



Multistage wet lipid extraction from fresh water stressed *Neochloris oleoabundans* slurry – Experiments and modelling

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ABSTRACT

Algae are considered an important renewable feedstock for lipid extraction to produce biofuels. Algae strain *Neochloris oleoabundans* used in this research can yield a high lipid content under stressed conditions. N-ethyl butylamine (EBA) as a switchable solvent has previously shown outstanding performance on energy efficient lipid extraction from non-broken wet algae slurry. In this work, a model was developed that describes the equilibrium state of lipid extraction from fresh water (FW)-stressed *Neochloris oleoabundans* algae slurry using EBA as solvent. When assuming that the cell interior is almost completely filled with the solvent phase during extraction, the model estimated extraction yields showed good agreement with those obtained in experiments. The developed model can predict the amount of crude lipid being recovered from any stage of a multistage extraction process.

1. Introduction

The global demand for energy is rapidly increasing with increasing human population, urbanization and modernization [1]. Hence, abundant, affordable and sustainable liquid fuel alternatives to fossil energy sources are necessary, especially in order to reduce the impact on the environment [2]. Biomass based energy production systems can partly replace the presently employed energy systems.

Microalgae as an important feedstock for biofuels are receiving increasing attention [3–7]. They have rapid growth rate, and thus high productivity, less competition with arable land and freshwater as compared to other crops and a high CO₂ fixation rate [8]. The algae *Neochloris oleoabundans* used in this research is a freshwater species that has been shown capable of producing 35–54% lipid of algae dry weight [9]. *Neochloris oleoabundans* was stressed to improve the lipid content under different growth conditions, such as other nitrogen sources [10], nitrogen starvation [9,11,12], mixotrophy (the use of phototrophy and heterotrophy in combination) [13], pH and salt concentration [14]. The research findings provide an interesting outlook on its application as alternative feedstock for biofuel production.

Lipid extraction is one of the main topics in the research of algae biorefinery process. Organic solvent extraction [15–25] and supercritical fluid extraction [21,26–34] are the most common methods being used for algae lipid extraction. In recent years, a method named CO₂-switchable solvent extraction aroused the interest of many researchers [8,35–39]. With this technology lipids can be extracted, after

which solvent recovery is accomplished by switching the solvent hydrophilicity with CO₂, thereby inducing phase splitting. These studies also showed the possibility of extracting lipids from wet algae slurries, hence without the need for harvesting and drying the algal biomass prior to extraction. In the work of Du et al. [40], it was found that using N-ethyl butylamine (EBA) for lipid extraction from fresh water (FW) stressed *Neochloris oleoabundans* reached extraction equilibrium within 18 h and the lipid extraction yield in that study was 47.0 wt%. They also found that the yield after four stages of extraction for the FW-stressed *Neochloris oleoabundans* was as high as 61.3 wt% lipids. However, why not all lipids were recovered during the first extraction step, hence: incomplete extraction, is still unknown. Also in literature very little attention has been devoted to the equilibrium status of algal lipid extraction and to the possibility and potential of multistage extraction.

In this paper, we aim to develop a model that describes the equilibrium state of lipid extraction using EBA from wet microalgae slurry. The model should provide a qualitative insight in the extraction process, and be able to predict the amount of crude lipid being recovered from FW-stressed *Neochloris oleoabundans* at any stage of a multistage EBA extraction process. For this purpose extraction experiments of using different solvent to feed ratio were carried out. Several model assumptions were made based on experimental results or visual observations and cross-checked against the generated experimental data to detect the validity of the proposed model. Sensitivity analysis of the assumed parameters further proves the reliability of the newly developed model, discussed in this work.

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2. Material and methods

2.1. Chemicals

The solvents and chemicals used in this study were as follows: N-ethyl butylamine (EBA) ($\geq 98.0\%$, Aldrich), chloroform ($\geq 99.9\%$, Sigma-Aldrich), methanol ($\geq 99.9\%$, Fluka), hexane ($\geq 95\%$, Sigma-Aldrich), methyl nonadecanoate ($\geq 99.5\%$, Fluka), sulfuric acid ($95.0\text{--}98.0\%$, Sigma-Aldrich), FAME column evaluation mix ($1000\ \mu\text{g}/\text{mL}$ each component in methylene chloride, analytical standard, Supelco).

2.2. Preparation and characterization of algae solutions

Algae of the strain *Neochloris oleoabundans* were obtained from AlgaePARC (NL). Algae paste was mixed with water to get $\sim 5\ \text{wt}\%$ algae slurry that can be used in extraction. The water content in algae slurries was determined by weighing a sample before and after drying at $105\ ^\circ\text{C}$ for 24 h.

2.3. Extraction and recovery of lipids from algae

Extraction of lipid from algae slurries was done according to the EBA extraction method used in previous research [39]. Here 20 g of algae slurries were extracted with varying amounts of EBA for at least 18 h to ensure extraction equilibrium has been reached. After the extraction experiments, the mixtures were centrifuged to separate the phases efficiently. When a second extraction was applied to the extracted algae, the amine layer containing the algal lipids was isolated and replaced by an equal amount of fresh solvent. This procedure was applied multiple times to achieve four extraction stages. To assist the lipid recovery from the isolated organic layers, equal amount of water were added, improving the phase separation as a result of improving the switching efficiency. During the switching procedure, CO_2 was bubbled in a flow rate of 2 VVM (volume per volume per minute) for 60 min, during which the solvent switched into its hydrophilic form. A small amount of chloroform was used to recover the lipid layer with a syringe, due to the small scale of experiments. Please 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) and reported as percentage on algae dry weight basis (defined as crude lipid yield). All experiments were performed at least twice. The reported error bars correspond to an accuracy of $\pm 2.8\%$ yield, which is the averaged relative standard deviation of all experiments (> 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 [39].

The TFA yield is defined as:

$$\text{TFA yield (\%)} = \frac{m_{\text{TFA}}}{m_{\text{dry algae}}} \times 100\% \quad (1)$$

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

$$\text{TFA fraction in crude lipid (\%)} = \frac{m_{\text{TFA}}}{m_{\text{crude lipid}}} \times 100\% \quad (2)$$

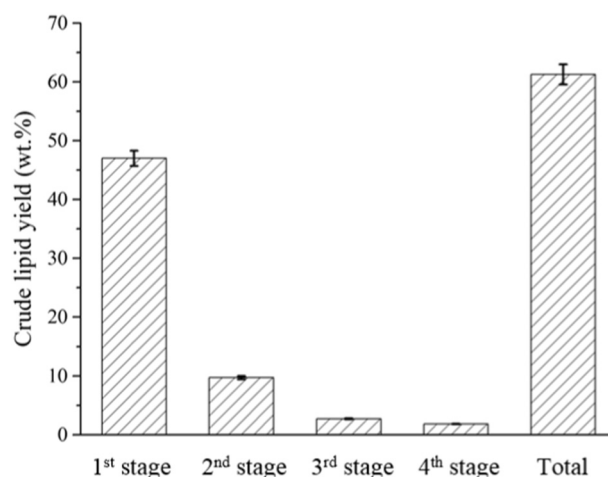


Fig. 1. Crude lipid yield of *Neochloris oleoabundans* extracted by N-ethyl butylamine method for multistage extractions. Data points in this figure are averages of at least two replicates, and the indicated error bars represent the averaged relative standard deviation (2.8%) over all 114 experiments of this type in the study.

