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RESEARCH PAPER

Fractionation and Lipase-catalyzed Conversion of Microalgal Lipids to Biodiesel

Se Won Kim, Mizhang Xiao, and Hyun-Jae Shin

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Abstract This study was conducted to evaluate the lipid fractionation and purification procedures of lipase-catalyzed conversion of neutral lipids to microalgal biodiesel. Microalgae lipids were efficiently recovered and purified by a combined extraction method and crude lipid extracts were separated into neutral lipids, glycolipids, and phospholipids by solid-phase extraction. The high purity of the neutral lipids fraction was confirmed by its low concentration of phosphorous (< 2.0 ppm). Transesterification was catalyzed by immobilized *Candida antarctica* lipase for 72 h with stepwise addition of methanol. The reaction displayed Michaelis–Menten kinetics and produced high yields of microalgal biodiesel (91.2% in the case of *Dunaliella salina*) with a high content of unsaturated fatty acids (81.5%). Neutral lipids were converted to biodiesel by three-step transesterification, while the removal of polar lipids maintained the activity of the immobilized lipase by reducing both reaction mixture viscosity and contamination risk.

Keywords: lipid fractionation, microalgal biodiesel, immobilized lipase, transesterification, *Chlorella* sp., *Dunaliella salina*

1. Introduction

Microalgal biodiesel or FAME (fatty acid methyl ester) is attracting much attention as a potential alternative fuel with the advantages of biodegradability, renewability, and improved exhaust emissions. The key procedures in the production of microalgal biodiesel include microalgae screening, economical cultivation, as well as efficient extraction and conversion of lipids [1-4]. In the biomass pretreatment step that takes place prior to lipid extraction, hydrolytic enzymes such as lipase or chemicals such as sodium dodecyl sulfate are used to lyse cell walls; however, not all cells are equally susceptible to enzymatic or chemical lysis. Milling microalgae slurry in the presence of glass beads or other fine ceramic particles releases the intracellular proteins of even the most resistant cells. High yields are obtained from biomass containing large amounts of free fatty acids (FFAs) and water, both of which can cause problems for the traditional alkaline transesterification process [5]. FFAs in the feedstock can directly react with the alkaline catalyst to form soaps, thus preventing the separation of biodiesel from the glycerol fraction [6]. By definition, lipids are biological molecules soluble in organic solvents, and can be classified into two general categories based on the polarity of the molecular head group: (1) neutral lipids, which comprise acylglycerols and FFAs and (2) polar lipids, which can be sub-categorized into phospholipids and glycolipids. Neutral lipids are used by microalgae cells primarily for energy storage, while polar lipids are packed in parallel to form bilayer cell membranes. Acylglycerol are fatty acids ester-bonded to glycerol backbone, and are further divided according to its number of fatty acids in triacylglycerols (triglycerides), diacylglycerols, and monoacylglycerols. FFAs are fatty acids bonded to a hydrogen atom [7]. Lipid fractionation and purification is necessary to prepare high purity FAME suitable for use as a foodstuff and energy source.

Although the enzymatic lysis of biomass is currently less economically competitive than chemical treatment because of the high cost of the enzymes, lipase-based lysis can be

Se Won Kim, Mizhang Xiao, Hyun-Jae Shin*
Department of Biochemical and Polymer Engineering, Chosun University, Gwangju 61452, Korea
Tel: +82-62-230-7518; Fax: +82-62-230-7226
E-mail: shinhj@chosun.ac.kr

improved through technical innovations [8]. Many types of lipases have been used in biodiesel production from a variety of oils, fats, and acyl acceptors, producing high yields of biofuel [9]. The efficient reuse of the enzymes, which is critical for cost reduction, depends upon the maintenance of the high initial enzyme activity by preventing inactivation or inhibition, *i.e*., by ensuring stability of the enzymes under the reaction conditions. Lipase-based transesterification has already been demonstrated to provide high yields, large numbers of reuse cycles, and short reaction times. Further improvements, such as the optimization of pretreatment steps and reactor designs, could make enzymatic transesterification a viable option for the production of microalgal biodiesel in the future [10]. The efficient reuse of a given enzyme batch through several reaction cycles has a positive impact on total productivity, and thus lowers the cost of microalgal biodiesel production.

In this study, we aimed to optimize lipid extraction from microalgae to improve the efficiency of the transesterification for biodiesel production. We introduced the use of pigment contents as indicators for confirming the removal of polar components, including lipids. By comparing different methods of lipid extraction with respect to their efficiencies in reducing the content of components that inhibit lipase catalysis, we determined the key steps for the recovery of neutral lipids. We also explored the lipase-catalyzed transesterification of microalgae-extracted lipids to biodiesel, with a focus on lipid extraction and purification.

