols than water (Fig. 1).

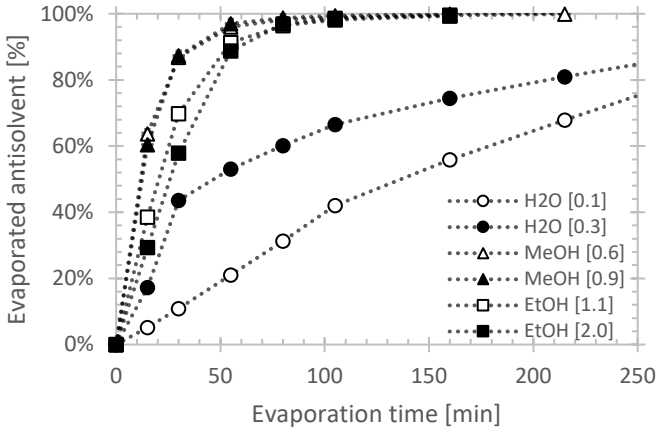


Fig. 5. Evaporation kinetics of water (circle), methanol (triangle), and ethanol (square) when mixed with imidazole/hexanoic acid (1:3) in low (open) and high (closed) concentration. The exact concentrations of antisolvents (in g g_{ES}^{-1}) are written inside brackets. 95% of water was removed after 600 minutes.

3.4. Recycle of the eutectic solvent

The final objective of this study is to investigate the feasibility of recycling the eutectic solvent. In this experiment, we performed three repeated cycles of lipid dissolution, recovery, and solvent regeneration using sunflower oil (Fig. 6a). This oil was chosen to simulate the worst-case scenario with lower recovery since the ES has a higher affinity to sunflower oil than algae oil. In each cycle, the extract was maintained to contain 38 w% of oil in the eutectic solvent. Then, the lipid recovery was performed by adding methanol to reach 0.96 g/g eutectic solvent. This concentration was chosen for its high separation efficiency with the least methanol amount. The mass composition of different phase throughout the entire process can be seen in Table 1. Throughout the cycles, no significant change in performance was observed. For example, the remaining unrecovered sunflower oil in the solvent-rich phase was consistent at ~5%.

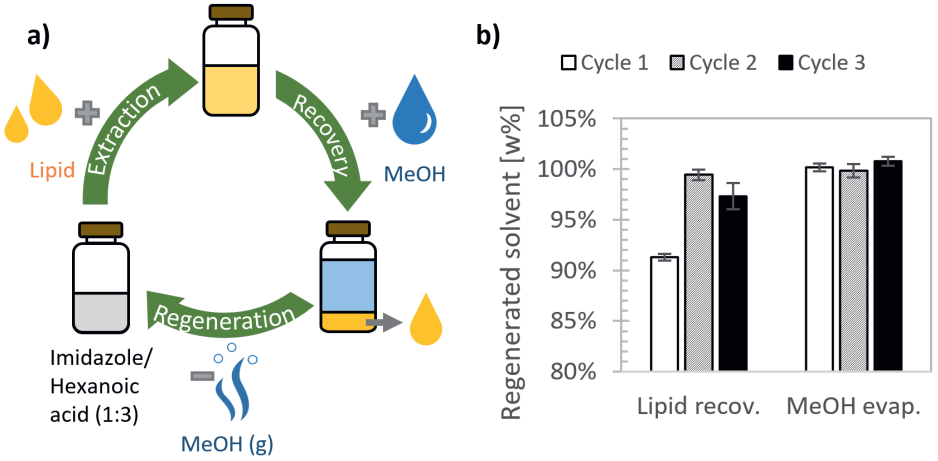


Fig. 6. a) Schematic process of the extraction cycle using imidazole/hexanoic acid ES, b) Fraction of eutectic solvent regenerated in each process step in three cycles (cycle 1 – white, cycle 2 – striped, cycle 3 – black).

Furthermore, a loss of hexanoic acid was observed after each step, reflected by the increased molar ratio of imidazole to hexanoic acid (Table 1). It started from 0.336 towards 0.408 $mol_{imidazole}/mol_{hexanoic\ acid}$, which is equivalent to (1:3) and (2:5) molar composition, respectively. The losses occurred mainly during the lipid recovery (phase split) step, at which a substantial fraction of hexanoic acid migrated to the oil-rich phase. Whereas during the evaporation step, a little amount of hexanoic acid evaporated despite the negligible vapor pressure, which could be due to the prolonged evaporation time (16 h instead of 3 h based on Fig. 5). The rising trend of imidazole molar ratio indicates the declining hydrophobicity of the eutectic solvent, hence less capacity of lipid dissolution.[6] To overcome this shortness, fresh hexanoic acid needs to be fed as make-up for the loss. Furthermore, the loss can be minimized by shortening the evaporation time and optimizing the condition (e.g., temperature) during the lipid recovery step to reduce the migration of hexanoic acid further. Despite the loss, > 90% eutectic solvent was regenerated after each step based on the mass balance (Fig. 6b).

Table 1. Relative composition of each phase (recovered oil, solvent-rich phase, and regenerated ES) throughout the three extraction cycles

Run	Phase	Hexanoic acid [w%]	Imidazole [w%]	Methanol [w%]	Sunflower oil [w%]	$mol_{Imid.}$ / $mol_{Hex.ac.}$
0	ES + Oil	52.1 ± 0.7	10.3 ± 0.1	0.0 ± 0.0	38.9 ± 1.7	0.336
1	Recov. oil	5.5 ± 0.1	0.2 ± 0.0	3.4 ± 0.2	93.7 ± 2.8	0.071
	Solvent-rich	41.0 ± 0.4	8.4 ± 0.1	47.6 ± 0.5	5.4 ± 0.1	0.350
	Regen. ES	75.7 ± 0.3	15.7 ± 0.2	0 .0 ± 0.0	9.7 ± 0.2	0.353
2	Recov. oil	7.3 ± 0.0	0.5 ± 0.0	5.4 ± 0.1	93.1 ± 2.7	0.121
	Solvent-rich	40.3 ± 0.0	8.7 ± 0.0	49.4 ± 2.6	4.8 ± 0.3	0.369
	Regen. ES	75.3 ± 0.9	16.4 ± 0.1	0.0 ± 0.0	9.5 ± 0.3	0.372
3	Recov. oil	6.1 ± 0.2	0.4 ± 0.1	6.2 ± 1.2	100.9 ± 0.2	0.114
	Solvent-rich	37.6 ± 0.4	8.8 ± 0.2	46.8 ± 0.0	5.4 ± 0.7	0.401
	Regen. ES	66.7 ± 0.6	15.9 ± 0.0	0.0 ± 0.9	11.1 ± 1.0	0.408

4. Conclusion

The presence of the antisolvents water, methanol, and ethanol in imidazole/hexanoic acid ES reduced the solubility of model lipids. The solubility reduction positively correlated with the antisolvent polarity and amount. The antisolvents were then applied to recover the dissolved sunflower and algae oil in the ES, resulting in high purity oils with > 90% recovery. Furthermore, the eutectic solvent can be regenerated via antisolvent evaporation. It was observed that there was a small loss of hexanoic acid, which mainly occurred during the phase split. Nevertheless, with this approach, > 90% of the solvent can be regenerated.

References

1. Halim, R., Danquah, M. K. & Webley, P. A. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnol. Adv.* **30**, 709–732 (2012).
2. Kumar, J., Garlapati, V. K., Dash, A., Scholz, P. & Banerjee, R. Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Res.* **21**, 138–147 (2017).
3. Lee, S. Y., Cho, J. M., Chang, Y. K. & Oh, Y.-K. K. Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresour. Technol.* **244**, 1317–1328 (2017).
4. Lu, W. *et al.* A new approach of microalgal biomass pretreatment using deep eutectic solvents for enhanced lipid recovery for biodiesel production. *Bioresour. Technol.* **218**, 123–128 (2016).
5. Ruesgas-Ramón, M., Figueroa-Espinoza, M. C. & Durand, E. Application of Deep Eutectic Solvents (DES) for Phenolic Compounds Extraction: Overview, Challenges, and Opportunities. *J. Agric. Food Chem.* **65**, 3591–3601 (2017).
6. Lo, C., Semerel, J., van den Berg, C., Wijffels, R. H. & Eppink, M. H. M. Eutectic solvents with tuneable hydrophobicity: lipid dissolution and recovery. *RSC Adv.* **11**, 8142–8149 (2021).
7. Anouti, M. *et al.* Aggregation behavior in water of new imidazolium and pyrrolidinium alkycarboxylates protic ionic liquids. *J. Colloid Interface Sci.* **340**, 104–111 (2009).
8. Maugeri, Z. & Domínguez De María, P. Novel choline-chloride-based deep-eutectic-solvents with renewable hydrogen bond donors: levulinic acid and sugar-based polyols. *RSC Adv.* **2**, 421–425 (2012).
9. Procentese, A. *et al.* Deep eutectic solvent pretreatment and subsequent saccharification of corncob. *Bioresour. Technol.* **192**, 31–36 (2015).
10. Grudniewska, A. *et al.* Enhanced Protein Extraction from Oilseed Cakes Using Glycerol-Choline Chloride Deep Eutectic Solvents: A Biorefinery Approach. *ACS Sustain. Chem. Eng.* **6**, 15791–15800 (2018).
11. Chen, Z. & Wan, C. Ultrafast fractionation of lignocellulosic biomass by microwave-assisted deep eutectic solvent pretreatment. *Bioresour. Technol.* **250**, 532–537 (2018).
12. Smink, D., Kersten, S. R. A. & Schuur, B. Recovery of lignin from deep eutectic solvents by liquid-liquid extraction. *Sep. Purif. Technol.* **235**,

116127 (2020).

13. Grudniewska, A. & Popłoński, J. Simple and green method for the extraction of xanthohumol from spent hops using deep eutectic solvents. *Sep. Purif. Technol.* **250**, 117196 (2020).
14. Kumar, A. K., Parikh, B. S. & Pravakar, M. Natural deep eutectic solvent mediated pretreatment of rice straw: bioanalytical characterization of lignin extract and enzymatic hydrolysis of pretreated biomass residue. *Environ. Sci. Pollut. Res.* **23**, 9265–9275 (2016).
15. Das, A. K., Sharma, M., Mondal, D. & Prasad, K. Deep eutectic solvents as efficient solvent system for the extraction of K-carrageenan from Kappaphycus alvarezii. *Carbohydr. Polym.* **136**, 930–935 (2016).
16. Osawa, C. C., Gonçalves, L. A. G. & Ragazzi, S. Correlation between free fatty acids of vegetable oils evaluated by rapid tests and by the official method. *J. Food Compos. Anal.* **20**, 523–528 (2007).
17. Lee Kuek, S. *et al.* Contribution of lipid towards acrylamide formation during intermittent frying of French fries. *Food Control* **118**, 107430 (2020).
18. Đokić, M., Kesić, Ž., Krstić, J., Jovanović, D. & Skala, D. Decrease of free fatty acid content in vegetable oil using silica supported ferric sulfate catalyst. *Fuel* **97**, 595–602 (2012).
19. Zhang, T., Shahbaz, K. & Farid, M. M. Glycerolysis of free fatty acid in vegetable oil deodorizer distillate catalyzed by phosphonium-based deep eutectic solvent. *Renew. Energy* **160**, 363–373 (2020).
20. Dortmund Databank. Saturated Vapor Pressure. (2020). Available at: http://ddbonline.ddbst.com/AntoineCalculation/AntoineCalculationCGI.exe.


NEG
EG_CL
EG_ 8-4-2019
POS
EG_CL
8-4-2019
Imz-HxA
EG_CL
EG_ 8-4-2019
Imz-HxA
EG_CL
EG_ 8-4-2019
ChCl-Ac.A
EG_CL
EG_ 8-4-2019
ChCl-U
EG_CL
8-4-2019
Chcl-EG
EG_CL
EG_ 8-4-2019

Chapter 4

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

This chapter has been submitted as:

Lo, C., Wijffels, R. H. & Eppink, M. H. M. Lipid extraction from fresh Nannochloropsis oceanica using semi-hydrophobic eutectic solvents.

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, have been applied on microalgae for extraction of lipids. However, in extractions used so far an additional hydrophobic solvent was still needed due to the hydrophilicity of the ES. In this work, we performed wet and dry extraction of lipids from intact *Nannochloropsis oceanica* in a single-solvent step using ES with tuneable hydrophobicity by the combination of imidazole and hexanoic acid. A total fatty acid yield, comparable to the traditional chloroform/methanol method, was obtained without cell-disruption pre-treatment. The effect of process parameters on extraction yield, such as ES composition, temperature, extraction time, solvent/biomass ratio, and water content, were investigated. Interestingly, the wet biomass gave higher extraction yields than the dry biomass. With wet biomass, > 80% lipids were extracted by the ES imidazole/hexanoic acid (15:85) at 50 °C within 2 hours. Whereas, the extraction yield of dry biomass was lower, reaching only 65% even after 12 hours under the same condition. Supplementation of water to 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 directly be applied on wet biomass.

1. Introduction

The current lipid extraction process from microalgae involve 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.[1–3] Furthermore, harmful organic solvents, such as hexane and chloroform, are often used during the actual extraction step.[4,5]

Alternative solvent-based technologies have been proposed to improve the lipid extraction from microalgae. Supercritical fluid extraction (SFE) and the use of bio-derived solvents, such as terpenes and dimethyl ether, can replace the use of harmful solvents.[2,4,6] However, SFE operations need a complex setup for the high pressure, and the bio-derived solvents are not yet largely available. Additionally, ionic liquids have been applied for their capability of weakening and permeabilizing the cell wall. Nevertheless, ionic liquids are associated with toxicity and high cost due to the complex synthesis and purification.[4,7–11]

Eutectic solvents (ES) are often considered as alternatives for ionic liquids as they exhibit similar benefits.[4,12,13] 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.[14] For example, Abbott et al.[15] discovered that choline chloride and urea (mp: 302 and 132 °C, consecutively) could stay liquid when mixed with a 1:2 molar ratio. Furthermore, ES can be prepared from various compounds, including amino acids, sugars and polyols, and carboxylic acids.[16–19]

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., resulting in a 1.5-fold lipid yield compared to untreated biomass.[20] Furthermore, a combination of ES and microwave improved the extraction speed and final yield in *Phaeodactylum tricornutum*.[21] 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.[22] On the other hand, the use of a switchable ES system as extraction solvent on disrupted *Scenedesmus dimorphus* was also reported.[23] 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.[23]

Here we aim to integrate pretreatment and extraction using a single solvent of semi-hydrophobic ES. In our recent study, the ES composed of imidazole/hexanoic acid was shown to exhibit tailorable hydrophobicity, i.e., dissolves both water (hydrophilic) and sunflower oil (hydrophobic).[24] The tailorable hydrophobicity is important since the lipids and ES cannot be separated by the solvent evaporation as ES lack of vapor pressure.[13] 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.[25–27] 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.[28–30] Therefore, the avoidance of cell disruption would be beneficial for the processing of this microalga. 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* (culture 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 μmol_{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 for a maximum of 10 days prior to the extraction. The removal of moisture for preparation of dried biomass and quantification of dry weight was performed by freeze-drying the harvested algal paste.

2.2. Materials and ES preparation

The materials used in this study were hexanoic acid (Sigma-Aldrich, ≥ 99%), imidazole (Sigma-Aldrich, ≥ 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.

2.3. Lipid extraction from microalgae paste and analysis

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. For fatty acid analysis of ES extract, the samples (50 µL) were methylated and run on the gas chromatography with a flame ionization detector (GC-FID) system following the method described in our previous work.[24] As for the control, the total FA from biomass was extracted using

chloroform/methanol (4:5 v/v) and analysed by following the protocol described by Remmers et al.[31]

2.4. Extraction efficiency

Extraction efficiency (EE) was calculated using this formula:

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

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 biomass (~7/3 g g_{dw}^{-1}) contributed to the final solvent volume as water is soluble in the used ES.[24] The correction factor f was used to take the water miscibility into account. Here we assumed that the water/ES miscibility depended on imidazole content (solvent hydrophobicity) and S/B ratio (Table 1). If the presence of water exceeded the water solubility, it formed a 3rd bottom phase and did not participate in the extract phase.

Table 1. Solubility of water in ES and the correction factor f used in this study

Imidazole [mol%]	S/B [mL g_{DW}^{-1}]	5	10	20	35	50	Water solubility [g g_{ES}^{-1}] [24]
0	f	1.1	1.1	1.1	1.1	1.0	0.1
15		1.3	1.2	1.1	1.1	1.0	0.3
25		-	1.2	-	-	-	0.5
35		-	1.2	-	-	-	> 1
50		-	1.2	-	-	-	> 1

3. Results & Discussion

An outline of the results and discussion is given below. Initially, the fatty acid content of the cultivated biomass was analyzed 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.