3. Results and discussion

3.1. Model assumptions

The modelling approach to describe the multistage lipid extraction is based on some experimental observations that are discussed prior to setting up the model in order to make the approach more comprehensive. It was found in the results of lipid extraction from fresh water (FW) stressed *Neochloris oleoabundans* that the extraction reached equilibrium within 18 h [40], and by applying multiple extraction steps to the same batch of algal biomass, the lipid yield could be increased, as is shown in Fig. 1. Two extraction stages extract $> 92\%$ of the total lipids obtained after 4 times extraction (61.3 wt%). Because of the limited amount of recovered lipids in the third and fourth stages, only the lipids from first and second extractions were analyzed by GC–MS. It can be found from the results in Fig. 2 that although the TFA fraction in crude lipid was lower in the second extraction stage, the composition of the fatty acids is very similar, if not identical.

From the light microscope images of FW-stressed *Neochloris*

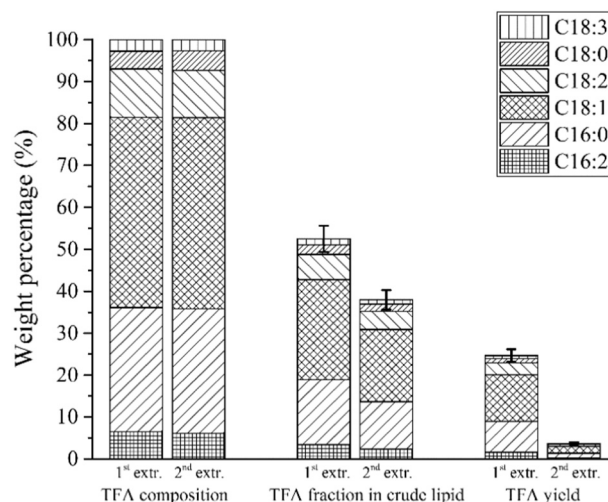


Fig. 2. TFA (total fatty acid) composition, TFA fraction in crude lipid and TFA yield of lipids from *Neochloris oleoabundans* obtained by N-ethyl butylamine 1st and 2nd extraction (Solvent/Feed = 1:1). Data points in this figure are averages of at least two replicates, and the indicated error bars represent the averaged relative standard deviation (5.7%) over all 60 experiments of this type in the study.

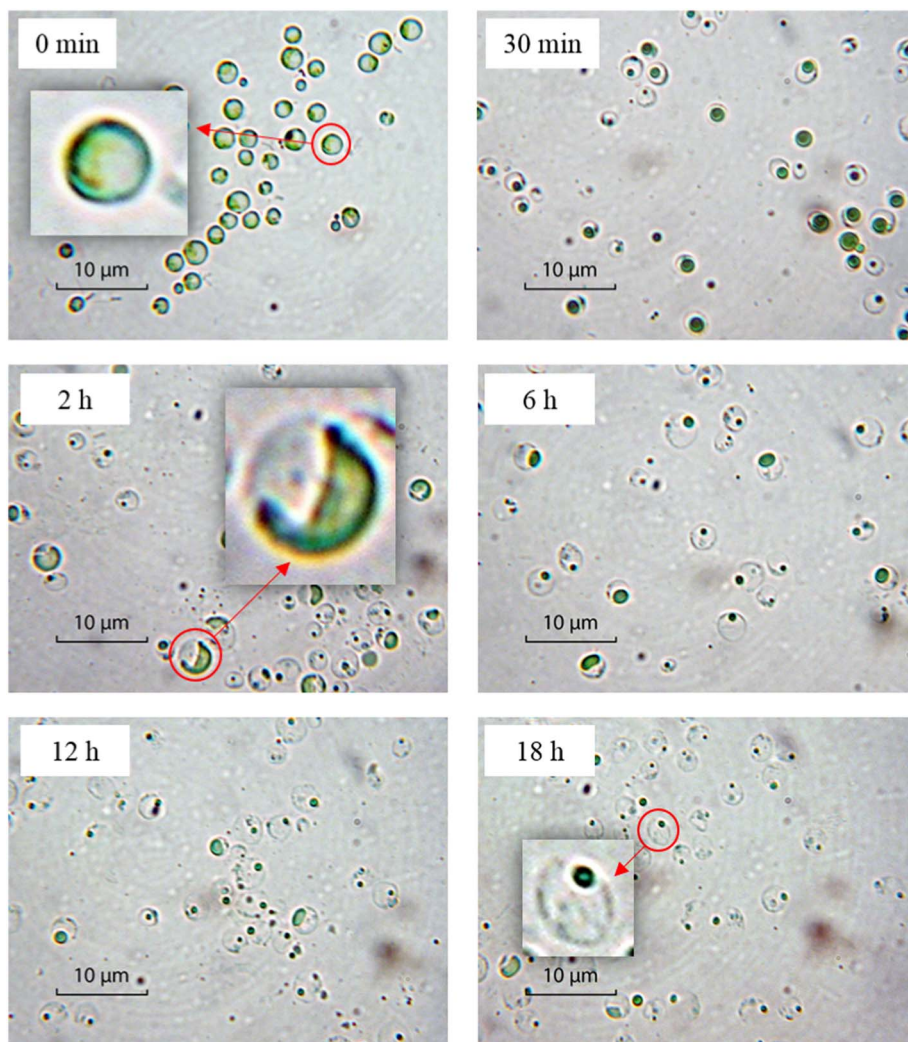


Fig. 3. Light microscope images of fresh water-stressed *Neochloris oleabundans* at different extraction time.

