Concentration of Lipase from Aspergillus oryzae Expressing Fusarium heterosporum by Nanofiltration to Enhance Transesterification

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Keywords: fatty acid methyl ester, nanofiltration, lipase, Fusarium heterosporum

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Nanofiltration membrane separation is an energy-saving technology that was used in this study to concentrate extracellular lipase and increase its total activity for biodiesel production. Lipase was produced by recombinant Aspergillus oryzae expressing Fusarium heterosporum lipase (FHL). A sulfonated polyethersulfone nanofiltration membrane, NTR-7410, with a molecular weight cut-off of 3 kDa was used for the separation, because recombinant lipase has a molecular weight of approximately 20 kDa, which differs from commercial lipase at around 30 kDa for CalleraTM Trans L (CalT). After concentration via nanofiltration, recombinant lipase achieved a 96.8% yield of fatty acid methyl ester (FAME) from unrefined palm oil, compared to 50.2% for CalT in 24 h. Meanwhile, the initial lipase activity (32.6 U/mL) of recombinant lipase was similar to that of CalT. The composition of FAME produced from recombinant concentrated lipase, i.e., C14:1, C16:0, C18:0, C18:1 cis, and C18:2 cis were 0.79%, 34.46%, 5.41%, 45.90%, and 12.46%, respectively, after transesterification. This FAME composition, even after being subjected to nanofiltration, was not significantly different from that produced from CalT. This study reveals the applicability of a simple and scalable nanofiltration membrane technology that can enhance enzymatic biodiesel production.

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Concentration of Lipase from *Aspergillus oryzae* Expressing *Fusarium heterosporum* by Nanofiltration to Enhance Transesterification

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Abstract: Nanofiltration membrane separation is an energy-saving technology that was used in this study to concentrate extracellular lipase and increase its total activity for biodiesel production. Lipase was produced by recombinant *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase (FHL). A sulfonated polyethersulfone nanofiltration membrane, NTR-7410, with a molecular weight cut-off of 3 kDa was used for the separation, because recombinant lipase has a molecular weight of approximately 20 kDa, which differs from commercial lipase at around 30 kDa for Callera^{TM} Trans L (CalT). After concentration via nanofiltration, recombinant lipase achieved a 96.8% yield of fatty acid methyl ester (FAME) from unrefined palm oil, compared to 50.2% for CalT in 24 h. Meanwhile, the initial lipase activity (32.6 U/mL) of recombinant lipase was similar to that of CalT. The composition of FAME produced from recombinant concentrated lipase, i.e., C14:1, C16:0, C18:0, C18:1 cis, and C18:2 cis were 0.79%, 34.46%, 5.41%, 45.90%, and 12.46%, respectively, after transesterification. This FAME composition, even after being subjected to nanofiltration, was not significantly different from that produced from CalT. This study reveals the applicability of a simple and scalable nanofiltration membrane technology that can enhance enzymatic biodiesel production.

Keywords: nanofiltration; lipase; *Fusarium heterosporum*; fatty acid methyl ester

1. Introduction

Over the past decade, interest in biodiesel as an alternative to diesel fuel continues to increase throughout the world due to concerns about global climate change. Increasingly, there is a desire for renewable/sustainable energy sources, and an interest in developing domestic supplies of fuel that are more secure [1]. Biodiesel (fatty acid alkyl esters) is produced from renewable natural sources such as vegetable oils (e.g., palm oil), animal fats, and microalgal oil [2,3]. Biodiesel can be used directly in existing diesel engines without major modifications, or as a mixture with petroleum diesel, and the burning of biodiesel produces gas emissions such as sulfur oxide, which are less harmful than those emitted by the burning of petroleum-based fuels [4].

