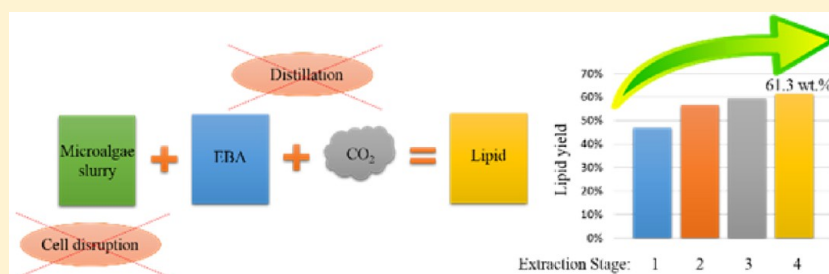


Maximizing Lipid Yield in *Neochloris oleoabundans* Algae Extraction by Stressing and Using Multiple Extraction Stages with N-Ethylbutylamine as Switchable Solvent

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ABSTRACT: The extraction yield of lipids from nonbroken *Neochloris oleoabundans* was maximized by using multiple extraction stages and using stressed algae. Experimental parameters that affect the extraction were investigated. The study showed that with wet algae (at least) 18 h extraction time was required for maximum yield at room temperature and a solvent/feed ratio of 1:1 (w/w). For fresh water (FW), nonstressed, nonbroken *Neochloris oleoabundans*, 13.1 wt % of lipid extraction yield (based on dry algae mass) was achieved, which could be improved to 61.3 wt % for FW stressed algae after four extractions, illustrating that a combination of stressing the algae and applying the solvent N-ethylbutylamine in multiple stages of extraction results in almost 5 times higher yield and is very promising for further development of energy-efficient lipid extraction technology targeting nonbroken wet microalgae.

1. INTRODUCTION

Microalgae have been considered as one of the most promising sustainable feedstocks in recent years. Algae primarily are composed of lipids, proteins, and carbohydrates and are widely used in the area of biofuels,¹ pharmaceuticals² and cosmetics,³ food, and feed.⁴ Microalgae are receiving increasing attention due to their rapid growth rate and thus high productivity, less competition with arable land and freshwater as compared to other crops, and high CO₂ consumption rate.⁵ Several process steps are needed to produce products from algae, such as algal cultivation, harvesting and dewatering, extraction and fractionation, distribution, and utilization.

Lipid extraction is one of the main topics in the research on algae biorefinery processes. Organic solvent extraction has the benefits of inexpensive solvents and high lipid recovery yield.⁶ Supercritical CO₂ (scCO₂) extraction is considered as an efficient and “green” extraction method for lipid extraction.⁷ Both the methods have their own advantages but also some drawbacks. The chemicals used in organic solvent extraction such as hexane are often highly flammable and toxic, and the solvent recovery is energy intensive.⁸ Supercritical CO₂ extraction requires high pressure equipment which is difficult to scale up because of the combination of high pressure equipment with dry solids handling and leads to high operating cost.⁹ Moreover, these methods generally require drying of the

biomass,^{10–15} which accounts for more than 70% of the energy that can be produced by algae.¹⁶

To avoid the considerable costs from the requirement for drying, the use of wet algae biomass as feedstock for lipid extraction is desirable. Different solvents and solvent combinations have been studied for lipid extraction from wet algae biomass,^{17–28} e.g., hexane, hexane/2-propanol, hexane/methanol, chloroform/methanol, dimethyl ether, ethyl acetate/methanol/water, 1,2-dimethoxyethane/water, etc. Also several supercritical fluids have been investigated for lipid extraction from wet algae biomass,^{10,13–15,18,29–33} such as CO₂, methanol, and ethanol. Besides these techniques, a method named CO₂-switchable solvent extraction aroused the interest of many researchers in recent years.^{5,16,34–36} With this technology, lipids can be extracted, after which solvent recovery is accomplished by switching the solvent hydrophilicity with CO₂, which induces phase splitting. In the research by Samori et al., it was found that switchable solvents with the DBU/1-octanol system exhibited a better extraction yield than *n*-hexane, both with dried and wet *Botryococcus braunii* samples (7.8 and 5.6 wt % hydrocarbons yield, respectively).³⁶ Tertiary amine N,N-

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dimethylcyclohexylamine (DMCHA) was also reported for extraction of lipids from microalgae. In the research of Jessop et al., the lipid extraction yields of lyophilized *Botryococcus braunii* were 19 wt % (room temperature) and 22 wt % (60 to 80 °C) of the algal dry weight³⁴ while using DMCHA as extractant. Samori et al. used DMCHA for extracting and recovering lipids directly from wet algae samples (about 80% water content) of three microalgae strains: *Nannochloropsis gaditana*, *Tetraselmis suecica*, and *Desmodesmus communis* and obtained 29.2, 57.9, and 31.9 wt % lipid yield, respectively.³⁵ In the work of Du et al., secondary amines N-ethylbutylamine (EBA) and dipropylamine (DPA) were found to be able to extract 16.8 and 15.4 wt % lipids, respectively, from aqueous slurries of fresh, nonbroken microalgae *Desmodesmus sp.*⁵ A more detailed study on extraction conditions showed that at least 18 h is required for extraction from wet, nonbroken algae and showed the total fatty acid (TFA) composition in the extracts.³⁷ An energy evaluation showed that the switchable solvent extraction method is a very promising method for extracting lipids from algae for use in energy applications.¹⁶ The net energy yield increases 5 to 6 times when the lipid yield increases from 10 to 45 wt %.¹⁶ This increase may be achieved by stressing the algae, and hence, in this paper, we explore extraction of lipids from stressed algae and study how extraction conditions should be optimized for this new scenario.

In this paper, studies on multistage lipid extraction from both nonstressed and stressed (fresh water (FW) stressed and artificial seawater (ASW) stressed) nonbroken *Neochloris oleoabundans* slurry (~5% dry weight) using secondary amine EBA are reported. Lipid yields of EBA extraction were compared with the Bligh and Dyer (B&D) lipid extraction method, commonly applied for analytical purpose. Fatty acid compositions of lipids were analyzed and compared. Optimization study for extraction was established with various parameters, such as cell disruption, extraction time, extraction temperature, and solvent to feed ratio to obtain maximum lipid extraction.

