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Lipase-catalyzed ethanolysis for biodiesel production of untreated palm oil mill effluent in water-containing system

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Lipase-catalyzed ethanolysis for biodiesel production of untreated palm oil mill effluent

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ABSTRACT

Palm oil mill effluent (POME), a liquid waste from palm oil industry, presents an alternative source for biodiesel production without interfering with food supply. This study attempted to produce biodiesel from untreated POME with aqueous ethanol using *Thermomyces lanuginosus* lipase as a biocatalyst. The effects of enzyme concentration, alcohol to oil ratio, and ethanol concentration were considered in the transesterification reaction. The optimum conditions were 2100 U lipase loading, 4:1 ethanol to oil molar ratio, and 45 % (v/v) ethanol concentration at 40 ^{o}C reaction, and under 24 hours. The maximum fatty acid ethyl ester (FAEE) yield reached 97.43 % (w/w) under these conditions. Integration of dilute ethanol for the conversion of POME to biodiesel could be promising as both feedstocks could be obtained from the same location, and thus reducing the logistical burden on biodiesel production.

Keywords: palm oil mill effluent; aqueous ethanol; *Thermomyces lanuginosus* lipase; ethanolysis; biodiesel.

INTRODUCTION

Biodiesel is a renewable, biodegradable and environmentally-friendly fuel produced by trans/esterification of vegetable oils with an acyl acceptor.[1] However, compared to petroleum diesel, biodiesel has a higher production cost with the cost of raw materials accounting for 60 - 70% of the total cost.[2,3] Investigation of alternative raw materials for biodiesel synthesis has attracted much interest in the last decade. According to Palm Oil Analytics (POA), Indonesia is the highest producer of palm oil in the world.[4] In 2016, Indonesia's palm oil production was around 35 million tons. With such a large production, a significant amount of palm oil waste results in waste streams and dumps.[5]

Palm oil production route involves sterilization, crude oil clarification and cracked mixture separation using high volumes of water. Palm oil mill effluent (POME) is generated through these processes, and contains high amounts of organic matter, grease, suspended solids, and high free fatty acids components.[6] About 5-7.5 tons of POME is generated from the production of 1 ton crude palm oil (CPO).[7] POME is currently freely discharged in open ponds and at landfill sites. Methane emission, freshwater pollution, and the unpleasant smell associated with POME require immediate mitigation. Currently, there is no sustainable utilization of POME. POME can be a sustainable feedstock for producing biodiesel because of its huge volumes, and its utilization does not interfere with the food supply chain. On the other hand, large amounts of free fatty acids (FFA) and water in feedstocks such as POME inhibits trans/esterification reaction and negatively affects current technology employed in biodiesel production. In literature, many attempts have been made. However, pretreatment methods such hexane

Soxhlet extraction are applied to separate the oil-grease containing fraction from the wastewater fraction (Table 1).

Methanol is currently the most common acyl acceptor in plant oil transesterification. In addition to its high environmental toxicity and flammability, the massive use of methanol is also hampered by its origin, which is mainly a limited fossil resource. Conversely, ethanol can be an alternative acyl acceptor for biodiesel production. Ethanol can easily be obtained from alcoholic fermentation of renewable agricultural resources, in the form of bioethanol. The replacement of methanol with bioethanol as acyl acceptor is an appropriate step towards sustainability and green production. However, research on the utilization of bioethanol towards biodiesel production is still inadequate as the application of bioethanol is hindered by high amounts of water. Water content in crude bioethanol from fermentation can be as high as 80 % (w/w).[8] Thus, the exploration of the use of low concentrated ethanol which correlates to bioethanol as proposed in this study is crucial.

High amounts of FFA and water are considered drawbacks in conventional biodiesel synthesis as they result in soap formation, reduce the yield of biodiesel, and complicate the separation process.[9,10] To overcome the problems associated with the use of chemical catalysts, a lipase-catalyzed process has been proposed and extensively researched in the last few years.[11–14] The ability of lipases to catalyze feedstocks from alternative sources is promising for biodiesel production. The use of liquid lipases instead of immobilized forms is effective in the trans/esterification process with its high water tolerance.[15]

This study investigates the use of liquid lipase in the transesterification reaction between “untreated POME” and aqueous ethanol. This concept is employed to

investigate the possibility of producing biodiesel from the untreated POME which contains high FFA with an exceptionally high amount of water. The novelty of this study is the effective utilization of POME without pre-separation for the production of biodiesel with dilute ethanol which demonstrates the possibility of using bioethanol that can be produced from another waste fraction (empty fruit bunch) from the palm oil industry.

MATERIALS AND METHODS

Materials

POME was obtained from PT. Agricinal (Bengkulu, Indonesia). Callera Trans L, a liquid formulation of *Thermomyces lanuginosus* lipase (CalT) was obtained from Novozymes (Bagsverd, Denmark). Biodiesel fuel-palm oil based as a comparison fuel for this study was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). All other reagents were purchased from Nacalai Tesque Inc (Kyoto, Japan) and Sigma-Aldrich (Tokyo, Japan).

