Study on the Utilization of Palm Oil Mill Effluent (POME) for Sustainable Chemical and Bioenergy Production

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博士論文

Study on the Utilization of Palm Oil Mill Effluent (POME) for Sustainable Chemical and Bioenergy Production

持続可能な化学品とバイオ燃料の POME からの生産に関する研究

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PREFACE

This thesis is submitted by the author to Kobe University in partial fulfilment of the requirements for the degree of Doctor of Engineering. The studies were carried out between the period of 2017 and 2021 under supervision of Professor Chiaki Ogino in the Laboratory of Biochemical and Bioprocess Engineering, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University.

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CONTENTS

CHAPTER I
Introduction

I.1. Palm Oil Industry

Oil palm, known as *Elaeis guineensis*, is the most productive oil-producing plant in the world [1]. The most significant oil palm sector in the world is Southeast Asia, in which more than 80 % of oil palm in the world is produced in Indonesia, Malaysia, and Thailand [2,3]. Indonesia is the largest producer and exporter, with 14.7 million ha cultivated area producing 51.4 million tons of palm oil in 2019 [4]. The mechanical process consists on the extractions fresh fruit bunches (FFB) to produce oil palm [5]. Fig. 1 showed how the plantation site is prepared for cultivating the oil palm tree and the harvesting of the FFB from the oil palm tree.

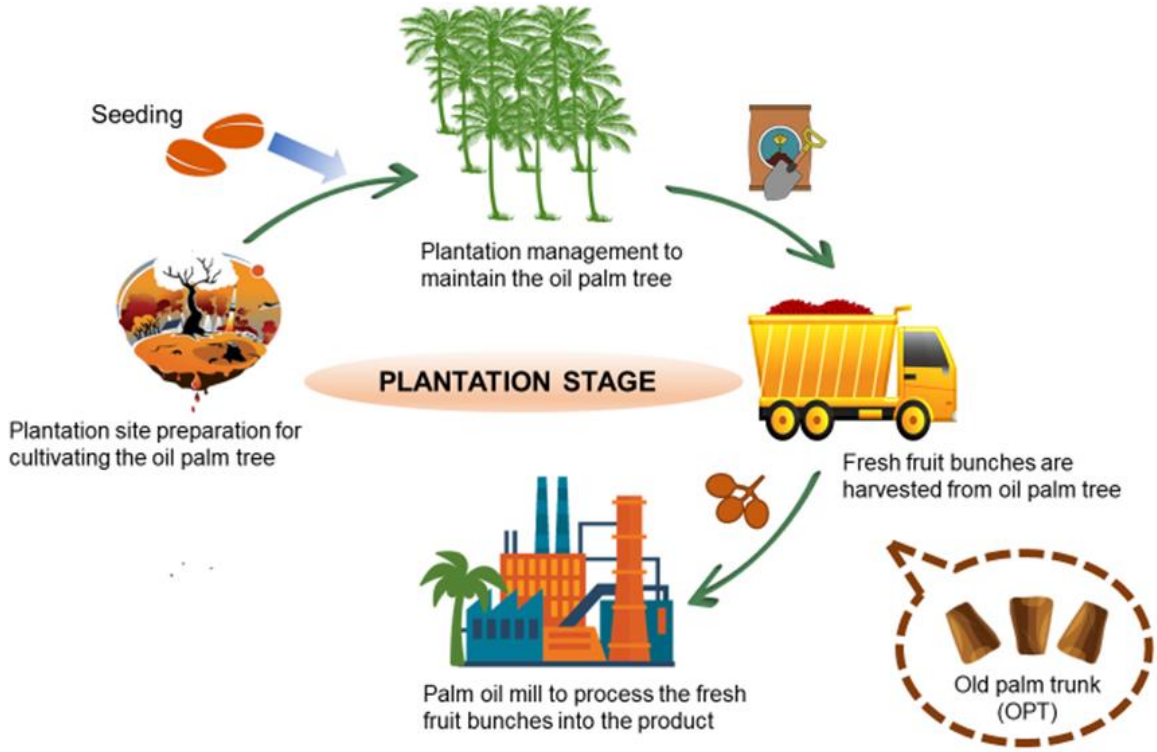

Fig. 1. Overview of palm oil processing in the plantation site

The FFB is transported to the palm oil mills to produce the oil, as presented in Fig. 2. FFB is autoclaved to make it easy to remove the fruit from bunches in the threshing process and break down the gums and resins that caused the oil to foam during frying [5–7]. After sterilization, FFB is beaten off by the threshing machine, which causes the palm fruit and the empty fruit bunches (EFB) separation [8,9]. The separated palm fruit is put into a digester to be mashed under steam-heated conditions. In this stage, the mesocarp oil-bearing cells are broken down, and the oil is passed through the screw press followed by the press cake [8,9]. The crude palm oil (CPO) is fed into a clarification tank to settle and filter the oil fiber [8]. Finally, the CPO is purified before sending it to a storage tank. On the other hand, the press cake discharged from the screw press is broken down by the breaking screw conveyor for the nuts and fiber separation [10]. The nuts are sent to the cracked mixture separating system, and most of the shell and kernel will be separated in the clay bath [9].