The viscosity of vegetable oils is improved via a transesterification pathway which involves triglycerides and alcohols of lower molecular weights and homogenous or heterogenous substances that are used as catalysts to yield biodiesel and glycerol [4]. Transesterification via enzymatic catalysis has attracted much attention because it is an eco-friendly process that produces no by-products, features easy product recovery, and requires a low reaction temperature [5]. However, the process is expensive, and has a relatively slow reaction rate [6,7]. A variety of lipases (EC 3.1.1.3) from various microorganisms (*Candida antarctica, Rhizopus oryzae*, *Pseudomonas cepacia*, *Thermomyces lanuginosus*, etc.) have been used to accomplish both transesterification and esterification [8–10]. Many researchers have attempted to solve the limitations of lipase-catalyzed biodiesel production by immobilizing the enzymes or cells on a suitable matrix [8,11] or via the use of a lipase cocktail [12]. In contrast, at least one previous study has successfully conducted biodiesel production using recombinant *Aspergillus oryzae* that expresses *Fusarium heterosporum* lipase (FHL), which has demonstrated a high level of tolerance to water [13]. With the use of that particular enzyme, however, the conversion of oil to fatty acid methyl ester (FAME) remained low. To increase the conversion rate, the total activity of this enzyme was increased using a simple process such as a concentrating method. In general, the conversion increases proportionally with the increase of lipase concentration [14]. Concentration methods such as precipitation [15,16] require costly chemicals such as ammonium sulfate. Another concentration method is membrane separation technology, which has advantages that include energy savings, selectivity, no chemical requirement, and simplicity of operation and scale-up [17].

Reinehr et al. [17] previously reported a membrane concentration of lipase that could be produced from *Aspergillus niger* by using microfiltration and ultrafiltration separation processes. The present study is the first to apply a nanofiltration membrane to simply concentrate lipase produced from recombinant *A. oryzae* (expressing FHL) and enable a high level of transesterification compared to a commercially available lipase, Callera^{TM} Trans L (CalT) (Novozymes, Bagsvaerd, Denmark). In this study, we use unrefined palm oil as a model substrate for transesterification. Palm oil is well known as one of the most suitable sources for biodiesel production. Indonesia and Malaysia produce approximately 85% of global crude palm oil, which is likely to increase in the future [3,18]. The aim of the present study was to efficiently produce FAME from unrefined palm oil using membrane-concentrated lipase.

2. Materials and Methods

2.1. Materials and Microorganisms

Unrefined palm oil was purchased as a substrate from Malang, East Java, Indonesia. *Aspergillus oryzae* expressing FHL used in this study was obtained as described previously [19,20].

2.2. Lipase Production

Sakaguchi flasks (500 mL) containing 100 mL of DP medium (2% glucose, 2% polypeptone, 1% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4{\cdot}7H_2O$) were aseptically inoculated with spores from *A. oryzae* expressing FHL in Czapek-Dox (CD)-NO_2-methionine selection plate agar [20]. The flasks were incubated at 30 °C and shaken at 150 rpm for 96 h on a reciprocal shaker. The culture broth was collected and then centrifuged at 6000× *g* for 15 min at 4 °C to recover the supernatant. The culture supernatant was dialyzed in MEMBRA-CEL® dialysis tubing with a molecular weight cut-off (MWCO) of 3500 Da (RC, SERVA Electrophoresis GmbH, Heidelberg, Germany), followed by filtrations through different filter papers in the following order: (1) a polycarbonate filter (0.8 µm pore size); (2) a polycarbonate filter (0.5 µm pore size); and (3) a polystyrene filter (0.22 µm pore size). The supernatant was then subjected to nanofiltration-membrane concentration. Lipase from Callera^{TM} Trans L, a liquid lipase from *Thermomyces lanuginosus* lipase (CalT) (Novozymes, Bagsvaerd, Denmark), was used as a control.

2.3. Nanofiltration Membrane Separation Processes

A sulfonated polyethersulfone nanofiltration membrane, NTR-7410, with a 3000 Da MWCO was obtained from the Nitto Denko Corporation (Osaka, Japan). The membrane was cut into a circle (diameter: 7.5 cm; effective area: 32 cm^2). The nanofiltration (NF) process was carried out at room temperature using a flat membrane test cell (model C40-B, Nitto Denko Corporation, Osaka, Japan) [21]. The lipase supernatant was then subjected to the test cell. The inside of the test cell was stirred at 300 rpm at a pressure of 2.5 MPa under nitrogen gas for one hour.