Lipase-Catalyzed Alcoholysis

The lipase catalyzed ethanolysis was performed in a borosilicate glass tube. The reaction mixture consisted of 4 g POME, 12 mg CalT (2100 U activity), and 0.2 g distilled water. The reaction was initiated via the addition of the initial amount of ethanol (1:1 molar ratio of the oil) diluted in five concentrations; 15, 45, 75, 92, and 99.5 % (v/v). The reaction proceeded in a water bath equipped with a Teflon coated magnetic stirrer. The reaction was carried out at 40 ^{o}C and 500 rpm for 24 h. Generally,

to avoid the deactivation of the lipase by ethanol, 1:1 molar ratios of the oil to ethanol were added step-wise at 2, 4, and 6 h leading to a total of 1:4. 100 µl samples were taken at specified times to determine the amount of free fatty acids and fatty acid alkyl ester over the course of the reaction.

Analytical Method

Fatty acid methyl ester (FAME) or fatty acid ethyl ester (FAEE) produced during the course of the reaction were measured via gas chromatography. Samples taken at specified times were centrifuged at 12,000 x g for 5 min at 15 °C, and the upper layer was analyzed using GC-2010 (Shimadzu, Kyoto, Japan) equipped with a ZB-5HT Interfeno capillary column (15m x 0.25 mm x 0.15 mm) (Phenomenex Inc, USA), an auto-sampler, and a flame ionization detector. During the analysis, the temperature conditions of injector and detector were set at 320 and 370 °C, respectively. Helium was employed as the carrier gas at a flow rate of 57.5 ml/min. The column was configured at a temperature program starting at 130 °C for 2 min, increased to 350 °C at a gradient of 10 °C/min, then 370 °C at 7 °C/min. It was maintained at this temperature for 10 min. The retention times for FAME and FAEE were identified using standard solutions of the respective fatty acid alkyl esters. The FAME and FAEE composition were reported as the percentage of alkyl ester in the sample using tricaprylin as an internal standard. FAME and FAEE yields were calculated using the gradient of the curves of the respective esters and the following equations; [16]

$$FAAE\ amount\ (mg) = \frac{Peak\ Area\ of\ FAAE\ \ x\ Weight\ of\ Internal\ Standard}{Standard\ Gradient\ (m)\ x\ Peak\ Area\ of\ Internal\ Standard}$$

$$\%\ FAAE\ yield\ (\%\ w/w) = \frac{FAAE\ amount\ (mg)}{Reaction\ sample\ (mg)}\ x\ 100\%$$

The functional group of biodiesel fuel was characterized by Attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR) analysis that was performed using a Shimadzu AIM-900 Infrared Microscope equipped with an ITRraces-100 (Shimadzu Corp., Tokyo, Japan). Biodiesel properties including density, viscosity, acid value, iodine value and cetane number were analyzed using standard ASTM methods.

Statistical Analysis

For the statistical analysis, the data presented were the averages of triplicate readings. The values were expressed as mean ± standard deviation. The experiments were conducted three times to further verify the results. The data were subjected to one-way ANOVA using Minitab® 19 (Minitab Inc., USA) to evaluate the significant differences where $p \leq 0.05$.

RESULTS AND DISCUSSION

Palm Oil Mill Effluent (POME) Characterization

POME is produced in high volumes at no extra cost in palm oil mills. Besides water, it contains high amounts of oil and grease. Among the characteristics of POME cited in literature, FFA, acid value, saponification value, and iodine value are the essentials in determining effectiveness for biodiesel production.[17] Table 1 shows the characteristics of POME that was used in this study. The initial FFA was 76.16 ± 0.13 % (w/w). This high amount of FFA would be problematic in the conventional biodiesel production via alkaline catalyzed transesterification.[18] Moreover, the high acid value

and saponification value (153.73 ± 2.11 and 211.70 ± 8.51 mg KOH/g, respectively) indicates that, it will be difficult to neutralize the free fatty acids that are present in the oil using acid catalyzed esterification. Thus, enzymatic trans/esterification would be more preferable for catalyzing the production of biodiesel from POME. Enzymatic transesterification has been successfully used for converting highly heterogeneous feedstock containing mixtures of FFA and triglycerides into biodiesel.[19]

Furthermore, iodine value was measured to observe the average degree of unsaturation of the oil. The higher the iodine value, the greater the number of C=C double bonds.[20] The iodine value (53.54 g I_2/100 g oil) of POME was observed to be in the range of palm oil (44 – 58 g I_2/100 g oil).[21] This low iodine value indicates that, POME is rich in saturated fatty acids such as palmitic (C16:0) and stearic (C18:0) acids. Further analysis of POME revealed that the substrate contained 59.22 % (w/w) saturated fatty acid. This level of saturation is known to contribute to a better oxidative stability of the resulting biodiesel fuel.

