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Improved aqueous extraction of microalgal lipid by combined enzymatic and thermal lysis from wet biomass of *Nannochloropsis oceanica*

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HIGHLIGHTS

- Method developed for extracting lipid from wet microalgae with 96.0% moisture content.
- Upto 88.3% of total lipids are extracted from wet algae biomass at optimal conditions.
- Microalgae cells were completely disrupted by combined thermal and enzymatic lysis.
- Excessive extraction of polar lipid was found for wet biomass.

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ABSTRACT

High moisture content in wet algal biomass hinders effective performance of current lipid extraction methods. An improved aqueous extraction method combining thermal and enzymatic lysis was proposed and performed in algal slurry of *Nannochloropsis oceanica* (96.0% moisture) in this study. In general, cell-wall of *N. oceanica* was disrupted via thermal lysis and enzymatic lysis and lipid extraction was performed using aqueous surfactant solution. At the optimal conditions, high extraction efficiencies for both lipid (88.3%) and protein (62.4%) were obtained, which were significantly higher than those of traditional hexane extraction and other methods for wet algal biomass. Furthermore, an excessive extraction of polar lipid was found for wet biomass compared with dry biomass. The advantage of this method is to efficiently extract lipids from high moisture content algal biomass and avoid using organic solvent, indicating immense potential for commercial microalgae-based biofuel production.

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1. Introduction

Biofuels have drawn an increasing attentions in recent years for its inherent advantages, including carbon dioxides sequestration and environmental harmlessly (Hu et al., 2008; Williams and Laurens, 2010). A number of raw materials have been used for biofuel production, of which the oleaginous microalgae are considered as the most promising alternative feedstock in virtue of their fast growth rate, high oil content, high oil productivity and adaptability to live in most of environmental conditions (Hu et al., 2008; Williams and Laurens, 2010).

Nannochloropsis sp. has been considered as one of the most promising reference oleaginous microalgae. Although recent works have studied the entire process of biofuel production of this alga,

including omics and physiological aspects (Pal et al., 2011; Wang et al., 2014), mass cultivation (Moazami et al., 2012), as well as oil extraction and conversion (Koberg et al., 2011; Chen et al., 2012a,b), technological barriers for commercial microalgae-biodiesel production are still remained. Among these, lipid extraction from *Nannochloropsis* biomass, in particular wet biomass, is inefficient, energy intensive and costly (Richardson et al., 2014). It should be emphasized that traditional lipid extraction methods for dry biomass encountered very low extraction efficiency when it is applied to wet biomass (Halim et al., 2011; Koberg et al., 2011). Currently, many lipid extraction methods, such as supercritical methanol (Patil et al., 2011), subcritical ethanol/hexane (Chen et al., 2012b) and hydrodynamic cavitation (Lee and Han, 2015), have obtained high oil extraction efficiency for processing wet biomass containing moisture upto 90%. However, an energy intensive step to remove moisture was still required in subsequent desolvation process, since moisture was extracted together with lipid from

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wet biomass. On the other hand, large volumes of organic solvents are consumed in these methods, resulting in environmental risks.

As a method of using water as an extraction and separation solution, aqueous extraction method (AEM) poses an advantage of avoiding the use of organic solvent. Several researchers have demonstrated that aqueous extraction method had been successfully applied to extract oil from oil-bearing seeds/fruits in both laboratory and industrial scales (Zhang et al., 2007; Campbell and Glatz, 2009; Naksuk et al., 2009). However, applying aqueous extraction method in oleaginous microalgae to extract lipid has not been attempted. In addition, cells walls, owing to their robustness, probably represent the biggest barrier to target-compound extraction. Previous studies suggested that cell disruption was necessary before/during aqueous extraction of lipid. But many cell disruption methods for oilseeds such as milling and flaking (Campbell and Glatz, 2009) have been proven ineffective for microalgae cells due to their tiny size and specific property of cell walls (Scholz et al., 2014). Hence, selection of an appropriate cell disruption method is largely dependent on microalgal distinct biology and cell-wall characteristics, and this method will also be applicable for technology scale-up.