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Zymography

The *A. oryzae* supernatant expressing FHL was either directly applied to polyacrylamide gel electrophoresis in the presence of SDS-PAGE, or applied after filtration through a 5K MWCO Spin-X Ultrafiltration Concentrator (Corning, UK) to concentrate the lipase. CalT was applied to SDS-PAGE after dilution. The proteins were then stained with Coomassie brilliant blue R-250. Zymography analysis was carried out to detect lipase activity, as described previously [22]. The SDS-PAGE gels were incubated for one hour at room temperature in developing solution consisting of 3 mM α-naphthyl acetate, 1 mM Fast Red TR (Sigma, St. Louis, MO, USA), and 100 mM sodium phosphate buffer, pH 8.0. Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA, USA) were used as a standard marker.

2.5. Measurement of Lipase Activity and Protein Assay

The hydrolytic activities of lipase were tested using *p*-nitrophenyl butyrate (pNPB) as a chromogenic substrate. A stock solution was prepared by dissolving 5 µL of pNPB in 250 µL of ethanol, with a further dilution to 50 mL using distilled water. The stock solution was then incubated in a Bioshaker (Taitec, Saitama, Japan) for 10 min at 30 °C to allow the development of lipase-hydrolytic activity. After incubation, 5% trichloroacetate was added to terminate the reaction. The absorbance of para nitrophenol (pNP) that was produced was measured at 400 nm (UV-Vis spectrophotometer, Shimadzu, Kyoto, Japan). One unit (U_2) of lipase activity was defined as the amount of lipase that liberates 1 µmol of pNP from pNPB per minute [12]. Protein concentrations were measured using a Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Rockford, IL, USA).

2.6. FAME Production by Enzyme

FAME production was carried out in triplicate using a 10 mL glass tube with a silicon cap equipped with a stirrer (Thermo Scientific VARIOMAG Magnetic Stirrers, Waltham, MA, USA) for circulation at 800 rpm. The glass tube was then immersed in a heated water bath Thermo Minder EX TAITEC (Taitec, Saitama, Japan) that was set to 30 °C. The reaction mixture consisted of 4 g of unrefined palm oil and 1.2 mL of lipase (unconcentrated and concentrated lipases from recombinant *A. oryzae* and CalT diluted 245-fold). The transesterifications were carried out at 32.6 U/mL lipase activity for concentrated lipase from recombinant *A. oryzae* and for that from CalT diluted 245-fold (Table 1). To avoid deactivation of the lipase, 186 µL of methanol (corresponding to a 1:1 molar ratio of unrefined palm oil to methanol) was added step-wise at 0, 2, 4, and 6 h. Samples were taken at 0, 2, 4, 6, 9, and 24 h [13].

Table 1. Comparison of lipase activity and protein content.

Process	Volume (mL)	Lipase Activity (U/mL)	Protein (mg/mL)
Recombinant lipase before concentration by NTR7410	350	6.4 ± 0.1	0.7 ± 0.0
Recombinant lipase after concentration by NTR7410	65	32.6 ± 3.1	4.0 ± 0.2
Permeate from NTR7410	280	0	0
Callera™ Trans L (CalT)		8396.7 ± 378.4	25.1 ± 1.4

2.7. Analytical Methods

Fatty acid methyl ester (FAME) produced during the course of the transesterification reaction was measured via gas chromatography. Samples taken at specified times were centrifuged at 12,000× *g* for 5 min at 5 °C, and the upper layer was analyzed via a GC-17A (Shimadzu, Kyoto, Japan) equipped with a ZB-5HT capillary column (0.25 mm × 15 m) (Phenomenex, Torrance, CA, USA), an auto-sampler, and a flame ionization detector, as previously described [12]. During the analysis, the temperatures of the injector and detector were set at 320 °C and 380 °C, respectively, using helium as a carrier gas at a flow rate of 58 mL/min. The column was configured with an initial temperature of 130 °C for 2 min, which was raised to 350 °C at 10 °C/min, and then to 370 °C at 7 °C/min. The FAME composition in each reaction mixture was reported as the percentage of the oil in the reaction mixture using tricaprylin as an internal standard [11].