Alcoholysis towards Biodiesel Synthesis from POME

Biodiesel synthesis is generally performed by the transesterification of plant oil with short chain alcohols such as methanol and ethanol. Both methanol and ethanol are usually used in the transesterification reaction with good yields of biodiesel. Fig 1. shows the obtained alkyl ester content for FAME and FAEE. The experiments were carried out using 2100 U lipase loading based on oil weight and 1:1 molar ratios of the oil at the stepwise addition time interval of 2 h leading to a total of 1:4. In the initial attempt, 99.5% (v/v) grade methanol and ethanol were used without dilution. The results showed that, FAME yield (93.33 ± 2.63 % w/w) was higher than FAEE yield

(83.64 ± 1.93 % w/w). Fatty acid alkyl ester content in both cases were low with respect to the 96.5 % (w/w) ester content specification ~~from~~ as stipulated by EN 14214 standards

Alcohols inhibit the functionality of enzymes through competitive inhibition. Methanol, the most widely used alcohol for enzymatic biodiesel production, gives a higher yield than ethanol due to its better reactivity. The higher reactivity of methanol was not observed in the initial 2-hour reaction period where ethanol had produced 45.59 ± 1.53 % (w/w) FAEE. Nonetheless, these results show that the reaction conditions could be improved to enhance FAEE production. Ethanol with its longer non-polar region has less deactivating effect on lipase. An improved initial reaction rate for ethanolysis was investigated to improve the overall FAEE yield while ensuring limited inhibition effect on the lipase.

Effect of Lipase Loading on FAEE Production from POME

The influence of lipase loading was investigated for FAEE production from POME where the amount of liquid lipase was varied from 700 to 7000 U. The other parameters (including temperature and agitation) were fixed for the optimization studies. The reaction conditions were; ethanol to oil ratio (4:1), excess water (5 % v/w) and 24 hours reaction time. The effect of lipase loading towards biodiesel synthesis is shown in Fig 2a. The yield increased with increasing lipase loading. FAEE gradually increased from 64.81 ± 1.01 to 83.64 ± 1.93 % (w/w) when the lipase loading was varied from 700 to 2100 U. The biodiesel yield at the highest loading, 7000 U, was similar to that of 4200 U (87.46 ± 1.91 and 87.40 ± 0.29 % w/w). The results indicate that the increase of lipase concentration can increase the initial synthesis rate and the final yield. The

199 statistical analysis showed that the yield of FAEE was significantly affected ($p < 0.05$)
200 by the different concentration of lipase. Based on the results, 7000 U lipase was optimal
201 loading for the production of FAEE. However, high enzyme loading results in high
202 production cost of biodiesel, therefore, the 2100 U lipase loading, which showed a close
203 yield of 83.64 ± 1.93 % (w/w), was used for subsequent experiments.
204
205 **Effect of Oil to Ethanol Molar Ratio on FAEE Production from POME**
206 Experiments were performed to evaluate the synthesis of biodiesel by varying the
207 molar ratio of oil to ethanol at four different levels, 1:3, 1:4, 1:5, and 1:6.
208 Stoichiometrically, a 1:3 (TAG: ethanol) molar ratio is required for complete
209 conversion to FAEE. As shown in Fig 2b., the FAEE conversion at 1:3 molar ratio was
210 59.61 ± 2.64 % (w/w). This was significantly lower than the theoretical yield.
211 Enzymatic transesterification is known to be a reversible reaction, thus, as ester content
212 increases, the equilibrium shifts to the dissociation of the products back to the reactants.
213 An excess amount of ethanol is used to drive the equilibrium to the production of
214 esters.[22] FAEE yield from POME improved significantly to 83.64 ± 1.93 % (w/w) by
215 the addition of an extra molar equivalent of ethanol. The yield of FAEE was
216 significantly affected ($p < 0.05$) by the different molar ratio between oil and ethanol.
217 However, according to the Ping-Pong Bi Bi mechanism which generally explains
218 the enzymatic transesterification of oils, alcohol molecules can directly bind with
219 enzyme and block the binding of substrate leading to a dead-end enzyme-alcohol
220 complex in a competitive inhibition mechanism.[23,24] Short chain alcohols are also
221 known to denaturize proteins, which are the main component of lipase. Consequently,
222 the addition of 5 and 6 molar equivalents of ethanol resulted in a drastic reduction in

FAEE production (82.81 ± 0.75 and 64.81 ± 1.01 % (w/w), respectively). Therefore, 1:4 molar ratios of POME and ethanol was applied for subsequent experiments.