In the present work, the feasibility of aqueous extraction for extracting oil from wet microalgae biomass was identified. A primary screen of aqueous solution based on lipid extraction efficiency was investigated firstly, which was followed by optimizing cell disruption processes of thermal and enzymatic lysis, to improve the efficiency of aqueous extraction. In addition, the extraction efficiency and the quality of extracted oil between this proposed method and conventional methods were also compared.

2. Materials and methods

2.1. Materials

The microalgal strain used in this study was *Nannochloropsis oceanica* IMET1, cultivated in a 15 L panel photobioreactor containing 12 L of BG11 medium prepared with sea water, with ambient air and 1% CO₂ agitating. The cultivations were carried out indoor at temperature of 25 °C and illumination intensity of 400 μmol m⁻² s⁻¹ provided by cool white fluorescent for 14 days. Then, all microalgal cells were harvested using a centrifuge for 10 min at 6000 rpm to obtain algal paste, and were kept in refrigerator at -80 °C until use. The obtained algal paste has a moisture content of ca. 65% and a total lipid content of 45.6% in dry weight.

2.2. Aqueous extraction method for algal lipid

Microalgal cells in form of paste were resuspended in various aqueous extraction solution including DI water and surfactant aqueous solutions at certain microalgal cell densities of 40–200 g L⁻¹, and then the algal slurry was kept stirring at 100 rpm until the set time at 50 °C. The blank experiment was carried out with the same procedure, but using silica sand as raw material instead of algal biomass. One anionic surfactant (Sodium dodecylsulphate, SDS), three non-ionic surfactants (Triton X-100, Tween 20 and Polyacrylamide, PAM), and mixtures of anionic and non-ionic surfactants were tested. For surfactants screening test, the final surfactant concentration of SDS was set at 2.5% (w: v) and that of each non-ionic surfactant was set at 0.1% (w: v). All the surfactants at analytical reagent were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

After extraction, the processed slurry was centrifuged at 8000 rpm for 10 min and separated into three phase, including lipid phase, aqueous phase and solid phase from top to bottom. The lipid phase was carefully collected with assistance of a small

volume of hexane for completely collection and transferred into a pre-weighted tube, in which the lipid was further purified to remove residual protein following Bligh and Dyer's method (Bligh and Dyer, 1959). The amount of extracted lipids was determined as the mass difference of tubes. The aqueous phase was also collected for lipids analysis. The lipid yield of each treatment was calculated as dividing mass of extracted lipid by mass of dried microalgae, and was subsequently confirmed based on the lipid content difference between raw algal biomass and residual.

The lipids extraction efficiency was calculated by Eq. (1):

$$\text{Lipids extraction efficiency, \%} = \frac{\text{Extracted lipids}}{\text{Total lipids in algae}} \times 100\% \quad (1)$$

2.3. Process for microalgal cells disruption

Thermal lysis and enzymatic lysis for cell disruption of *N. oceanica* were carried out in this study, both singly and in combination. For thermal lysis, microalgal slurry was conducted in a laboratory steam generator (Shanghai Huazheng special boiler manufacture Co., Ltd., China) coupled with twin-channel atomizer. The algal slurry was pumped into atomizer from one channel at a flow rate of 50 ml s⁻¹, and meanwhile the steam at 0.4 MPa was supplied from the other channel into atomizer. Then, the mixture of algal slurry and steam were released from outlet and collected for further analyses. Alternatively, the thermal lysis of microalgae was conducted in a laboratory autoclave (Hirayama HVE-50) at 121 °C for 20 min. For enzymatic hydrolysis, a concentrated enzyme solution was added into aforementioned algal slurry at a certain concentration and then gently stirring at a moderate temperature (25–50 °C) and pH (4–10) for variable time. The tested enzymes include cellulose from *Aspergillus niger*, lipase (Solarbio Co. Ltd.) and protease (Novozyme Co. Ltd.).

After cell disruption treatment, the processed slurry was separated into a solid phase of algal residue and an aqueous phase by centrifuged at 8000 rpm for 10 min. Both phases were collected for lipids and protein analysis.