2. Material and Methods

2.1. Materials

Microalgae (*Chlorella* sp., *Dunaliella salina, Spirulina* sp., and *Nannochlopsis salina*) were cultured in Chosun University (Gwangju, Korea). The algal cultured using modified Erd-Schreiber's medium. Growth conditions were 9/15 h of dark/light cycle under 200 μmol/m^2/ sec of photosynthetically active radiations, shaking, and 2% CO_2. The biomass was harvested using a continuous feed fixed-bowl centrifuge (Mega 17R, HANIL, Incheon, Korea). The resulting paste consisting of 15 ~ 20% dry matter was immediately frozen. The paste was dried in a vacuum oven at 70 and 500 torr and stored at -15°C. Immobilized *Candida antarctica* lipase B, which was used for the transesterification of neutral lipids, was obtained from GenoFocus Inc. (Daejeon, Korea).

2.2. Gravimetric analysis of lipid content

The total lipid content was determined by gravimetric analysis [11]. Approximately 100 mg of dried algae powder was mixed with 5 mL chloroform/methanol (1:2, v/v) at 65°C for 1 h. The mixture was then centrifuged at 6,000 × *g* for 15 min. The supernatant was collected, and the residual biomass was extracted twice. After the supernatants were combined, and chloroform and 1% NaCl aqeous solution were added to a final volume ratio of 1:1:0.9 (chloroform/methanol/water). The mixture was allowed to separate, and the organic phase was transferred to a vial and dried to a constant weight at 60°C under nitrogen flow. The total lipid content was calculated as the percentage of the microalgal dry weight. The molar oil content was calculated based on its saponification value.

2.3. Lipid extraction with organic solvents

Extraction with a chloroform/methanol mixture was based on the methodology of Folch *et al.* [12]. The organic phase was collected and subjected to two identical extraction cycles.

2.4. Detergent extraction of microalgal lipids

The procedure was performed as previously described [13]. The extraction mixture was centrifuged at 6,000 × *g* for 15 min at 4°C. The upper layer was collected, mixed with Triton™ X-114 (10:1, v/v), incubated, and centrifuged as described above. The upper layer was then collected.

2.5. Silica extraction of lipids

The procedure was performed according to a modified version of the protocol described by Neoh *et al.* [14]. The extraction mixture was incubated for 2 h under constant agitation, and then centrifuged at 6,000 × *g* for 15 min at 4°C. A total of 360 mg of silica was added to the supernatant, and the mixture was incubated for 24 h at ambient temperature, followed by centrifugation under the same conditions.

2.6. Lipid extraction using a soxhlet extractor

Prior to the hexane extraction, dried algal flakes were finely ground and placed in a Soxhlet extractor until the hexane became colorless (24 ~ 48 h). The lipid-containing hexane solution was then filtered through a 1.2 μm Whatman® GF/C glass microfiber filter (Whatman, Maidstone, Kent, UK). Hexane was removed using a rotary evaporator. The resulting black-colored hexane-soluble lipid mixture was labeled "crude oil." The crude oil was then re-dissolved in hexane and filtered through activated carbon to remove pigments. The resulting neutral lipids were transparent and light yellow to amber in color.

2.7. Lipid separation by thin layer chromatography (TLC)

About 50 mg of lipid extract was loaded on a preparative silica gel G TLC plate (thickness: 500 μm) for neutral lipid

separation. The plate was developed by chloroform/methanol/acetone/acetic acid/water (65:10:20:10:3, v/v) through half of the TLC plate. After drying, a solvent mixture of hexane/diethyl ether/acetic acid (80:20:1, v/v) was run through the whole plate.

2.8. Transesterification and gas chromatography analysis

The reaction mixture consisted of the extracted microalgal lipids, methanol, hexane, and immobilized lipase (1 ~ 4%, w/w of oil). Hexane was used as solvent. The reaction of lipids and methanol was conducted in a 1.5 mL vessel at 45 °L with occasional shaking. The initial molar ratio of the methanol/oil was set at 3:1. The highest temperature was 50°C. The content of methyl esters in the reaction mixture was quantified using an HP 5890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a 0.32 mm (ID) × 30 m HP-INNOWax capillary column (Agilent Technologies). Heptadecanoic acid methyl ester was used as an internal standard. The column temperature was raised from 50 to 200°C at a rate of 1°C/min, and then was maintained at 200°C for 9 min; the temperature was then increased to 250°C and held there for 2 min. The temperatures of the injector and oven were 200 and 250°C, respectively.