2. MATERIAL AND METHODS

2.1. Chemicals. The solvents and chemicals used in this study were as follows: N-ethylbutylamine (EBA) (≥98.0%, Aldrich), chloroform (≥99.9%, Sigma-Aldrich), methanol (≥99.9%, Fluka), hexane (≥95%, Sigma-Aldrich), methyl nonadecanoate (≥99.5%, Fluka), sulfuric acid (95.0–98.0%, Sigma-Aldrich), and FAME column evaluation mix (1000 µg/mL each component in methylene chloride, analytical standard, Supelco).

2.2. Preparation and Characterization of Algae solutions. Algae of the strain *Neochloris oleoabundans* and stressed *Neochloris oleoabundans* (under nitrogen limitation condition) were obtained from AlgaePARC (NL) as paste. Both fresh and frozen paste have been applied, with indifferent results.³⁷ Algae paste was mixed with water to get ~5 wt % algae slurry that can be used in extraction. The broken fresh algae slurries were prepared by bead milling. The water content in algae slurries was determined by weighing a sample before and after drying at 105 °C for 24 h.

2.3. Extraction and Recovery of Lipids from Algae. Extraction of lipid from algae slurries was done either according to the original B&D method³⁸ or using EBA as solvent. According to the original B&D method, 20 g of an algae sample (5 wt %) was mixed for 8 min with a mixture of 48 mL of methanol and 24 mL of chloroform. To the mixture was then

added another 24 mL of chloroform, and after mixing for 1 min, 24 mL of water was added and mixing continued for another 1 min. The homogenate is centrifuged, and the chloroform layer containing the algae oil was collected. To ensure extraction equilibrium, a prolonged extraction time (120 min) was applied in the experiments with multiple extractions. All experiments were performed at least twice.

For measurements with EBA, the method was kept the same as the previous research.³⁷ Here, 20 g of algae slurries were extracted with EBA for extraction times varying from 10 min up to 24 h. Different EBA to algae slurry weight ratios, e.g., 2:1, 1:1, 1:2, and 1:5, were applied. After the extraction experiments, the mixtures were centrifuged, and the amine layer containing the algal lipids was isolated. An equal amount of H₂O was added to the isolated organic layer to improve the phase separation by improving the switching efficiency. CO₂ was bubbled in a flow rate of 2 VVM (volume per volume per minute) for 60 min, during which the solvent switched into the hydrophilic form. Chloroform was used to recover the lipid layer due to the small scale of experiments. Note that this step is not required when working with larger volumes. The two phases thus created were separated by centrifugation (9000 rpm, 5 min), and the total amount of the extracted product was measured gravimetrically (after evaporating the chloroform recovery solvent) and reported as a percentage on algae dry weight basis (defined as crude lipid yield). The solvent evaporation from the recovered product was complete, and all EBA was removed from the lipids recovered. This was validated in an earlier paper.⁵ All experiments were performed at least twice. The reported error bars correspond to an accuracy of ±2.8% yield, which is the averaged relative standard deviation of all experiments (more than 110 extraction experiments).

2.4. Lipid Transesterification and GC-MS Analysis. The algae lipid extracts were analyzed by GC-MS on total fatty acids (TFAs) after transesterification of the lipids which contain fatty acids into the corresponding fatty acid methyl esters (FAMES). The transesterification and GC-MS method were the same as previous research.³⁷

The TFA yield is defined as

$$\text{TFA yield (\%)} = \frac{m_{\text{TFA}}}{m_{\text{dry algae}}} \times 100\%$$

In this research, TFA fraction in crude lipid is also used for evaluation and it is defined as

$$\text{TFA fraction in crude lipid (\%)} = \frac{m_{\text{TFA}}}{m_{\text{crude lipid}}} \times 100\%$$

3. RESULTS AND DISCUSSION

3.1. Effect of Cell Breaking and Time. The B&D extraction method was taken as a reference in this research. Several different extraction times were applied to study the required time to reach the extraction equilibrium. The results are shown in Figure 1(A). For both nonbroken algae and broken algae, the crude lipid extraction yield is comparable at approximately 13 wt % of the dry mass for lower extraction times. Over time, from 10 min to 24 h, the maximum crude lipid yield for nonbroken algae hardly increased (max 13.9 wt % of dry algae), while for the broken algae the yield increased slightly in the first 60 min to 15.3 wt % of dry algae. Apparently, from the broken algae some compounds are extracted that are

- Crude lipid yield (non-broken algae) ○ Crude lipid yield (broken algae)
▲ TFA yield (non-broken algae) ▲ TFA yield (broken algae)

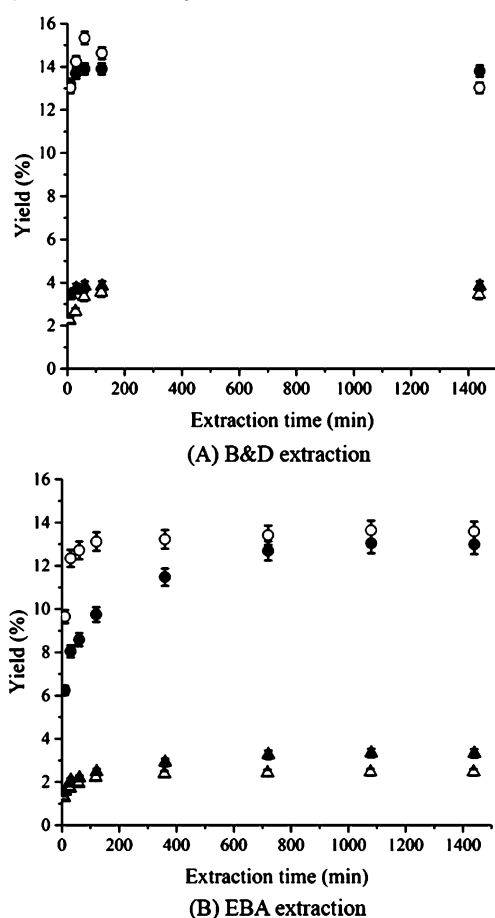


Figure 1. Crude lipid yield (in percentage of the dry algae mass) and TFA yield of *Neochloris oleoabundans* extracted by (A) B&D extraction and (B) EBA extraction. Data in this figure were published before.³⁷