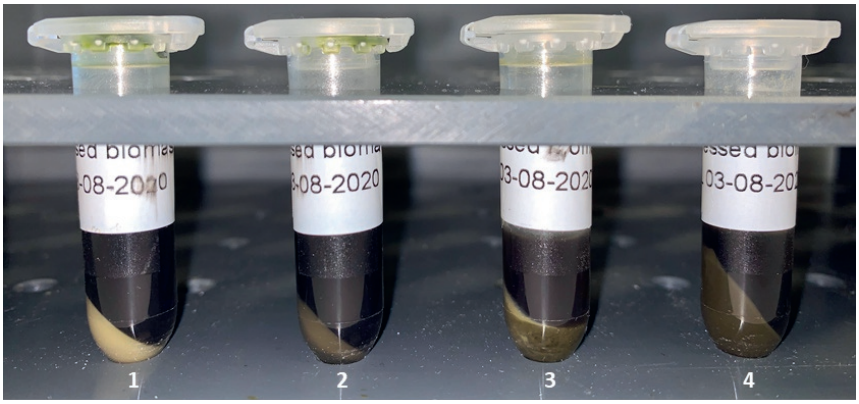


Fig. 1. Direct extraction of lipid from intact microalga *Nannochloropsis oceanica* with different conditions: hexanoic acid on wet (1) and dried biomass (3), and imidazole/hexanoic acid (15:85 mol/mol) on wet (2) and dried biomass (4). The extraction was performed at 50 °C.

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 2. Batch 1 biomass contained a higher amount of lipid than Batch 2 biomass due to the prolonged nitrogen-starved 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 2. Fatty acid profile 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	Sum	PL fraction	NL fraction	Sum
C12:0	0.0%	0.1%	0.1%	0.0%	0.1%	0.1%
C13:0	0.1%	0.0%	0.1%	0.1%	0.0%	0.1%
C14:0	0.4%	1.4%	1.7%	0.7%	1.1%	1.7%
C14:1 cis-9	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C15:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C16:0	1.6%	14.1%	15.6%	2.2%	5.6%	7.8%
C16:1	0.9%	9.0%	9.9%	1.7%	5.7%	7.4%
C17:0	0.0%	0.1%	0.1%	0.0%	0.1%	0.1%
C16:3	0.0%	0.1%	0.1%	0.0%	0.1%	0.1%
C18:0	0.0%	0.3%	0.3%	0.0%	0.2%	0.2%
C18:1	0.2%	6.1%	6.3%	0.7%	4.7%	5.5%
C18:2	0.0%	0.2%	0.2%	0.1%	0.2%	0.4%
C18:3	0.1%	0.0%	0.1%	0.0%	0.1%	0.1%
C20:4	0.4%	0.6%	1.1%	0.7%	0.5%	1.3%
C20:5	1.6%	1.1%	2.6%	1.4%	0.6%	2.0%
Total FA	**5.2%**	**33.1%**	**38.3%**	**7.7%**	**19.1%**	**26.8%**

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 hydrophobicity of imidazole/hexanoic acid ES depends on the ES composition.[24] 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. 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 destabilizing the cell wall.[32] Furthermore, Medronho and coworkers[33] suggested that the presence of amphiphilic compounds could solvate cellulose, increasing the aqueous solubility and permeability of cellulose.[33]

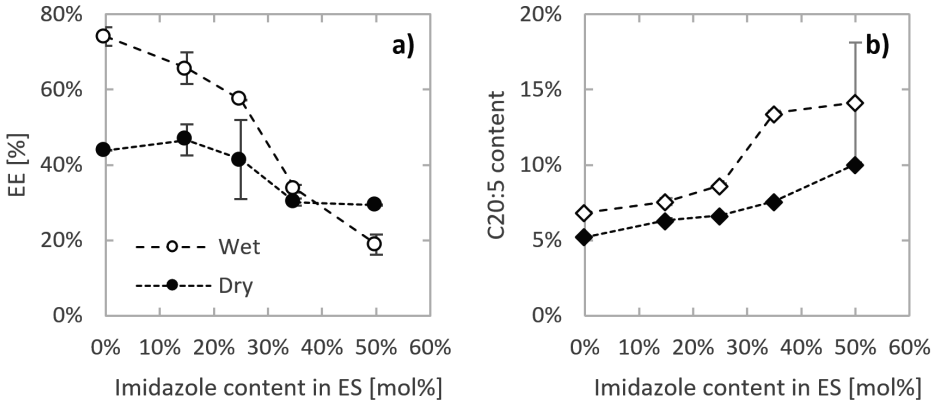


Fig. 2. a) EE from wet (open) and dry (filled) stressed *N. oceanica* Batch 1 using ES with different imidazole content at 35 °C. b) Weight fraction of C20:5 (EPA) extracted from wet (open) and dry (filled) biomass with the same condition as above.

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, it is expected that imidazole content also influences the selectivity of unsaturated fatty acid. It is known that unsaturated bonds can form π-π interaction with aromatic compounds, including imidazole.[34] In our previous work, this ES was found to dissolve more sunflower oil (rich in C18:2) than culinary algae oil (rich in C18:1).[24] Fig. 2b shows the distribution of FA C20:5 (eicosapentaenoic acid, EPA) in the different extracts. As expected, C20:5 content increased, reaching up to 2.5- and 1.5-fold for wet and dry extraction,

respectively. This knowledge can be applied to produce lipid fraction with an enriched content of omega-3 FA since this type of FA (C20:5) 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 favorable 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 were used to extract biomass from Batch 1 at different temperatures (Fig. 3a). For the wet extraction, the yield increased with higher temperature for both solvents, reaching $\mathrm{EE} \cong 100\%$. For the dry extraction, as expected that ES with 15 mol% imidazole gave higher EE than hexanoic acid. 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.

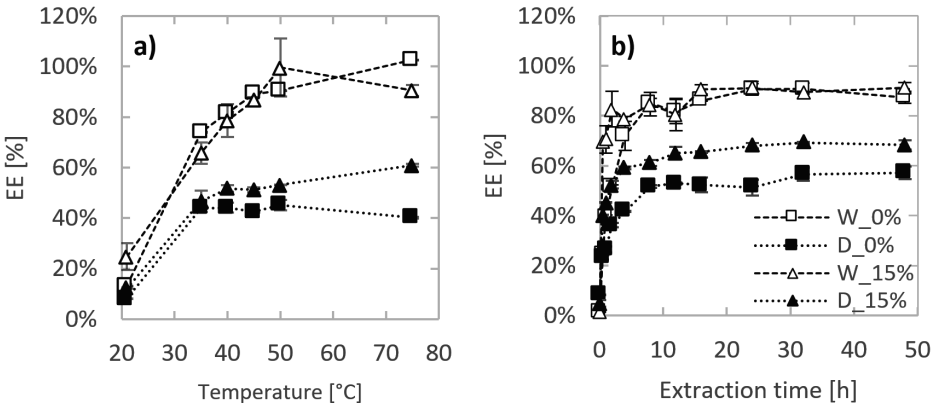


Fig. 3. EE of hexanoic acid (square) and 15% imidazole ES (triangle) from wet (open) and dried (filled) biomass under different conditions: a) different temperature (Batch 1, 16 h), and different extraction time (Batch 2, 50 °C). The beginning of steady state was approximately at 8 h.

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.[35] Besides that, protein from the biomass may lose its three-dimensional structure and eventually its functionality, which is not desired in multi-product biorefinery.[36,37]

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 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 hours, respectively). In comparison, extraction using hexanoic acid reached a steady-state after approx. 8 hours 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 been used to hydrolyze cellulose and chemically disintegrate the cell wall of microalgae.[29] Furthermore, cellulose tends to be more water-soluble and permeable when ionized at low or high pH.[33]

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.

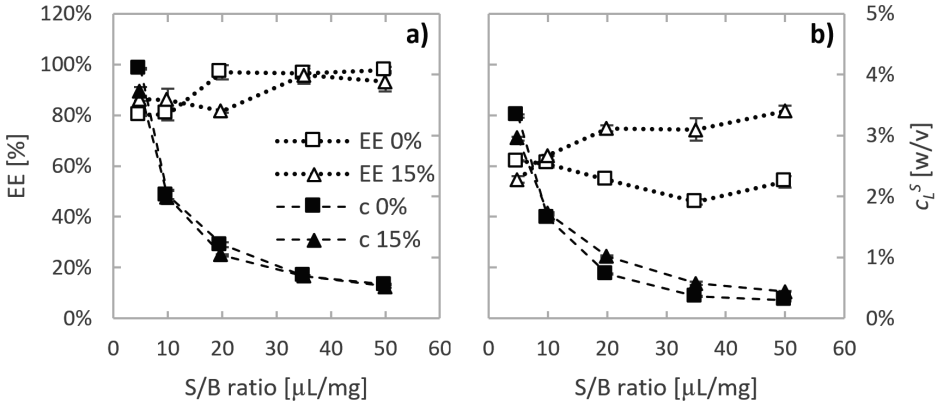


Fig. 4. Effect of solvent-to-biomass ratio (S/B, v/w) on EE (open, left y-axis) and c_L^S (closed, right y-axis). The extraction was performed at 50 °C on wet (a) and dry (b) biomass Batch 2 using hexanoic acid (square) and ES with 15 mol% imidazole (triangle) for 16 h.

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.[38,39] 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. In this experiment, the extraction was performed on dried biomass with water supplementation. It was observed that water supplementation increased the extraction yield, even reached the same EE as the extraction with wet biomass (Fig. 5). It implied that the water-enhancement mechanism would work regardless the water origin. 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.

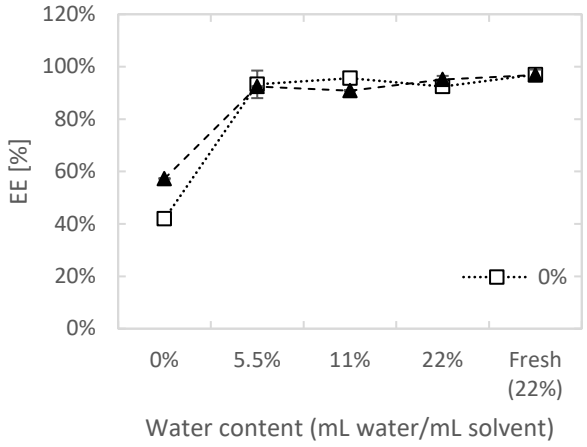


Fig. 5. Effect of water supplementation on lipid extraction from dried biomass (Batch 2) using ES with 0 (clear) and 15 mol% (striped) imidazole. Fresh (22%) indicates the extraction from wet algae paste.

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 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.[28,40,41] Furthermore, prior to dissolution, the lipids (especially the polar ones) must be disentangled from the neighboring biomolecules. The lipids, particularly the polar lipids, are bound to other biomolecules (i.e., proteins and carbohydrates) *via* electrostatic forces.[2,41] 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.[42,43] The high polarity of water facilitates ion dissociation and solvation, whereas hexanoic acid promotes ion association;[44] the dielectric constants of water and hexanoic acid are 80 and 2.6, respectively.[45] This fact implies that water enhances the dissociation of hexanoic acid and improves its acidity. Acidic environment (solvent) destabilizes 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.

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 hydrolyzed 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.[46–48]

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,[49] but it should improve the extraction from dried biomass, which was not observed here. Based on the result within this study, we instead proposed the opposite, which is the cell wall compaction increased the cell wall strength. Günther et al.[50] reported the increase of required energy to mechanically disrupt microalga *Chlorella vulgaris* when the cell was exposed to medium with high osmolality. The high osmolality forced intracellular water to escape from the cytoplasm, causing the decline of turgor pressure and enhanced cell wall flexibility.[50]

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.

Besides that, 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 comparable extraction efficiency to the benchmark (chloroform/methanol) 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.

References

1. Xu, L., Wim Brilman, D. W. F., Withag, J. A. M., Brem, G. & Kersten, S. Assessment of a dry and a wet route for the production of biofuels from microalgae: Energy balance analysis. *Bioresour. Technol.* **102**, 5113–5122 (2011).
2. Halim, R., Danquah, M. K. & Webley, P. A. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnol. Adv.* **30**, 709–732 (2012).
3. Lee, S. Y., Cho, J. M., Chang, Y. K. & Oh, Y.-K. K. Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresour. Technol.* **244**, 1317–1328 (2017).
4. Kumar, J., Garlapati, V. K., Dash, A., Scholz, P. & Banerjee, R. Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Res.* **21**, 138–147 (2017).
5. Li, Y. *et al.* Evaluation of alternative solvents for improvement of oil extraction from rapeseeds. *Comptes Rendus Chimie* (2014). doi:10.1016/j.crci.2013.09.002
6. Sicaire, A.-G. *et al.* Alternative Bio-Based Solvents for Extraction of Fat and Oils: Solubility Prediction, Global Yield, Extraction Kinetics, Chemical Composition and Cost of Manufacturing. *Int. J. Mol. Sci* **16**, 8430–8453 (2015).
7. Eppink, M. H. M., Ventura, S. P. M., Coutinho, J. A. P. & Wijffels, R. H. Multiproduct Microalgae Biorefineries Mediated by Ionic Liquids. *Trends Biotechnol.* (2021). doi:10.1016/j.tibtech.2021.02.009
8. Orr, V. C. A., Plechkova, N. V., Seddon, K. R. & Rehmann, L. Disruption and Wet Extraction of the Microalgae Chlorella vulgaris Using Room-Temperature Ionic Liquids. *ACS Sustain. Chem. Eng.* **4**, 591–600 (2016).
9. Liu, Z. W., Yue, Z., Zeng, X. A., Cheng, J. H. & Aadil, R. M. Ionic liquid as an effective solvent for cell wall deconstructing through astaxanthin extraction from Haematococcus pluvialis. *Int. J. Food Sci. Technol.* **54**, 583–590 (2019).
10. Ventura, S. P. M. *et al.* Ionic-Liquid-Mediated Extraction and Separation Processes for Bioactive Compounds: Past, Present, and Future Trends. *Chem. Rev.* **117**, 6984–7052 (2017).
11. Desai, R. K., Streefland, M., Wijffels, R. H. & Eppink, M. H. M. Novel astaxanthin extraction from Haematococcus pluvialis using cell

permeabilising ionic liquids. *Green Chem* **18**, 1261–1267 (2016).

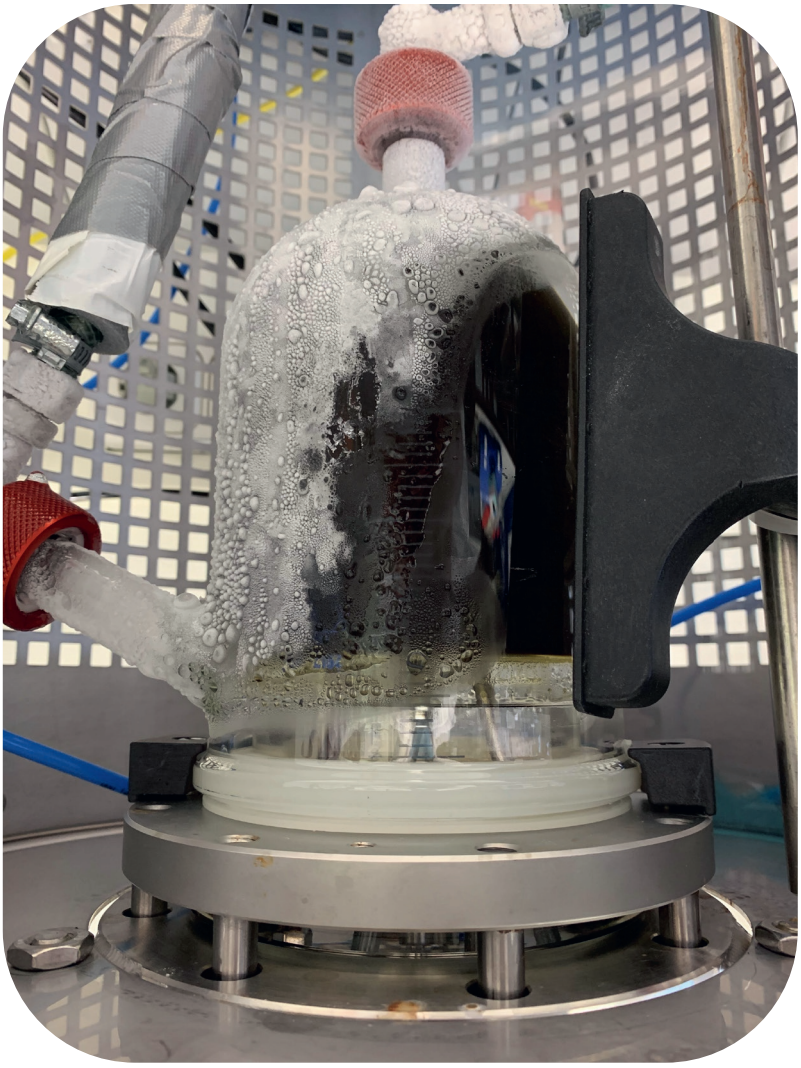
12. Zhang, Q., De, K., Vigier, O., Royer, S. & Jeoe, F. Deep eutectic solvents: syntheses, properties and applications. *Chem. Soc. Rev.* **41**, 7108–7146 (2012).
13. Smith, E. L., Abbott, A. P. & Ryder, K. S. Deep Eutectic Solvents (DESs) and Their Applications. *Chem. Rev.* **114**, 11060–11082 (2014).
14. Martins, M. A. R., Pinho, S. P. & Coutinho, J. A. P. Insights into the Nature of Eutectic and Deep Eutectic Mixtures. *J. Solution Chem.* **48**, 962–982 (2018).
15. Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K. & Tambyrajah, V. Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* 70–71 (2003). doi:10.1039/b210714g
16. Francisco, M., Van Den Bruinhorst, A. & Kroon, M. C. Low-transition-temperature mixtures (LTTMs): A new generation of designer solvents. *Angew. Chemie - Int. Ed.* **52**, 3074–3085 (2013).
17. Jeong, K. M. *et al.* Tailoring and recycling of deep eutectic solvents as sustainable and efficient extraction media. *J. Chromatogr. A* **1424**, 10–17 (2015).
18. Macário, I. P. E. *et al.* Unraveling the ecotoxicity of deep eutectic solvents using the mixture toxicity theory. *Chemosphere* **212**, 890–897 (2018).
19. Radošević, K. *et al.* Natural deep eutectic solvents as beneficial extractants for enhancement of plant extracts bioactivity. *LWT - Food Sci. Technol.* **73**, 45–51 (2016).
20. Lu, W. *et al.* A new approach of microalgal biomass pretreatment using deep eutectic solvents for enhanced lipid recovery for biodiesel production. *Bioresour. Technol.* **218**, 123–128 (2016).
21. Tommasi, E. *et al.* Enhanced and Selective Lipid Extraction from the Microalga P. tricornutum by Dimethyl Carbonate and Supercritical CO 2 Using Deep Eutectic Solvents and Microwaves as Pretreatment. *ACS Sustain. Chem. Eng.* **5**, 8316–8322 (2017).
22. Pan, Y. *et al.* One-step production of biodiesel from wet and unbroken microalgae biomass using deep eutectic solvent. *Bioresour. Technol.* **238**, 157–163 (2017).
23. Sed, G., Cicci, A., Jessop, P. G. & Bravi, M. A novel switchable-hydrophilicity, natural deep eutectic solvent (NaDES)-based system for bio-safe biorefinery. *RSC Adv.* **8**, 37092–37097 (2018).