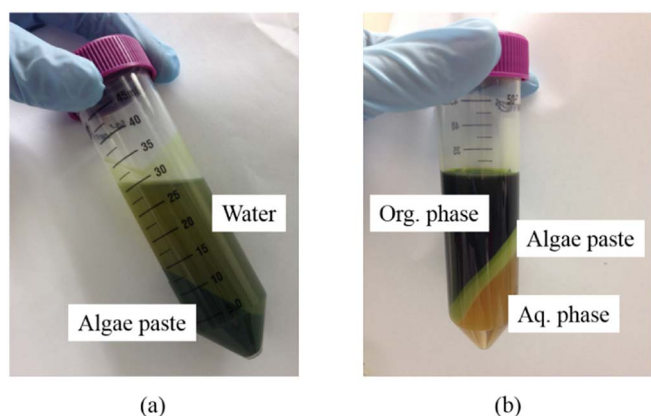


Fig. 4. (a) algae slurry and (b) algae slurry mixed with N-ethyl butylamine for 18 h after centrifugation (9000 rpm, 5 min).

oleabundans at different extraction times (Fig. 3) it can be seen that the extraction did not change the shape of the algae cells, and even after 18 h, clear cells similarly of shape as compared to the cells before extraction can be identified. However, it is also clearly visible that the chloroplast of algae cell, which is the dark green part, shrank during the extraction process and became a tiny part ('dot') at the end of extraction. In Fig. 4, it can be observed that the algae cells just after being contacted with solvent, thus before significant extraction took place,

move to the bottom of the tube during centrifugation, while algae cells after extraction (18 h) stayed between the organic layer and aqueous layer during centrifugation. This indicates that the density of algae cells changed during extraction. Based on this observation, it can be hypothesized that organic solvent went into the cells after extraction. After isolation of the EBA-layer and recovery of the lipids from the solvent, the lipids that are solubilized in the solvent that is still inside the cell walls is not measured in the yield calculation for the first extraction step, but the composition of these lipids resembles the composition of the lipids isolated after the first extraction step. A significant amount of these lipids is then liberated from the cells during the second extraction step, and results in the similarity between the lipid compositions in both extraction steps. The model was developed to account for this effect, in order to accurately predict the extraction yields in the various extraction steps.

To estimate the total lipid content ($Y_{lipid, tot}$), as shown in Fig. 5, an empirical exponential curve was fitted to the experimental results which were shown in Fig. 1. Algae have a finite amount of lipid, so it is assumed that when the number of steps would be increased to 10 steps, all the lipid should be extracted. Based on this hypothesis, the $Y_{lipid, total}$ is calculated to be 64.0 wt% of the algae dry weight.

The model should describe the equilibrium states of lipid extraction using EBA from wet microalgae slurry, and the parameters that may be used in calculation are illustrated in Fig. 6. The following assumptions are made:

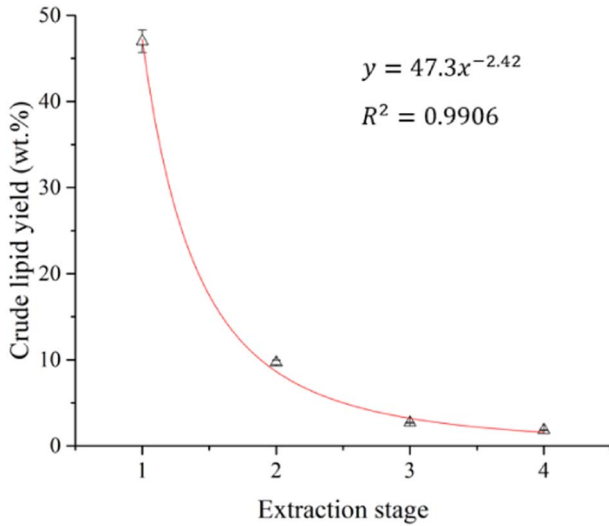


Fig. 5. Crude lipid yield of *Neochloris oleoabundans* extracted by N-ethyl butylamine method in different stages. Data points in this figure are averages of at least two replicates, and the indicated error bars represent the averaged relative standard deviation (2.8%) over all 114 experiments of this type in the study.

- 1) The cells are assumed to be spherical with a constant volume $V_{cell, in}$ before and after extraction.
- 2) The total volume of algae cells ($V_{cell, tot}$) was assumed to be the same as the volume of algae paste after centrifugation. The algae solution was centrifuged for 10 min at 9000 rpm to get an algae paste. The algae paste was dried at 105 °C for 24 h to determine the algae content, $C_{algae\ paste}$ was found to be 20.6 wt % \pm 0.5 wt%.
- 3) The algae in the extraction mixture are assumed to have an initial crude lipid amount $m_{lipid, tot}$. The dark green part inside the cell is chloroplast which contains lipid and shrinks during extraction as shown in Fig. 3. When the extraction reaches equilibrium, the volume of chloroplast is assumed to be negligible.
- 4) It is assumed that in each extraction stages, different amount of lipid will be released from cellular material. The ratio of lipid released from cellular material in stage i ($m_{lipid, rel, i}$, $i = 1, 2, 3, 4$ is the stage number) to the total lipid amount ($m_{lipid, tot}$) is defined as:

$$R_{rel, i} = \frac{m_{lipid, rel, i}}{m_{lipid, tot}} \quad (3)$$

- 5) The cell wall is not selectively permeable for solvent and lipids. Therefore it is assumed that at equilibrium state, the solvent phase inside and outside the cells has the same composition and the crude lipid concentration in the solvent phase inside the cell ($C_{lipid, in}$) and outside ($C_{lipid, out}$) are also the same.
- 6) Organic solvent goes into the cells during extraction. This assumption was made based on the density change of algae paste before and after extraction. However, it is uncertain if there is any aqueous phase inside the cells at equilibrium and we did not see a possibility

to measure this under the current experimental conditions. So it is assumed that at equilibrium state, the ratio of organic phase volume ($V_{cell, org}$) inside the cell to the total volume inside the cell ($V_{cell, in}$) is:

$$R_{vol, i} = \frac{V_{cell, org, i}}{V_{cell, in}} \quad (4)$$

where i is the stage number, $i = 1, 2, 3, 4$.

- 7) In each stage, only the solvent outside cells can be separated for lipid recovery. In subsequent stages, the remaining solvent containing lipid inside cells is washed out.