not extractable in the case of nonbroken algae. After 60 min, the yield slowly went down with prolonged extraction time to fall eventually back to the 13 wt % dry mass that was initially also extracted. It may be that breaking the algae enables disclosure of some extractable compounds that at longer extraction times do (partially) decompose or evaporate. It is known that mechanical cell disruption is nonselective,³⁹ and cell disruption studies by Pinto and co-workers have shown that after disruption evaporation of matter occurred already at modest temperatures.⁴⁰ Overall, it can be concluded that both the crude lipid yield and the TFA yields from broken algae and from nonbroken algae are comparable. The B&D 120 min extraction from nonbroken algae results will be taken as a reference in further studies.

Both nonbroken and broken algae (from the same batch of algal biomass) were also extracted by EBA at 22 °C with a solvent/feed ratio of 1:1 (w/w), again applying different extraction times. The results in Figure 1(B) show that for nonbroken algae the crude lipid yield increased gradually with time and reached equilibrium (~13 wt %) at 18 h. For broken algae, the increase in crude lipid yield happened mostly in the first 2 h. This is because the cell breaking step released the lipids which were inside the algae cell and made them easier to be extracted by EBA. However, the higher crude lipid yield did not result in higher TFA yield. The TFA yield of nonbroken

algae was approximately 3 wt % and slightly higher than broken algae (~2.5 wt %). From the results of the TFA fraction in the crude lipid shown in Table 1, it can be seen that the TFA

Table 1. TFA Fraction in Crude Lipid of *Neochloris oleoabundans* Extracted by EBA Method^a

extraction time (min)	TFA fraction in crude lipid (%)	
	nonbroken	broken
10	24.5	13.8
30	25.3	14.4
60	25.1	15.7
120	25.0	17.3
360	25.1	18.4
720	25.4	18.4
1080	25.3	18.5
1440	25.3	18.5

^aResults are mean \pm standard deviation (SD), $n \geq 2$, $SD \leq 1.3$.

fraction in the crude lipid of nonbroken algae kept constant while that of broken algae increased with extraction and reached equilibrium in 3 h. The low TFA fraction in the total crude lipid of broken algae in the first 3 h was not due to less extraction of lipids containing fatty acids but due to high nonfatty acid lipid yield which was caused by the lipids released from cell breaking. This also shows that there is no benefits to break the algae cell if fatty acid is considered as the main product. From the results in Figure 2, it is apparent that the fatty acid compositions of lipids from EBA extractions were constant with increasing extraction time. Although there are some slight differences, the composition is in both cases comparable. When compared to the B&D method, it shows that the maximum crude lipid yield and TFA yield using EBA is comparable, but the time required with this solvent is longer. From this, it can be concluded that with the EBA solvent, it is not necessary to break the algae, which is an energy intensive step, but when nonbroken algae are used as feedstock for lipids, considerable longer extraction times should be accounted for.

3.2. Effect of Extraction Temperature. Nonbroken *Neochloris oleoabundans* were extracted by EBA for 18 h with the solvent/feed ratio of 1:1 (w/w) at different temperatures to study the effect. Crude lipid yield and TFA yield obtained at 22 and 50 °C are summarized in Figure 3. The lipid extraction yield was 13.0 wt % at 22 °C. When the extraction temperature was increased to 50 °C, a higher lipid extraction yield of 14.3 wt % could be achieved. The higher temperature may contribute to the distribution of the lipids to the EBA phase. The lipids that largely present in cell membranes are supramolecular structures.⁴¹ Keeping these structures in tact is more difficult at higher temperature; thus, it becomes more easy to extract single molecules from the supramolecular aggregates. At the meantime, there was almost no change in the TFA yield when increasing the extraction temperature. There are more unsaturated fatty acids present in the lipids of 22 °C extraction than 50 °C extraction. Considering the energy input and output, there is no obvious benefits of doing the extraction at an elevated temperature. Hence, an extraction temperature of 22 °C (room temperature) was chosen for further use in this study.

3.3. Effect of Solvent/Feed Ratio. Lipid extraction experiments using broken *Neochloris oleoabundans* (5% dw) at 22 °C were also carried out with different solvent/feed ratios (2:1, 1:1, 1:2, and 1:5 w/w). After 18 h of extraction, the