24. Lo, C., Semerel, J., van den Berg, C., Wijffels, R. H. & Eppink, M. H. M. Eutectic solvents with tuneable hydrophobicity: lipid dissolution and recovery. *RSC Adv.* **11**, 8142–8149 (2021).
25. Chua, E. T. & Schenk, P. M. A biorefinery for Nannochloropsis: Induction, harvesting, and extraction of EPA-rich oil and high-value protein. *Bioresour. Technol.* **244**, 1416–1424 (2017).
26. Zanella, L. & Vianello, F. Microalgae of the genus Nannochloropsis: Chemical composition and functional implications for human nutrition. *Journal of Functional Foods* **68**, 103919 (2020).
27. Yao, L., Gerde, J. A., Lee, S.-L., Wang, T. & Harrata, K. A. Microalgae Lipid Characterization. *J. Agric. Food Chem.* **63**, 1773–1787 (2015).
28. Scholz, M. J. *et al.* Ultrastructure and Composition of the Nannochloropsis gaditana Cell Wall. *Eukaryot. Cell* **13**, 1450–1464 (2014).
29. Günerken, E. *et al.* Cell disruption for microalgae biorefineries. *Biotechnology Advances* (2015). doi:10.1016/j.biotechadv.2015.01.008
30. Halim, R. *et al.* Towards sustainable microalgal biomass processing: anaerobic induction of autolytic cell-wall self-ingestion in lipid-rich Nannochloropsis slurries. *Green Chem.* **21**, 2967–2982 (2019).
31. Remmers, I. M. *et al.* Continuous versus batch production of lipids in the microalgae Acutodesmus obliquus. *Bioresour. Technol.* **244**, 1384–1392 (2017).
32. Van Osch, D. J. G. P. *et al.* Ionic liquids and deep eutectic solvents for lignocellulosic biomass fractionation. *Phys. Chem. Chem. Phys.* **19**, 2636–2665 (2016).
33. Medronho, B., Romano, A., Miguel, M. G., Stigsson, L. & Lindman, B. Rationalizing cellulose (in)solubility: reviewing basic physicochemical aspects and role of hydrophobic interactions. *Cellul. 2012 193* **19**, 581–587 (2012).
34. Aleem, A. M. *et al.* Probing the Electrostatic and Steric Requirements for Substrate Binding in Human Platelet-Type 12-Lipoxygenase. *Biochemistry* **58**, 848–857 (2019).
35. Liu, K., Liu, Y. & Chen, F. Effect of storage temperature on lipid oxidation and changes in nutrient contents in peanuts. *Food Sci. Nutr.* **7**, 2280 (2019).
36. Suarez Ruiz, C. A., Emmery, D. P., Wijffels, R. H., Eppink, M. H. M. & van den Berg, C. Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems. *J. Chem. Technol. Biotechnol.*

93, 2774–2783 (2018).

37. ’t Lam, G. P., Vermuë, M. H., Eppink, M. H. M., Wijffels, R. H. & van den Berg, C. Multi-Product Microalgae Biorefineries: From Concept Towards Reality. *Trends Biotechnol.* **36**, 216–227 (2017).
38. Dai, Y., Witkamp, G.-J., Verpoorte, R. & Choi, Y. H. Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. *Food Chem.* **187**, 14–19 (2015).
39. Meng, X., Ballerat-Busserolles, K., Husson, P. & Andanson, J.-M. Impact of water on the melting temperature of urea + choline chloride deep eutectic solvent. *New J. Chem.* **40**, 4492–4499 (2016).
40. Zhang, Z. & Volkman, J. K. Algaenan structure in the microalga Nannochloropsis oculata characterized from stepwise pyrolysis. *Org. Geochem.* **104**, 1–7 (2017).
41. Yap, B. H. J., Crawford, S. A., Dumsday, G. J., Scales, P. J. & Martin, G. J. O. A mechanistic study of algal cell disruption and its effect on lipid recovery by solvent extraction. *Algal Res.* **5**, 112–120 (2014).
42. Jaworski, J. S. *et al.* Interactions of polar hydrogen bond donor solvents with ions: a theoretical study. *Struct. Chem.* **27**, 1279–1289 (2016).
43. Johnson, K. E., Pagni, R. M. & Bartmess, J. Brønsted Acids in Ionic Liquids: Fundamentals, Organic Reactions, and Comparisons. *Monatshefte für Chemie* **138**, 1077–1101 (2007).
44. Nordness, O. & Brennecke, J. F. Ion Dissociation in Ionic Liquids and Ionic Liquid Solutions. *Chem. Rev.* **120**, 12873–12902 (2020).
45. Engineering ToolBox. Dielectric Constants of Liquids. (2001). Available at: https://www.engineeringtoolbox.com. (Accessed: 7th April 2021)
46. Osawa, C. C., Gonçalves, L. A. G. & Ragazzi, S. Correlation between free fatty acids of vegetable oils evaluated by rapid tests and by the official method. *J. Food Compos. Anal.* **20**, 523–528 (2007).
47. Lee Kuek, S. *et al.* Contribution of lipid towards acrylamide formation during intermittent frying of French fries. *Food Control* **118**, 107430 (2020).
48. Zhang, T., Shahbaz, K. & Farid, M. M. Glycerolysis of free fatty acid in vegetable oil deodorizer distillate catalyzed by phosphonium-based deep eutectic solvent. *Renew. Energy* **160**, 363–373 (2020).
49. Stirk, W. A. *et al.* Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass. *J. Biotechnol.* **307**, 35–43 (2020).

50. Günther, S., Gernat, D., Overbeck, A., Kampen, I. & Kwade, A. Micromechanical properties and energy requirements of microalgae Chlorella vulgaris for cell disruption. *Chem. Eng. Technol.* **39**, 1693–1699 (2016).



Chapter 5

Multi-product extraction from microalga *Nannochloropsis oceanica* based on semi-hydrophobic eutectic solvents

This chapter is in preparation for submission as:

Lo, C., Boboescu, I. Z., Haemers, S., Wijffels, R. H. & Eppink, M. H. M. Multi-product from microalga *Nannochloropsis oceanica* based on semi-hydrophobic eutectic solvents.

Abstract

Semi-hydrophobic eutectic solvents (ES) possess a great potential as lipid extraction solvent from untreated microalgae. However, the low volatility 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 and scalability) were studied. About 60% of the extracted lipids could be recovered *via* selective precipitation of saturated and monounsaturated fatty acids. Furthermore, based on 3 iterative extractions, the solvents were demonstrated to be reusable with a consistent lipid yield despite the accumulation of polyunsaturated fatty acid-rich lipids. Finally, aqueous extraction of protein and carbohydrate from the defatted biomass was feasible under alkaline condition. Based on the protein analysis, the proposed ES process was not sufficiently mild to maintain the protein native structure. On the other hand, opportunities are discussed to create new functionalities for proteins and carbohydrates so that a multiproduct biorefinery is feasible for ES.

1. Introduction

Microalgae biorefinery is a set of downstream processing units aiming for the complete valorization of microalgal biomass. Hence, an ideal biorefinery would translate the various biomolecules to multiple products.[1–4] Moreover, the biorefinery typically involves sequential process steps, i.e., harvesting, pre-treatment, extraction, and fractionation.[4,5] Besides being complex, these processes are also often energy demanding.[6–9] Furthermore, non-renewable, harmful solvents are often used for lipid extraction.[10,11] 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.[12–15] 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.[16–19] Furthermore, in our previous work, we developed semi-hydrophobic ES made of imidazole and hexanoic acid for lipid extraction from fresh microalga *Nannochloropsis oceanica* (Chapter 4).

Despite the simplified process, the use of ES for microalgae biorefinery still faces challenges. One of the major challenges is the non-volatility of the solvent, which makes solvent-lipid separation and solvent regeneration complex. Previously, we used polar antisolvents, such as water and methanol to induce phase split between the ES and model lipids. Moreover, the ES was successfully regenerated by evaporating the antisolvents (Chapter 3). However, the model systems were highly simplified as the purified model lipids would behave differently with the lipid extracts from the microalgae. Additionally, the effect of the ES extraction on the other biomolecules 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.

In this study, the recovery of lipids extracted from *N. oceanica* was performed. We investigated several factors affecting the recovery, i.e., antisolvent type, amount, imidazole content and temperature. Cooling is commonly used in the oleochemical industry to fractionate lipids based on their solubility and melting point. Typically, before the refrigeration (also termed winterization), the lipids are dissolved in alcohol, which is highly similar to the ES-methanol mixture. Besides that, the recyclability of the solvent and the scalability of the extraction and recovery were discussed. Furthermore, the process mildness was evaluated. Mild processes should maintain the protein structure and functionality, which highly correlates to the added value of this fraction. Moreover, the remaining proteins and carbohydrates were isolated from the defatted biomass.

2. Materials & Methods

2.1. Microalgae cultivation

Microalga *Nannochloropsis oceanica* (kindly provided by Necton, Portugal) was cultivated in a 10-L stirred tank photobioreactor under constant illumination of 500 mol_{ph} m^{-2} s^{-1}. The growth medium 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 under nitrogen limitation to induce lipid accumulation. Air with 5% CO_2 was continuously supplied as a carbon source and pH regulator. After 7 weeks of cultivation, the culture was centrifuged to obtain biomass paste (32.4% dry weight) 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 (1:3) was prepared by dissolving the pre-weighed imidazole flakes in hexanoic acid at room temperature until a clear homogenous solution was obtained.

2.3. Lipid extraction, recovery, and solvent regeneration

The lipid extraction from the microalgae was performed following the method described in Chapter 4. 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:

$$\mathrm{EE} = \frac{f\, m_S\, (c_E - c_L^*)}{m_X\, c_X}$$

where f is the factor for water-solvent miscibility (= 1.1 & 1.2, for pure hexanoic acid and 15 mol% imidazole solution), m_S is the amount of solvent or previously regenerated solvent [g], c_E is the fatty acid concentration in the extract [$g_{FA}\ g^{-1}$], c_L^* represents the fatty acid concentration in the previously regenerated solvent [$g_{FA}\ g^{-1}$] (= 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 lipid was recovered by adding a proper amount of water or methanol 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 at proper temperatures. Furthermore, the liquid phase was vacuum evaporated for 5 h at 8 mbar, 40 °C to remove the methanol and thus regenerate the solvent.

Since the amount of precipitated lipid could not be easily quantified, the efficiency of recovery (η) was calculated based on the remaining lipid dissolved in the solvent, following equation:

$$\eta = \frac{m_E\, c_E - (m_E + m_M)\, c_L}{(1 - c_L)\, m_E\, c_E}$$

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

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 type and amount, and temperature) under different process constraints (Table 1). The experimental design approaches were developed and analyzed using the Design-Expert 13 software suite (StatEase Inc., USA). In this study, the imidazole molar content and the antisolvent type and amount were based on the results of our previous studies for optimal extraction and lipid recovery (Chapter 3 and 4). We did not use more methanol since methanol would require more energy to evaporate. The temperature points were selected based on the operational convenience.

Table 1. Variables of interest for the Design of Experiment

Variable	Type	Low point	Middle point	High point
Imidazole content [mol%]	Categorical	0	-	15
Amount of water [g g_{ES}^{-1}]	Numerical	0.01	0.1	0.3
Amount of methanol [g g_{ES}^{-1}]	Numerical	0.2	0.4	1.5
Temperature [°C]	Numerical	-20	3	22

2.5. Scale-up experiment

In the scale-up experiment, the batch extraction and recovery were performed in a 1-L stirred glass vessel. Temperature control was provided through a temperature jacket outside the vessel, which was connected to a thermoregulator. Most of the conditions were kept the same as the original scale, e.g., solvent ratio, temperature, and incubation time. However, agitation was lower for the extraction and higher for the recovery when compared to the smaller scale. Prior to the lipid recovery step, the liquid extract was separated from the defatted biomass by centrifugation (4000 rcf). Methanol was added to the glass container (1.5 g methanol/g ES extract), and the temperature was set at -20 °C. About 10 mL of suspension was sampled out of the vessel after 16 hours of stabilized temperature and analyzed.

2.6. Analysis of fatty acids and solvent components

The fatty acids were quantified with gas chromatography coupled with a flame ionization detector (GC-FID). The preparation of samples from biomass and the hexanoic acid and ES extracts were described in our previous work (Chapter 4) and Remmers et al.[20] Furthermore, the solvent components were also analyzed using GC-FID system with the previously developed protocol (Chapter 3). 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 the centrifugation of extraction mixtures, the defatted biomass was obtained as the heavy phases. 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 x 6000 rpm. For protein determination, a *DC* protein assay kit (Bio-Rad, US) based on the Lowry method[21] was used with bovine serum albumin as standard. The carbohydrate content was measured with the phenol-sulfuric acid method[22] using glucose as standard.

3. Results and Discussion

The results and discussion section is outlined as follows. First, the recovery of algal lipid by antisolvent addition was investigated. Then, three cycles of repetitive extraction and a scale-up experiment were performed to demonstrate the recyclability and scalability of the eutectic solvent. Furthermore, we attempted to extract the proteins and carbohydrates for obtaining multi-product fractions based on this ES process.

3.1. Lipid recovery by methanol-induced precipitation

Separation of the extracted lipid from the solvent is necessary for solvent regeneration. In our previous work, it was found that the addition of water and methanol could reduce the solubility of model lipids from imidazole/hexanoic acid (1:3) (Chapter 3). Hence, it was hypothesized that the antisolvents would induce the same effect on the microalgal lipid. To prove this hypothesis, various amounts of water and methanol were added to hexanoic acid and ES extracts of *N. oceanica*, which gave the highest extraction yields in the previous study (Chapter 4). Moreover, the temperature is known to influence the lipid solubility and induce phase transition of lipids.[23] 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 (Chapter 3), 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 added water only formed another liquid phase. For the ES extract, the water content was ~20% w/w, while the saturation point is ~30% w/w.[24] 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 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, increasing the concentration of 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.[24] In previous work, the model lipids were recovered from ES with 25 mol% imidazole (Chapter 3), 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.

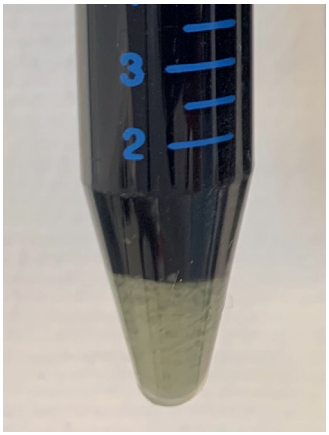


Fig. 1. Precipitation of lipids from the ES extract after addition of methanol at -20 °C.