3.2. Modelling

The model is developed for 4 stages extraction of lipid from FW-stressed *Neochloris oleoabundans* slurry with ~ 5 wt% algae content ($C_{algae\ slurry}$). At the start of an extraction, EBA is added to the algae slurry. Directly after mixing, two liquid phases are formed: one organic phase and one aqueous phase, each of them containing both EBA and water because of the partial mutual solubility. The weight of organic phase and aqueous can be calculated by using the equations below.

$$S_{water\ in\ EBA} = \frac{m_{water, org}}{m_{EBA, org} + m_{water, org}} \quad (5)$$

$$m_{water, org} = \frac{S_{water\ in\ EBA} \times m_{EBA, org}}{1 - S_{water\ in\ EBA}} \quad (6)$$

$S_{water\ in\ EBA}$ is the solubility of water in EBA. $m_{water, org}$ is the mass of water in organic phase. $m_{EBA, org}$ is the mass of EBA in organic phase.

$$S_{EBA\ in\ water} = \frac{m_{EBA, aq}}{m_{EBA, aq} + m_{water, aq}} \quad (7)$$

$$m_{EBA, aq} = \frac{S_{EBA\ in\ water} \times m_{water, aq}}{1 - S_{EBA\ in\ water}} \quad (8)$$

$S_{EBA\ in\ water}$ is the solubility of EBA in water. $m_{EBA, aq}$ is the mass of EBA in aqueous phase. $m_{water, aq}$ is the mass of water in aqueous phase.

$$m_{EBA} = m_{EBA, org} + m_{EBA, aq} \quad (9)$$

$$m_{water} = m_{water, aq} + m_{water, org} \quad (10)$$

m_{EBA} is the mass of EBA in total. m_{water} is the mass of water in total.

$$m_{EBA, org} = \frac{m_{EBA} \times (1 - S_{EBA\ in\ water}) \times (1 - S_{water\ in\ EBA}) - m_{water} \times S_{EBA\ in\ water} \times (1 - S_{water\ in\ EBA})}{1 - S_{water\ in\ EBA} - S_{EBA\ in\ water}} \quad (11)$$

$$m_{water, aq} = \frac{m_{water} \times (1 - S_{EBA\ in\ water}) \times (1 - S_{water\ in\ EBA}) - m_{EBA} \times S_{water\ in\ EBA} \times (1 - S_{EBA\ in\ water})}{1 - S_{water\ in\ EBA} - S_{EBA\ in\ water}} \quad (12)$$

$$m_{org} = m_{EBA, org} + m_{water, org} \quad (13)$$

$$m_{aq} = m_{water, aq} + m_{EBA, aq} \quad (14)$$

m_{org} is the mass of organic phase. m_{aq} is the mass of aqueous phase.

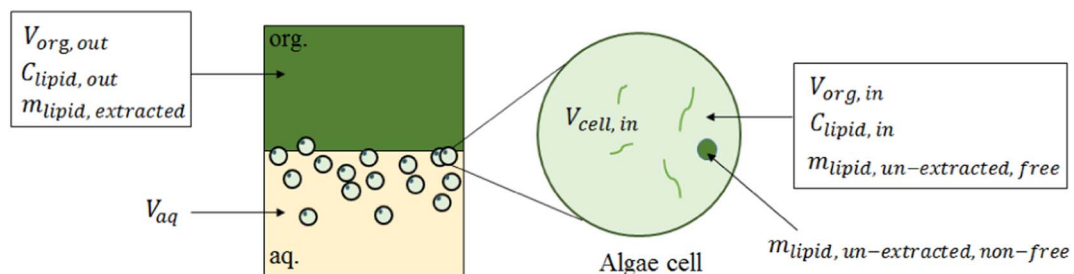


Fig. 6. A schematic diagram of the crude lipid extraction in equilibrium state.

Here the mass of lipid is considered as negligible.

It was checked experimentally that the total volume didn't change significantly with mixing EBA and water together. So the density of organic phase (ρ_{org}) and aqueous phase (ρ_{aq}) can be approximated as below:

$$\rho_{org} = \frac{m_{org}}{\frac{m_{EBA,org}}{\rho_{EBA}} + \frac{m_{water,org}}{\rho_{water}}} \quad (15)$$

$$\rho_{aq} = \frac{m_{aq}}{\frac{m_{water,aq}}{\rho_{water}} + \frac{m_{EBA,aq}}{\rho_{EBA}}} \quad (16)$$

where the ρ_{EBA} and ρ_{water} are the densities of pure EBA and water respectively.

The total amount of lipid ($m_{lipid, tot}$) is calculated by

$$m_{lipid, tot} = m_{dry\ algae} \times Y_{lipid, tot} \quad (17)$$

Besides the lipid, there are some parts of the algae that cannot be extracted by EBA ($m_{non-extractable}$).

$$m_{non-extractable} = m_{dry\ algae} - m_{lipid, tot} \quad (18)$$

Total volume of the algae cells $V_{cell, total}$ is calculated by

$$V_{cell, tot} = \frac{m_{dry\ algae}}{C_{algae\ paste} \times \rho_{algae}} \quad (19)$$

where $m_{dry\ algae}$ is the dry weight of algae added in the system, $C_{algae\ paste}$ is the algae content of algae paste, and ρ_{algae} is the average density of algae cells. Based on our observation that the cells of algae *Neochloris oleoabundans* flocculate very slowly without centrifugation, the density ρ_{algae} is assumed to be the same as the density of water.

The volume inside the cells ($V_{cell, in}$) is calculated by the equation below:

$$V_{cell, in} = V_{cell, tot} - \frac{m_{non-extractable}}{\rho_{algae}} \quad (20)$$

where, due to lack of additional information, the density of non-extractable is assumed to be the same as the average density of algae.

Since it is assumed that at equilibrium, the algae cells are filled with the same mixture as the organic phase, the volume of organic solution outside the cells ($V_{org, out, i}$) is calculated by

$$V_{org, out, i} = V_{org, i} - R_{vol, i} \times V_{cell, in} \quad (21)$$

The amount of lipid released from cellular material in stage i is calculated by

$$m_{lipid, rel, i} = R_{rel, i} \times m_{lipid, tot} \quad (22)$$

where R_i which is defined in Eq. 3 is the ratio of lipid released from cellular material in stage i to the total lipid amount. And $m_{lipid, tot}$ is the total lipid amount.

The lipid concentration is calculated by

$$C_{lipid} = \frac{m_{lipid, rel}}{V_{org}} \quad (23)$$

The amount of extracted lipid $m_{lipid, extr}$ is calculated by

$$m_{lipid, extr} = C_{lipid} \times V_{org, out} \quad (24)$$

Therefore the lipid yield is calculated by

$$Y_{lipid} = \frac{m_{lipid, extr}}{m_{dry\ algae}} \times 100\% \quad (25)$$

The un-extracted lipid amount in extraction stage i is named $m_{lipid, unextr, i}$

$$m_{lipid, unextr, i} = m_{lipid, unextr, i-1} - m_{lipid, extr, i} \quad (26)$$

For the first stage extraction, the $m_{lipid, unextr, i-1}$ is the total lipid amount in algae $m_{lipid, tot}$. $m_{lipid, unextr, i}$ contains two parts: one part is the lipid that hasn't dissolved into the organic solvent ($m_{lipid, unextr, non-free}$,

i), the other part is the lipid fraction that did dissolve in the organic solvent but is still present in the organic phase inside the cell wall ($m_{lipid, unextr, free, i}$).

$$m_{lipid, unextr, free, i} = R_{vol, i} \times V_{cell, in} \times C_{lipid, i} \quad (27)$$

After the first stage extraction, the organic layer was separated, some makeup water ($m_{water, makeup}$) was added to the remaining aqueous phase and algae residue to make it the same weight as the starting feed ($m_{algae\ slurry}$).

$$m_{water, makeup, i} = m_{algae\ slurry} - m_{aq, i-1} - m_{non-extractable} - R_{vol, i-1} \times V_{cell, in} \times \rho_{org} \quad (28)$$

Then, fresh EBA (m_{EBA}) was added and the second stage extraction started.