Transesterification was conducted by following the protocol from the fatty acid methylation kit (Nacalai Tesque Inc., Kyoto, Japan). The FAME composition was analyzed using a gas chromatography-mass spectrometer (GC-MS) (Shimadzu, Kyoto, Japan). The GC-MS was equipped with a 0.25 mm × 30 m DB-23 capillary column (J&W Scientific, Folsom, CA, USA). The carrier was helium gas with a flow rate of 0.8 mL/min at 1:5 split ratios. The initial column temperature was 250 °C, which was increased to 50 °C for 1 min and then increased 25 °C/min to 190 °C and 5 °C/min to 235 °C for 4 min. An internal standard C8:0 (octanoic acid) was included in each sample and FAME was detected at the provided retention time (Supplementary Table S1). The amount of FAME (%) was calculated as the percentage of each fatty acid to the total weight of fatty acids produced [23].

3. Results and Discussion

3.1. Characterization of Lipases before and after Membrane Concentration

As described previously, the molecular weights of lipases ranged from 20 to 80 kDa [17,24] or up to 150 kDa [25]. Thus, NTR-7410 with a MWCO of 3 kDa [26] was selected as the membrane that would best concentrate lipase. Nanofiltration concentration was performed for one hour at 2.5 MPa.

Both concentrated and unconcentrated lipases produced from recombinant *A. oryzae* were characterized and compared with commercial lipase, CalT. At first, the molecular weight of the lipase produced by recombinant *A. oryzae* was determined via SDS-PAGE. Then, lipase enzyme activity was detected using the Zymography technique. As a result, the supernatant of *A. oryzae* contained plural proteins with molecular weights that ranged from 20 to 50 kDa (Figure 1A). However, lipase enzyme was detected as a single band at around 20 kDa (Figure 1B). As expected, the concentration of lipase had definitely increased. By comparison, the CalT contained a major protein at around 30 kDa along with some minor proteins that also showed lipase activity (Figure 1C,D). Therefore, we assumed that the observed bands other than that at around 30 kDa also represented small amounts of lipase (Figure 1D). These results suggest that the FHL produced in *A. oryzae* was a smaller molecule compared with the lipases produced in CalT. In addition, recombinant *A. oryzae* produced some other unknown proteins that were not lipase. Due to this contamination by other enzymes, the lipase produced from recombinant *A. oryzae* showed low specific activity (Table 1) compared with lipase reported elsewhere (more than 66 U/mg) [25]. However, the nanofiltration concentration of lipase produced by recombinant *A. oryzae* successfully increased its total activity from 6.4 U/mL to 32.6 U/mL (five-fold concentration factor) in the short time of one hour. Enzyme activity losses in this study were low (5.5%, Table 2), and may have occurred due to adsorption on the membrane surface as a function of fouling [17]. The activity loss was generally lower, because the use of NTR-7410 (MWCO of 3 kDa) could retain more lipase protein than that produced by recombinant *A. oryzae* at around 20 kDa. The denaturation did not occur due to the pressure applied lower than 400 MPa as used in [27].