2.4. Characterization of cell disruption

The glucose, a main (almost the only) degradation product for cellulose of *Nannochloropsis* cell wall (Scholz et al., 2014; Mirsiaghi and Reardon, 2015), was assayed by a SBA-40 biosensor analyzer (Shandong Academy of Sciences, China) equipped with the glucose oxidase immobilized membrane, to evaluate lysis efficiency of cell walls.

The morphology of microalgal cells were monitored under microscope before and after cell disruption processes. Images were taken with an Olympus BX 51 microscope equipped with DP 72 digital camera.

2.5. Traditional hexane extraction

Hexane extraction was carried out using Soxhlet approach. A 2.0 g of lyophilized algae powder was loaded into Soxhlet extraction thimble, extracted with 100 ml hexane at boiling temperature until the algal color turned gray. Mixture of solvent and extracted lipids was taken out and solvent was evaporated at low pressure to recover lipids.

2.6. Lipids analysis

The triglyceride content in total extracted lipids was determined by the glycerol-3-phosphate-oxidase-p-chlorophenol

method (Triglycerides reagent, Sinnowa Medical Science & Technology Co., Ltd., China), with triolein as the standard (Takagi et al., 2000). This method was calibrated by column chromatography with silica gel as immobile phase and eluted by chloroform (Kates, 1972).

TAG extraction efficiency was calculated by Eq. (2):

$$\text{TAG extraction efficiency, \%} = \frac{\text{TAG in extracted lipids}}{\text{Total TAG in algae}} \times 100\% \quad (2)$$

The total lipids content in raw algal biomass and residue after extraction were determined by chloroform-methanol ($\text{CHCl}_3/\text{MeOH}$) extraction, as a description of Bligh and Dyer (1959).

2.7. Protein analysis

The total protein concentration in aqueous extraction medium was assayed by Bradford protein assay kit (Beyotime Institute of Biotechnology, China), in measuring absorbance at 595 nm with a spectrophotometer (Varian 50 Bio, US), with bovine serum albumin (BSA) as a standard. The protein extraction efficiency (%) was calculated by dividing mass of extracted protein by mass of total protein in raw algal biomass. The total protein content of *N. oceanica* was analyzed by the Kjeldhal method (AOAC, 1984), using 6.25 as a conversion factor.

2.8. Statistic method

The statistic significance of the result was analyzed using one-way ANOVA analysis by SPSS 11.0. The effects on lipid recovery are considered significant when $p < 0.05$.

3. Result and discussion

3.1. Directly aqueous extraction of algal oil

Firstly, *N. oceanica* slurry was directly adopted for oil extraction using DI water, but no obvious algal oil could be extracted (Fig. 1). Considering surfactant could enhance oil solubility in aqueous solution (Naksuk et al., 2009), and be useful to cell disruption (Huang and Kim, 2013), several surfactants were added singly or jointly into water to promote oil extraction. The results indicated that most tested surfactants increased total lipids extraction efficiency, except Triton X-100 (Fig. 1). The highest total lipid extraction efficiency of 13.5% was obtained by using a mixture of

SDS and Tween-20 (SDS + Tween-20), but the TAG extraction efficiency was significantly reduced. Compared with SDS + Tween-20, a surfactant mixture of SDS and PAM (referred to as SDS + PAM) could provide a much higher TAG extraction efficiency and relatively high extraction efficiency of total lipid. As a result, the combination of SDS and PAM seemed to be a priority option.

The effect of different SDS + PAM concentrations on total lipid extraction efficiency was studied thereafter. The lipid extraction efficiency enhanced obviously with increase of SDS + PAM concentrations, and the maximum extraction efficiency of algal lipids (6.5%) was reached at 1.25% SDS and 0.05% PAM, beyond which the extraction efficiency would not increase anymore (Fig. 2A). The best lipid extraction was achieved at a SDS concentration (equal to 43 mmol L^{-1}) greater than its critical micelle concentration (10 mmol L^{-1}), which is in accordance with the view proposed by Lai et al. (2016).