The lipid extracted in the second stage extraction was formed by part of the lipid which was already in the organic solvent inside the algal cells during previous extraction stage ($m_{lipid, unextr, free, i-1}$) and part of the lipid that was released from cellular material during the second stage extraction ($m_{lipid, rel, i}$).

In $m_{lipid, extra, i}$, some of the lipid was released during the first stage extraction $m_{lipid, unextr, free, from\ stage\ 1, i-1}$. In the first stage extraction, $m_{lipid, unextr, free, from\ stage\ 1}$ is the same as $m_{lipid, unextr, free}$. In the subsequent extraction stages, $m_{lipid, extr, from\ stage\ 1, i}$ can be calculated by

$$m_{lipid, extr, from\ stage\ 1, i} = m_{lipid, unextr, free, from\ s\ 1, i-1} \times \frac{m_{lipid, extr, i}}{m_{lipid, unextr, free, i-1} + m_{lipid, rel, i}} \quad (29)$$

The remaining lipid which was from stage 1 ($m_{lipid, unextr, free, from\ stage\ 1, i}$) can be calculated by

$$m_{lipid, unextr, free, from\ stage\ 1, i} = m_{lipid, unextr, free, from\ stage\ 1, i-1} - m_{lipid, extr, from\ stage\ 1, i} \quad (30)$$

The key parameter $R_{vol, i}$ (the ratio of organic phase volume inside the cell to the total volume inside the cell) and $R_{rel, i}$ (ratio of lipid released from cellular material in stage i to the total lipid amount) in this model were identified based on the minimization of difference between estimated and experimental lipid yield, representing the absolute error. Three groups of experiment results were used in the error calculation. The solvent to feed ratio was the same for four extraction stages which was 1:1 in experiment 1 and 1:2 in experiment 2. In experiment 3, the solvent to feed ratio was 2:1 for stage 1, 1:2 for stage 2, 1:1 for stage 3 and 4, respectively. The absolute error between estimated and experimental lipid yield at stage i were calculated by:

$$Error_i = \sum_{n=1}^3 |Y_{lipid, est, i} - Y_{lipid, exp, i}|_n \quad (31)$$

where $Y_{lipid, est, i}$ and $Y_{lipid, exp, i}$ is the estimated and experimental lipid yield at stage i respectively. And n is the number of experiment group.

The effect of $R_{vol, i}$ and $R_{rel, i}$ to $Error$ at each extraction stage is illustrated in Fig. 7.

The optimum value of $R_{vol, i}$ and $R_{rel, i}$ was taken when $Error$ reached the minimum. From the calculation results shown in Fig. 7 it can be observed that in all four stage extraction, the optimum value of $R_{vol, i}$ is 1, which means that the cells were filled with 100% organic phase at equilibrium state. The optimum values of $R_{rel, i}$ ($i = 1, 2, 3, 4$) are listed below:

$$\begin{aligned} R_{rel, 1} &= 87.5\% \\ R_{rel, 2} &= 3.7\% \\ R_{rel, 3} &= 2.3\% \\ R_{rel, 4} &= 2.2\% \end{aligned}$$

The proposed model was used to predict the crude lipid yield for a four stages extraction when the solvent to feed ratio was 1:1. The estimated model results for the four stages lipid extraction from FW-stressed *Neochloris oleoabundans* using EBA are listed in Table 1.

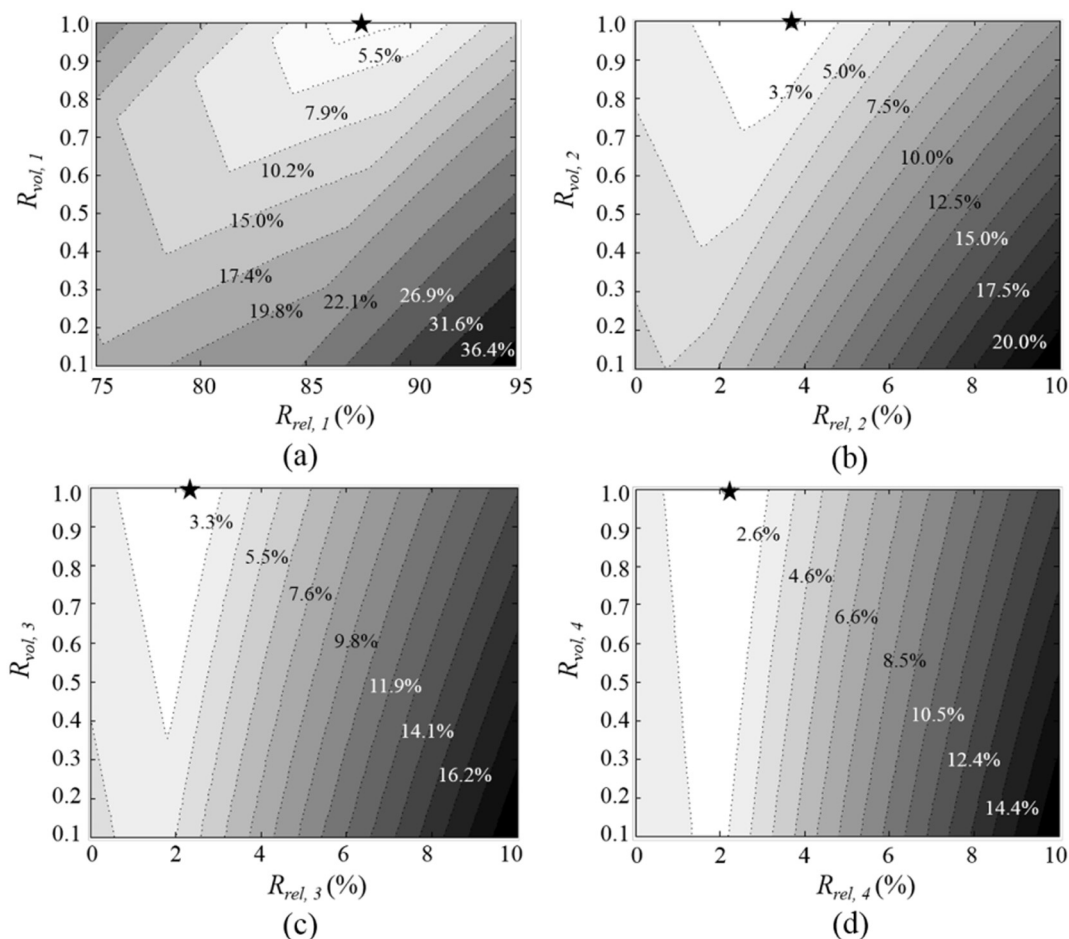


Fig. 7. Effect of $R_{vol,i}$ and $R_{rel,i}$ to Error (absolute error between estimated and experimental lipid yield) at (a) stage 1, (b) stage 2, (c) stage 3 and (d) stage 4. The point where Error reached minimum value is marked by \star .