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*.[25,26] Furthermore, from the visual observation, the precipitate seemed to be less colored, indicating the lower content of chlorophyll (Fig. 1). The recovery efficiency was found to be dependent on the methanol amount and temperature (Fig. 2a). 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 1.5 g/g extract). The precipitation yield was somewhat unsatisfactory compared to the previous work using model lipids

(Chapter 3). It is worth noting that the initial lipid concentration in this study was 10-fold lower compared with the previous study (Chapter 3). More methanol might improve the recovery but would also require more energy to evaporate.

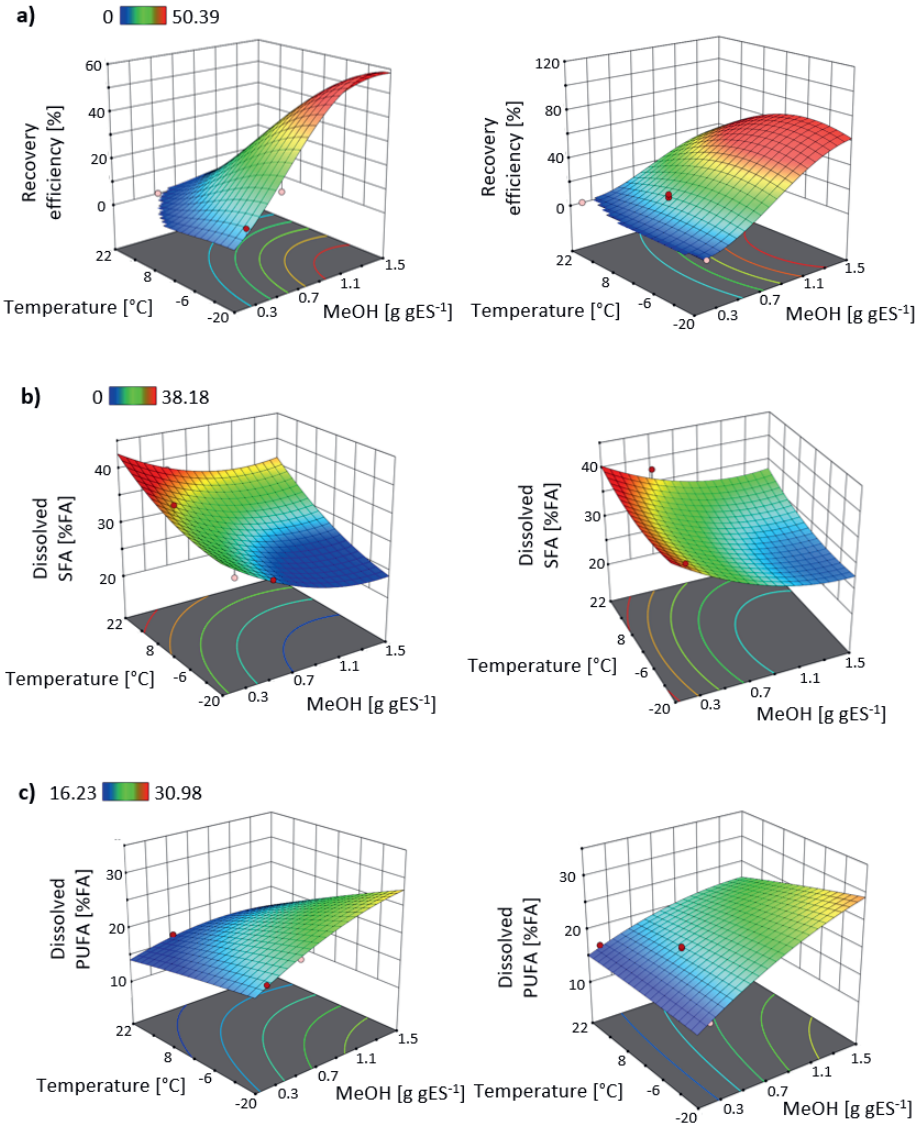


Fig. 2. a) Recovery efficiency η [%], b) soluble saturated fatty acids (SFA), and c) soluble polyunsaturated fatty acids (PUFA) [% total FA] from hexanoic acid (left) and imidazole/hexanoic acid (15:85) (right)

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. Furthermore, the strength of hydrogen bonds increases at lower temperatures.[27] 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 is rather impractical for large-scale production; the latter gave a trade-off with lower lipid extraction efficiency (Chapter 4).

Moreover, the profile of soluble fatty acids was also affected by temperature and methanol content (Fig. 2b & c). 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.

The selectivity of major unsaturated fatty acids was also influenced by the presence of imidazole (Fig. 3). Compared to hexanoic acid extract, the ES extract yielded higher dissolved PUFA (C20:4 and C20:5) content and reduced content of MUFA (monounsaturated fatty acids, indicated by C16:1 and C18:1) after the recovery. Furthermore, it has been known that imidazole within the ES could establish π-π interactions with unsaturated bonds.[24] This interaction could explain why PUFA dissolved better than MUFA, thus, higher recovery of MUFA.

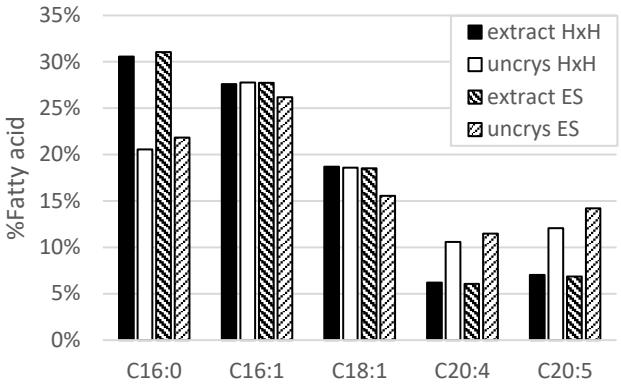


Fig. 3. Profile of soluble fatty acids in hexanoic acid (solid) and ES (striped) before and after the lipid recovery at -20 °C (shown by darker and lighter colors, respectively).

A deeper look at the fatty acid distribution and lipid classes gave a further hint on the selectivity. Majority of PUFA in the biomass was detected in the polar lipid (PL) fraction, whereas more than 75% of SFA and MUFA were present in the neutral lipid fraction (Table 2). Polar solvents such as alcohols are able 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%, Chapter 3). 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 and water concentrations 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 the higher recovery. Moreover, like the lipid, the moisture from the wet biomass was also dissolved during the extraction. The presence of water, an antisolvent, would raise the recovery efficiency (Chapter 3). For instance, lower

solvent-to-biomass ratios would increase both lipid and water content in the extract, thus potentially lead to higher recovery.

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

	FA content [$g_{FA}\ g_{DW}^{-1}$]		
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%

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. Previously, we successfully recycled the ES after the dissolution and recovery of model lipids. The ES regeneration was performed by vacuum evaporating the antisolvents (Chapter 3). 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.

Throughout the cycles, the lipids accumulated due to the incomplete lipid removal during recovery (Fig. 4). In accordance with the above 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 iterations, the soluble PUFA content reached 2-fold of the initial extract.

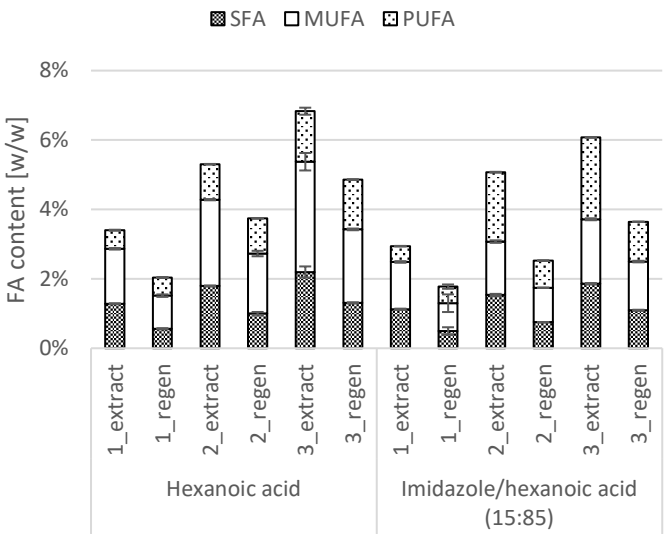


Fig. 4. The fatty acid content in the solvent extracts and the regenerated solvents throughout the different cycles.

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. 5). 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 from our previous study[24] were ≥ 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.

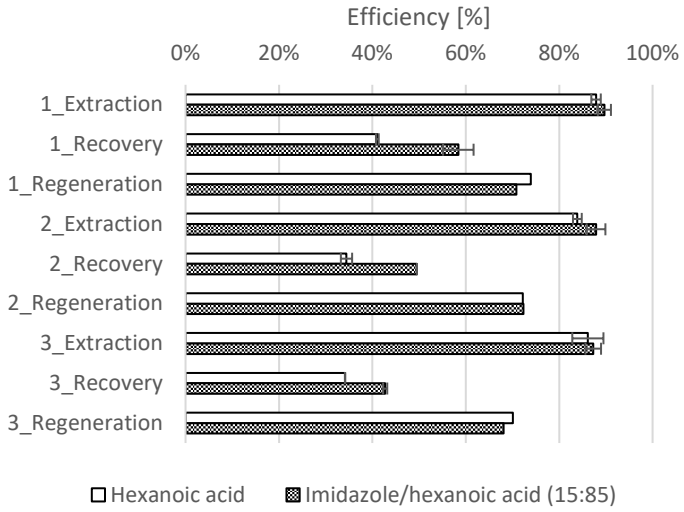


Fig. 5. The efficiency of lipid extraction, recovery, and solvent regeneration in three cycles.

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 (Chapter 3). 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. 5). 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.

Besides that, about 70% of the solvents were regenerated within a single iteration (Fig. 5). 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 was not scaled-up since methanol evaporation is a widely implemented technology, i.e., *via* distillation. In this experiment, the solvent used was the ES imidazole/hexanoic acid (15:85), and the working volume increased by larger than 100-fold. While most conditions were kept constant, the agitation was not the same in both scales 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). 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;[28] 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.

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

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
Extraction efficiency	1	0.92
Recovery		
Extract volume [mL]	1	250
Methanol volume [mL]	2	500
Temperature [°C]	-20	-20
Agitation [rpm]	-	< 100 (low)
Recovery duration [h]	16	16
Concentration of remaining FA [w%]	0.48	0.55
Recovery efficiency	0.58	0.46

The lipid recovery of the larger scale was also lower than that of the smaller scale (Table 3). 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.[29] 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.[29]

3.4. Outlook for multi-product biorefinery

As a preliminary work of microalgae biorefinery, we also tried to valorize the proteins and carbohydrates from *N. oceanica*. The proteins and carbohydrates were possibly distributed in the solvent extracts and in the residual biomass, which was separated via centrifugation at 4000 rcf. Fig. 6 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 (20000 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 were homogenized before further treatments.

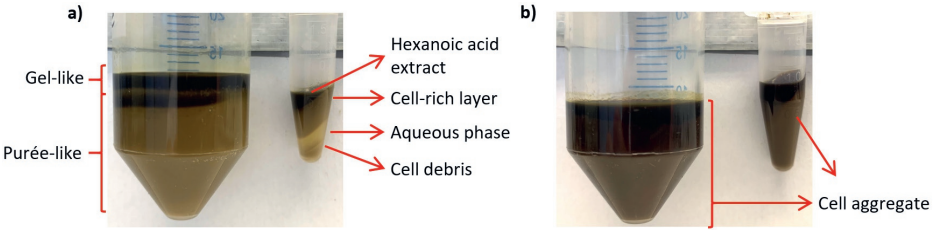


Fig. 6. The suspension of residual biomass which were defatted by hexanoic acid (top) and imidazole/hexanoic acid ES (bottom). The suspensions were centrifuged at 4000 rcf (in larger tubes) and at 20000 rcf (in smaller tubes).

The composition of biomolecules from the obtained fractions in the process is shown in Table 3. Due to the nitrogen limitation, the major content of microalgae was fatty acids (32%), followed by protein (25%), and carbohydrate (12%). Interestingly, some proteins were also detected in the hexanoic acid and ES extract. As hexanoic acid and the ES were both relatively hydrophobic, the dissolved proteins should also be hydrophobic. From the total proteins, 20% of the total proteins were found to be water-soluble, which was similarly

reported in the literature.[30,31] Hence, about 80% of the proteins were hydrophobic, membrane-bound proteins. Due to the low economic value, little attention has been given to these proteins. Till recently, Dai and coworkers[32] valorized the insoluble proteins from *Chlorella protothecoides* as foam stabilizer.

Moreover, the major content of the defatted biomass were proteins and carbohydrates (Table 3). The latter was most likely cell wall components and storage polysaccharides called laminarin.[33,34] The proteins could be hydrophilic proteins or large proteins which could not pass the cell wall porosity. The protein content of the defatted biomass (0.8 – 1.2%) was rather lower than the reported protein content from defatted *Nannochloropsis* spp., which contained about 3%.[35] The difference could be associated with the presence of residual hexanoic acid and the ES (together with water), whereas in the reported study, the biomass was thoroughly dried.

Table 3. Biochemical compositions of the obtained fractions

	FA	Protein	Carbohydrate
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.d.*
ES extract [mg mL^{-1}]	27.7 ± 0.5	12.2 ± 2.1	*n.d.*
Hexanoic acid-defatted biomass [mg g_{susp}^{-1}]	*n.d.*	68.7 ± 2.9	46.8 ± 13.7
ES-defatted biomass [mg g_{susp}^{-1}]	*n.d.*	84.8 ± 4.2	57.5 ± 10.1

n.d. = not determined

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 cell disruption. For protein extraction, both alkalinity and cell disruption improved the extraction efficiency (Fig. 7a). It is widely known that alkaline conditions drive proteins to be negatively charged and 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. Moreover, the cell disruption opened the cell wall and liberated the large proteins or protein aggregates. However, for carbohydrates, the pH effect was only distinctive at the highest pH (Fig. 7b). Similar to proteins, carbohydrates could also be ionized and hydrolyzed at such pH.

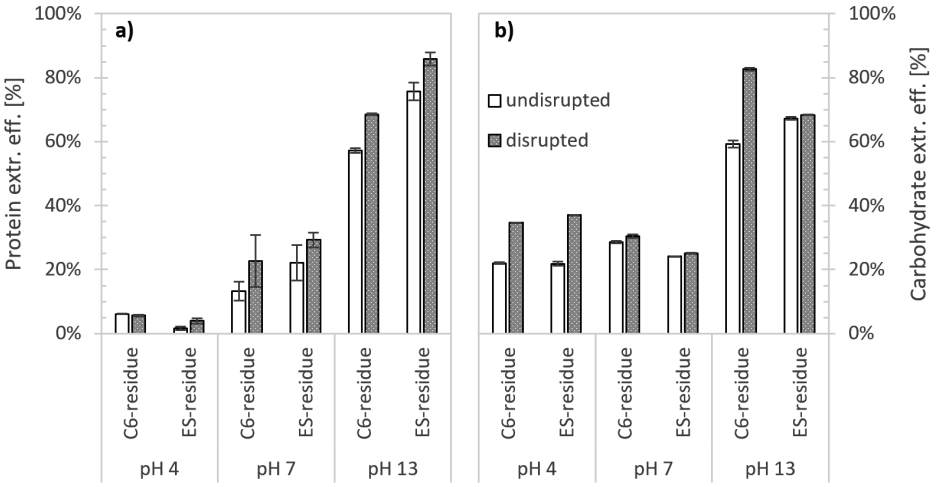


Fig. 7. The extraction efficiency of protein (a) and carbohydrate (b) from the defatted biomass.

The process mildness has been one of the central issues in multi-product biorefinery as it guarantees low energy consumption and the quality of biomolecules. In mild conditions, biomolecules, especially proteins, do not lose their three-dimensional structure and thus the functionality.[2,36,37] Therefore, we also evaluated the quality of the solubilized proteins from the defatted biomass and in the solvent extracts using SDS-PAGE. Based on the molecular size, 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.[38] 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.[39,40] 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 a mild process. 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. Even further, the solvent molecules might also bind strongly to 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.

4. Conclusion

This study was a preliminary feasibility study of microalgae biorefinery based on semi-hydrophobic eutectic solvents. 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 slight lower efficiency and recovery. Moreover, for the other fractions, the suspensions of defatted biomass contained proteins and carbohydrates. However, the lipid extraction using the solvents was not sufficiently mild as the proteins lost their native structure based on the protein analysis. Therefore,

further investigations on the critical points discussed in this study are required to develop a viable process.