The kinetic curve of aqueous extraction with aqueous solution of SDS + PAM solution showed that extraction efficiency of total lipid quickly reached the maximum value after extraction for 10 min, but was no more than 7.5% even after extraction for 120 min (Fig. 2B). The lipid extracting speed of aqueous solution of SDS + PAM was quite quickly compared to slow equilibrium occurred in organic solvent extraction (Balasubramanian et al., 2011), and the similar result was also proved in the performance for palm kernel oil by aqueous surfactant (Naksuk et al., 2009).

The microscopic observation of extracted algal residues showed that only a few microalgal cells (less than 10%) were disrupted while most cells remained intact. Since stored lipid (mainly of TAG) are located in specialised organelles termed lipid bodies (LBs) and are closed in a network of cytoplasm (Hu et al., 2008; Klok et al., 2014), cellular stored lipid could not be released into aqueous solution when algal cells are intact or just partly disrupted. Although our result indicated that lipid could be released into aqueous extraction solution from disrupted cells probably due to reducing interfacial tension between lipids and bulk solution and formation of microemulsion with assistance of surfactant (Do et al., 2009; Naksuk et al., 2009), the physical barrier of the cell wall and bonding protein around the lipid bodies are still be the major bottlenecks limiting lipid release. Some effective methods for weakening and/or breaking the cell-wall may be helpful for

Fig. 1. Effect of surfactant species on extraction efficiency of total lipids and TAG. The cells were extracted at 50°C for 1 h, and the liquid to solid (dry weight) ratio was 15:1 (mL:g). The final concentrations of SDS was set at 2.5% (w:v) and of the other ones were set at 0.1% (w:v).

Fig. 2. Effect of surfactants concentration (A) and extraction time (B) on lipid extraction efficiency. The biomass was extracted at 50°C , and the liquid to solid (dry weight) ratio was 15:1 (mL:g). The final concentration of PAM was adjusted to 4% of SDS concentration. The concentration profile was expressed by curve fitting obeying a DoseResp model. Data are expressed as means $\pm$ SD of three replicates.

enhancing oil extraction efficiency (Lee et al., 2010), in particular for aqueous extraction.

3.2. Improved aqueous extraction of algal oil with assistant of thermal lysis or enzymatic lysis

Considering the scale up practicability of cell disruption approaches, thermal lysis and enzymatic lysis were adopted and evaluated in this study. Cell-wall of *Nannochloropsis* sp. (Scholz et al., 2014; Mirsiaghi and Reardon, 2015) is largely composed of cellulose, the main monosaccharide of which is glucose, thus the content change of glucose could be considered to indicate degradation degree of cell walls.

The thermal lysis was carried out in a laboratory autoclave due to the little dosage of biomass. The lipid extraction efficiency obtained from aqueous extraction combined with thermal lysis (24.5%) increased to ca. 3-fold of the value from controls. However, no obvious broken of cell wall was observed, because no glucose was determined during thermal lysis process (data not shown) and most cells of *N. oceanica* remained intact after thermal lysis when observed at normal microscope (Fig. 3B). The high lipid extraction efficiency seems to be contradictory to the intact of cell wall. One possible explanation is that the physical barrier for intracellular lipid was just weakened or partly damaged by thermal lysis employed in this study, and this damage probably occurred

in ultrastructure but not in microstructure, resembling that for *Haematococcus pluvialis* (Mendes-Pinto et al., 2001).

For enzymatic lysis, different kinds of enzyme on algal cells disruption and lipid extraction were initially carried out and the results suggested that the effect of cellulase on the cell-wall disruption was better than those by protease and lipase (data not shown). Thereafter, the parameters involved enzymatic hydrolysis, including incubation time, temperature, pH, as well as enzyme dosage and liquid/solid ratio, were investigated. The optimal incubation temperature and pH in this study was 37 °C and pH 5.0. Although a higher degradation degree of cell walls achieved at a bigger cellulase dosage until at the concentration reached 2.5% (Fig. 4A), 1.3% of enzyme dosage was still chosen for following work in virtue of efficiency and cost. In this dosage, the effect of liquid/solid ratio on cell wall degradation was also investigated (Fig. 4C). A higher concentration of glucose could be detected with decrease of liquid/solid ratio, while the highest glucose yield was achieved when liquid/solid ratio was 15 ml g⁻¹. Experiments for incubation time indicated that the glucose concentration kept increasing until incubated for 5 h (Fig. 4B). As a result, the optimal conditions for enzymatic lysis of microalgae *N. oceanica* were determined as a cellulase dosage of 1.3%, an incubation time of 5 h, and an incubation temperature of 37 °C. Under this condition, the lipid extraction efficiency was upto 28.8%, which increased to ca. 4-fold of the value from controls (Run 7 in Table 1).