From calculation it can be found that in the second stage extraction, 79% of the extracted lipid was released already in the first stage (but remained in the solvent phase inside the cells). Because of the limited amount of recovered lipids in the third and fourth stages, only the lipids from first and second extractions were analyzed by GC-MS. The total fatty acid (TFA) compositions are presented in Fig. 2. The fatty acid profile of *Neochloris oleoabundans* was dominated by palmitic (C16:0), hexadecadienoic (C16:2), oleic (C18:1), linoleic (C18:2), stearic (C18:0) and linolenic (C18:3) acids, and (as will now be clear because of the 79% mentioned above) no significant differences were found between the first and second extraction stage.

The TFA yield in crude lipid of first stage extraction (52.9 wt%) was much higher than the second stage extraction (38.3 wt%). The extracted crude lipid from second stage was formed by the lipid released from both first stage and second stage extraction. Taking the error into account, the TFA yield in second stage released lipid was calculated to be 0 to 1 wt%, suggesting that (near) all TFA is released in the first stage (but not all is recovered!). This also confirms that the lipid released in first and second stage had different compositions which may result in different extractability. Most of the lipid that contained fatty acid were released in the first stage extraction. About 99.9 wt% of the lipid from first stage release was extracted after four stages while > 97.5 wt% was

Table 1
Estimated results of 4 stages lipid extraction from FW-stressed *Neochloris oleoabundans* using EBA (S/F = 1:1).

Source	Name	Unit	Stage 1	Stage 2	Stage 3	Stage 4
Input	S/F	–	1:1	1:1	1:1	1:1
Model – Eq. 25	Y_{lipid}	–	47.1%	9.5%	2.7%	1.6%
Model – Eq. 22	$m_{lipid,rel}$	g	0.6683	0.0283	0.0176	0.0168
Model – Eq. 23	C_{lipid}	g/mL	0.0191	0.0037	0.0011	0.0006
Model – Eq. 24	$m_{lipid,extr}$	g	0.5623	0.1137	0.0322	0.0192
Model – Eq. 26	$m_{lipid,unextr}$	g	0.2015	0.6501	0.7316	0.7446
Model – Eq. 27	$m_{lipid,unextr,free}$	g	0.1060	0.0206	0.0059	0.0035
Model – Eq. 29	$m_{lipid,extr,from\ stage\ 1}$	g	0.5623	0.0898	0.0137	0.0021
Model – result	$m_{lipid,extr,total}$	g	0.7275			
Model – result	$m_{lipid,rel,total}$	g	0.7310			
Model – result	$m_{lipid,extr,from\ stage\ 1}$	–	100.0%	79.0%	42.6%	11.1%
	$m_{lipid,extr}$					
Model – result	$\sum_{i=1}^n m_{lipid,extr,from\ stage\ 1}$	–	84.1%	97.6%	99.6%	99.9%
	$m_{lipid,rel,stage\ 1}$					

Table 2
Estimated results of 4 stages lipid extraction from FW-stressed *Neochloris oleoabundans* using EBA (S/F = 1:2).

Source	Name	Unit	Stage 1	Stage 2	Stage 3	Stage 4
Input	S/F	–	1:2	1:2	1:2	1:2
Model – Eq. 25	Y_{lipid}	–	38.1%	14.4%	5.2%	2.5%
Model – Eq. 22	$m_{lipid, rel}$	g	0.6381	0.0270	0.0168	0.0160
Model – Eq. 23	C_{lipid}	g/mL	0.0386	0.0127	0.0047	0.0023
Model – Eq. 24	$m_{lipid, extr}$	g	0.4339	0.1642	0.0591	0.0287
Model – Eq. 26	$m_{lipid, unextr}$	g	0.2953	0.5651	0.6702	0.7005
Model – Eq. 27	$m_{lipid, unextr, free}$	g	0.2042	0.0670	0.0247	0.0120
Model – Eq. 29	$m_{lipid, extr, from stage 1}$	g	0.4339	0.1450	0.0417	0.0123
Model – result	$m_{lipid, extr, total}$	g	0.6859			
Model – result	$m_{lipid, rel, total}$	g	0.6979			
Model – result	$m_{lipid, extr, from stage 1}$	–	100.0%	88.3%	70.6%	42.8%
	$m_{lipid, extr}$					
Model – result	$\frac{\sum_{i=1}^n m_{lipid, extr, from stage 1}}{m_{lipid, rel, stage 1}}$	–	68.0%	90.7%	97.3%	99.2%

extracted in two stages. It was therefore concluded that two extraction stages is sufficient for TFA recovery, when using the same 1:1 S/F ratio.

Besides the four stages extraction experiment which solvent to feed ratio was 1:1, another two experiments were carried out in which the solvent to feed ratio was 2:1 for one experiment and changed for each stage (2:1 for stage 1, 1:2 for stage 2, 1:1 for stage 3 and 4) for another. The model results are presented in Table 2 and Table 3.

3.3. Experimental validation of the extraction model

Fig. 8 shows both the experimental and model estimated values of crude lipid yield of four extraction stages. For both the experiments at constant S/F (Fig. 8 (a) and (b)) and those with changing S/F per stage (Fig. 8 (c)), the model estimated values were shown to be in very good agreement with those obtained in experiments. The correlation between estimated and experimental values was excellent. This is clearly illustrated by the parity plot provided in Fig. 8 (d). When the solvent to feed ratio was 2:1, 93% of the lipid released from cellular material in stage 1 was extracted, while only 68% for solvent to feed ratio 1:2. Since most of the TFA were released in the first stage, the more solvent used, the more TFA were recovered from first stage extraction.