References

1. Wijffels, R. H., Barbosa, M. J. & Eppink, M. H. M. Microalgae for the production of bulk chemicals and biofuels. *Biofuels, Bioprod. Bioref.* **4**, 287–295 (2010).
2. Vanthoor-Koopmans, M., Wijffels, R. H., Barbosa, M. J. & Eppink, M. H. M. Biorefinery of microalgae for food and fuel. *Bioresour. Technol.* (2013). doi:10.1016/j.biortech.2012.10.135
3. Chew, K. W. *et al.* Microalgae biorefinery: High value products perspectives. *Bioresour. Technol.* **229**, 53–62 (2017).
4. 't Lam, G. P., Vermuë, M. H., Eppink, M. H. M., Wijffels, R. H. & van den Berg, C. Multi-Product Microalgae Biorefineries: From Concept Towards Reality. *Trends Biotechnol.* **36**, 216–227 (2017).
5. Halim, R., Danquah, M. K. & Webley, P. A. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnol. Adv.* **30**, 709–732 (2012).
6. Xu, L., Wim Brilman, D. W. F., Withag, J. A. M., Brem, G. & Kersten, S. Assessment of a dry and a wet route for the production of biofuels from microalgae: Energy balance analysis. *Bioresour. Technol.* **102**, 5113–5122 (2011).
7. Angles, E., Jaouen, P., Pruvost, J. & Marchal, L. Wet lipid extraction from the microalga Nannochloropsis sp.: Disruption, physiological effects and solvent screening. *Algal Res.* **21**, 27–34 (2017).
8. Günerken, E. *et al.* Cell disruption for microalgae biorefineries. *Biotechnology Advances* (2015). doi:10.1016/j.biotechadv.2015.01.008
9. Show, K. Y., Lee, D. J., Tay, J. H., Lee, T. M. & Chang, J. S. Microalgal drying and cell disruption – Recent advances. *Bioresour. Technol.* **184**, 258–266 (2015).
10. Kumar, J., Garlapati, V. K., Dash, A., Scholz, P. & Banerjee, R. Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Res.* **21**, 138–147 (2017).
11. Chua, E. T. & Schenk, P. M. A biorefinery for Nannochloropsis: Induction, harvesting, and extraction of EPA-rich oil and high-value protein. *Bioresour. Technol.* **244**, 1416–1424 (2017).
12. Francisco, M., Van Den Bruinhorst, A., Zubeir, L. F., Peters, C. J. & Kroon, M. C. Equilibria A new low transition temperature mixture (LTTM) formed by choline chloride + lactic acid: Characterization as solvent for CO2 capture.

Fluid Phase Equilib. **340**, 77–84 (2013).

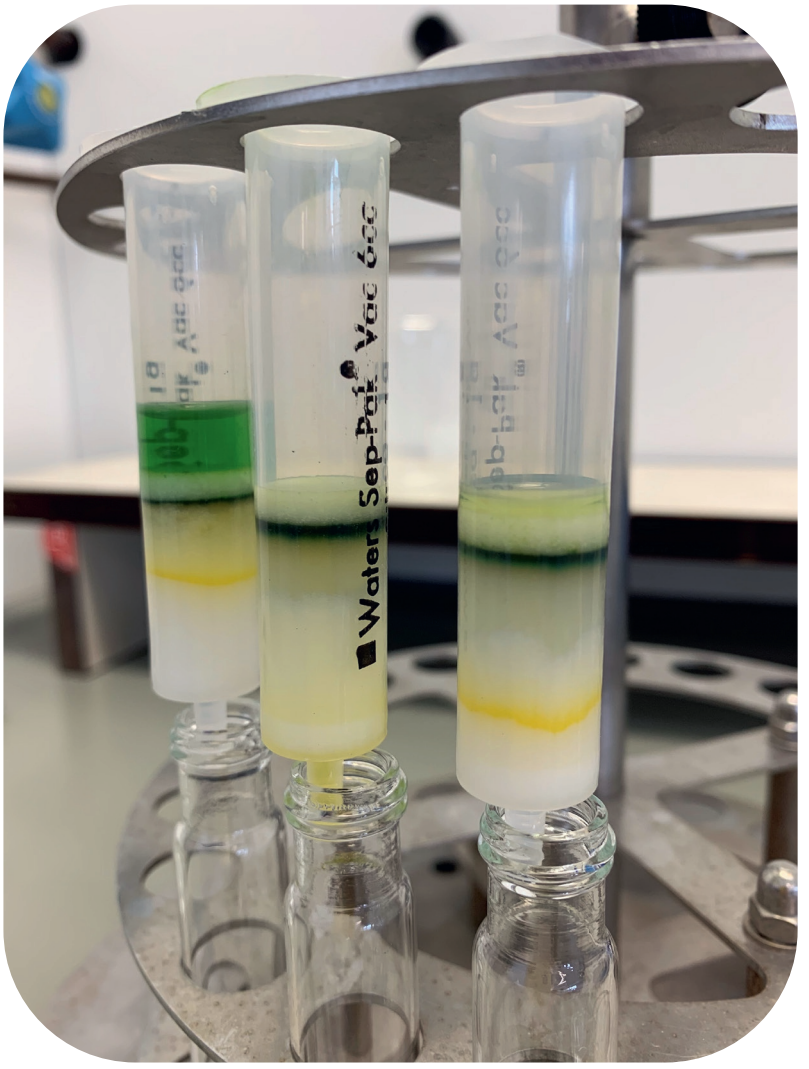
13. Zhang, Q., De, K., Vigier, O., Royer, S. & Jeoe, F. Deep eutectic solvents: syntheses, properties and applications. *Chem. Soc. Rev.* **41**, 7108–7146 (2012).
14. Paiva, A. *et al.* Natural Deep Eutectic Solvents − Solvents for the 21st Century. *ACS Sustain. Chem. Eng.* **2**, 1063–1071 (2014).
15. Martins, M. A. R., Pinho, S. P. & Coutinho, J. A. P. Insights into the Nature of Eutectic and Deep Eutectic Mixtures. *J. Solution Chem.* **48**, 962–982 (2018).
16. Lu, W. *et al.* A new approach of microalgal biomass pretreatment using deep eutectic solvents for enhanced lipid recovery for biodiesel production. *Bioresour. Technol.* **218**, 123–128 (2016).
17. Tommasi, E. *et al.* Enhanced and Selective Lipid Extraction from the Microalga P. tricornutum by Dimethyl Carbonate and Supercritical CO 2 Using Deep Eutectic Solvents and Microwaves as Pretreatment. *ACS Sustain. Chem. Eng.* **5**, 8316–8322 (2017).
18. Pan, Y. *et al.* One-step production of biodiesel from wet and unbroken microalgae biomass using deep eutectic solvent. *Bioresour. Technol.* **238**, 157–163 (2017).
19. Cicci, A., Sed, G. & Bravi, M. Potential of Choline Chloride-Based Natural Deep Eutectic Solvents (NaDES) in the Extraction of Microalgal Metabolites. *Chem. Eng. Trans.* **57**, (2017).
20. Remmers, I. M. *et al.* Continuous versus batch production of lipids in the microalgae Acutodesmus obliquus. *Bioresour. Technol.* **244**, 1384–1392 (2017).
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275 (1951).
22. Dubois, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal Chem* **28**, 350–356 (1956).
23. Zaliha, O., Chong, C. L., Cheow, C. S., Norizzah, A. R. & Kellens, M. J. Crystallization properties of palm oil by dry fractionation. *Food Chem.* **86**, 245–250 (2004).
24. Lo, C., Semerel, J., van den Berg, C., Wijffels, R. H. & Eppink, M. H. M. Eutectic solvents with tuneable hydrophobicity: lipid dissolution and

recovery. *RSC Adv.* **11**, 8142–8149 (2021).

25. Yao, L., Gerde, J. A., Lee, S.-L., Wang, T. & Harrata, K. A. Microalgae Lipid Characterization. *J. Agric. Food Chem.* **63**, 1773–1787 (2015).
26. Lu, Y. *et al.* Regulation of the cholesterol biosynthetic pathway and its integration with fatty acid biosynthesis in the oleaginous microalga Nannochloropsis oceanica. *Biotechnol. Biofuels 2014 71* **7**, 1–15 (2014).
27. Ross, P. D. & Rekharsky, M. V. Thermodynamics of Hydrogen Bond and Hydrophobic Interactions in Cyclodextrin Complexes. *Biophys. J.* **71**, 2144–2154 (1996).
28. Halim, R., Rupasinghe, T. W. T., Tull, D. L. & Webley, P. A. Modelling the kinetics of lipid extraction from wet microalgal concentrate: A novel perspective on a classical process. *Chem. Eng. J.* **242**, 234–253 (2014).
29. Herrera, M. L. & Hartel, R. W. Effect of processing conditions on crystallization kinetics of a milk fat model system. *J. Am. Oil Chem. Soc.* **77**, 1177–1188 (2000).
30. Schwenzfeier, A., Wierenga, P. A. & Gruppen, H. Isolation and characterization of soluble protein from the green microalgae Tetraselmis sp. *Bioresour. Technol.* **102**, 9121–9127 (2011).
31. Suarez Garcia, E. *et al.* Selective and energy efficient extraction of functional proteins from microalgae for food applications. *Bioresour. Technol.* **268**, 197–203 (2018).
32. Dai, L., Shivananda, R., Hinrichs, J. & Weiss, J. Foaming of Acid-Hydrolyzed Insoluble Microalgae Proteins from Chlorella protothecoides. *Food Biophys. 2020 153* **15**, 368–375 (2020).
33. Vogler, B. W., Brannum, J., Chung, J. W., Seger, M. & Posewitz, M. C. Characterization of the Nannochloropsis gaditana storage carbohydrate: A 1,3-beta glucan with limited 1,6-branching. *Algal Res.* **36**, 152–158 (2018).
34. Scholz, M. J. *et al.* Ultrastructure and Composition of the Nannochloropsis gaditana Cell Wall. *Eukaryot. Cell* **13**, 1450–1464 (2014).
35. Gerde, J. A. *et al.* Optimizing protein isolation from defatted and non-defatted Nannochloropsis microalgae biomass. *Algal Res.* **2**, 145–153 (2013).
36. Desai, R. K. *et al.* One-step mild biorefinery of functional biomolecules from microalgae extracts. *React. Chem. Eng.* (2017). doi:10.1039/c7re00116a
37. Suarez Ruiz, C. A., Emmery, D. P., Wijffels, R. H., Eppink, M. H. M. & van den Berg, C. Selective and mild fractionation of microalgal proteins and

pigments using aqueous two-phase systems. *J. Chem. Technol. Biotechnol.* **93**, 2774–2783 (2018).

38. Djagny, K. B., Wang, Z. & Xu, S. Gelatin: A Valuable Protein for Food and Pharmaceutical Industries: Review Gelatin: A Valuable Protein for Food and Pharmaceutical Industries: Review. *Crit. Rev. Food Sci. Nutr.* **41**, 481–492 (2001).
39. Montone, C. M. *et al.* Peptidomic strategy for purification and identification of potential ACE-inhibitory and antioxidant peptides in Tetradesmus obliquus microalgae. *Anal. Bioanal. Chem. 2018 41015* **410**, 3573–3586 (2018).
40. Ejike, C. E. C. C. *et al.* Prospects of microalgae proteins in producing peptide-based functional foods for promoting cardiovascular health. *Trends Food Sci. Technol.* **59**, 30–36 (2017).


Waters Sep-Pak Vac

Chapter 6

General discussion

1. Introduction

With the threatening issues of climate change and biodiversity loss, there is an urgent need to produce lipids in an environmental-harmless way. Compared to oleaginous terrestrial plants (like oil palm), several microalgae exhibit higher lipid productivity without the need for arable land.[1,2] Therefore, there is growing attention on microalgae as lipid feedstock. However, the current lipid extraction methods from microalgae still involve series of energy-intensive pretreatments and nonrenewable organic solvents.[3–7] These concerns make this process not only cost ineffective but unsustainable as well. Furthermore, to avoid biomass underutilization, the biorefinery concept (i.e., valorization of the entire valuable compounds from biomass) is necessary to be implemented.

Eutectic solvents (ES), including "deep eutectic solvents", hold great potential as an extraction system for microalgae. Besides being virtually inflammable, ES can be readily prepared from bio-derived and biodegradable compounds. Several hydrophilic ES can form pores or cracks in the cell wall of various microalgae, which enhanced the lipid extraction yield even without the cell disruption step.[8,9] The tailorable properties of ES may also open possibilities for process integration. For instance, hydrophobic ES can be used to pretreat the biomass and extract the lipids simultaneously. However, the low volatility of ES complicates the process due to the challenging separation of lipids from the solvent. Therefore, this thesis aimed to develop suitable ES for lipid extraction from microalgae and further evaluate the feasibility of microalgae biorefinery. In this chapter, the major breakthroughs and challenges are summarized. Moreover, the future outlook on the ES application for microalgae biorefinery is discussed.

2. Begin with the end in mind: altering ES hydrophobicity

To develop a functional solvent process, the focus should address the extraction step and include solvent regeneration. Murphy's law of solvent states that the best solvent in any process would be bad for the subsequent step. This principle implies that while the strong affinity of hydrophobic ES

towards the lipid solutes would benefit the extraction process, it would also cause the separation of lipids from the ES practically impossible. Therefore, in this thesis, we propose to use semi-hydrophobic ES, which were prepared by pairing hydrophobic and hydrophilic compounds (**Chapter 2**). For instance, the combination of imidazole and hexanoic acid dissolved model lipids and water. The solvent hydrophobicity was found to decrease with increasing imidazole concentration. Thus, by adding imidazole, model lipids could be recovered with relatively high purity (> 85%). However, solvent regeneration via imidazole removal is not straightforward with this approach due to the strong association between imidazole and hexanoic acid.

Another approach shifting the solvent hydrophobicity is to use polar antisolvents, e.g., water, methanol, and ethanol (**Chapter 3**). The presence of antisolvents accentuated the ES hydrophilicity and thus reduced the solubility of model lipids. This approach offered a significantly simpler method to regenerate the ES, i.e., by evaporating the antisolvents. Since a large amount of antisolvent can be loaded into the system, this approach reached higher recovery (> 90%) and purity of the obtained lipids compared to the previous method (**Chapter 2**). Considering the recovery yield and the ease of regeneration, methanol was selected to be the best antisolvent.

3. ES on microalgae: lipids and beyond

Imidazole/hexanoic acid ES was found to extract lipid from undisrupted *Nannochloropsis oceanica*, which confirmed the hypothesis of using ES as 'pretreatment' and extraction solvent (**Chapter 4**). At low imidazole content (≤ 15 mol%), the extraction yield using ES at 50 °C was comparable to the benchmark chloroform/methanol method. Interestingly, extraction on wet algae gave higher yields than the dried biomass. This finding suggests that both cell disruption and complete dehydration is unnecessary for the ES extraction, simplifying the microalgae processing. Besides that, it also implies that water enhanced the ES performance, contradicting the finding from **Chapter 3**. Hence, it is hypothesized that water facilitates the ES penetration to the cell matrix. However, the interaction between the solvent with the biomolecules

and how ES penetrated the cell wall is still unknown. Moreover, since cell wall structure is species-dependent, the ES penetration might be different between microalgal species. Additionally, the extracted lipid might undergo undesired reactions, such as hydrolysis, transesterification, or oxidation, which were undetected with the used analytical method.

Initial feasibility study of microalgae biorefinery based on the developed ES was performed in **Chapter 5**. The proposed biorefinery process is shown in Fig. 1, which was based on the **Chapter 3 & 4**. Solvent reusability and process scalability were evaluated for lipid extraction. Unlike the model lipids, the recovery of algal lipids with methanol reached a lower yield (~60%) even at low temperature (-20 °C). The lower recovery was due to the lower starting concentration of lipids; the concentration of model lipids was 10-fold higher than the extracted algal lipids. Besides that, the different fatty acid distribution between the algal extract and the model lipids might contribute to the lower recovery as well as the ES had a higher affinity towards certain fatty acids. Moreover, unlike the model lipids, which were mainly refined triacylglycerols (TAG), the extract from the algae could also contain polar lipids (PL), sterols, waxes, and pigments. These compounds might interact differently with the ES and interfere the lipid recovery. Furthermore, despite the incomplete recovery, the ES could be reused for the three extraction cycles with consistent performance. Some losses of the solvents were observed during solid-liquid separations (i.e., after the extraction and the lipid recovery). In scaling up, the heterogeneity is a recurring issue for the extraction, while agitation and cooling rate influenced the recovery.

Besides lipids, biomass contains proteins and carbohydrates, which remain inside the defatted biomass after the ES extraction (**Chapter 5**). Based on the protein analysis, the ES extraction was not mild since the proteins lost the native conformations and aggregated. The denaturation might be caused by the acidic and amphiphilic nature of the solvents, combined with the elevated extraction temperature. Moreover, the proteins and carbohydrates were isolated through aqueous extraction, which was enhanced by pH manipulation.

However, despite giving the highest yield, the alkaline condition (i.e., pH 13) hydrolyzed the remaining proteins.