Fig. 3. The morphology of *Nannochloropsis oceanica* cells. (A) Control, intact cells; (B) cells after thermal lysis; (C) cells after combined thermal and enzymatic lysis. Scale bars = 10 μm.

Fig. 4. Parameters optimization for enzymatic lysis of *Nannochloropsis oceanica*, including dosage of cellulase (A), incubation time (B) and liquid to solid (dry weight) ratio (C). The experiments were operated at pH 5.0 and 37 °C. The extraction profile was expressed by curve fitting obeying a DoseResp model. Data are expressed as means ± SD of three replicates.

Table 1
Effect of cell disruption processes on lipid and protein extraction efficiency.

Run No.	Thermal lysis	Enzymatic lysis	Aqueous extraction	Extraction efficiency (%)	
				Lipid	Protein
1	–1	–1	–1	2.2 ± 0.6	6.9 ± 0.1
2	1	–1	–1	13.9 ± 2.1	28.5 ± 0.6
3	1	1	–1	54.6 ± 5.2	53.3 ± 4.2
4	1	1	1	88.3 ± 0.8	62.4 ± 1.9
5	–1	1	–1	18.7 ± 5.3	26.1 ± 0.1
6	–1	1	1	28.8 ± 0.0	29.9 ± 0.0
7	–1	–1	1	7.2 ± 2.4	12.6 ± 2.3
8	1	–1	1	24.5 ± 1.5	31.8 ± 0.6

Note: “1” indicated positive treatment, “–1” indicated negative treatment.

3.3. Improved aqueous extraction of algal oil by combined thermal and enzymatic lysis

Although the cell disruption by single thermal or enzymatic lysis has demonstrated the improvement of lipid extraction, the efficiency is still unsatisfied. Based on single-factor experiments tested afore, a complete factorial experiment was employed to analyze the efficiency of aqueous surfactants combining with thermal and enzymatic lysis on cell-wall disruption, lipid extraction and protein extraction from wet microalgal biomass. The experimental arrangement was shown in Table 1. The lipid extraction efficiency for combined cell lysis prior to aqueous surfactant extraction (88.3%) was significantly higher than that for either single thermal lysis process (24.5%) or single enzymatic lysis process (28.8%). Remarkably, a similar situation occurred in the case of using water rather than surfactant as extraction medium. These results confirmed that the two cell lysis processes have a synergistic effect on cell-wall disruption. As the cellulosic cell wall was damaged by thermal lysis (Chandra et al., 2015), the effect of enzymatic lysis process became more obviously, in a cooperative manner, resulting in completely destroy in cell-wall structure (Fig. 3C) and a significant increase in lipid extraction efficiency. In addition, ca. 53.3% of intracellular protein was extracted after the combined cell lysis process (Run 3 in Table 1), certifying the good crush effect of microalgal cells. This great proportion of extracted protein might damage glycoprotein bonding at cell wall and linkage between lipid bodies and cytoplasm (Gerken et al., 2013), and thus facilitate releasing intracellular lipid from disrupted cells (Zhang et al., 2007).

Table 2
Comparison of lipids extraction efficiency with different extraction methods.