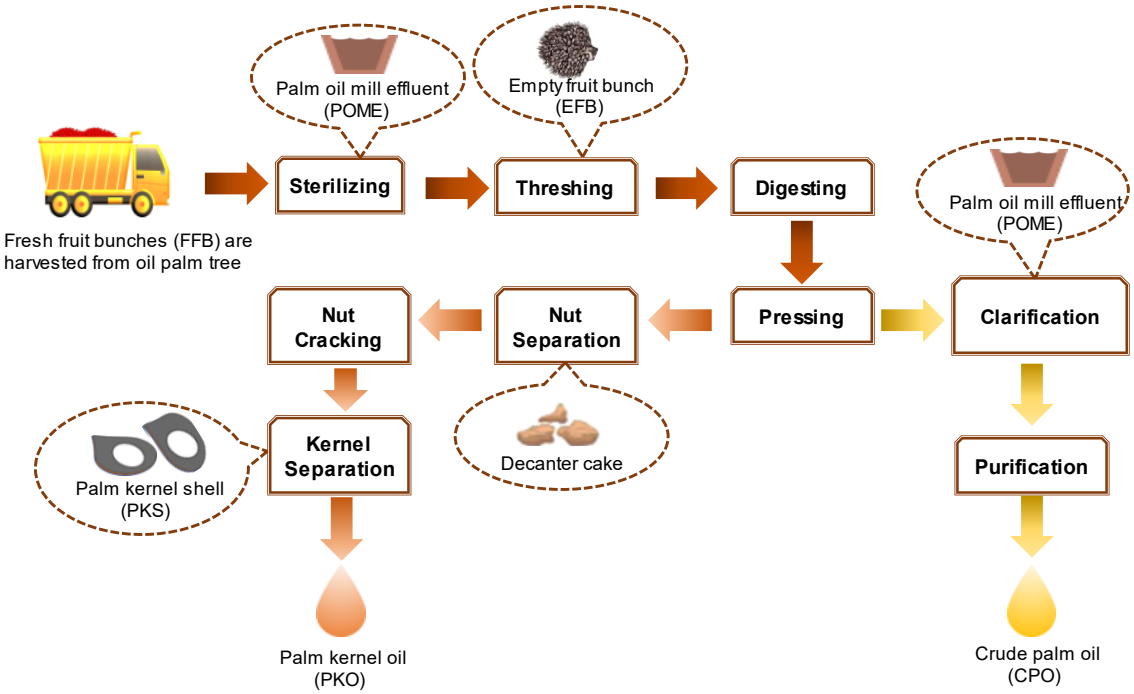

Fig. 2. The schematic process in the milling stage of oil palm

I.2. Waste Classification and Generation in Palm Oil Mills

Palm oil mills produce crude palm oil (CPO) from the flesh of the fruit (the outer part) and palm kernel oil (PKO) from the seed or kernel (the inner part) as primary products, and a significant number of agricultural wastes such as palm kernel shells (PKS), oil palm trunks (OPT), empty fruit bunches (EFB), and palm oil mill effluent (POME) were simultaneously generated during the processing of oil palm as co-products as presented in Fig. 3.