The extracted lipids were analyzed by GC–MS when the solvent to feed ratio was 1:2 for four stages and 2:1, 1:2, 1:1 and 1:1 for stage 1 to 4 respectively. The TFA analysis results in Fig. 9 show that when the solvent to feed ratio was 1:2, the TFA yield in crude lipid of first and second stage extraction are 53.9 wt% and 47.1 wt% respectively. When the solvent to feed ratio was 2:1 for first stage and 1:2 for second stage, the TFA yield in crude lipid of first and second stage extraction are 53.3 wt% and 33.6 wt% respectively. Neither the solvent to feed ratio nor the extraction stages had influence on the TFA composition. TFA

content in crude lipid in the second extraction stage was lower than the first. After calculation, it was found out that the lipid released from cellular material in second extraction stage contains little/no fatty acid, which was the same as the results earlier when the solvent to feed ratio was 1:1. If the lipid released in the second stage was assumed containing no fatty acid, the estimated value of TFA yield in crude lipid extracted in second stage was equal to the measured value. The proposed model was thus found to successfully describe the extraction equilibrium using EBA for lipid extraction from FW-stressed *Neochloris oleoabundans*. Unfortunately, direct experimental evidence of the mechanism, especially the presence of the organic phase inside the cells, was not possible. However, the good fit of model data with experimental findings, in combination with the sensitivity study, shows that our assumptions are not in conflict with the data and likely to be a fair representation of the actual extraction mechanism.

3.4. Sensitivity analysis

The model discussed and successfully applied in the above section contains several parameter values and assumptions. As different assumptions and different parameter values assumed may lead to different estimated values, a sensitivity analysis towards several key assumptions made is considered valuable. Therefore a simulation was performed for the crude lipid yields of the four extraction stages with changing the volume of algae cells.

The parameter $C_{algae\ paste}$ is the algae content of algae paste, which was used for calculating the volume of algae cells. Before extraction, the $C_{algae\ paste}$ was around 20 wt% and this value was used for the modelling. Whether the volume of algae cells changed during extraction is difficult to identify experimentally and still not known. So the crude

Table 3
Estimation results of 4 stages lipid extraction from FW-stressed *Neochloris oleoabundans* using EBA while solvent to feed ratio was different for each stage.

Source	Name	Unit	Stage 1	Stage 2	Stage 3	Stage 4
Input	S/F	–	2:1	1:2	1:1	1:1
Model – Eq. 25	Y_{lipid}	–	51.8%	4.5%	3.0%	1.6%
Model – Eq. 22	$m_{lipid, rel}$	g	0.6381	0.0270	0.0168	0.0160
Model – Eq. 23	C_{lipid}	g/mL	0.0089	0.0044	0.0011	0.0006
Model – Eq. 24	$m_{lipid, extr}$	g	0.5910	0.0509	0.0342	0.0186
Model – Eq. 26	$m_{lipid, unextr}$	g	0.1383	0.6784	0.6951	0.7106
Model – Eq. 27	$m_{lipid, unextr, free}$	g	0.0471	0.0232	0.0058	0.0032
Model – Eq. 29	$m_{lipid, extr, from stage 1}$	g	0.5910	0.0323	0.0126	0.0018
Model – result	$m_{lipid, extr, total}$	g	0.6947			
Model – result	$m_{lipid, rel, total}$	g	0.6979			
Model – result	$m_{lipid, extr, from stage 1}$	–	100.0%	63.6%	36.9%	9.8%
	$m_{lipid, extr}$					
Model – result	$\frac{\sum_{i=1}^n m_{lipid, extr, from stage 1}}{m_{lipid, rel, stage 1}}$	–	92.6%	97.7%	99.7%	100.0%

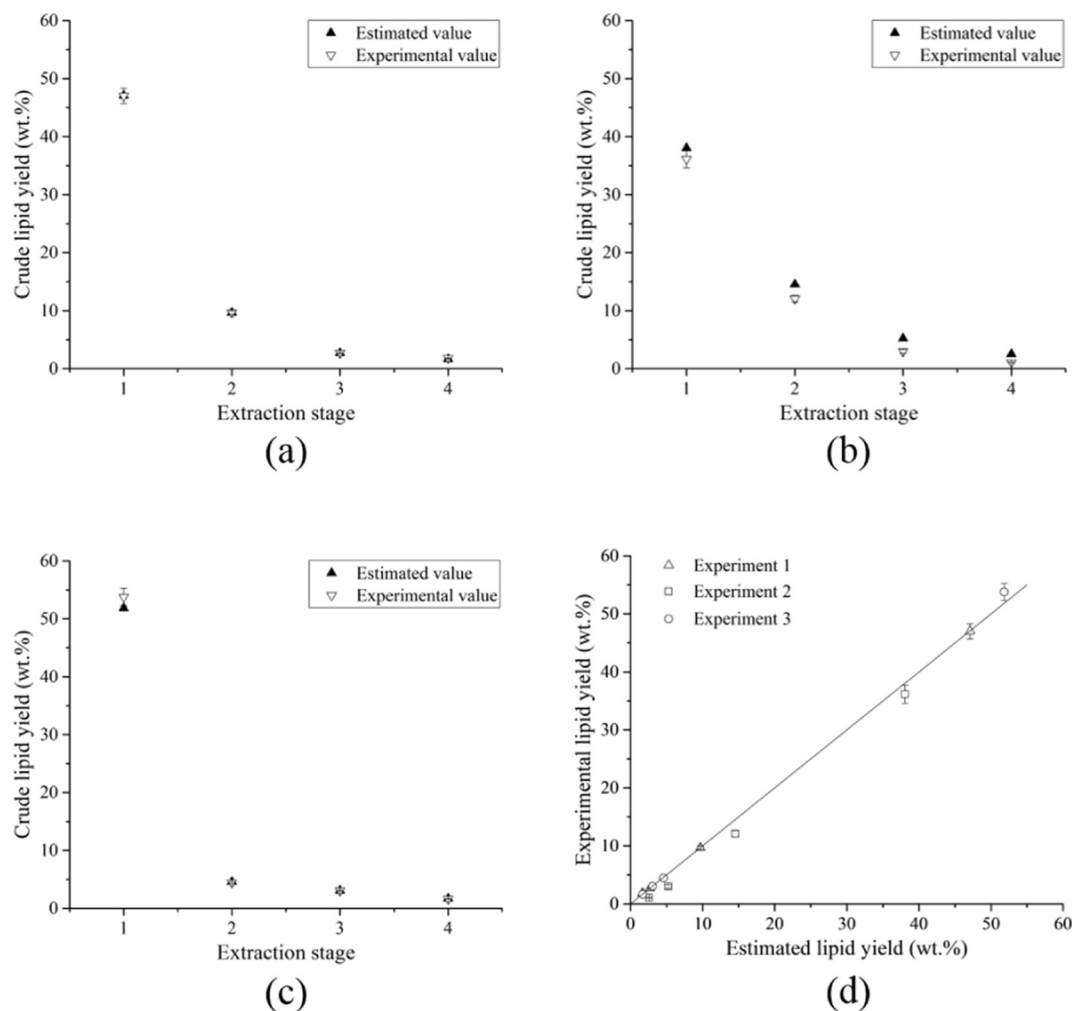


Fig. 8. Estimated and experimental values of crude lipid yield of *Neochloris oleoabundans* extracted by N-ethyl butylamine method for multistage extractions, (a) S/F = 1:1, (b) S/F = 1:2, (c) S/F = 2:1 for stage 1, 1:2 for stage 2, 1:1 for stage 3 and 4, (d) parity plot. Data points in this figure are averages of at least two replicates, and the indicated error bars represent the averaged relative standard deviation (2.8%) over all 114 experiments of this type in the study.