Lipid extraction method	Cell-wall disruption	Lipid extraction efficiency		References
		Wet biomass [moisture (%)] ^a	Dry biomass	
Chloroform/methanol	Lyophilized	N/A	100%	This study
Improved AEM	Thermal & emzymatic	88.3% [96]	N/A	This study
Hexane	Lyophilized	N/A	57.0%	This study
Hexane ^b		21.1–45.1% [60]	14.1%	Halim et al. (2011)
Hexane ^c		20.0% [93]	100%	Taher et al. (2014)
Hexane ^c	Emzymatic	78.7% [93]	N/A	Taher et al. (2014)
Hexane	Autoclave	16.2–66.5% [98]	N/A	Lee and Han (2015)
Hexane	Ultrasonic	5.4–26.9% [98]	N/A	Lee and Han (2015)
Hexane	Hydrodynamic cavitation	25.9–99.0% [98]	N/A	Lee and Han (2015)
Hexane	Acid and base hydrolysis	77.0% [84]	N/A	Sathish et al. (2015)
Hexane	Steam explosion	56.7% [78]	N/A	Lorente et al. (2015)
Hexane/isopropanol	Surfactant	32% [N/A]	N/A	Lai et al. (2016)
Supercritical CO ₂ ^b		100% [60]	81.7%	Halim et al. (2011)
Supercritical methanol		88.9% [90]	N/A	Patil et al. (2011)
Subcritical hexane–ethanol		88.2% [65]	N/A	Chen et al. (2012)

N/A, not available or not applicable.

^a Moisture content was expressed as percent of wet basis.

^b Lipid extraction efficiency was expressed in comparison with supercritical CO₂ extraction from wet biomass (as 100%).

^c Lipid extraction efficiency was expressed in comparison with Soxhlet extraction using *n*-hexane from lyophilized biomass (as 100%).

3.4. Comparison of lipid extraction methods

The lipid extraction efficiency of aqueous surfactant combining with thermal and enzymatic lysis was compared with that by conventional solvent extraction methods for dry biomass and other methods for wet biomass (Table 2). The improved aqueous extraction method gave a total lipid extraction efficiency of 88.3%. Though the obtained total lipid extraction efficiency is lower than the highest value given by CHCl₃/MeOH extraction, which was generally assigned as extraction efficiency of 100% in many previous reports (Chen et al., 2012b; Cheng et al., 2015), it is significantly higher than hexane extraction for dry biomass. Many methods have successfully performed lipid extraction from wet microalgal biomass, which are presented in Table 2. Compared with these methods, this improved aqueous extraction method gave a moderate lipid extraction efficiency, which was not as high as that by hexane extraction following hydrodynamic cavitation (Lee and Han, 2015), but comparable to those obtained by supercritical methanol (Patil et al., 2011) and subcritical ethanol/hexane (Chen et al., 2012b), and much higher than those obtained by hexane

Fig. 5. Yield of major lipid fractions of *Nannochloropsis oceanica* according to extraction methods. Data are expressed as means ± SD of three replicates.

extraction following ultrasonication (Lee and Han, 2015) or double steps of acid–base hydrolysis (Sathish et al., 2015).

The yield of major lipid fraction of the tested microalgae with different extraction methods were shown in Fig. 5. The TAG yield achieved with this improved aqueous extraction method was comparable with that of hexane method, however a ca. 2.3-fold of polar lipid yield (mainly of phospholipids) was simultaneously achieved, leading to a notable high extraction efficiency of polar lipid (128.3%) and a relatively low TAG purity (51.8%) for this method. One possible explanation for excessive extraction of polar lipid is the presence of high polarity solvent, such as water and surfactant aqueous solution, enhancing the resolvability of membrane-bound lipids (mainly of polar lipid), which had been previously reported in extracting lipid from wet biomass of *Chlorococcum* and *Botryococcus braunii* (Halim et al., 2011; Paudel et al., 2015).

4. Conclusions

In recent work, an improved aqueous extraction with combined thermal and enzymatic lysis was constructed for cell-wall disruption and lipid extraction from wet microalgal biomass. Under optimal conditions, lipid extraction efficiency of 88.3% was achieved. This method possesses many advantages over other methods, for example, direct acting on algal slurry containing 96.0% of moisture, non-toxic characteristic compared to organic solvents, co-extraction of both lipids and protein in a single extraction process, lowering extraction cost and enhancing economic success. Therefore, the results showed that this method presents great potential for microalgae-based biofuel production.

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