Effect of Ethanol Dilution on FAEE Production from POME

On an integrated biorefinery concept where bioethanol can be of essence, the effect of ethanol dilution was investigated for the improvement of FAEE production from POME. In this study, 5 different ethanol concentrations were explored (15, 45, 75, 92, and 99.5 % v/v), by diluting pure ethanol with water. The lower concentrations are similar to ethanol concentrations in crude bioethanol mixtures. ~~The~~ ANOVA also showed that the yield of FAEE was significantly affected ($p < 0.05$) by the different concentration of ethanol dilution. The highest FAEE yield (97.43 ± 1.24 % w/w) shown in Fig. 3 was obtained with 45% (v/v) diluted ethanol. This indicates that the dilution of ethanol suppressed the deactivation effect on lipase. Even though the dilution of ethanol reduced the initial reaction rate, a comparable FAEE yield was achieved in the end. The lower concentrations of ethanol, thus, maintained the lipase activity. For 15 % (v/v) ethanol, the content of water was so high that, a competitive hydrolysis occurred (Supplementary Fig. S1), leading to a much slower rate and low final yield.

With 45% (v/v) ethanol dilution showing a higher yield, the time interval (frequency) of ethanol addition was examined. At a molar ratio of 1:1, various time intervals (10 min, 30 min, 60 min, 120 min) were independently investigated (Supplementary Fig. S2). 10, 30 and 60-min time intervals as well as 1-time addition at 0 h yielded less than 80 % (w/w) FAEE in 6 h. By extending the addition interval over 120 min almost 80 % (w/w) FAEE was achieved in 6 hours. The addition of the 4^{th} molar equivalent after the 6^{th} hour resulted in the highest final yield of 97.43 ± 1.24 %

FAEE. This addition rate was therefore the most suitable for the introduction of highly diluted (45 % v/v) ethanol to POME for FAEE production.

Biodiesel Properties from POME

In this work, POME biodiesel is also characterized by the mid-infrared spectral data (4000 - 400 cm^{-1}) to identify the functional group of organic and inorganic bonds in sample. Fig 4. shows peak identified from the spectra of commercial biodiesel (a) and POME biodiesel (b). The functional group in biodiesel from POME and commercial biodiesel indicates similar spectra features. The peaks consisted of symmetric and asymmetric stretching vibrations of –C–H alkane groups at 2912-2845 cm^{-1}, –C=O stretching at 1741-1735 cm^{-1} attributed to carbonyl group of the formed ester in biodiesel synthesis, $–CH_3$ groups in fuel at 1452-1441 cm^{-1}, the bending vibration of C–O and $O–CH_3$ at 1274-1105 cm^{-1}, and =C–H group indicating the methylene functional group in biodiesel at 721 cm^{-1}. [25]

Biodiesel from POME as feedstock was characterized according to ASTM standards. Table 3 depicts the fuel properties of optimized produced biodiesel from POME. The results show some biofuel properties were found to be in acceptable range with the ASTM standard specifications. The acid value of biodiesel was 0.50 ± 0.03 mg KOH/g biodiesel with FFA content 0.25 ± 0.02 % w/w. In addition, a small amount of MAG (0.85 ± 0.07 % w/w) and DAG (0.72 ± 0.08 % w/w) is remained. The ester yield compared with other investigations showed a comparable yield (Table 1).

CONCLUSION

The concept of untreated POME, liquid lipase and diluted ethanol was successfully used for biodiesel production. FAEE yield of 97.43 ± 1.24 % (w/w) was achieved within 24 hours when 45 % (v/v) aqueous ethanol was utilized under the optimal reaction conditions of 40 ˚C, 500 rpm and 1:4 oil to ethanol molar ratio. The ester yield fulfills the EN 14214 standard specification for ester content which is 96.5 % (w/w), minimum. The concept of using dilute ethanol and POME could make a crucial contribution to sustainable production of biodiesel, as they provide an integrative approach to the utilization of agricultural waste.

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FIGURE LEGEND

Fig 1. Comparison of methanolysis and ethanolysis towards biodiesel synthesis from POME. Reaction conditions: lipase loading (2100 U), water (5 % v/w), total reaction time (24 h), oil to alcohol ratio (1:4), temperature (40 °C) and stirring speed (500 rpm).

Fig 2. FAEE production from POME with varying (a) lipase loading and (b) feedstock to ethanol molar ratio. Reaction conditions: excess water (5 % v/w), total reaction time (24 h), temperature (40 °C) and stirring speed (500 rpm).

Fig 3. Effect of ethanol dilution on FAEE production from POME. Reaction conditions: lipase loading (2100 U), excess water (5 % v/w), the total reaction time (24 h), temperature (40 °C) and stirring speed (500 rpm).

Fig 4. ATR-FTIR spectra from (a) commercial biodiesel and (b) biodiesel from POME.