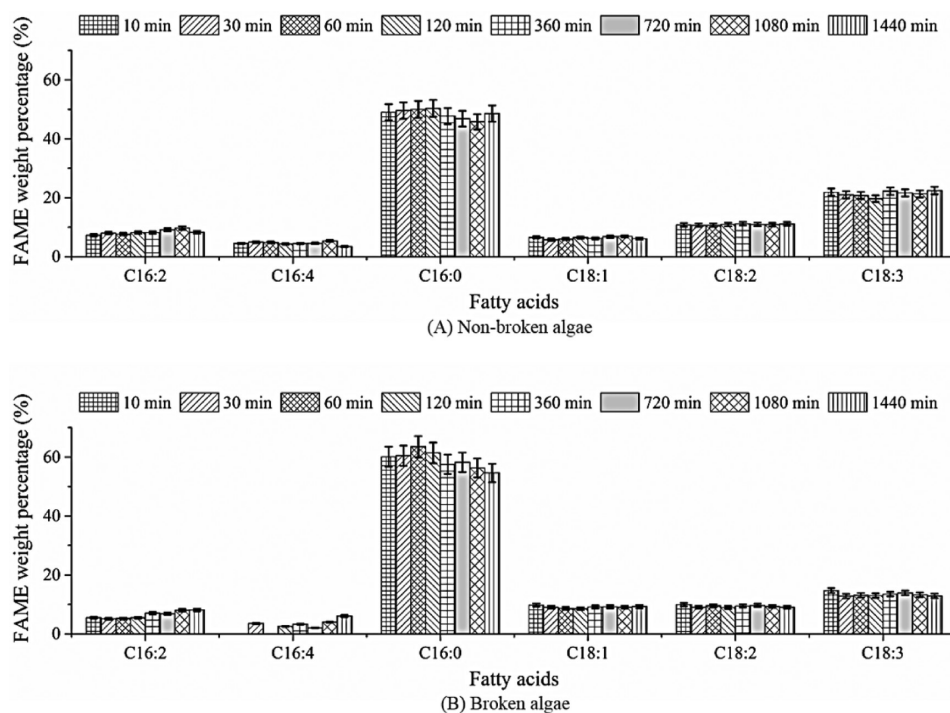


Figure 2. TFA compositions of lipids as determined after transesterification from (A) nonbroken and (B) broken *Neochloris oleoabundans* extracted by EBA method. Data in this figure were published before.³⁷

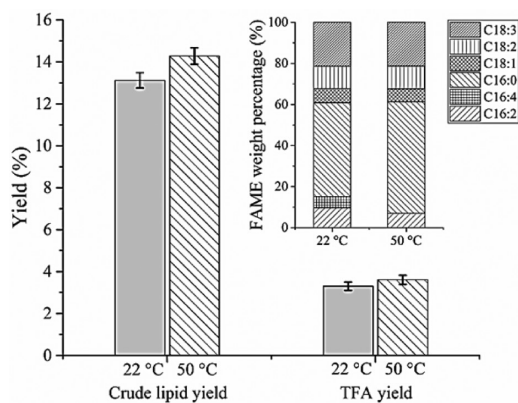


Figure 3. Crude lipid yield, TFA yield, and TFA compositions as determined after transesterification of *Neochloris oleoabundans* extracted by EBA method at 22 and 50 °C.

highest crude lipid yield (14.2 wt %) and TFA yield in algae dry weight (3.6 wt %) were obtained at the highest solvent/feed ratio of 2:1 (Figure 4). Lowering the solvent/feed ratio resulted in a continuously lowered crude lipid yield. An equilibrium validation experiment using a 72 h extraction time was done for the solvent/feed ratio of 1:5, showing that indeed equilibrium was reached after 18 h extraction (7.3 wt % \pm 0.2 wt %). The TFA compositions were compared for all tested solvent/feed ratios and are shown in Figure 5. The results indicate that the lipids obtained from different solvent/feed ratios had exactly the same FAME profiles. Thus, the solvent/feed ratio only influences the lipid extraction yield and not the lipid composition. Solvent/feed ratios higher than 2:1 were not studied because the highest lipid yields at ratios of 1:1 and 1:2 are already very close, suggesting that a higher solvent/feed ratio will not lead to much higher lipid extraction yields. Because more solvent also means a higher heat duty in the lipid

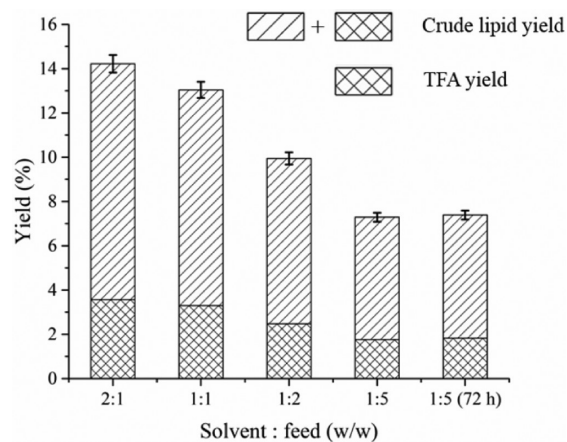


Figure 4. Crude lipid yield and TFA yield of *Neochloris oleoabundans* extracted by EBA method at different solvent/feed ratios.

recovery from the solvent, from an energy balance point of view, a solvent/feed ratio of 1:1 was chosen in this study.

3.4. Multistage Extraction. To investigate whether higher yields could be obtained than in single stage extractions, multiple extraction steps were applied to the same batch of algal biomass. After an EBA extraction of nonbroken algae for 18 h (and for B&D after 2 h), a second extraction was applied to the extracted algae. The solvent layer was separated after centrifuging, removed, and replaced by an equal amount of fresh solvent. This procedure was applied multiple times to achieve four extraction stages. The results obtained for various S/F ratios and for the B&D method are illustrated in Figure 6. In all cases, the cumulative crude lipid yield increased significantly during the second to fourth stages. The highest crude lipid yield (22.1 wt %) obtained in the EBA 1:1 ratio extraction was 6.7 wt % higher than the B&D four stages extraction (15.4 wt %). Because of the limited amount of

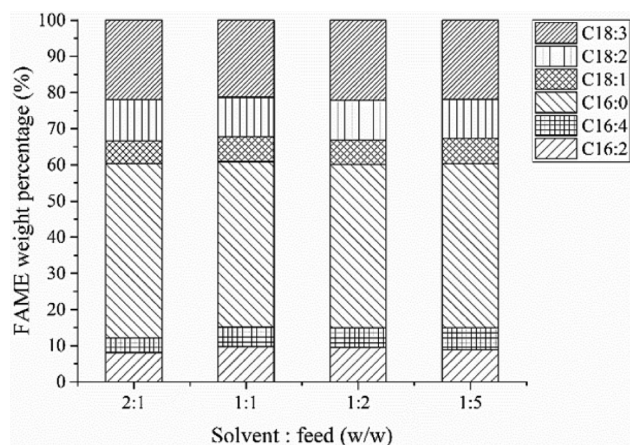


Figure 5. TFA compositions of lipids from *Neochloris oleabundans* extracted by EBA method at different solvent/feed ratios as determined after transesterification.