2.9. Analysis of neutral lipids, glycolipids, and phospholipids

The neutral lipid fraction was subjected to one-dimensional thin-layer chromatography using silica gel 60-coated plates (Merck Millipore, Billerica, MA, USA). Plates were activated at 100°C for 2 h prior to use. A solvent mixture containing hexane/diethyl ether/acetic acid (70:30:1, v/v) was used. After co-chromatography with pure standards, lipid bands were stained with 2,7-dichlorofluorescein and visualized under UV light.

2.10. Neutral lipids analysis

Neutral lipids were scraped from the TLC plate (scraping were in the weight range of 1 mg) and extracted by the addition of 20 mL of chloroform:methanol (1:2, v/v). Then, 1.25 mL of methanol was added followed by 1.25 mL of dH_2O, and the solution was centrifuged at 750 × *g* for 5 min. Contamination by compounds present at the interface between phases was avoided by trying to recover only about 90% of the volume of the lower phase.

3. Results and Discussion

3.1. Lipid extraction and purification

Lipids produced by microalgae generally include neutral lipids, polar lipids, wax esters, sterols, and hydrocarbons, as well as prenyl derivatives (such as tocopherols, carotenoids, terpenes, and quinines), and pyrrole derivatives (such as the chlorophylls). They can be grouped into two categories: storage lipids (non-polar lipids) and structural lipids (polar lipids) [15]. Four different methods (Bligh & Dyer, detergent, silica and Soxhlet extraction) were tested for lipid extraction from a various microalgae species (*Chlorella* sp., *Dunaliella salina, Spirulina* sp., and *Nannochlopsis salina*). Table 1 presents the yields for each microalgae species and each extraction method. The total lipid (TL) yield of each method was evaluated by lipid recovery and ranged from 12.3 to 55.9% among the different methods and species tested. High levels of TL were extracted from *N. salina* by the Bligh & Dyer method [11]. On the other hand, the lowest lipid yields were obtained by Soxhlet extraction. Among these four techniques, the Bligh & Dyer method seems to be the most efficient for the extraction of lipids from *N. salina* as it resulted in higher lipid recovery. Lipids were dried under nitrogen gas, weighed (weight range 1 ~ 1.5 mg), and re-suspended in 0.1 mL of chloroform. Lipids were fractionated by TLC using two solvent mixtures sequentially. Firstly, chloroform/methanol/acetone/acetic acid/water (65:10:20:10:3, v/v) was run through half of the TLC plate. After drying, hexane/diethyl ether/acetic acid (80:20:1, v/v) was run through the whole plate. The triglyceride band was then scraped from the TLC plate and eluted with chloroform/methanol (2:1, v/v). The solvent was then evaporated under vacuum.

Repeated purification by phase separation could improve the purity, but decrease the overall yield of total lipids because of losses in each purification step (data not shown). Chlorophyll was partially removed by evaporation of excess methanol at a relatively low boiling point, but some residual chlorophyll remained in the biodiesel product. In column chromatography, solid particles (Celite 560 coarse, containing diatomaceous earth and silicon dioxide) that adsorbed chlorophyll moved faster than microalgal lipids because of their higher density. Subsequent centrifugation at 6,000 × *g* for 15 min did not result in the formation of either foam on the upper surface or visible precipitates at the bottom; however, centrifugation should be performed prior to column chromatography in order to remove solid particles from the lipid solution.

By using a colorimetric assay with phenol-sulfuric acid (absorbance was measured at 450 nm), the polysaccharide content in all microalgal biomass samples was estimated to be about 90% of the dry weight after removal of proteins and lipids. Concentrated sulfuric acid destroys microalgal cells and releases carbohydrate components, with polysaccharides being the dominant fraction. Microalgal polysaccharides are composed mostly of rhamnose, with a minor presence of galactose, arabinose, glucose, and glucuronic acid [1].