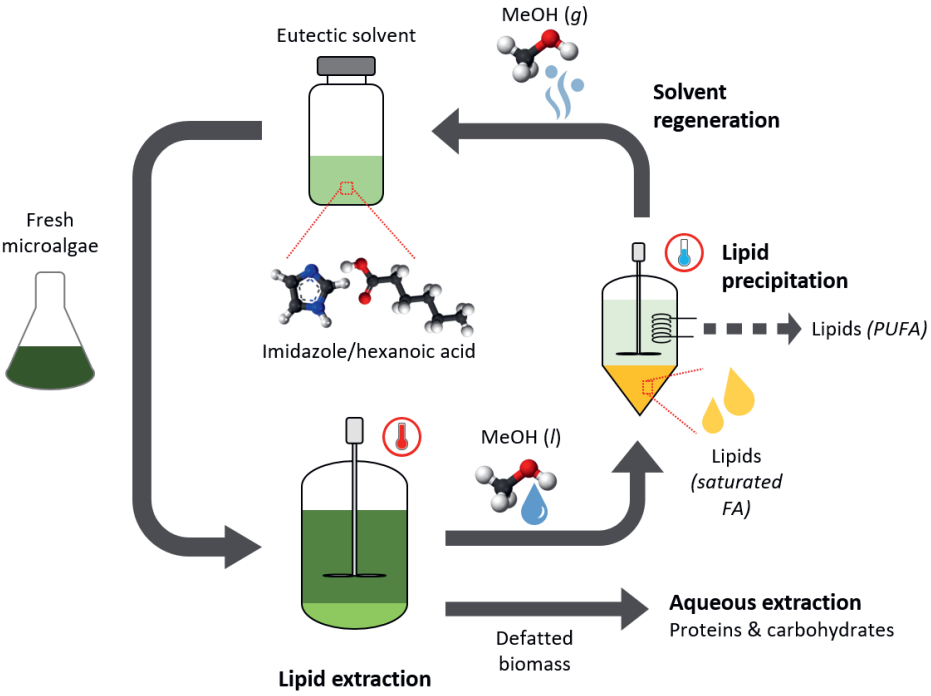


Fig. 1. Schematic overview of the proposed biorefinery of *Nannochloropsis oceanica* based on the imidazole/hexanoic acid eutectic solvent.

4. Challenges and Future perspectives

As mentioned above, applications of eutectic solvents on microalgae processes are still relatively new. In this work, several relevant challenges for biorefinery processing were discovered. Hence, we outline below the challenges to be addressed and future perspectives on microalgae biorefinery based on ES.

In this thesis, we mainly focused on the semi-hydrophobic ES as the basis of the microalgae biorefinery. This process, unfortunately, rendered the proteins denatured, which is associated with compromised functionality (**Chapter 5**). Arguably, the denatured proteins could also possess new functionalities, such as a gelling agent or as amino acids precursor. Moreover, from the Lowry analysis, some proteins were detected in the hexanoic acid and ES phase,

which might indicate the isolation of hydrophobic proteins. If hydrophobic proteins were indeed extracted, then this study would be the first to extract hydrophobic proteins using ES. To date, little attention is given to this protein fraction, although the majority of microalgal proteins are insoluble (membrane-bound).[10] Recently, the insoluble proteins from microalga *Chlorella protothecoides* were used as an emulsifier.[10] However, it is clear that this approach is not suitable to extract water-soluble proteins, like phycobiliproteins, the main pigment-protein complex for as light absorber in cyanobacteria, or Rubisco (Ribulose-1,5-biphosphate carboxylase oxygenase), the responsible enzyme for carbon assimilation in all photosynthetic organisms.

The protein denaturation might be caused by several factors, such as the solvent chemical property, low water activity, high extraction temperature, or their combinations. Depending on the triggering factors, strategies to improve the proposed process to be milder should be developed. Therefore, it is necessary to determine the actual cause of the denaturation.

Imidazole/hexanoic acid ES exhibited amphiphilicity (consisting of both hydrophilic and hydrophobic moieties), which is like detergent, could promote protein unfolding. Typically, the water-soluble proteins have a hydrophobic core and hydrophilic surface. Thus, the presence of amphiphiles could destabilize the protein structure and unfold the proteins.[11] Besides that, the high concentration of hexanoic acid, as a pure solvent or in ES, implies that the system is highly acidic. At high acidity (or low pH values in aqueous solutions), proteins are mostly positively charged and may change their conformation as similar charges repel each other (the electrostatic interaction). Furthermore, certain ions and compounds could also influence the surface charge of protein by either promoting (kosmotrope) or breaking (chaotrope) the hydrogen bonding network in water, Hofmeister effect.[12,13] Typically, the protein stability is promoted by kosmotropic compounds or pairs of chaotropic cation and kosmotropic anion or kosmotropic compounds.[14] However, the categorization of ES based on the Hoffmeister effect has not yet been widely researched despite the extensive studies done on ionic liquids.[15] In ionic liquids,

imidazolium cations with shorter side chains tend to be chaotropic[14,15], while carboxylate anion with longer alkyl chains became less kosmotropic.[16] Therefore, there is a chance that the used ES destabilized the proteins.

That said, in the situation where the native structure of water-soluble proteins is desired, the use of hydrophilic ES could be beneficial. Not only they can weaken the microalgae cell wall[8], but they can stabilize proteins as well.[17–19] For instance, lipase could remain stable at choline chloride/urea ES despite the denaturing effect of urea.[20] Moreover, ES choline chloride/urea and choline chloride/glycerol were reported to facilitate thermal refolding of lysozyme.[21] Hydrophilic ES have also been applied for protein extraction in an aqueous two-phase system (ATPS). ATPS based on phosphate buffer and ES that made of organic salts (choline chloride or betaine) and hydrogen bond donors (polyols, sugars, or urea) were used to extract > 98% of bovine serum albumin (BSA).[22,23] The ATPS was further improved to enhance back-extraction of the protein (reaching 72% of efficiency) using a ternary ES tetramethylammonium chloride/glycerol/urea.[24]

Another approach would be implementing a biphasic system made of hydrophobic and hydrophilic phases. Such a system would simultaneously extract both hydrophobic and hydrophilic biomolecules without compromising the functionality. For instance, the combination of the semi-hydrophobic ES with salt or polymeric solution would form such a biphasic system. Thus, while the ES could directly permeabilize the cell wall and extract the lipids, the water-soluble components would migrate to the aqueous solution. Such a system was implemented for the separation of both hydrophobic and hydrophilic bioactive compounds from *Ginkgo biloba* leaves.[25] The leaves contained flavonoids, terpene trilactone, procyanidine – which are hydrophilic – and polyprenyl acetate (hydrophobic). Using a biphasic system that was formed using three different ES: choline chloride/lactic acid (hydrophilic), choline chloride/malonic acid (hydrophilic), and methyltrioctylammonium chloride/octanol/octanoic acid (hydrophobic) were used to separate those metabolites and reached ~80 – 95% of extraction efficiencies.[25] However, to the furthest of our knowledge,

the ES-based biphasic system has not yet been implemented for protein extraction.

As mentioned before, solvent acidity is an important parameter that can affect the protein charge and conformation. The use of a high concentration of unbuffered acid should be avoided. However, ES are a mixture of pure compounds where water is undesired. Thus, ES made of the less acidic compound could greatly enhance the protein stability. For instance, instead of using hexanoic acid as the Brønsted acid, perhaps neutral hydrogen bond donors such as menthol could be used. However, since the concept of acidity in an aqueous solution would be different in the ES-rich environment, the charge dynamic of the protein surface needs to be studied.

Besides that, the Hofmeister effect – ion-specific interaction – should be considered.[26,27] For this purpose, further studies of how the solvent components interact with each other, water, and proteins should be understood. However, since the system is multicomponent, even the starting ES are already a mixture, instead of pure salts like ionic liquids, studying this system would be incredibly complex. Thus, it is important to implement a step-by-step approach with a model system. Starting with possibly formed kosmotropes and chaotropes in the ES, including their synergized effect, is recommended. Then, continue with the concentration of water.

Low water activity could also denature proteins. The polar groups of ES compete with proteins for water, while the latter requires hydration to stabilize their hydrophilic surface.[27] Moreover, without sufficient hydration, the ES polar groups (e.g., hexanoate anion) would interact strongly with the protein surface. Thus, at low water content, the hexanoate anion would be a chaotropic anion – destabilizing proteins – despite the kosmotropic effect in dilute aqueous solutions.[27] Furthermore, BSA and lysozyme were observed to be partially folded in pure ES of choline chloride/glycerol. When the ES was hydrated, the proteins retained their folded structure as in a phosphate buffer saline.[18] Thus, the strategy to tackle would be to remove less water during the harvesting or use one or more aqueous phases in the biphasic or ATPS system.

The former would be economically attractive since less water removal would require less energy during this step. The latter could also be achieved by implementing ATPS or the biphasic system with a water-rich phase.

Protein denaturation may also be a thermal effect since hydrogen bond is weakened at higher temperatures. The ES extraction was performed optimally at 50 °C, and the lipid yield decreased at lower temperatures (**Chapter 4**). Thus, it is a trade-off between the lipid yield and the protein native state since lower temperature (≤ 35 °C) is necessary to ensure the latter. However, the actual upper limit for temperature might not be 35 °C and should be the denaturation temperature of the algal proteins with the presence of the ES. Thus, this upper limit needs to be determined. For instance, differential scanning calorimetry (DSC) could be used to study the thermal stability (T_m) of proteins.[28]

Simultaneously, the effect of heat for the ES extraction needs to be investigated so that the extraction temperature could be lowered without compromising the lipid yield. The lipid solubility in the ES with low imidazole content was already high, indicating the high solvent carrying capacity, even at room temperature. Thus, the higher temperature might improve the solvent penetration and the segregation of lipids from other biomolecules. Besides decreasing the ES viscosity[29–31], the high temperature could also weaken the cell wall, which served as the main barrier for solvent penetration. Hence, if the cell wall could be removed, disintegrated, or significantly weakened by physical or mechanical energy input, the compromised lipid yield could be compensated.[6,7,32] Previously, microwave treatments were used to enhance the cell wall-weakening effect of hydrophilic ES[33] and induce cell disruption before the extraction with a switchable hydrophilicity ES.[34] However, the temperature could reach up to ≥ 100 °C during the microwave treatment, which would render the proteins denatured.[33]

Other milder external forces, such as acoustic or electric fields, may be applied to reduce the cell wall integrity or to disrupt the cells. During ultrasonication, high-frequency acoustic waves decompress the liquid and induce cavitation, eventually collapsing and rupturing the cell wall.[7,35,36] The cell wall-weakening

effect of the ES might also reduce the energy required to damage the cell wall. However, it is not yet clear how the cavitation in the ES would work since cavitation requires a pressure lower than the vapor pressure of ES, which are relatively nonvolatile. Besides that, the propagation of the sound wave, the optimal frequency and intensity, and the transmission of shear forces in the ES media should be investigated. On the other hand, electroporation by pulsed electric field (PEF) offers an alternative option to accelerate the solvent penetration. PEF require media with high conductivity to ensure the propagation of the electric field.[7,37,38] Thus, the ES, when mixed with water, may give a beneficial effect due to the presence of ionic solutes with low viscosity.[39] Alternatively, a short mechanical cell disruption by the conventional high-pressure homogenizer or bead milling technique could also be used.[40] However, it is important to note that this additional treatment would increase the energy demand and the production cost. It is worth noting that with this extra treatment, the extraction time could be shortened. Currently, the lipid extraction from the intact biomass took place > 8 hours. With the ruptured cell wall, however, the lipids would be liberated and readily accessible for the solvent.[32]

In addition, several improvement points on lipid extraction are also discussed. The operational parameters used in **Chapter 5** were rather chosen arbitrarily due to the lack of information and it is highly likely to cause a suboptimal overall process. For instance, the solvent-to-biomass ratio of 10 mL g_{DW}^{-1} was used, resulting in a low lipid concentration and the low efficiency of lipid recovery. To design an optimal biorefinery process, process modelling is a powerful tool to predict the process outcome. In that regard, a combination of experimental data and robust mathematical models could be a good starting point. Our preliminary result using non-random two liquids (NRTL) thermodynamic model showed that the model could describe well the equilibria in the lipid extraction and precipitation.

Furthermore, in **Chapter 5**, the PUFA-rich polar lipid fraction remained dissolved in the ES-rich fraction, with accumulation went on with the extraction cycles. It is economically and technically important to obtain this PUFA-rich

fraction since the fraction would have a high added value and eventually decrease the yield of the next extraction cycle. Therefore, a strategy for this lipid recovery is required. High performance liquid chromatography (HPLC) techniques, both normal (polar stationary phase) and reverse phase (nonpolar stationary phase), may be useful to separate the lipid fractions from the solvent phase.[41]

Besides that, the main advantage of designer solvents, including ES, is their tailorable properties. In this thesis, we demonstrated that ES's physicochemical properties, particularly hydrophobicity, were influenced by the nature of their constituents and the composition (**Chapter 2**). Furthermore, additions of other compounds, such as lipids or water, would definitely affect the system property (**Chapter 3**). These insights were obtained through the empirical trial and error method. With this approach, although the molecular interactions could be deduced from the observable property, the exact interaction at the molecular level remains unknown. For instance, theoretically, imidazole and hexanoic acid could interact via several ways: 1) proton transfer, producing a protic ionic liquid;[42,43] 2) hydrogen bonding, which is typical for eutectic solvents;[44,45] 3) formation of other complexes, such as homoassociation of hexanoate anion and hexanoic acid;[43,46,47] and 4) combinations of above. Each of the mentioned interactions would implicate different lipid solubilization mechanisms and even recovery strategies.

In contrast to the empirical approach, the mechanistic approach could predict the observable macroscopic property based on the intermolecular forces. Thus, besides having a higher chance of designing the task-specific solvent, insight into the solvation mechanism could be acquired. Typically, solvatochromism is used to experimentally determine the molecular property of the ES, such as hydrogen bond donating and accepting capacity and polarity.[48–51] On the other hand, the molecular interactions in the ES system can also be simulated *via* computational chemistry modellings, such as molecular dynamics[52,53] and quantum chemical calculation.[45,54,55] The latter, particularly COSMO-RS (Conductor-like Screening Model for Realistic Solvents), has been widely used in the field of ES.[51,56–58]

Computational chemistry like COSMO-RS is a powerful tool to study the interaction of ES components with biomolecules, e.g., proteins[26] and plant secondary metabolites,[59,60] and cytotoxicity.[61] Moreover, COSMO-RS has been used to develop biphasic eutectic solvents (hydrophilic: choline chloride/hexafluoroispropanol; and hydrophobic: methyltrioctylammonium chloride/menthol) for the extraction and separation of both polar and nonpolar natural compounds from *Artemisia annua* leaves.[62] Eventually, the obtained knowledge might also be used to predict the interaction of the solvents with more complex biomolecules, such as proteins and cell wall components. This prediction would enable designing the task-specific ES which suits the need of microalgae biorefinery.

Finally, solvent sustainability should not be taken for granted.[63] Proper toxicity studies and life cycle assessments still need to be performed. Currently, few studies are available in the literature about the actual environmental impact and toxicity of ES. One study reported that ES made of choline chloride/acetic acid is more cytotoxic than the ionic liquid cholinium acetate.[64] Ironically, ionic liquids are more commonly associated with potential toxicity, whereas ES are perceived as environmentally benign. Furthermore, not all ES components in this thesis are categorized as renewables. While hexanoic acid is biodegradable and can be produced *via* fermentation, imidazole, despite being biodegradable, is currently fossil-derived and considered toxic for humans. Hence, it is necessary to find more sustainable and safe alternatives. Therefore, besides understanding the role of each component *via* COSMO-RS, having a database of sustainable and naturally available compounds would be advantageous for designing the task-specific green ES for microalgae biorefinery.

5. Conclusion

This thesis demonstrated the use of a new type of ES, semi-hydrophobic ES made of imidazole and hexanoic acid, for lipid extraction from microalgae without biomass pretreatments. Furthermore, the dissolved lipids can be recovered from the ES by the addition of methanol, which was later

evaporated to regenerate the ES. We also performed a preliminary investigation of the use of the ES for microalgae biorefining. The solvent recyclability and scalability were feasible. However, despite the successful lipid extraction, the process was not sufficiently mild to maintain the native structure of proteins. Possible combinations of the ES chemical properties, the high extraction temperature, and the low water content might cause denaturation, which is undesired in the biorefinery context. Therefore, this issue is extensively discussed and several perspectives for the process improvement are suggested. The use of a biphasic system (hydrophobic and hydrophilic ES) with less acidic constituents, lower extraction temperatures, and the application of external physical fields might alleviate the problem and even accelerate the extraction process. The lipid extraction can be improved by recovering the PUFA-rich polar lipid fraction from the ES phase. Moreover, the process parameters need to be optimized based on the process modelling (i.e., equilibrium-based liquid-liquid extraction). Besides that, the need to study the molecular interaction between the ES components, antisolvents, and biomolecules are emphasized. Computational chemistry modelling, like COSMO-RS, is an effective tool to understand the system's chemistry and design and tailor the suitable ES for microalgae biorefinery. Last, green and safe ES are pre-requisite to have a sustainable microalgae biorefinery.