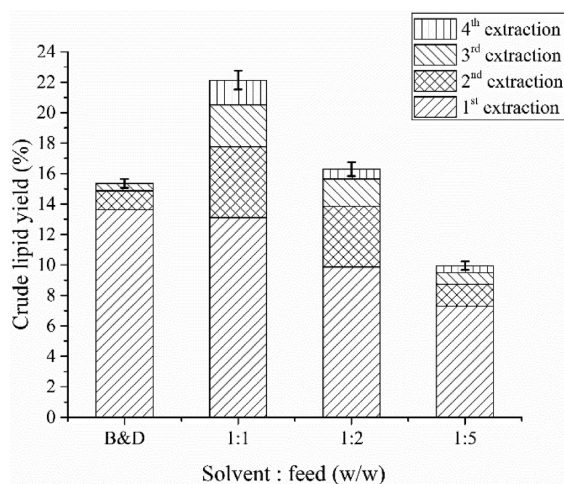


Figure 6. Crude lipid yield of *Neochloris oleabundans* extracted by EBA method for multistage extractions.

recovered lipids in the third and fourth stages, only the lipids from the first and second extractions were analyzed by GC-MS. The TFA compositions are presented in Figure 7. The fatty acid profile of *Neochloris oleabundans* was dominated by palmitic (C16:0), hexadecadienoic (C16:2), hexadecatetraenoic (C16:4), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids, and no significant differences were found between the extraction methods and extraction stages tested.

Lower solvent/feed ratios resulted in lower crude lipid yields. This is not only valid for single extraction experiments but was also found for the multistage extractions. Part of the reduced yields may be caused by losses of EBA remaining inside the algae cell after extraction. Assuming there is a distribution of lipids over the mixture inside the cell and the EBA phase, it will show that the more solvent is used, the lower the equilibrium concentrations are in both the cells and in the bulk EBA phase and the less lipid is lost remaining inside the algae cell. This might at least partly explain the results shown in Figure 8. For further elucidation, an extraction starting with a solvent/feed ratio of 1:5 followed by a second extraction with a solvent/feed ratio of 1:1 was carried out. In the second extraction step, it was found that 5.2 wt % lipid was extracted. This was more than the corresponding second extraction lipid yield of both combina-

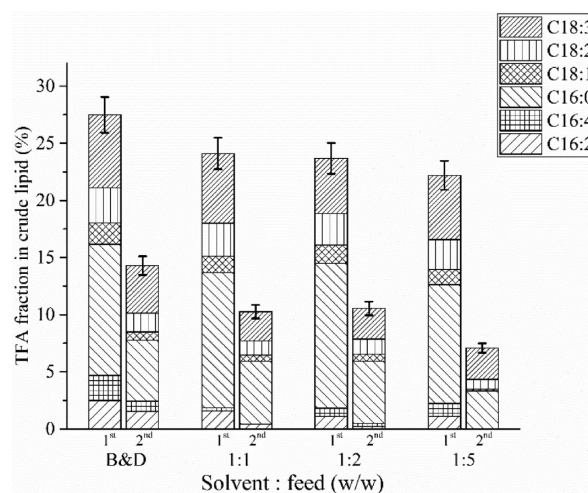


Figure 7. TFA compositions of lipids from *Neochloris oleabundans* obtained by EBA first and second extraction.

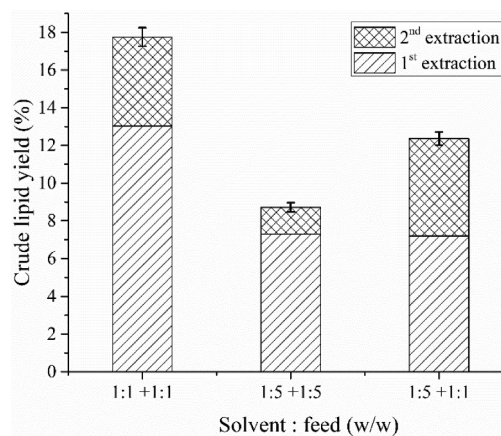


Figure 8. Crude lipid yield of *Neochloris oleabundans* extracted by EBA method for different solvent/feed ratio combinations.

tions “1:1 + 1:1” (4.7 wt %) and “1:5 + 1:5” (1.4 wt %). The results above indicate that a simple phase distribution model is not sufficient. It further shows that there is always some lipid material left in the algae cell after an extraction. It also shows clearly, and from Figure 6 especially for the EBA extraction method, that the overall yield of lipids could be further increased significantly by applying a multistage extraction.

3.5. Lipid Extraction from Stressed *Neochloris oleabundans*. From the results above, it can be found that EBA has good performance in lipid extraction from wet, nonbroken algae. The algae used in a previous study were cultivated in fresh water without stressing. To investigate to what extent this procedure is applicable to *Neochloris oleabundans* cultivated in different ways, the crude lipid yield and TFA yield were determined for both the B&D extraction and for the EBA extraction. The results are summarized in Figure 9. Compared with extraction from nonstressed *Neochloris oleabundans*, a much higher crude lipid yield and TFA yield were obtained from freshwater cultivated, stressed (FW-stressed) algae for both B&D and EBA extraction. Moreover, EBA can extract 80% more lipids than via the B&D method, while the TFA fraction of the crude lipid products is similar in these two cases. The results indicate that EBA has an excellent performance for lipid extraction from FW-stressed algae.

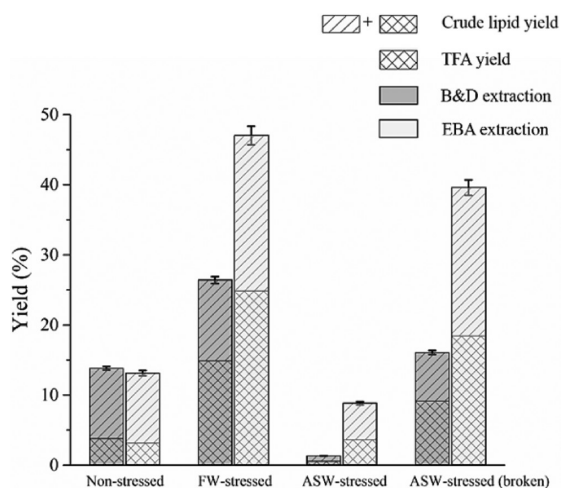


Figure 9. Crude lipid yield and TFA yield of nonstressed, FW-stressed, ASW-stressed, and ASW-stressed (broken) *Neochloris oleoabundans* extracted by B&D and EBA method. The nonstressed, FW-stressed, and ASW-stressed series were measured for nonbroken algae.