Table 1. Extraction methods explored and their effectiveness in lipid recovery

Extraction method		Microalga strains			
		Chlorella sp.	*Dunaliella salina*	*Spirulina* sp.	*Nannochlopsis salina*
Bligh & Dyer method	Total lipids (wt%, DB[1])	19.83 ± 1.69	20.21 ± 3.81	19.42 ± 4.25	55.91 ± 8.54
	Neutral lipids (wt%, DB)	14.61 ± 2.87	15.07 ± 2.82	9.01 ± 1.58	46.06 ± 3.48
	Neutral lipids (wt%, TB[2])	73.80 ± 1.21	74.60 ± 0.58	62.60 ± 2.58	82.40 ± 2.18
	Glycolipids (wt%, TB)	18.40 ± 0.57	12.10 ± 1.48	14.80 ± 1.28	10.60 ± 1.54
	Phospholipids (wt%, TB)	6.60 ± 1.54	3.80 ± 2.84	11.20 ± 2.35	9.00 ± 0.51
	Free fatty acid (wt%, TB)	8.80 ± 1.62	7.40 ± 0.81	3.20 ± 0.85	2.42 ± 1.08
Detergent extraction	Total lipids (wt%, DB)	15.60 ± 3.52	16.31 ± 4.25	16.80 ± 3.75	50.23 ± 3.51
	Neutral lipids (wt%, DB)	11.75 ± 3.15	11.72 ± 2.15	11.56 ± 2.84	40.91 ± 2.48
	Neutral lipids (wt%, TB)	75.30 ± 2.18	71.90 ± 1.58	68.80 ± 3.58	81.53 ± 2.51
	Glycolipids (wt%, TB)	16.80 ± 2.58	13.70 ± 1.05	15.70 ± 2.09	8.72 ± 1.52
	Phospholipids (wt%, TB)	4.80 ± 1.24	5.50 ± 0.85	13.22 ± 2.81	12.33 ± 1.84
	Free fatty acid (wt%, TB)	6.70 ± 0.84	6.62 ± 0.76	2.84 ± 0.78	2.63 ± 0.97
Silica extraction	Total lipids (wt%, DB)	14.73 ± 2.15	14.9 ± 1.15	18.13 ± 2.51	53.11 ± 3.60
	Neutral lipids (wt%, DB)	10.32 ± 1.12	10.98 ± 2.53	11.02 ± 2.35	41.52 ± 2.61
	Neutral lipids (wt%, TB)	70.23 ± 2.36	73.72 ± 1.51	60.92 ± 2.58	78.22 ± 5.21
	Glycolipids (wt%, TB)	11.82 ± 1.52	13.12 ± 0.81	16.61 ± 2.62	13.60 ± 1.52
	Phospholipids (wt%, TB)	4.83 ± 0.84	5.22 ± 1.05	15.70 ± 1.05	7.04 ± 1.52
	Free fatty acid (wt%, TB)	5.51 ± 0.57	7.11 ± 0.53	1.9 ± 0.82	1.53 ± 0.65
Soxhlet extraction	Total lipids (wt%, DB)	12.32 ± 3.31	12.8 ± 3.56	17.81 ± 3.51	48.8 ± 2.68
	Neutral lipids (wt%, DB)	9.21 ± 2.15	10.09 ± 3.58	13.30 ± 3.18	43.38 ± 3.98
	Neutral lipids (wt%, TB)	74.92 ± 3.52	78.83 ± 4.25	74.72 ± 4.25	88.93 ± 6.84
	Glycolipids (wt%, TB)	9.82 ± 0.83	7.93 ± 0.19	10.32 ± 1.56	6.72 ± 1.25
	Phospholipids (wt%, TB)	3.22 ± 0.62	3.02 ± 0.35	14.21 ± 3.57	4.32 ± 0.59
	Free fatty acid (wt%, TB)	7.91 ± 0.68	5.52 ± 0.85	1.70 ± 0.26	1.72 ± 0.63

[1]DB: dry algae base; [2]TB: Total lipid base.

Semi-purified microalgal polysaccharides were obtained by a combination of solvent partitioning and alcohol precipitation. More than 90% of the ash content in the dry mass can be attributed to protein, carbohydrate, and lipid components of the biomass [16]. It is therefore strongly recommended to wash marine algae samples before determination of their total biomass, as well as their protein, carbohydrate, and lipid contents [17].

It was easier to obtain transparent lipids from *Spirulina* sp. and *N. salina* than from *Chlorella* sp. and *D. salina*. In case of the last two species, additional purification steps are required, which may reduce the yield of lipid extraction and increase the risk of lipase inactivation during catalysis. Gel-like precipitates (*e.g.*, monogalactosyl and digalactosyl diglycerides, sulfolipids, sulfoquinovosyl diglyceride, and phosphatidylglycerol) tend to contain pigments and proteins (*e.g.*, thylakoid pigment-protein complexes and bilayer thylakoid membranes with a high content of non-bilayer forming lipids) that increase the viscosity and opacity of crude microalgal lipids [8,18].