References

1. Wijffels, R. H., Barbosa, M. J. & Eppink, M. H. M. Microalgae for the production of bulk chemicals and biofuels. *Biofuels, Bioprod. Bioref.* **4**, 287–295 (2010).
2. Ruiz Gonzalez, J. *et al.* Towards industrial products from microalgae. *Energy Environ. Sci.* (2016). doi:10.1039/C6EE01493C
3. Halim, R., Danquah, M. K. & Webley, P. A. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnol. Adv.* **30**, 709–732 (2012).
4. Kumar, J., Garlapati, V. K., Dash, A., Scholz, P. & Banerjee, R. Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Res.* **21**, 138–147 (2017).
5. Lee, S. Y., Cho, J. M., Chang, Y. K. & Oh, Y.-K. K. Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresour. Technol.* **244**, 1317–1328 (2017).
6. Halim, R. *et al.* Towards sustainable microalgal biomass processing: anaerobic induction of autolytic cell-wall self-ingestion in lipid-rich Nannochloropsis slurries. *Green Chem.* **21**, 2967–2982 (2019).
7. Günerken, E. *et al.* Cell disruption for microalgae biorefineries. *Biotechnology Advances* (2015). doi:10.1016/j.biotechadv.2015.01.008
8. Lu, W. *et al.* A new approach of microalgal biomass pretreatment using deep eutectic solvents for enhanced lipid recovery for biodiesel production. *Bioresour. Technol.* **218**, 123–128 (2016).
9. Pan, Y. *et al.* One-step production of biodiesel from wet and unbroken microalgae biomass using deep eutectic solvent. *Bioresour. Technol.* **238**, 157–163 (2017).
10. Dai, L., Reichert, C. L., Hinrichs, J. & Weiss, J. Acid hydrolysis behavior of insoluble protein-rich fraction extracted from Chlorella protothecoides. *Colloids Surfaces A Physicochem. Eng. Asp.* **569**, 129–136 (2019).
11. Otzen, D. E. Protein Unfolding in Detergents: Effect of Micelle Structure, Ionic Strength, pH, and Temperature. *Biophys. J.* **83**, 2219–2230 (2002).
12. Mazzini, V. & Craig, V. S. J. What is the fundamental ion-specific series for anions and cations? Ion specificity in standard partial molar volumes of electrolytes and electrostriction in water and non-aqueous solvents. *Chem. Sci.* **8**, 7052–7065 (2017).
13. Hyde, A. M. *et al.* General Principles and Strategies for Salting-Out

Informed by the Hofmeister Series. *Org. Process Res. Dev.* **21**, 1355–1370 (2017).

14. Zhao, H. Protein Stabilization and Enzyme Activation in Ionic Liquids: Specific Ion Effects. doi:10.1002/jctb.4837
15. Umapathi, R., Reddy, P. M., Rani, A. & Venkatesu, P. Influence of additives on thermoresponsive polymers in aqueous media: a case study of poly(N-isopropylacrylamide). *Phys. Chem. Chem. Phys.* **20**, 9717–9744 (2018).
16. Sultana, N. & Ismail, K. Specific ion effects of chloride vis-à-vis acetate, propionate and butyrate counterions on the cetylpyridinium headgroup at the micelle–solution and air–solution interfaces. *J. Mol. Liq.* **213**, 145–152 (2016).
17. Gertrudes, A. *et al.* How Do Animals Survive Extreme Temperature Amplitudes? The Role of Natural Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **5**, 9542–9553 (2017).
18. Sanchez-Fernandez, A., Edler, K. J., Arnold, T., Venero, D. A. & Jackson, A. J. Protein conformation in pure and hydrated deep eutectic solvents. *Phys. Chem. Chem. Phys.* **19**, 8667–8670 (2017).
19. Sanchez-Fernandez, A. & Jackson, A. J. Proteins in deep eutectic solvents: Structure, dynamics and interactions with the solvent. *Adv. Bot. Res.* **97**, 69–94 (2021).
20. Monhemi, H., Housaindokht, M. R., Moosavi-Movahedi, A. A. & Bozorgmehr, M. R. How a protein can remain stable in a solvent with high content of urea: insights from molecular dynamics simulation of Candida antarctica lipase B in urea : choline chloride deep eutectic solvent. *Phys. Chem. Chem. Phys.* **16**, 14882–14893 (2014).
21. Esquembre, R. *et al.* Thermal unfolding and refolding of lysozyme in deep eutectic solvents and their aqueous dilutions. *Phys. Chem. Chem. Phys.* **15**, 11248–11256 (2013).
22. Xu, K., Wang, Y., Huang, Y., Li, N. & Wen, Q. A green deep eutectic solvent-based aqueous two-phase system for protein extracting. *Anal. Chim. Acta* **864**, 9–20 (2015).
23. Li, N. *et al.* Development of green betaine-based deep eutectic solvent aqueous two-phase system for the extraction of protein. *Talanta* **152**, 23–32 (2016).
24. Zhang, H. *et al.* Ternary and binary deep eutectic solvents as a novel extraction medium for protein partitioning. *Anal. Methods* **8**, 8196–8207

(2016).

25. Cao, J. *et al.* Two-phase systems developed with hydrophilic and hydrophobic deep eutectic solvents for simultaneously extracting various bioactive compounds with different polarities. *Green Chem.* **20**, 1879–1886 (2018).
26. Mehringer, J. *et al.* Salting-in and salting-out effects of short amphiphilic molecules: a balance between specific ion effects and hydrophobicity. *Phys. Chem. Chem. Phys.* **23**, 1381–1391 (2021).
27. Zhao, H. Protein stabilization and enzyme activation in ionic liquids: specific ion effects. *J. Chem. Technol. Biotechnol.* **91**, 25–50 (2016).
28. Schön, A., Clarkson, B. R., Jaime, M. & Freire, E. Temperature stability of proteins: Analysis of irreversible denaturation using isothermal calorimetry. *Proteins* **85**, 2009 (2017).
29. Hayyan, A. *et al.* Glucose-based deep eutectic solvents: Physical properties. (2013). doi:10.1016/j.molliq.2012.11.025
30. Abbott, A. P., Boothby, D., Capper, G., Davies, D. L. & Rasheed, R. K. Deep Eutectic Solvents Formed between Choline Chloride and Carboxylic Acids: Versatile Alternatives to Ionic Liquids. *J. Am. Chem. Soc.* **126**, 9142–9147 (2004).
31. Abbott, A. P. *et al.* Glycerol eutectics as sustainable solvent systems. *Green Chem.* **13**, 82–90 (2011).
32. Yap, B. H. J., Crawford, S. A., Dumsday, G. J., Scales, P. J. & Martin, G. J. O. A mechanistic study of algal cell disruption and its effect on lipid recovery by solvent extraction. *Algal Res.* **5**, 112–120 (2014).
33. Tommasi, E. *et al.* Enhanced and Selective Lipid Extraction from the Microalga P. tricornutum by Dimethyl Carbonate and Supercritical CO 2 Using Deep Eutectic Solvents and Microwaves as Pretreatment. *ACS Sustain. Chem. Eng.* **5**, 8316–8322 (2017).
34. Sed, G., Cicci, A., Jessop, P. G. & Bravi, M. A novel switchable-hydrophilicity, natural deep eutectic solvent (NaDES)-based system for bio-safe biorefinery. *RSC Adv.* **8**, 37092–37097 (2018).
35. Kurokawa, M. *et al.* Effect of sonication frequency on the disruption of algae. *Ultrason. Sonochem.* **31**, 157–162 (2016).
36. Zhang, R., Lebovka, N., Marchal, L., Vorobiev, E. & Grimi, N. Pulsed electric energy and ultrasonication assisted green solvent extraction of bio-molecules from different microalgal species. *Innov. Food Sci. Emerg.*

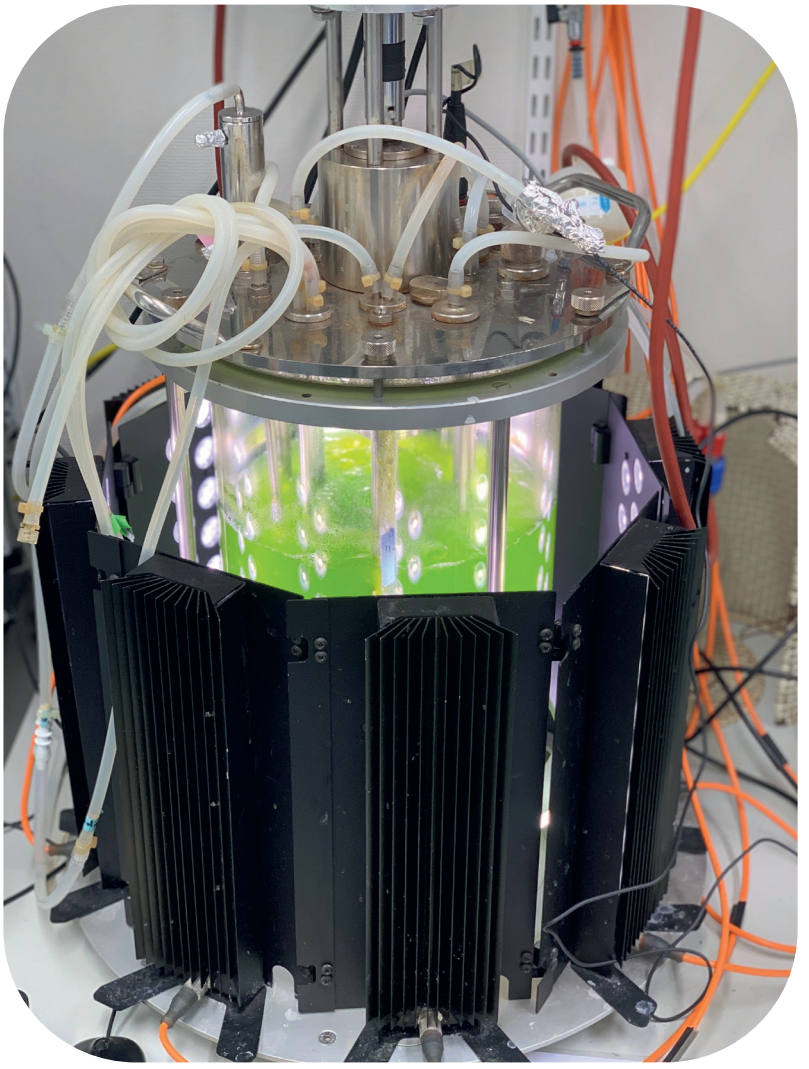
Technol. **62**, 102358 (2020).

37. Lam, G. P. 't *et al.* Pulsed Electric Field for protein release of the microalgae Chlorella vulgaris and Neochloris oleoabundans. *Algal Res.* **24**, 181–187 (2017).
38. Parniakov, O. *et al.* Pulsed electric field and pH assisted selective extraction of intracellular components from microalgae Nannochloropsis. *Algal Res.* **8**, 128–134 (2015).
39. Dai, Y., Witkamp, G.-J., Verpoorte, R. & Choi, Y. H. Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. *Food Chem.* **187**, 14–19 (2015).
40. Suarez Garcia, E. *et al.* Selective and energy efficient extraction of functional proteins from microalgae for food applications. *Bioresour. Technol.* (2018). doi:10.1016/J.BIORTECH.2018.07.131
41. Olsson, P., Holmbäck, J. & Herslöf, B. A single step reversed-phase high performance liquid chromatography separation of polar and non-polar lipids. *J. Chromatogr. A* **1369**, 105–115 (2014).
42. Anouti, M. *et al.* Aggregation behavior in water of new imidazolium and pyrrolidinium alkycarboxylates protic ionic liquids. *J. Colloid Interface Sci.* **340**, 104–111 (2009).
43. Yoshizawa, M., Xu, W. & Angell, C. A. Ionic Liquids by Proton Transfer: Vapor Pressure, Conductivity, and the Relevance of ΔpKa from Aqueous Solutions. *J. Am. Chem. Soc.* **125**, 15411–15419 (2003).
44. Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K. & Tambyrajah, V. Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* 70–71 (2003). doi:10.1039/b210714g
45. Ashworth, C. R., Matthews, R. P., Welton, T. & Hunt, P. A. Doubly ionic hydrogen bond interactions within the choline chloride–urea deep eutectic solvent. *Phys. Chem. Chem. Phys.* **18**, 18145–18160 (2016).
46. Martins, M. A. R., Carvalho, P. J., Santos, L. M. N. B. F., Pinho, S. P. & Coutinho, J. A. P. The impact of oligomeric anions on the speciation of protic ionic liquids. *Fluid Phase Equilib.* **531**, 112919 (2021).
47. Johansson, K. M., Izgorodina, E. I., Forsyth, M., MacFarlane, D. R. & Seddon, K. R. Protic ionic liquids based on the dimeric and oligomeric anions: [(AcO)xHx−1]−. *Phys. Chem. Chem. Phys.* **10**, 2972–2978 (2008).
48. Teles, A. R. R. *et al.* Solvatochromic parameters of deep eutectic solvents formed by ammonium-based salts and carboxylic acids. *Fluid Phase Equilib.*

448, 15–21 (2017).

49. Florindo, C., McIntosh, A. J. S., Welton, T., Branco, L. C. & Marrucho, I. M. A closer look into deep eutectic solvents: Exploring intermolecular interactions using solvatochromic probes. *Phys. Chem. Chem. Phys.* **20**, 206–213 (2017).
50. Pandey, A. & Pandey, S. Solvatochromic Probe Behavior within Choline Chloride-Based Deep Eutectic Solvents: Effect of Temperature and Water. *J. Phys. Chem. B* **118**, 14652–14661 (2014).
51. Martins, M. A. R. *et al.* Tunable Hydrophobic Eutectic Solvents Based on Terpenes and Monocarboxylic Acids. *ACS Sustain. Chem. Eng.* **6**, 8836–8846 (2018).
52. Zahn, S., Kirchner, B. & Mollenhauer, D. Charge Spreading in Deep Eutectic Solvents. *ChemPhysChem* **17**, 3354–3358 (2016).
53. Mohan, M. *et al.* Solubility of glucose in tetrabutylammonium bromide based deep eutectic solvents: Experimental and molecular dynamic simulations. *Fluid Phase Equilib.* **448**, 168–177 (2017).
54. Wagle, D. V, Deakyne, C. A. & Baker, G. A. Quantum Chemical Insight into the Interactions and Thermodynamics Present in Choline Chloride Based Deep Eutectic Solvents. *J. Phys. Chem. B* **120**, 6739–6746 (2016).
55. Stefanovic, R., Ludwig, M., Webber, G. B., Atkin, R. & Page, A. J. Nanostructure, hydrogen bonding and rheology in choline chloride deep eutectic solvents as a function of the hydrogen bond donor. *Phys. Chem. Chem. Phys.* **19**, 3297–3306 (2017).
56. Silva, L. P. *et al.* Design and Characterization of Sugar-Based Deep Eutectic Solvents Using Conductor-like Screening Model for Real Solvents. *ACS Sustain. Chem. Eng.* **6**, 10724–10734 (2018).
57. Fernandez, L. *et al.* Indirect assessment of the fusion properties of choline chloride from solid-liquid equilibria data. *Fluid Phase Equilib.* **448**, 9–14 (2017).
58. Kundu, D., Rao, P. S. & Banerjee, T. First-Principles Prediction of Kamlet–Taft Solvatochromic Parameters of Deep Eutectic Solvent Using the COSMO-RS Model. *Ind. Eng. Chem. Res.* **59**, 11329–11339 (2020).
59. Wojeicchowski, J. P., Ferreira, A. M., Abranches, D. O., Mafra, M. R. & Coutinho, J. A. P. Using COSMO-RS in the Design of Deep Eutectic Solvents for the Extraction of Antioxidants from Rosemary. *ACS Sustain. Chem. Eng.* **8**, 12132–12141 (2020).

60. Jeliński, T. & Cysewski, P. Application of a computational model of natural deep eutectic solvents utilizing the COSMO-RS approach for screening of solvents with high solubility of rutin. *J. Mol. Model. 2018 247* **24**, 1–17 (2018).
61. Hayyan, M. *et al.* Natural deep eutectic solvents: cytotoxic profile. *SpringerPlus 2016 51* **5**, 1–12 (2016).
62. Tang, W. & Row, K. H. Design and evaluation of polarity controlled and recyclable deep eutectic solvent based biphasic system for the polarity driven extraction and separation of compounds. *J. Clean. Prod.* **268**, 122306 (2020).
63. Chen, Y. & Mu, T. Revisiting greenness of ionic liquids and deep eutectic solvents. *Green Chem. Eng.* **2**, 174–186 (2021).
64. Morais, P. de, Gonçalves, F., Coutinho, J. A. P. & Ventura, S. P. M. Ecotoxicity of Cholinium-Based Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **3**, 3398–3404 (2015).



Summary

The common lipid extraction from microalgae involves sequential of energy-intensive processes and the use of harmful organic solvents. Eutectic solvents (ES), a novel class of designer solvents, hold a great potential as alternative solvents. Not only they are easy to prepare and have tailorable properties, but they are also able to permeabilize the cell wall of microalgae. However, the lack of vapor pressure makes the solvent regeneration difficult. Therefore, in this thesis, a semi-hydrophobic eutectic solvent was developed to extract lipids from microalgae, with a viable solvent regeneration step. Moreover, a preliminary biorefinery process was also explored.