lipid yields of the four extraction stages at different $C_{algae\ paste}$ were simulated and showed in Fig. 10. The crude lipid yield of first stage extraction increased when higher $C_{algae\ paste}$ applied. This is because higher $C_{algae\ paste}$ means lower water content in the algae paste, also

means the volume of algae cells are smaller. Less organic solvents containing lipid loss into the cells with smaller algae cells volume. The increasing of crude lipid yield in first stage results in the decreasing in second stage because of the finite lipid amount of algae. The influence

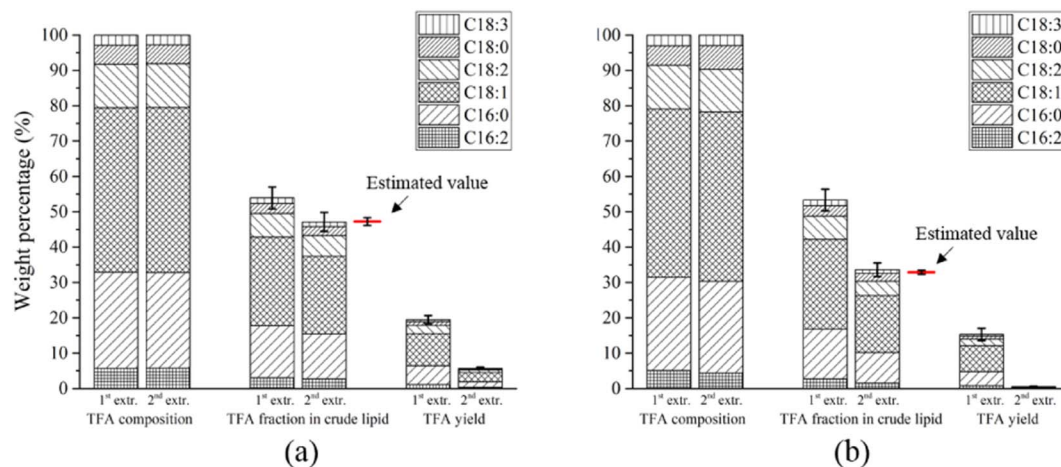


Fig. 9. TFA (total fatty acid) composition, TFA fraction in crude lipid and TFA yield of lipids from *Neochloris oleoabundans* obtained by N-ethyl butylamine 1st and 2nd extraction (a) Solvent/Feed (S/F) = 1:2, (b) S/F = 2:1 for stage 1 and S/F = 1:2 for stage 2. Data points in this figure are averages of at least two replicates, and the indicated error bars represent the averaged relative standard deviation (5.7%) over all 60 experiments of this type in the study.

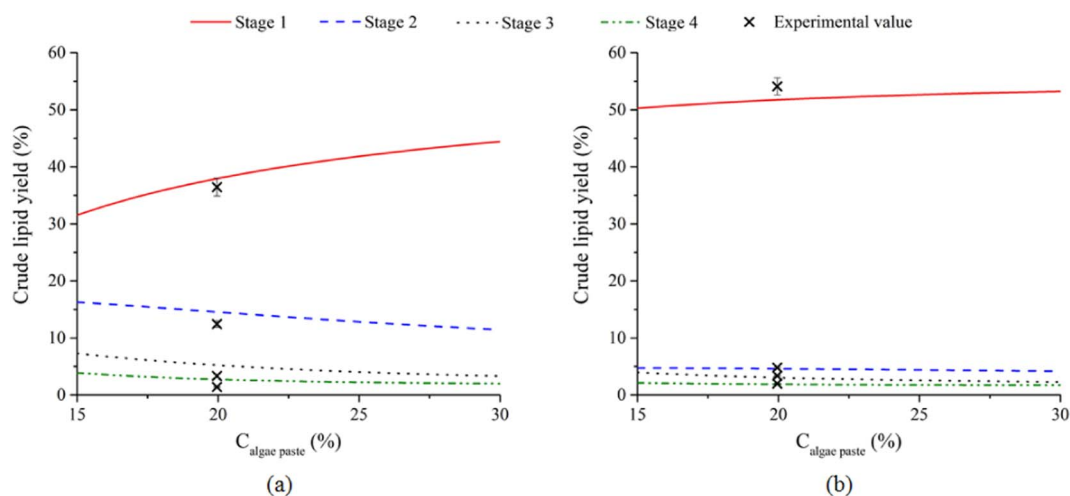


Fig. 10. Crude lipid yields of four extraction stages at different $C_{algae\ paste}$ when (a) Solvent/Feed (S/F) = 1:2, (b) S/F = 2:1, 1:2, 1:1 and 1:1 for stage 1 to 4 respectively. Data points in this figure are averages of at least two replicates, and the indicated error bars represent the averaged relative standard deviation (2.8%) over all 114 experiments of this type in the study.

of $C_{algae\ paste}$ to the crude lipid yield was not very strong when the solvent to feed ratio was 2:1 for first stage extraction. This may be because when more solvent was used for extraction, the lipid concentration in the organic solvent was lower. The difference in the algae cells volume hardly influences the amount of lipid left inside the cells. Considering the experimental results of crude lipid yields in the four stage extraction, it can be concluded that even if the volume of algae cells changes during extraction, the change will be < 10% of the original volume and this will not cause too much difference for the estimated values.

4. Conclusions

This study advanced our understanding on the equilibrium extraction of lipids from FW-stressed *Neochloris oleoabundans*. With the hypothesis that after extraction, the algae cells were completely filled with the organic solvent phase, having the same composition as the organic phase outside the cells, the model was successfully fitted to the experimental crude lipid yields of the four stage extractions at various solvent to feed ratios. By modelling it was found that nearly all fatty acids were released from the cell material, but not all is recovered due to the organic phase remaining inside the cell. This mechanism also explains the incomplete lipid recovery in a single extraction stage. For common applied solvent to feed ratios, two extraction stages is sufficient for recovering most of the lipid containing fatty acid.

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Author contributions

All authors have contributed significantly to this work, either as first author performing the experiments and modelling, or as supervisor interpreting the data and discussing on the design and course of the study.

Conflict of interest statement

None of the authors has any financial or other interest that could have influenced the outcomes of this work.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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