357 **Table 1**. Comparison study using POME as feedstock for biodiesel production

Substrate	Method	Catalyst	Acyl Acceptor	Condition	FAAE Content (%)	References
POME	Soxhlet Extraction	*Pacific white shrimp*	Methanol (99.5 %)	40 ^{o}C; 40 kU enzyme loading; 6:1 methanol to oil ratio; 3 % water content; 250 rpm; under 12 h	96.5 ± 0.90 (FAME)	Rakkan et al.[6]
POME	Soxhlet Extraction	Immobilized palm lipase	Methanol (99.5 %)	35 ^{o}C; 36 kU enzyme loading; 6:1 methanol to oil ratio; 200 rpm stirring speed; under 24 h	93.5 ± 0.5	Paichid et al.[26]
POME	Soxhlet Extraction	NaOH	Methanol (99.5 %)	60 ^{o}C; 1 % wt. alkali; 9:1 methanol to oil ratio; 800 rpm stirring speed; under 1 h	96.5 ± 1.01 (FAME)	Suwanno et al.[17]
POME	Soxhlet Extraction	Crude lipase from oil palm fruit	Methanol (99.5 %)	35 ^{o}C; 36 kU enzyme loading; 6:1 methanol to oil ratio; 200 rpm stirring speed; under 36 h	96.5 ± 0.90	Suwanno et al.[17]
POME	Solvent extraction	Immobilized *Candida rugosa*	Methanol (99.5 %)	40 ^{o}C; 2 g of immobilized beads weight; 6:1 methanol to oil ratio; 300 rpm stirring speed;5h	85	Matinja et al.[27]
POME	Direct	*Thermomyces lanuginosus*	Ethanol (45 %)	40 ^{o}C; 2100 U lipase loading; 4:1 ethanol to oil ratio; 5 % excess water; 500 rpm stirring speed; under 24 h	98.39 ± 0.80	This study

359 **Table 2**. Analyzed parameters for characterization of POME

Parameters	Unit	Content[a, b]
Free fatty acid (FFA)	% w/w	76.16 ± 0.13
Monoglyceride (MAG)	% w/w	2.18 ± 0.20
Diglyceride (DAG)	% w/w	9.20 ± 0.76
Triglyceride (TAG)	% w/w	13.02 ± 0.46
Acid value	mg KOH/g oil	153.73 ± 2.11
Saponification value	mg KOH/g oil	211.70 ± 8.51
Iodine value	g I_2/100 g lipid	53.54 ± 1.50

360 [a.] Each entry is expressed as the mean of three independent measurements ± standard
361 deviation (n = 3).

362 [b] $p < 0.05$.

363 **Table 3.** The specifications of Biodiesel from POME according to ASTM standards

Properties	Unit	Test Method (ASTM)	Biodiesel from POME[s, b]	ASTM Limits
Density at 15 ^{o}C	kg/m^3	D1298	868.29 ± 3.48	860 - 900
Viscosity at 40 ^{o}C	mm^2/s	D445	5.45 ± 0.67	1.9 - 6.0
Acid Value	mg KOH/g oil	D664	0.50 ± 0.03	0.5 (max)
Iodine Value	g I_2/100 g lipid	D5554	67.87 ± 1.59	120 (max)
Cetane Number		D613	59.68	47 (min)

364 [a.] Each entry is expressed as the mean of three independent measurements ± standard
365 deviation (n = 3).

366 [b] $p < 0.05$.