The lipid yield obtained from the artificial seawater cultivated, stressed algae (ASW-stressed) algae was even less than from (FW)-nonstressed algae. Especially, the lipid extraction yield of the B&D method (for nonbroken ASW-stressed algae) was only 1.3 wt %, which was much less than expected. Therefore, an extraction of lipids from broken ASW-stressed algae was carried out. With broken cells, the extraction yield was much higher than with nonbroken cells for the ASW-stressed algae, suggesting that the lipid extraction from ASW-stressed algae was limited by the fact that the cells were intact. Different culture media and culture conditions influence the properties of the cell and cell wall. Microscope images were taken from the cells and are displayed in Figure 10. As shown in Figure 10, FW-stressed algae have a smaller cell size than the

ASW-stressed algae. Algae cultivated in ASW have thicker cell walls which make extraction difficult. Cell disruption is needed for ASW-stressed algae in order to get a competitive lipid extraction yield. Thus, the FW-stressed algae, with the highest lipid yield and no need to be dried nor to be broken prior to extraction, are preferred.

The fatty acid compositions of the lipids obtained from the B&D and EBA extraction were determined (Figure 11). No

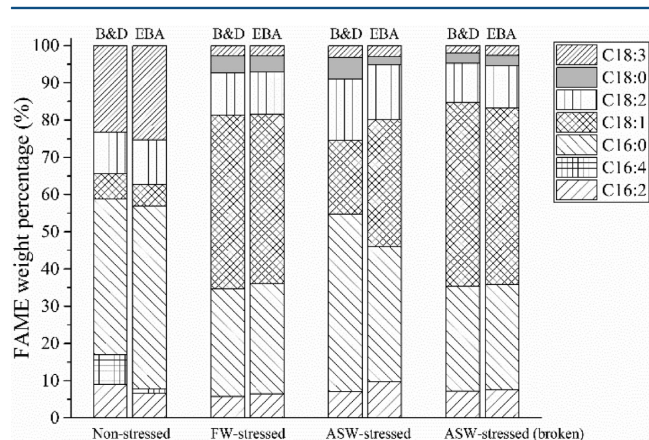


Figure 11. TFA compositions of lipids from nonstressed, FW-stressed, ASW-stressed, and ASW-stressed (broken) *Neochloris oleoabundans* extracted by B&D and EBA methods.

significant difference was observed, meaning that lipid from B&D and EBA extraction contained roughly the same fatty acids. Furthermore, from Figure 11, it follows that there are seven fatty acids that make up the overall composition; no other fatty acids were present in detectable amounts.

The four stage extraction procedure was also applied to nonbroken FW-stressed and nonbroken ASW-stressed *Neochloris oleoabundans*. The results are presented in Figure 12(A).

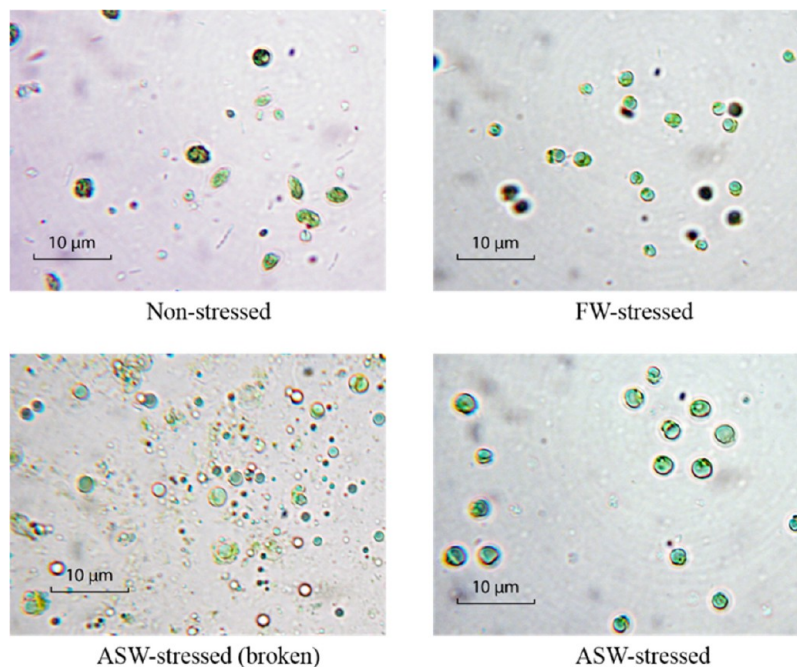


Figure 10. Light microscope images of nonstressed, FW-stressed, ASW-stressed, and ASW-stressed (broken) *Neochloris oleoabundans* prior to extraction.