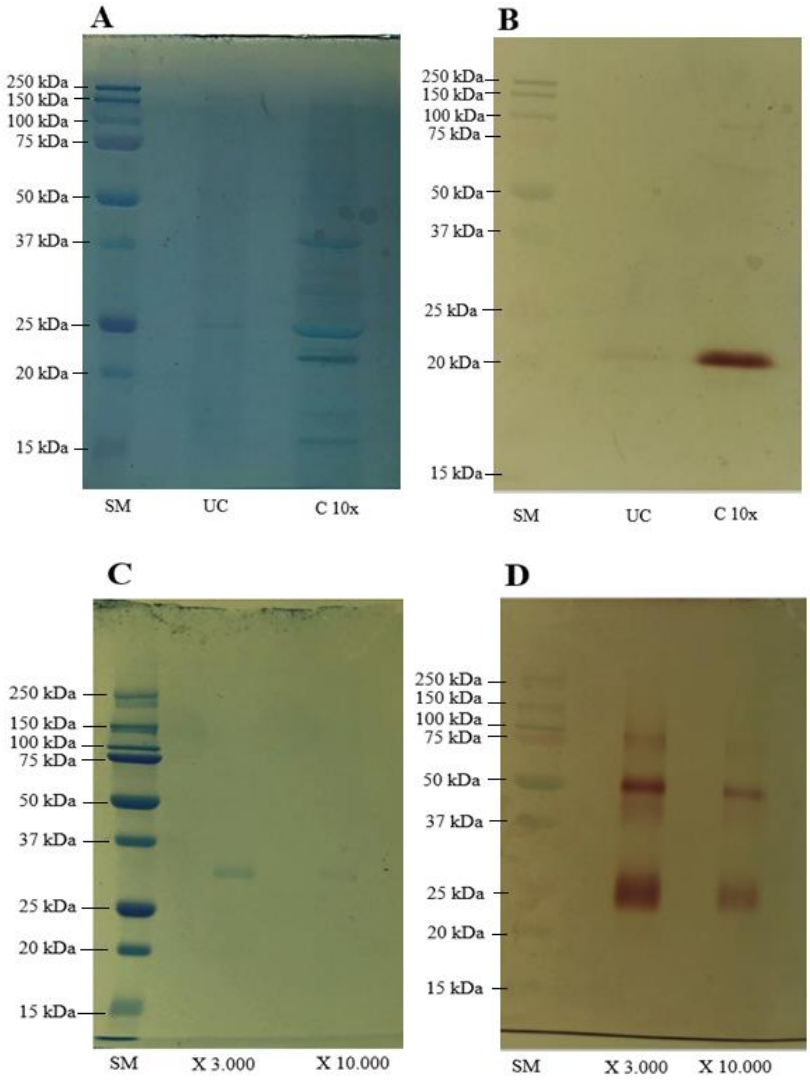

Figure 1. SDS-PAGE and Zymogram. (**A**) SDS-PAGE for the culture supernatant of *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase (FHL) (UC: unconcentrated, C 10×: concentrated 10-fold). (**B**) Zymogram for the detection of lipase produced from recombinant *A. oryzae*. (**C**) SDS-PAGE of the lipase from *Thermomyces lanuginosus* (CalT). (**D**) Zymogram of CalT. (X 3.000 at dilution 3.000-fold, X 10.000 at dilution 10.000-fold). SM: standard marker.

Table 2. Concentration factors and activity loss obtained in previous processes involving the concentration of proteins using membrane separation technologies.

Protein Concentrated	Process Used	Concentration Factor Obtained	Activity Loss	Ref.
Lipase (recombinant *Aspergillus oryzae*)	Nanofiltration (3 kDa)	5	5.5%	This study
Lipase (*Aspergillus niger*)	Sequential micro- and ultrafiltration	3	17% in microfiltration and 22% in ultrafiltration	[17]
Phytase (*Aspergillus niger*)	Ultrafiltration (10 kDa)	4.3	14%	[28]
Inulinase (*Kluyveromyces marxianus*)	Ultrafiltration (100 kDa)	5.5	18.4%	[29]
Lignin-peroxidase (*Streptomyces viridosporus*)	Ultrafiltration (10 kDa)	10	10%	[30]
Phycocyanin (*Spirulina* sp.)	Sequential micro- and ultrafiltration	2	13.6% in 1 µm pore size	[31]