Fig. 1

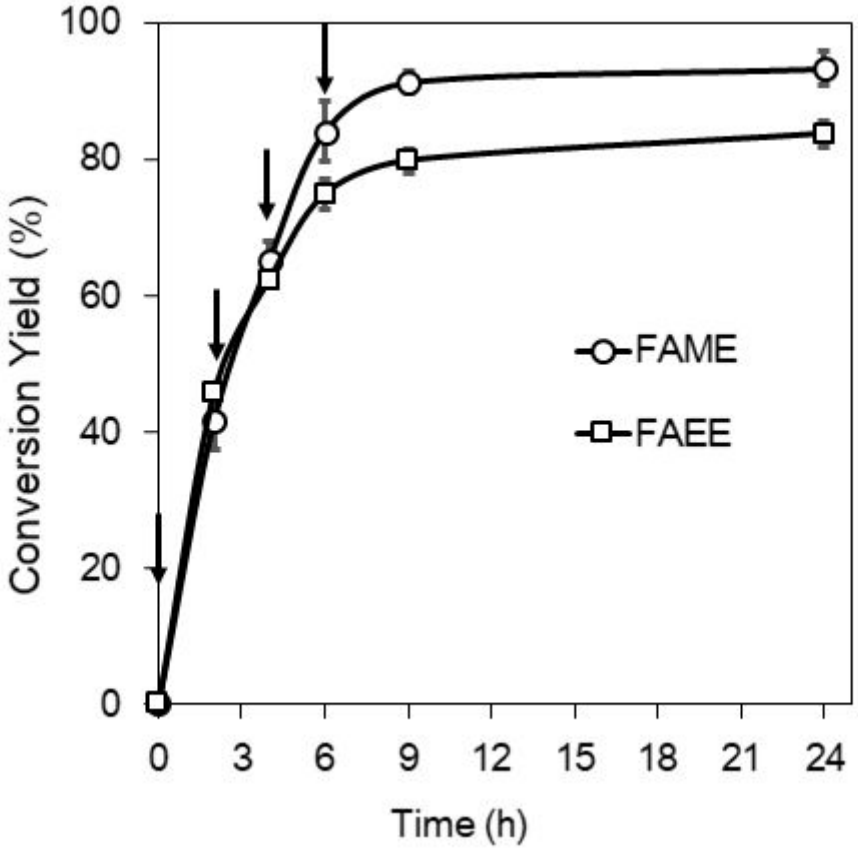

100
80
60
40
20
0
Conversion Yield (%)
0
3
6
9
12
15
18
21
24
Time (h)
FAME
FAEE

Fig. 2

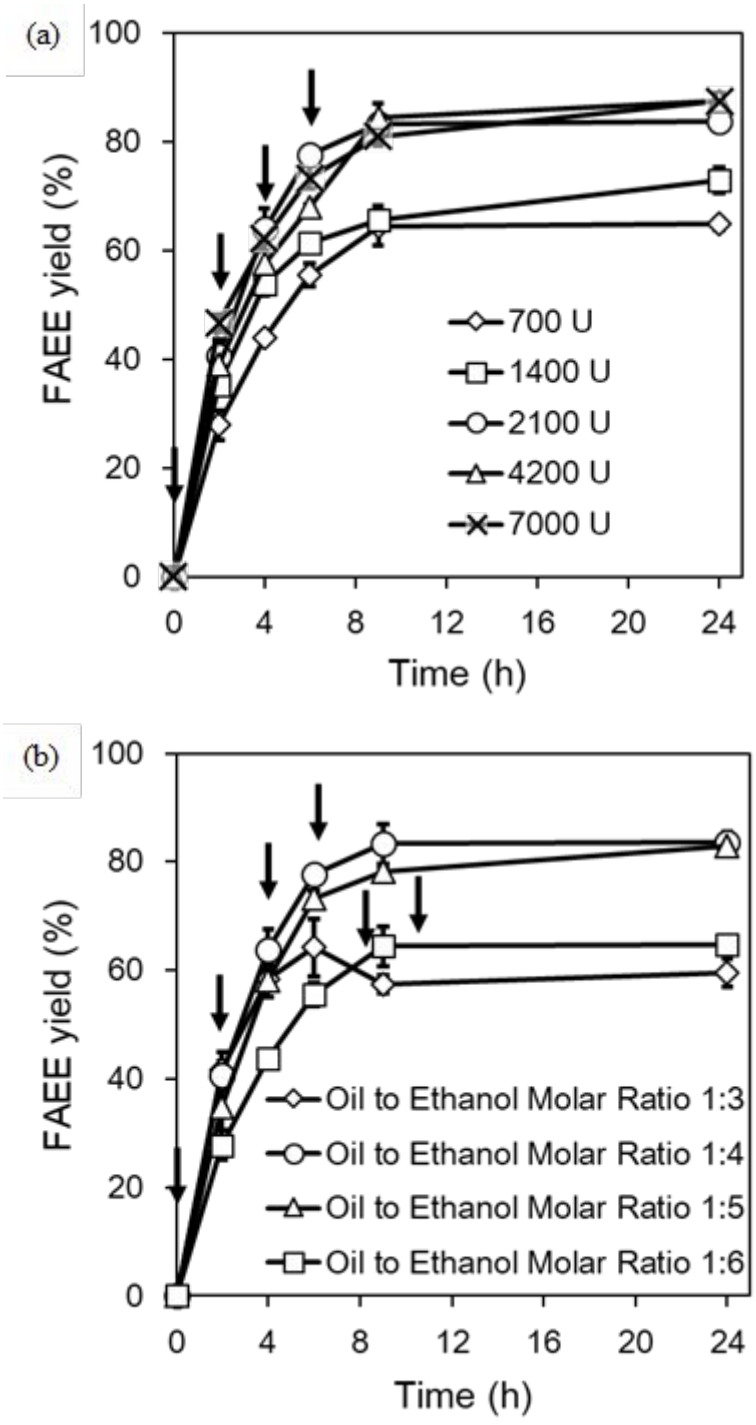

(a)
FAEE yield (%)
Time (h)
700 U
1400 U
2100 U
4200 U
7000 U
(b)
FAEE yield (%)
Time (h)
Oil to Ethanol Molar Ratio 1:3
Oil to Ethanol Molar Ratio 1:4
Oil to Ethanol Molar Ratio 1:5
Oil to Ethanol Molar Ratio 1:6