In **Chapter 2**, we screened several semi-hydrophobic ES. The combination of polar imidazole and nonpolar hexanoic acid showed tuneable hydrophobicity depending on the composition. At low imidazole presence, the mixture dissolved lipids and the lipid solubility decreased at higher imidazole content. This principle was then applied to separate the ES from the dissolved lipids. With this approach, about 75% of lipids could be recovered with a high purity (> 85%).

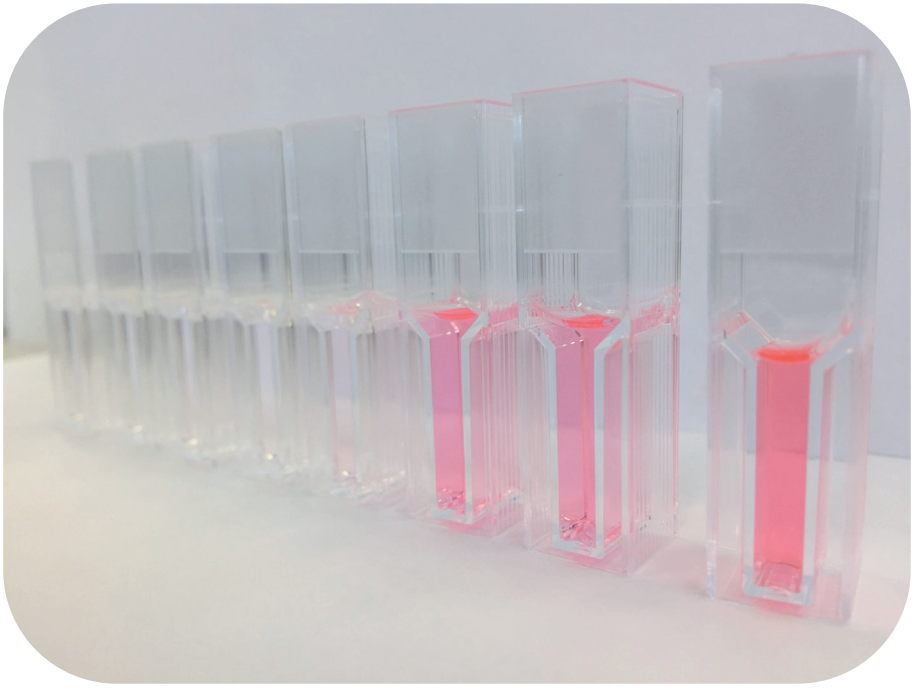
Since retrieving imidazole from the altered ES was difficult, we explored other auxiliaries which can also shift the ES hydrophobicity (**Chapter 3**). Polar antisolvents, such as water, methanol, and ethanol, were observed to reduce the lipid solubility in the ES. The reduction of lipid solubility increases with the antisolvent polarity and amount. Furthermore, since the antisolvents were (moderately) volatile, a large amount of antisolvents could be loaded to obtain higher purity (up to 100%) and recovery (> 90%) when compared to the previous approach. With this approach, more than 90% of ES could be regenerated through evaporating the antisolvents. Methanol was selected as the best antisolvent as it offered the ease of regeneration without sacrificing the lipid recovery.

The novel ES was then applied to intact *Nannochloropsis oceanica* for lipid extraction (**Chapter 4**). The yield of lipids extracted with the ES was comparable to the standard Bligh & Dyer method using chloroform/methanol. The extraction yield was found to benefit from the low imidazole content (0 – 15

mol%; hydrophobic state), a high temperature, longer incubation time, and high solvent-to-biomass ratio. Interestingly, the moisture from the wet biomass enhanced the lipid extraction, which was found to decrease with freeze-dried biomass. In addition, supplementation of water could reverse the adverse effect of drying. This result implied that the disruption and drying of biomass was not necessary to ensure the high lipid yield.

We also developed further the ES-based process towards microalgae biorefinery (**Chapter 5**). First, the separation of algae lipid from the ES was performed by addition of methanol at low temperature (-20 °C). With this approach, 60% of the lipids could be recovered, which was significantly lower than the finding in **Chapter 3**. Furthermore, the ES were reusable after three iterative cycles without significant drop of extraction efficiency. Besides that, the process was found to be scalable with slightly lower efficiency and recovery. Furthermore, denatured proteins and carbohydrates could be obtained from the defatted biomass through an aqueous extraction.

Several key findings and challenges from this thesis are summarized in **Chapter 6**. Besides that, some ideas for tackling the current drawbacks of the proposed process are also discussed. Additionally, we include recommendations for further research to gain understanding of molecular interactions between the ES components and target molecules. Ultimately, task-specific ES for biorefining could be tailored based on the obtained knowledge.



Acknowledgements

With this final section, this thesis has come to an end, and so has my PhD journey. Looking back at the past four years, I certainly have learned a lot, both personally and professionally. All of these would have been impossible without the help and support of people I cannot thank enough.

First of all, my supervisors, **René**, **Michel**, and **Corjan,** for their guidance, patience, wisdom, effort, and time. **René**, during my bachelor study, I came across your big name in the algae field, and I met you the first time in Bandung, which perhaps you do not even remember. Since then, I have aspired to be your student, and thus I came to Wageningen for my MSc. During the second half of the PhD, I got the opportunity to learn closely from you since you became my biweekly supervisor despite your busy agenda. You gave me a lot of valuable inputs, both scientifically and personally, for which I am deeply grateful.

Michel, you are always supportive and responsive wherever and whenever you are. Thank you for always providing me time among your super busy schedules and for your lightning-speed high-quality feedback on the manuscripts. Besides that, you are truly inspiring, as a profound scientist as well as a competent manager. You gave me the freedom to choose my research direction. But at the same time, you knew when exactly I needed your help, especially during the critical time of finishing this thesis.

Corjan, thanks for giving me a chance to embark on this journey right after my MSc. I really enjoyed all our discussions, regardless of the topics, about science and beyond. You constantly challenged me to get out of my comfort zones, to learn new things and improve myself, especially for my personal development. Although you left BPE (which I knew for a good reason), we continued to have good contact. I wish you good luck with your entrepreneurial adventure.

Besides that, a substantial portion of this thesis was based upon the hard work of these fantastic students: **Stephanie**, **Valerie**, **Jeltzlin**, **Jorien**, **Rob**, **Elena**, **Kindi**, **Erwin**, **Tamara**, and **Rutger.** Guys, you did really amazing jobs especially bearing with this unexplored and challenging world of DES. This thesis would

not have been as it is now without you. I thank all of you for the valuable lessons and wish you success in your present and future endeavour.

My research was a part of a larger European project, MAGNIFICENT. **Hans**, thank you for coordinating this magnificent team and for the nice conversations, especially during the project meetings. **Maria**, our magnificent coordinator, I appreciate the excellent organization and the lively atmosphere in the meetings and wherever you are. I also thank **all members of the MAGNIFICENT consortium** for their input, interest, and nice conversations during the project meetings, which stimulated me to improve my work. Thank you for the MAGNIFICENT experience.

Next, the Genie's of BPE, **Wendy**, **Snezana**, **Sebastiaan**, **Fred**, **Rick**, and **Rafa**. Thank you for always being ready to help and making my numerous demanding wishes came true: sophisticated analytical methods, inexhaustible consumables, assembled and functional reactors, a new computer, and instant biomass out of thin air. Special appreciation to **Wendy** and **Fred**, who calmly brought me to the hospital when I had the finger accident in the lab. Also, **Miranda** and **Marina**, thank you for all the outstanding administrative supports and the organizations of MAGNIFICENT meetings.

Also, I would like to thank particular colleagues. **Marian**, you were my MSc study advisor, teacher and fairy godmother. During my PhD, I still went to you for troubleshooting, especially regarding students. Thank you for all of that. I hope you enjoy your retirement life. **Giuseppe**, thank you for your advice, collaboration, effort, and time you gave my students and me. **Edgar**, you were my MSc supervisor, and because of you, I enjoyed doing science at BPE. You became a mentor-like figure to me, and I am pleased to have you as my paranymph. All the best for your empire-building journey! **Iulian** and **Antoinette**, you guys became my discussion partners in the field, and I appreciate that. **Narcís**, **Fengzheng (August)**, **Mihris**, and **Marta**, the MAGNIFICENT teams, thanks for the unforgettable fun trips and talk over nice food that we had after project meetings. **Marta**, our manager, thank you for being my paranymph. **Iago**, although you did not join our trips, I enjoy the

entertaining conversations with you especially with some drinks. **Camilo**, thanks for the suggestions, help, and magic touch in designing the thesis layout and cover. **Iris**, I still remember our conversation in the coffee corner that comforted me when I felt low. I enjoy our dinners, walks, and deep talks. **Bárbara**, thanks for chitchat and dinner with **Tom**.

I want to mention people I am happy to share the E2.137 office, **Youri**, **Agi**, **Guangyuan (Jin)**, **Kylie**, and **Jort**. Thanks for the pleasant and conducive atmosphere in the office, for the spontaneous talks and jokes now and then. **Henrik**, you recently started your PhD and chose a good spot; welcome to the best office.

I would like to also thank other colleagues at BPE for the nice coffee breaks, shared cakes, chats inside or outside the lab, lunches, dinners, borrels, fantastic barbecues, labuitjes, Christmas dinners (and PhD trip): **Dirk**, **Sofia**, **Jorijn**, **Fabian**, **Pieter**, **Nicola**, **Sabine**, **Anna**, **Chunzhe**, **Pauline**, **Enrico**, **Catalina**, **Rocca**, **Ana**, **Robin**, **Sebastian**, **Mitsue**, **Christian**, **Alex**, **Pedro**, **Renske**, **Nuran**, **Ruud**, **Arjen**, **Aamna**, **Marcel**, **Sarah**, **Carlos**, **Iris**, **Mark**, and **all of BPE family** that I could not mention one by one. Proost! to **Anna** and **Pauline** for being merciful trainers in drinking. Additionally, I would like to thank the **members of the VLAG PhD Council** for the nice time and lessons that I learned in the council.

For my friends, who have made my life warmer and brighter in this country for the past 6 years. Mierenneukers: **Dionne**, **Lisette**, **Ji**, and **Daniel**, I miss our Orion lunch and the drinking sessions; let's resume our biannual holiday again! **Martha**, **Pavel**, **Joana**, **Tiffany**, and **other friends from MSc studies**, cheers for the good time and laughter. To friends in **ICC choirs and members**, thanks the lovely time every weekend during the rehearsal and all other activities. **Meli**, **Fiona** and **Robert**, I will never forget our Christmas dinner. **Sam** and **Tammy**, I cherish our friendship, holidays, delicacies, boot camp, and the extra effort to help me finish this thesis. So, where are we going next? **Jessica**, many credits to you for designing my thesis cover. I am also thankful for **Indonesian**

community in Wageningen and **all my friends** scattered all over the globe whom I cannot mention all.

Heidi, my partner in gluttony, buddy in chores, travelling companion, caretaker, dumb & dumber, joy bringer, personal motivator, and forever mood booster. Thank you for always being there for me with your devoted support, accompanying me to start and end this journey. I am beyond happy to have you in my life. And looking forward to the coming adventures.

To my faithful support system and pillars: **Papa**, **Mama**, **Molly**, **Qiu Andi**, **Meh Tania**, **Olin**, **Will**, **Ie Hanny**, **Apo** and **the entire family**. I am eternally grateful for your relentless support, prayers, patience, wisdom, and love. Without you, I will never be the person I am now. I proudly present and dedicate this thesis to you.

Calvin

About the author

Biography

Calvin Lo was born on November 4th, 1992, in Cirebon, Indonesia. After completing his primary school in 2004, he left hometown and moved to Bandung (Indonesia) to follow the lower and upper secondary educations. Calvin obtained an undergraduate degree with honors in Bio-engineering at Institut Teknologi Bandung in 2014. Subsequently, he assisted in a research project to produce thermostable enzymes for biodiesel waste valorization.

In 2015, Calvin got a scholarship and moved to The Netherlands to pursue his MSc in Biotechnology (specialization: Process Technology) at Wageningen University. He did his MSc thesis at the Bioprocess Engineering (BPE) group, where he studied the disruption of algae cells with bead milling. At the same time, he was a student assistant in Transfer Process (MSc course). Then, he did an internship at Wetsus (European centre of excellence for sustainable water technology), Leeuwarden, The Netherlands. There he was involved in the project of nutrient recovery from human urine using cyanobacteria.

In August 2017, Calvin started his doctoral research at BPE. His project was a part of a large European research project, MAGNIFICENT. During this study, he developed eutectic solvents for microalgae biorefinery, focusing on lipid extraction. The results of his work are described in this thesis.

Calvin is currently working as a postdoctoral researcher at BPE where he further develops his PhD research. His current role is emphasized on the supervision of BSc and MSc theses.

Contact information: lo.calvin@outlook.com

List of Publications

Published

Lo, C., Eppink, M. H. M., van den Berg, C. & Wijffels, R. H. Extraction and back-extraction of hydrophobic compounds using deep eutectic solvent with tuneable hydrophobicity. *European patent application* no. EP3878534A1 (2021).

Lo, C., Semerel, J., van den Berg, C., Wijffels, R. H. & Eppink, M. H. M. Eutectic solvents with tuneable hydrophobicity: lipid dissolution and recovery. *RSC Adv.* **11**, 8142–8149 (2021).

Suarez Garcia, E., Lo, C., Eppink, M. H. M., Wijffels, R. H. & van den Berg, C. Understanding mild cell disintegration of microalgae in bead mills for the release of biomolecules. *Chem. Eng. Sci.* **203**, 380-390 (2019).

Lo, C., Manurung R. & Esyanti R. R. Enhancement of lycopene and β-carotene production in cherry tomato fruits (*Solanum lycopersicum* L. var. cerasiforme) by using red and blue light treatment. *Int. J. Tech. Res. Appl.* **2**, 7-10 (2014).

Submitted for publication

Lo, C., Wijffels, R. H. & Eppink, M. H. M. Lipid recovery from semi-hydrophobic eutectic solvents by polar antisolvents. *Submitted.*

Lo, C., Wijffels, R. H. & Eppink, M. H. M. Lipid extraction from fresh *Nannochloropsis oceanica* using semi-hydrophobic eutectic solvents. *Submitted.*

Lo, C., Boboescu, I., Haemers, S., Wijffels, R. H. & Eppink, M. H. M. Multi-product extraction from microalga *Nannochloropsis oceanica* based on semi-hydrophobic eutectic solvents. *In preparation.*

Overview of completed training activities

Discipline specific activities

Courses

VLAG Microalgae Biorefinery, Wageningen (NL)	2017
COSMOlogic COSMOTherm Workshop, Leverkusen (DE)	2017
BioTech Delft Advanced Course Downstream Processing, Delft (NL)	2018
University of Copenhagen - Food Proteins: Significance, Reactions, and Modifications, online	2020

Conferences and meetings

Algae Biorefinery for Europe, Brussels (BE)	2017
NBV DSP Symposium: Computers & Bioprocess Development, Amersfoort (NL)	2018
NBC-18: Biotechnology in Harmony, Ede (NL) [1]	2018
14th International Conference on Renewable Resources and Biorefineries (RRB), Ghent (BE) [1]	2018
NBV DSP Symposium: Next Generation Biorefinery, Amersfoort (NL)	2018
4th International Conference on Ionic Liquids in Separation and Purification Technology (ILSEPT), Sitges (ES) [1]	2019
Young Algaeneers Symposium 2021 (EABA), online [1]	2021
MAGNIFICENT project meeting, Doorwerth (NL), Olhão (PT), Madeira (PT), Nantes (FR), Lisbon (PT), online [2]	2017-2021

General courses

WGS Competence Assessment	2017
WGS Supervising BSc and MSc Thesis Students	2017
WGS Project and Time Management	2018
VLAG PhD Week, Baarlo (NL)	2018
WGS Presenting with Impact	2018
WGS Brain Friendly Working and Writing	2019
WGS Scientific Publishing	2019
WGS Scientific Writing	2019
WGS Career Orientation	2020

[1]Poster presentation, [2]Oral presentation

Optionals

Preparation of research proposal	2017
BPE PhD excursion to San Diego (US) [1,2]	2018
Wageningen Indonesia Scientific Exposure (organizer) [1]	2019
BPE Group Meeting [2]	2017-2021
BPE Monthly Biorefinery Meeting [2]	2017-2021
BPE Algae Theme Meeting [2]	2020-2021
VLAG PhD Council	2020-2021

Teaching

Transfer Process (MSc course)	2017-2018
Advanced Biorefinery (MSc course)	2018-2019
Microalgae Biorefinery (VLAG)	2019
Biorefinery (BSc course)	2020-2021
Supervising 8 MSc theses, 1 BSc thesis & 1 internship project	2017-2021

The research described in this thesis was performed within the MAGNIFICENT project, funded by Bio-Based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation program (grant agreement no. 745754).

Cover design by Jessica Tania

Printed by Digiforce || ProefschiftMaken