An optimal extraction procedure should provide maximal lipid yield (through efficient microalgal cell disruption and solvent extraction) and minimal interference by gel-like precipitates (using phase separation and particle absorption). Considering that none of the solvents could extract all microalgal lipids in a single step and that a relatively low amount of lipids was recovered by the 3rd extraction, we suggested using at least two sequential extractions. Although only triglycerides (the main component of neutral lipids) are valuable for biodiesel production, the complexity of the lipid composition in microalgae necessitates a multi-step extraction procedure to remove polar lipids (glycolipids and phospholipids), which are typically present in the extract even after multiple purification steps. Therefore, different extraction methods should be compared in order to achieve maximal lipid yields. When making such comparisons, pigment indicators provide an easy way of evaluating the triglyceride yield.

3.2. The indicators (chlorophyll and carotenoids) of lipid extraction

Chlorophyll was used as an indicator for the efficiency of extraction of microalgal neutral lipids because it can be detected more easily than the target triglycerides, by UV-

Vis spectrophotometry. Gel-like precipitates containing lysophosphatidylcholine and other phospholipids were observed between the organic and water phases during the purification of crude microalgal lipids. Chlorophyll dissolves in less polar solvents such as methanol, isopropanol, and acetone, which were effective at extracting chlorophyll as well as lipids. The contents of chlorophyll and total carotenoids were determined as described by Wellburn [19]. Since the retention times of chlorophyll in polarity and high pressure liquid chromatography are similar to those of neutral lipids (between those of diacylglycerols and monoacylglycerols), it was reasoned that complete lipid extraction from microalgae should also remove chlorophyll. Decolorization of extracted material was assessed as a preliminary indicator of the solvent extraction efficiency. The diluted sample was scanned in the range of 200 ~ 1,000 nm using a UV-Vis spectrophotometer. Carotenoids have maximum absorption at 418 nm, while their absorption at 640 nm (which is an absorption peak for chlorophyll) is low. It has been reported that the cell membranes have low contents of xanthophylls, β-carotene, and chlorophyll a. The major peaks in the absorption spectra of algal cell membranes (435, 455, and 487 nm) were attributed to carotenoids, while a minor peak (673 nm) was attributed to chlorophyll a, which is consistent with the yellow color of the cytoplasmic membranes [20]. The low pigment solubility in water (especially at pH < 7) results in a transparent, colorless water phase after centrifugation at 6,000 *g* for 15 min. Each time the solution was centrifuged under the aforementioned conditions, phospholipids were partially removed from the organic phase. The green phase would then undergo another round of centrifugation.

The complex composition of lipids in microalgae (Fig. 1) means that a series of purification methods is required for the extraction of high purity neutral lipids. Microalgal lipids extracted from *Chlorella* sp. and *D. salina* showed an absorption peak at 668 nm, while the upper yellow fraction of *D. salina* lipids had little absorption at this wavelength (Fig. 2), indicating a good separation. In addition, the overall absorption in the range of 300 ~ 700 nm was greatly reduced (from 3 to < 1), confirming a reduction in contamination in the microalgal lipid fraction. The absorption of the crude biodiesel produced from microalgal lipids has been previously investigated [21]. After rotary evaporation, the crude microalgal lipids fraction was dark green, and the color could not be removed by activated carbon. Efforts to improve the effectiveness of chloroform/methanol (2:1) extraction by sonication, pulverization bead mill, or refluxing in solvent resulted in an increase of about 10% in the purity of the extracted triglycerides following each step, but the extracts remained dark green even after

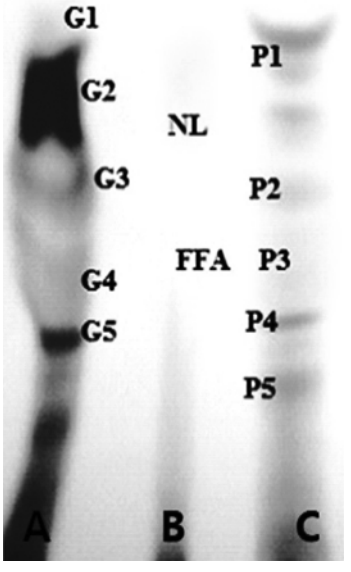

Fig. 1. Thin-layer chromatography of *Dunaliella salina* lipids using silica gel plates: A, glycolipids (G1–G5), B, neutral lipids (NL, triglycerides; FFA, free fatty acids), and C, phospholipids (P1–P5).