3.2. *Efficient FAME Production by Concentrated Lipase*

FAME yield was compared for concentrated and unconcentrated lipases produced from recombinant *A. oryzae* and commercial lipase (CalT). In these reactions, the total lipase activity of concentrated lipase produced by recombinant *A. oryzae* was arranged to be nearly the same as that of diluted CalT. The results for the FAME yield are shown in Figure 2. Interestingly, FAME production was enhanced by using concentrated lipase produced by recombinant *A. oryzae* (designated as AC), compared with diluted CalT (designated as CalT), and unconcentrated lipase (designated as BC). Other controls were 0.5 AC + 0.5 CalT, which contained half of the concentrated lipase produced by recombinant *A. oryzae* and half of diluted CalT, and 0.5 BC + 0.5 CalT, which contained half of the unconcentrated lipase produced by recombinant *A. oryzae* and half of diluted CalT. By comparing these data, it was apparent that activity for lipase produced by recombinant *A. oryzae* to convert unrefined palm oil to FAME was significantly enhanced by nanofiltration concentration, compared with that produced by CalT. The reason for this remains unclear. We hypothesized that the FHL produced by recombinant *A. oryzae* would have different characteristics from commercial lipase (CalT).

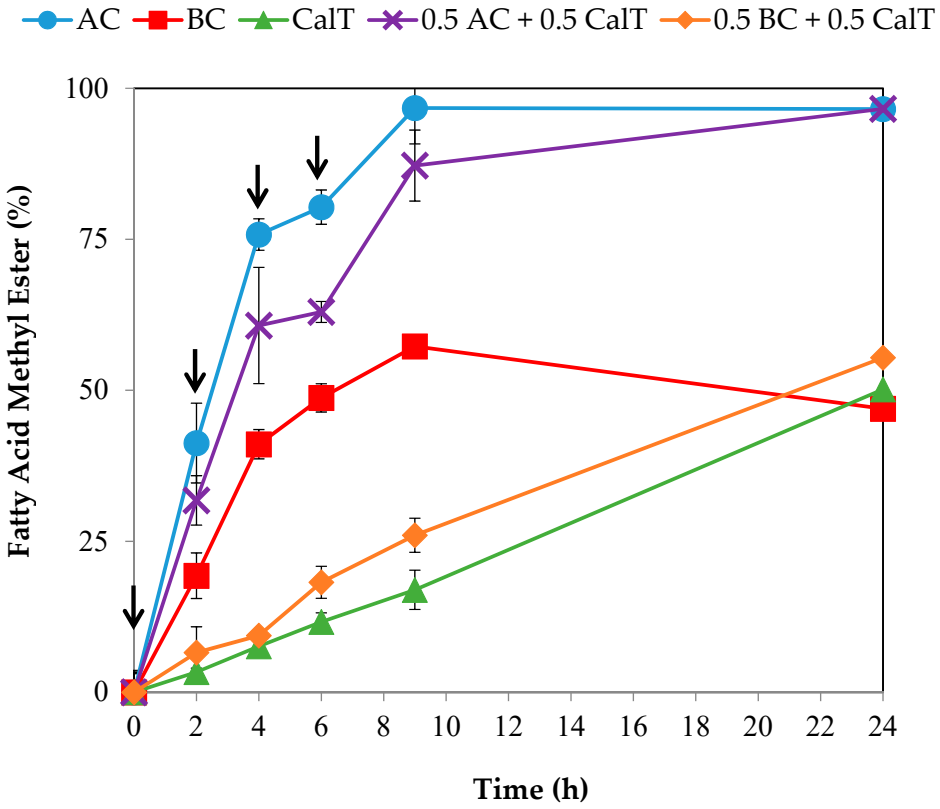

Figure 2. Fatty acid methyl ester (FAME) production by lipase enzymes. AC: lipase produced from recombinant *A. oryzae* after nanofiltration (NF) concentration; BC: lipase produced from recombinant *A. oryzae* before NF concentration; CalT: lipase from *Thermomyces lanuginosus* showing lipase activity similar to AC (diluted around 245-fold); 0.5 AC + 0.5 CalT: half AC and half CalT; and 0.5 BC + 0.5 CalT: half BC and half CalT. The arrows indicate the time to add methanol.