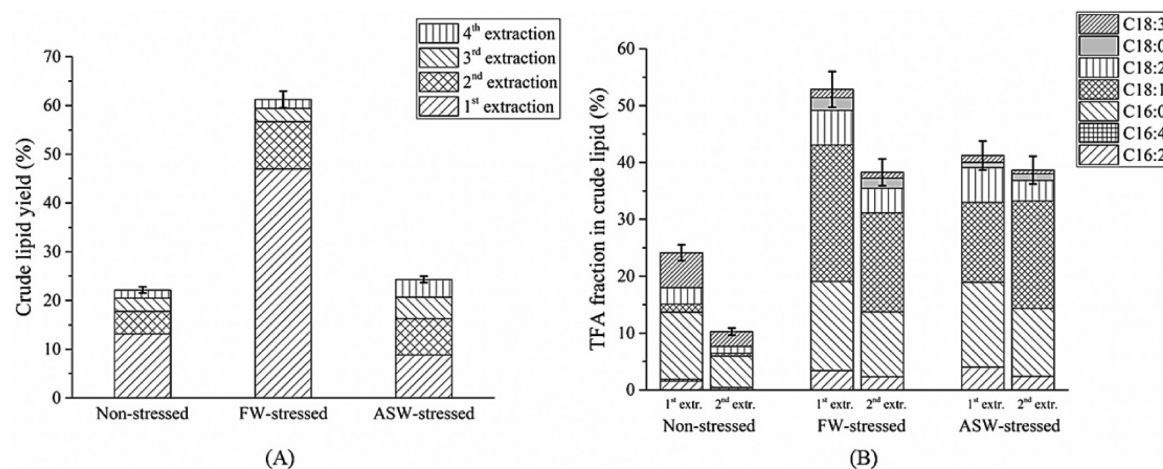


Figure 12. Crude lipid yield (A) and TFA compositions (B) of nonstressed, FW-stressed, and ASW-stressed *Neochloris oleoabundans* extracted by EBA method for multistage extractions.

After four times extraction, the lipid extraction yield of ASW-stressed algae (24.3 wt %) only accounts for 60% of the single stage extraction yield of ASW-stressed broken algae (39.6 wt %). This makes extraction from nonbroken, ASW-stressed algae not very attractive. For FW-stressed algae, two extraction steps extract more than 92% of the total lipids obtained after four times extraction (61.3 wt %), which is considered sufficient. Adding more extraction stages will require more solvent and more processing time, leading to more energy usage and lower production capacity, while the additional yield is limited. Also it can be found from the results in Figure 12(B) that the TFA content in the crude lipid is lower in the second extraction stage. It was therefore concluded that two extraction stages are sufficient.

4. CONCLUSIONS

In this work, extraction conditions for using EBA to extract lipids from *Neochloris oleoabundans* were optimized for both single stage extractions and for multistage extractions. It was shown that the EBA extraction is very suitable for high lipid contents, such as observed in FW-stressed algae *Neochloris oleoabundans*, and cell disruption was not needed, although a considerable longer extraction time (18 h) was required for nonbroken cells. Room temperature (22 °C) and solvent/feed ratio 1:1 (w/w) were selected as optimal conditions with respect to lipid yield (47 wt % in a single stage for FW-stressed algae). Multiple extractions increased the crude lipid yield, reaching for FW-stressed *Neochloris oleoabundans* a maximum yield of 61.3 wt % after four extractions. After two extractions, already 57 wt % was obtained, suggesting that two stages should be sufficient. This study shows that using both algae stressing techniques and effective lipid extraction technology for nonbroken wet microalgae enable high lipid yields.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

- Slade, R.; Bauen, A. Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. *Biomass Bioenergy* **2013**, *53*, 29.
- Torres, F. A.; Passalacqua, T. G.; Velásquez, A. M.; de Souza, R. A.; Colepicolo, P.; Graminha, M. A. New drugs with antiprotozoal activity from marine algae: a review. *Rev. Bras. Farmacogn.* **2014**, *24*, 265.
- Wang, H.-M. D.; Chen, C.-c.; Huynh, P.; Chang, J.-S. Exploring the potential of using algae in cosmetics. *Bioresour. Technol.* **2015**, *184*, 355.
- Enzing, C.; Ploeg, M.; Barbosa, M.; Sijtsma, L.; Vigani, M.; Parisi, C.; Cerezo, E. R. *Microalgae-Based Products for the Food and Feed Sector: An Outlook for Europe*; Publications Office of the European Union: Luxembourg, 2014.
- Du, Y.; Schuur, B.; Samori, C.; Tagliavini, E.; Brilman, D. W. F. Secondary amines as switchable solvents for lipid extraction from non-broken microalgae. *Bioresour. Technol.* **2013**, *149*, 253.
- Demirbas, A. Production of biodiesel from algae oils. *Energy Sources, Part A* **2008**, *31*, 163.
- Eller, F. J.; King, J. W. Supercritical carbon dioxide extraction of cedarwood oil: A study of extraction parameters and oil characteristics. *Phytochem. Anal.* **2000**, *11*, 226.
- Greenwell, H. C.; Laurens, L. M. L.; Shields, R. J.; Lovitt, R. W.; Flynn, K. J. Placing microalgae on the biofuels priority list: A review of the technological challenges. *J. R. Soc., Interface* **2010**, *7*, 703.
- Pawliszyn, J. Kinetic model of supercritical fluid extraction. *J. Chromatogr. Sci.* **1993**, *31*, 31.
- Mendes, R. L.; Fernandes, H. L.; Coelho, J.; Reis, E. C.; Cabral, J. M. S.; Novais, J. M.; Palavra, A. F. Supercritical CO₂ extraction of carotenoids and other lipids from *Chlorella vulgaris*. *Food Chem.* **1995**, *53*, 99.
- Lardon, L.; Hélias, A.; Sialve, B.; Steyer, J.-P.; Bernard, O. Life-Cycle Assessment of Biodiesel Production from Microalgae. *Environ. Sci. Technol.* **2009**, *43*, 6475.
- Teixeira, R. E. Energy-efficient extraction of fuel and chemical feedstocks from algae. *Green Chem.* **2012**, *14*, 419.