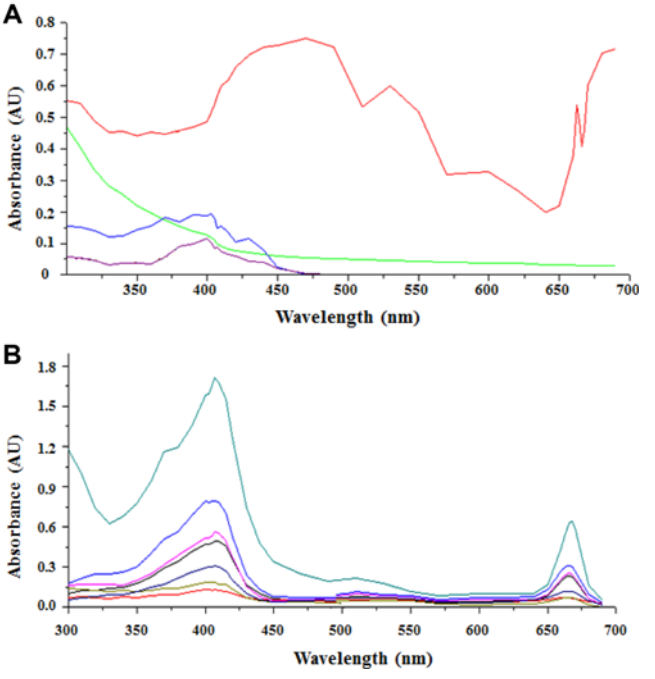

Fig. 2. (A) Absorption spectra of lipid fractions from *Dunaliella salina*. Glycolipids No. 1–3 corresponded to G1–G3 in Fig. 1. (—), crude oil, *Dunaliella salina*; (—), neutral lipid; (—), biodiesel from *Dunaliella salina*; (—), neutral lipid in hexane. (B) Glycolipid residuals (No. 1–3) are what remained of the glycolipid samples (No. 1–3) after washing with methanol. Glycolipids were fractionated by solid-phase extraction, and most fractions showed absorbance peaks at 405 and 662 nm. (—), glycolipid residual No.1 in methanol; (—), glycolipid residual No.2 in methanol; (—), glycolipid residual No.3 in methanol; (—), neutral lipids; (—), glycolipid No.1 in methanol; (—), glycolipid No.2 in methanol; (—), glycolipid No.2 in methanol.

centrifugation. At 80°C and under vacuum, 10 g of bleaching earth was added to 50 g of crude oil for 1 h under rotary evaporation to remove pigments and other impurities; bleaching earth was then precipitated by centrifugation at 6,000 × *g* for 15 min. Both the purified and crude microalgal lipids were diluted with heptane to a concentration of 2.7 g/L.

3.3. Three-step transesterification of microalgal lipids

Three-step transesterification of microalgal oil was conducted. Results are shown in Fig. 3. In the 1st step of transesterification (0 ~ 24 h), the conversion of microalgal lipids to FAME was 9.8% at 16 h, reaching 24.3% at 24 h. During a 72 h reaction period, 1.0 mL methanol was added at the start of the reaction, and again at 24 and 48 h. Lipase inactivation was partially avoided by the stepwise addition of methanol (data not shown). The initial rate of the 2nd step of transesterification (24 ~ 48 h) was much higher, while the reaction rate of the 3rd step (4 ~ 72 h) was slightly lower compared to that of the 1st step; this was illustrated by the numerical simulation of the reaction kinetics shown in Fig. 3. The reduction in reaction rate can be partially attributed to the accumulation of glycerol, which is the byproduct of the reaction. Glycerol coats the immobilized lipase, resulting in the initial rate of the 3rd step of transesterification being lower than that of the first 2 steps. After 72 h of reaction, a total yield of more than 90% was obtained, and the triacylglycerol content in the system reached negligible levels. Transesterification is a reversible reaction and the reaction product, FAME, can inhibit the process. Methanol was completely soluble in the reaction mixture after the 1st step of transesterification, which made the enzyme-substrate contact more efficient, even though FAME accumulated in the mixture after the 2nd step of transesterification. Biodiesel production under the conditions described above reached the yields of 90.8% (*Chlorella* sp.) and 91.2% (*D. salina*), which were very close to the theoretical maximum value.