Fig. 3

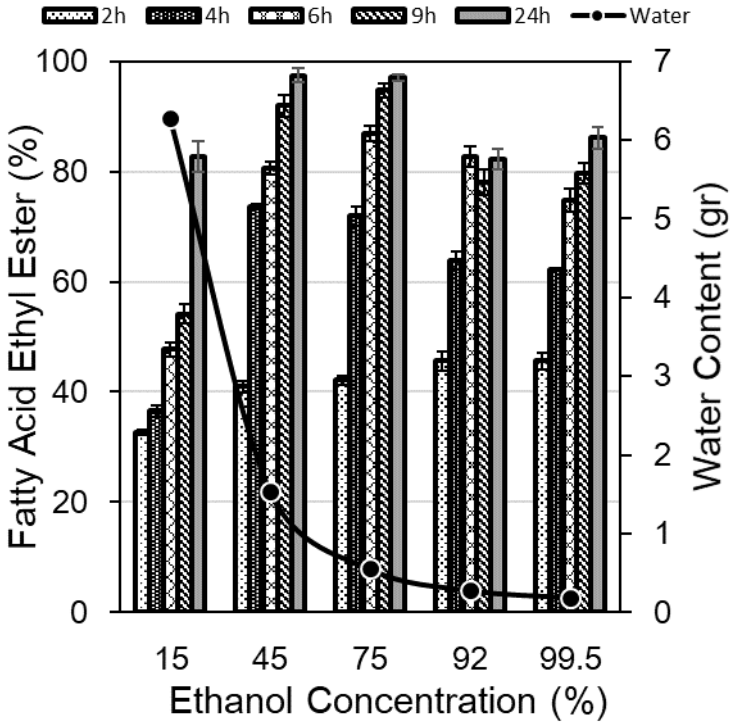
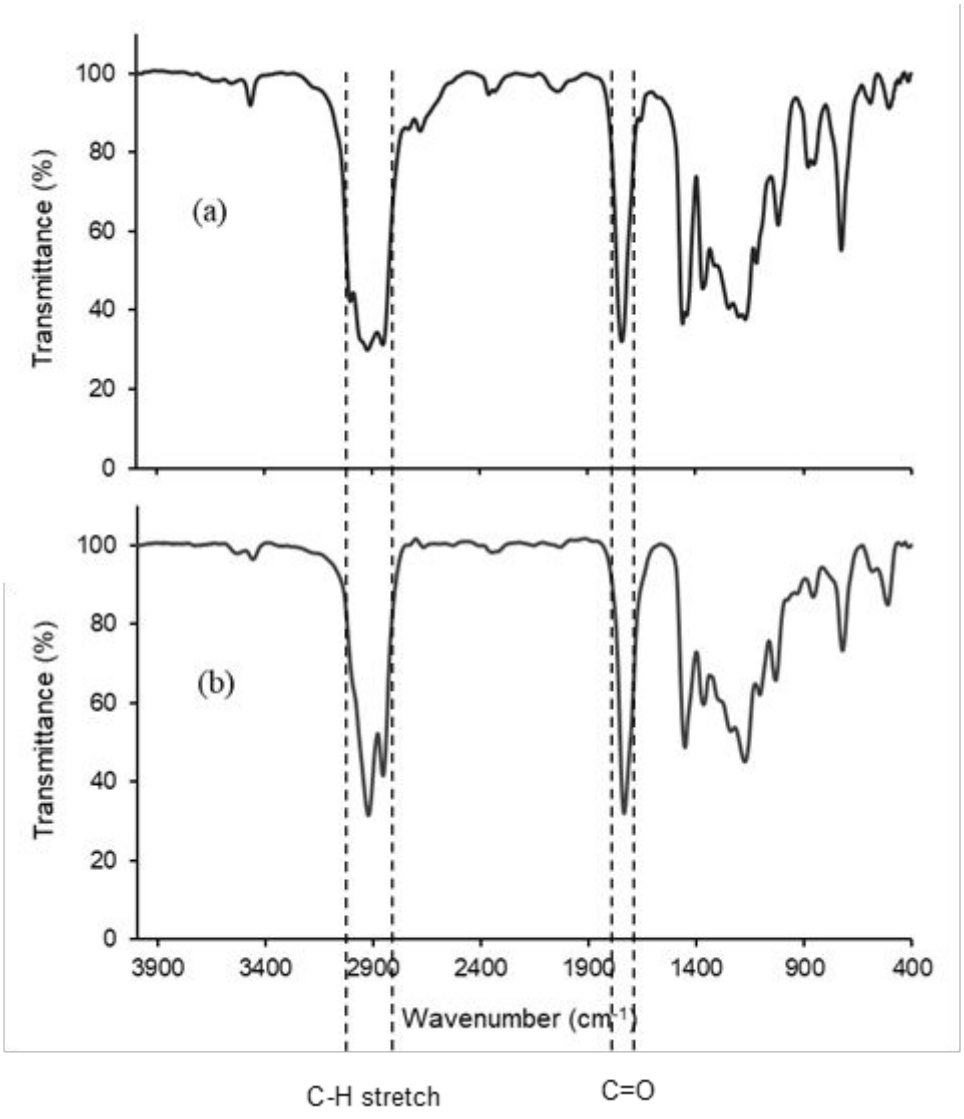
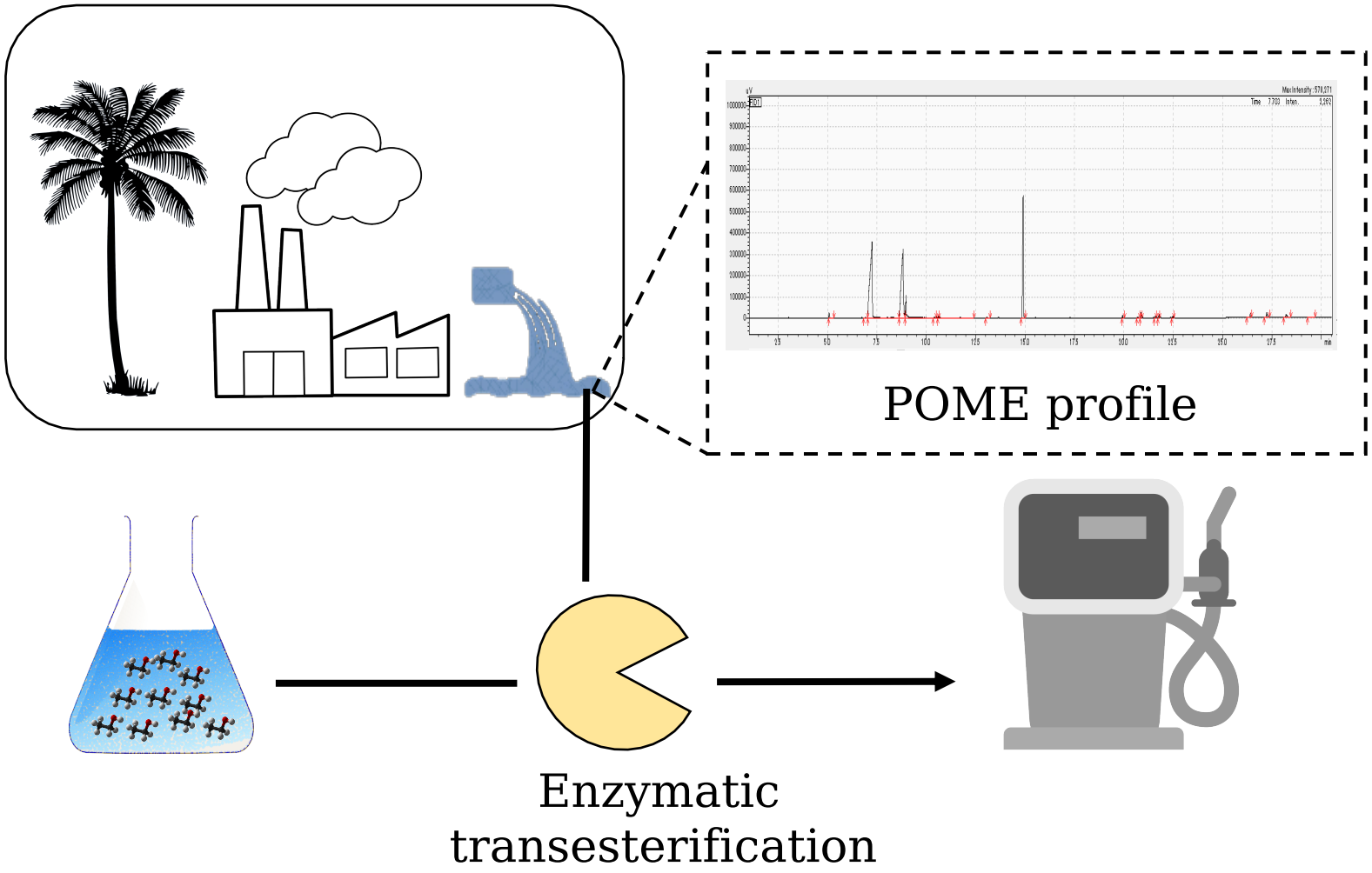

2h
4h
6h
9h
24h
Water
Fatty Acid Ethyl Ester (%)
Water Content (gr)
100
80
60
40
20
0
7
6
5
4
3
2
1
0
15
45
75
92
99.5
Ethanol Concentration (%)

Fig 4.


Transmittance (%)
100
80
60
40
20
0
(a)
(b)
3900
3400
2900
2400
1900
1400
900
400
Wavenumber (cm-1)
C-H stretch
C=O


POME profile
Enzymatic
transesterification