The FAME compositions after 24 h of enzymatic reaction were analyzed by GC-MS, as shown in Table 3. In general, there were no major differences in the FAME composition converted from unrefined palm oil by both concentrated and unconcentrated lipases, produced either by recombinant *A. oryzae* or CalT. These results suggest that the biodiesel produced using concentrated lipase from recombinant *A. oryzae* has high potential to be used in the same manner as biodiesel produced from

CalT. Furthermore, nanofiltration concentration can be used to enhance the quality of lipase used in biodiesel production without a loss of FAME quantity.

Previously, most of the research focusing on enzymatic catalysis has employed lipase immobilized on polymer support as a catalyst. However, the immobilization process is neither simple nor inexpensive [32]. Using the suggested membrane separation technology to concentrate lipase therefore simplifies the process and reduces the cost. In addition, concentrated lipase produced from recombinant *A. oryzae* can be used as a sole lipase or as a supplement to other commercially available lipases to reduce costs and improve biodiesel conversion yields from unconventional feedstock.

Table 3. FAME profiles of transesterification results.

Lipase Variations	FAME Compositions (%)				
	C14:1	C16:0	C18:0	C18:1 cis	C18:2 cis
AC	0.79 ± 0.15	34.46 ± 0.37	5.41 ± 1.01	45.90 ± 1.71	12.46 ± 0.29
BC	0.89 ± 0.04	35.45 ± 0.12	5.84 ± 0.08	44.88 ± 0.03	12.32 ± 0.09
CalT	0.81 ± 0.04	34.75 ± 0.44	5.85 ± 0.18	45.06 ± 0.12	12.48 ± 0.19
0.5 AC + 0.5 CalT	0.79 ± 0.01	34.46 ± 0.03	5.75 ± 0.08	45.14 ± 0.09	12.94 ± 0.12
0.5 BC + 0.5 CalT	0.76 ± 0.08	34.71 ± 0.57	5.76 ± 0.11	45.24 ± 0.44	12.66 ± 0.56

AC: lipase produced from *A. oryzae* expressing FHL after NF concentration, BC: lipase produced from *A. oryzae* expressing FHL before NF concentration, CalT: lipase from *Thermomyces lanuginosus* showing lipase activity similar to AC (diluted around 245-fold), 0.5 AC + 0.5 CalT: half AC and half CalT, and 0.5 BC + 0.5 CalT: half BC and half CalT.

4. Conclusions

In this study, the total activity of lipase produced by recombinant *A. oryzae* was successfully increased at about 5-fold using nanofiltration membrane separation technology. Concentrated lipase produced from recombinant *A. oryzae* showed a higher FAME yield of 96.8% from unrefined palm oil, compared to 50.2% for CalT in a 24 h period, although the lipase activity (32.6 U/mL) was nearly the same between concentrated lipase and CalT. FAME composition using concentrated lipase was unchanged, compared with that using CalT. The FAME, C14:1, C16:0, C18:0, C18:1 cis, and C18:2 cis were produced, respectively, at 0.79%, 34.46%, 5.41%, 45.90%, and 12.46% after transesterification at 30 °C for 24 h. In this study, a simple and inexpensive process was developed using a nanofiltration membrane that is expected to improve enzymatic and industrial biodiesel production.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/4/450/s1, Table S1: Retention time of target compound of fatty acid methyl ester obtained in Gas Chromatography-Mass Spectrometer analysis.

Author Contributions: Conceptualization, H.W., K.S., P.K., J.A., E.Q., N.R.; experiment, H.W., E.Q., N.R.; methodology, K.S., P.K., J.A., S.H., C.O., A.K.; validation, K.S., P.K., J.A.; data curation, H.W., K.S., P.K., J.A.; formal analysis, H.W., K.S., J.A., P.K.; writing—original draft preparation, H.W., K.S., P.K., J.A., E.Q.; writing—review and editing, K.S., P.K., J.A., S.H., C.O., A.K. All authors have read and agreed to the published version of the manuscript.

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