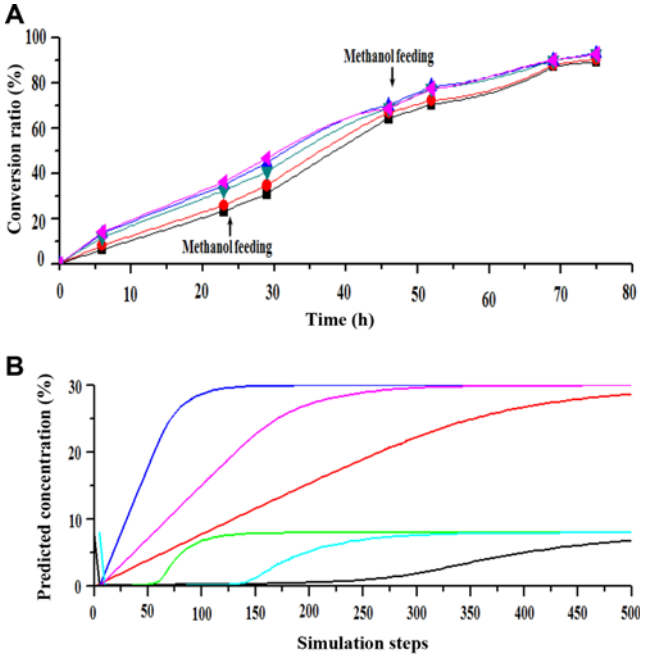

Fig. 3. (A) Time course of lipase-catalyzed transesterification. A reaction mixture of 9.2 g oil, 1.0 g methanol, and 1.2 g immobilized lipase was shaken at 50°C. A further 1.05 g of methanol was added at 24 h and 48 h. (■), *Chlorella* sp. oil; (●), *Dunaliella salina* oil; (▲), *Spirulina* sp. oil; (▼), *Nannochlopsis salina* oil; (◂), soybean oil. (B) The simulation was used to explore Michaelis–Menten enzyme kinetics, with the initial conditions as follows: E = 8; S = 30; P = 0; ES = 0; enzyme-substrate binding (forward reaction), E + S → ES, 0.2; enzyme-substrate separation (reverse reaction), ES → E + S, 0.1; product formation, ES → E + P, 0.01. The latter value (valid factor) is the kinetic constant for target product formation. (—), type A, product, valid factor 0.05; (—), type B, product, valid factor 0.02; (—), type C, product, valid factor 0.01; (—), type A, enzyme; (—), type B, enzyme; (—), type C, enzyme.

Immobilized lipase was used to catalyze the transesterification of microalgal lipids. Lipase addition increased the initial rates of transesterification, confirming that immobilized lipase functions as a recyclable biocatalyst and is highly efficient in catalyzing the transesterification of triglycerides under mild conditions, when high concentrations of hexane are present in the extraction mixtures. After centrifugation, organic complexes floated on the upper surface, while the residual microalgal cells had precipitated at the bottom. TLC revealed obvious spots at the positions corresponding to monoacylglycerols, diacylglycerols, and triacylglycerides, while gas chromatography (GC) analysis revealed FAME signals in diluted biodiesel samples. GC analysis showed that the upper liquid phase had a relatively high oil content compared to the transparent middle phase, owing to the preferential adsorption of organic complexes in the upper layer.

In our case, simplified kinetic models were not readily suitable for the interpretation of reaction kinetics because of interference by trace amounts of pigments, FFAs, glycolipids, and phospholipids [22]. A gradient with a small slope (< 1, labeled as "type C" in Fig. 3B) and relatively low product formation fits well with the time course, showing that the last reaction equation (ES → E + S) is rarely a dominant factor compared to the first two equations (*i.e.*, forward and reverse reactions between substrates and lipase). Increasing the methanol/oil molar ratio and the temperature had positive effects, while increasing the mixing rate negatively affected transesterification. After 72 h, the relative FAME content in the reaction mixture increased to 90.87% (*Chlorella* sp.) and 91.22% (*D. salina*), while the triglyceride content to 5.8% (*Chlorella sp.*) and 3.7% (*D. salina*). In fact, the reaction could be divided into two

slow steps (before 24 h and after 48 h), and one fast step (between 24 and 48 h), which may be attributed to the occasional shaking being performed more frequently during the middle stage.

The fatty acid (FA) composition of the biodiesel produced from microalgal lipids is presented in Table 2. Monounsaturated and polyunsaturated FAs were the dominant fractions, comprising 63.2 ~ 81.5% of the total biodiesel FAs. A positive correlation between the relative content of long-chain FAs ($\geq C_{20}$) and the distillation temperature could be observed. Medium-chain FAs ($\leq C_{18}$) constituted 66.4 and 60.2% of the biodiesel from *Chlorella* sp. and *D. salina*, respectively. In particular, biodiesel produced from *D. salina* contained 60.6% polyunsaturated FAs, which was quite remarkable compared to their percentage in biodiesel produced from *Chlorella* sp (32.9%). It has been reported that FA molecular characteristics, such as length of carbon chain and degree of unsaturation, greatly affected biodiesel properties, such as oxidative stability and ignition quality [23].