- (13) Andrich, G.; Nesti, U.; Venturi, F.; Zinnai, A.; Fiorentini, R. Supercritical fluid extraction of bioactive lipids from the microalga *Nannochloropsis* sp. *Eur. J. Lipid Sci. Technol.* **2005**, *107*, 381.
- (14) Couto, R. M.; Simões, P. C.; Reis, A.; Da Silva, T. L.; Martins, V. H.; Sánchez-Vicente, Y. Supercritical fluid extraction of lipids from the heterotrophic microalga *Cryptocodinium cohnii*. *Eng. Life Sci.* **2010**, *10*, 158.
- (15) Cheng, C. H.; Du, T. B.; Pi, H. C.; Jang, S. M.; Lin, Y. H.; Lee, H. T. Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO₂. *Bioresour. Technol.* **2011**, *102*, 10151.
- (16) Du, Y.; Schuur, B.; Kersten, S. R. A.; Brilman, D. W. F. Opportunities for switchable solvents for lipid extraction from wet algal biomass: An energy evaluation. *Algal Res.* **2015**, *11*, 271.
- (17) Dejoye Tanzi, C.; Abert Vian, M.; Chemat, F. New procedure for extraction of algal lipids from wet biomass: A green clean and scalable process. *Bioresour. Technol.* **2013**, *134*, 271.
- (18) Halim, R.; Gladman, B.; Danquah, M. K.; Webley, P. A. Oil extraction from microalgae for biodiesel production. *Bioresour. Technol.* **2011**, *102*, 178.
- (19) Jiménez Callejón, M. J.; Robles Medina, A.; Macías Sánchez, M. D.; Hita Peña, E.; Esteban Cerdán, L.; González Moreno, P. A.; Molina Grima, E. Extraction of saponifiable lipids from wet microalgal biomass for biodiesel production. *Bioresour. Technol.* **2014**, *169*, 198.
- (20) Kanda, H.; Li, P. Simple extraction method of green crude from natural blue-green microalgae by dimethyl ether. *Fuel* **2011**, *90*, 1264.
- (21) Lee, S. J.; Yoon, B. D.; Oh, H. M. Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnol. Tech.* **1998**, *12*, 553.
- (22) Liu, C. Z.; Zheng, S.; Xu, L.; Wang, F.; Guo, C. Algal oil extraction from wet biomass of *Botryococcus braunii* by 1,2-dimethoxyethane. *Appl. Energy* **2013**, *102*, 971.
- (23) Nagle, N.; Lemke, P. Production of methyl ester fuel from microalgae. *Appl. Biochem. Biotechnol.* **1990**, *24–25*, 355.
- (24) Nikolava-Damyanova, B.; Stefanov, K.; Seizova, K.; Popov, S. Extraction and rapid identification of low molecular weight compounds from marine organisms. *Comp. Biochem. Physiol., B: Comp. Biochem.* **1992**, *103*, 733.
- (25) Shen, Y.; Pei, Z.; Yuan, W.; Mao, E. Effect of nitrogen and extraction method on algae lipid yield. *Int. J. Agric. Biol. Eng.* **2009**, *2*, 51.
- (26) Yoo, G.; Park, W. K.; Kim, C. W.; Choi, Y. E.; Yang, J. W. Direct lipid extraction from wet *Chlamydomonas reinhardtii* biomass using osmotic shock. *Bioresour. Technol.* **2012**, *123*, 717.
- (27) Zbinden, M. D. A.; Sturm, B. S. M.; Nord, R. D.; Carey, W. J.; Moore, D.; Shinogle, H.; Stagg-Williams, S. M. Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae. *Biotechnol. Bioeng.* **2013**, *110*, 1605.
- (28) Kanda, H.; Li, P.; Goto, M.; Makino, H. Energy-saving lipid extraction from wet *Euglena gracilis* by the low-boiling-point solvent dimethyl ether. *Energies* **2015**, *8*, 610.
- (29) Herrero, M.; Cifuentes, A.; Ibañez, E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review. *Food Chem.* **2006**, *98*, 136.
- (30) Patil, P. D.; Gude, V. G.; Mannarswamy, A.; Cooke, P.; Nirmalakhandan, N.; Lammers, P.; Deng, S. Comparison of direct transesterification of algal biomass under supercritical methanol and microwave irradiation conditions. *Fuel* **2012**, *97*, 822.
- (31) Patil, P. D.; Reddy, H.; Muppaneni, T.; Ponnusamy, S.; Cooke, P.; Schuab, T.; Deng, S. Microwave-mediated non-catalytic transesterification of algal biomass under supercritical ethanol conditions. *J. Supercrit. Fluids* **2013**, *79*, 67.
- (32) Santana, A.; Jesus, S.; Larrayoz, M. A.; Filho, R. M. Supercritical Carbon Dioxide Extraction of Algal Lipids for the Biodiesel Production. *Procedia Eng.* **2012**, *42*, 1755.
- (33) Soh, L.; Zimmerman, J. Biodiesel production: The potential of algal lipids extracted with supercritical carbon dioxide. *Green Chem.* **2011**, *13*, 1422.
- (34) Boyd, A. R.; Champagne, P.; McGinn, P. J.; MacDougall, K. M.; Melanson, J. E.; Jessop, P. G. Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production. *Bioresour. Technol.* **2012**, *118*, 628.
- (35) Samori, C.; López Barreiro, D.; Vet, R.; Pezzolesi, L.; Brilman, D. W. F.; Galletti, P.; Tagliavini, E. Effective lipid extraction from algae cultures using switchable solvents. *Green Chem.* **2013**, *15*, 353.
- (36) Samori, C.; Torri, C.; Samori, G.; Fabbri, D.; Galletti, P.; Guerrini, F.; Pistocchi, R.; Tagliavini, E. Extraction of hydrocarbons from microalga *Botryococcus braunii* with switchable solvents. *Bioresour. Technol.* **2010**, *101*, 3274.
- (37) Du, Y.; Schuur, B.; Kersten, S. R. A.; Brilman, D. W. F. Microalgae wet extraction using N-ethyl butylamine for fatty acid production. *Green Energy Environ.* **2016**, *1*, 79.
- (38) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911.
- (39) Günerken, E.; D'Hondt, E.; Eppink, M. H. M.; Garcia-Gonzalez, L.; Elst, K.; Wijffels, R. H. Cell disruption for microalgae biorefineries. *Biotechnol. Adv.* **2015**, *33*, 243.
- (40) Pohndorf, R. S.; Camara, Á. S.; Larrosa, A. P. Q.; Pinheiro, C. P.; Strieder, M. M.; Pinto, L. A. A. Production of lipids from microalgae *Spirulina* sp.: Influence of drying, cell disruption and extraction methods. *Biomass Bioenergy* **2016**, *93*, 25.
- (41) Tsekos, I. The supramolecular organization of red algal cell membranes and their participation in the biosynthesis and secretion of extracellular polysaccharides: a review. *Protoplasma* **1996**, *193*, 10.