With a phosphorus concentration lower than 2.0 ppm, the biodiesel that was produced by both *Chlorella* sp. and *D. salina* met the standard set by the American Society for Testing and Materials (ASTM) [24]. The crude biodiesel contained lysophosphatidylcholines, which account for a big portion of the residual phosphorus. The problem of residual phosphorus in crude biodiesel was solved to some extent by our extraction method, as indicated by low absorption over a wide spectral range and the disappearance of the peak at 668 nm. In our study, a very low concentration of phospholipids in the upper yellow fraction facilitated the enzymatic reaction by eliminating the gel-like fraction during solid-phase extraction of crude microalgal lipids. It has been reported that the phosphorous concentration in crude biodiesel from *Scenedesmus* sp. was 295.6 ppm, which is much higher than the ASTM limit of 10 ppm. By using bleaching earth, the chlorophyll and carotene contents could be reduced from 4296.7 and 1918.9 ppm to 40.3 and 199.0 ppm, respectively [21].

In our study, the total yield of microalgal biodiesel from *N. salina* reached the maximum yield observed among the tested microalgae (16.5 ~ 34.6 g per 100 g of microalgae; Fig. 3), indicating that the production of microalgal biodiesel could reach 34.6% (w/w) of dried microalgae), assuming a distillation output of 95% and a 90% decrease in yield reduction due to lipid loss after multiple purification steps, both of which are reasonable estimates.

In general, the total yield of microalgal biodiesel is below 45% (w/w) of dried microalgae, assuming that the lipid content of microalgae is 20 ~ 50 wt%. The recovery rate of non-polar lipids after the removal of polar lipids is 80%, while the recovery rate during distillation is 95%. In fact, the production yield of total microalgae biomass is restricted by physiological mechanisms (photosynthesis limitation) and the size of production area (space limitation). Since the polar lipid content can be as high as 50% of the total lipids, the aforementioned biodiesel yield of 45 wt% is a theoretical maximum value, which in most microalgae species is reduced by half when scaling-up production. The reduction in lipid yield (5 ~ 10 wt%) during purification has commonly been neglected in most reports. This indicates that in the production of renewable fuels (biodiesel and fuel ethanol) from microalgal biomass, emphasis should be placed on achieving fast growth, a high lipid content, and a high content of valuable unsaturated FAs [1,6,8,18,22]. Furthermore, one may conclude that so far purification procedures have been commonly described in a very simplified way, with little discussion of lipid losses or the typically high content of residual polar lipids, precluding the application of those procedures for large-scale production. This situation also emphasizes the necessity of carrying out detailed comparisons among the different

Table 2. Fatty acid composition of biodiesels produced from microalgal lipids

	Chlorella sp.	*Dunaliella salina*
C14:0	0.07	0.57
C14:1	0.07	0.47
C16:0	12.06	0.32
C16:1	0.35	0.28
C18:0	1.58	0.75
C18:1	27.61	2.08
C18:2	23.2	25.70
C18:3	1.48	30.07
C20:0	5.30	0.43
C20:1	0.89	15.42
C20:2	5.01	0.03
C20:3	1.22	0.03
C20:4	2.78	1.78
C21:0	0.04	0.37
C22:0	2.78	1.25
C22:1	0.05	0.86
C22:6	8.17	3.01
C23:0	4.26	2.60
C24:0	0.78	12.23
C24:1	1.35	1.75
Others	4.55	1.86
Saturated	26.87	18.52
Mono-unsaturated	30.32	20.86
Poly-unsaturated	32.86	60.62

Lipid fractions from both microalga species have a high content of poly-unsaturated fatty acids. Conversion rate = $(I_5 - I_6) \div I_6 \times I_8 \times I_9 \div I_{10} \times 100$, where I_5 is the total area of all peaks; I_6 is the methyl heptadecanoic acid peak area; I_8 is the methyl heptadecanoic acid concentration (μM); I_9 is the volume of methyl heptadecanoic acid (mL); and I_{10} is the weight of solution sampled from the reaction mixture (mg).

extraction methods, similar to comparison performed in this study. These should result in the improvement of the following steps in biofuel production, *i.e.*, lipase catalysis and distillation.

4. Conclusion

Our results indicate the importance of the efficient separation of neutral lipids after lipid extraction from microalgae. Three-step esterification of microalgal lipids was performed to optimize the reaction conditions. GC revealed high conversion rate (90.9% for *Chlorella sp.* and 91.2% for *D. salina*), indicating that lipase catalysis fits a small-slope Michaelis–Menten kinetics model. Chlorophyll and carotenoids were used to estimate the content of residual polar lipids and conversion prior to lipid extraction and the removal of polar lipids. Moreover, all steps increased the efficiency of mass diffusion and surface renewal during lipase catalysis.

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