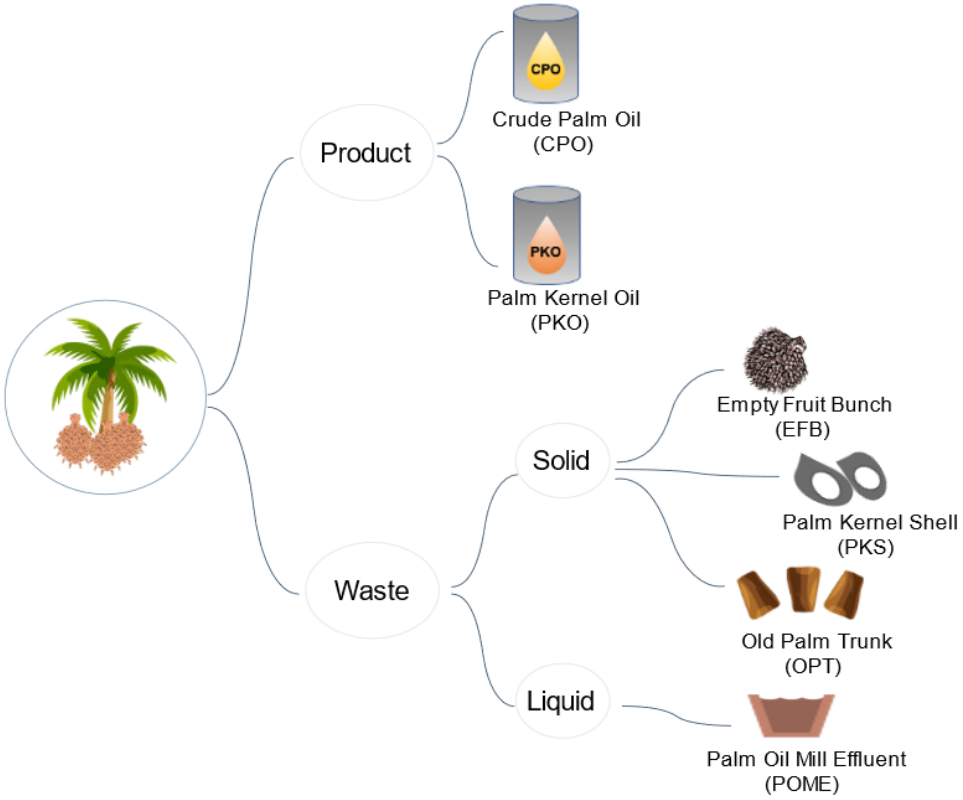

Fig. 3. The products and wastes of the palm oil industry

The wastes are divided into two classifications; solid wastes and liquid effluents.

I.2.1. Solid wastes

Oil palm solid wastes are generated from the plantation site (Fig. 1) and the milling stage processing (Fig. 2). OPT, EFB, and PKS are the most common by-products in

the oil palm industry (Table 1). OPT is generated during the replanting of the oil palm tree that happens in a 25-year interval due to a decreased fruit yield and oil productivity [11–14]. EFB is the highest biomass by-product generated in the oil palm milling process. Kong *et al*. [14] stated that FFB contains approximately 21% CPO, 6–7% PKO, 5.5–7% PKS, and 22–23% EFB on wet basis. 44.91 million tons of EFB and 11.23 million ton of PKS were generated from palm oil mills to produce 42.87 million tons of CPO in 2019 [4], as presented in Table 1.

I.2.2. Liquid effluents

POME is the effluent generated from the oil palm manufacture in the milling stage. POME leads for the most significant portion of all waste generated in the palm oil mill. Hambali *et al.* [13] assumed that around 58% of POME would be generated after processing. Therefore, 118.40 million tons of POME were generated from palm oil mills to produce 42.87 million tons of CPO in 2019 [4].

Table 1. The waste classification in the palm oil industry

Source of waste	Type of waste	Weight of the total source (%)[11–15]	Production of the wastes (million ton)
Fresh fruit bunch (from palm oil mill)	Palm kernel shell	5.5	11.23
	Empty fruit bunch	22	44.91
	Palm oil mill effluent	58	118.40
Oil palm tree (from plantation)	Trunk	74.5[a]	54.76
	Frond	14.5[b]	10.66

[a] Based on 74.5 t of dry OPT per ha and the estimated 5% oil palm planted area due for replanting (OPT felled once every 25–30 years) [11].

[b] Based on 14.5 t of dry OPF per ha and the estimated 5% oil palm planted area due to replanting [11].

I.3. Palm Oil Mill Effluent

Palm oil mill effluent (POME) refers to the liquid waste resulting from large quantities of steam and hot water used to clean the fruit and separate the shell and cake from the palm fruit in the milling process. POME is a thick, brownish, greasy, and non-toxic liquid effluent comprised of suspended solids, total dissolved solids, and highly degraded oil [16,17]. It consists of 95 – 96 % and made up with 0.6 – 0.7 % oil and 4 – 5 % total solids [18,19]. Moreover, a bad odour is emitted by POME due to organic matter content such as phenolic, pectin and carotene [20].

Table 2 shows the general properties, fatty acids, and mineral contents of POME. POME possesses high COD of 15,000 - 100,000 ppm and BOD of 10,250 - 43,750 ppm, which become a severe threat since its biodegradation could induce aquatic hypoxia [21,22]. In addition, the pH of POME is low about 3.4 - 5.5 due to the organic compounds and grease from partial degradation of palm fruits before processing [23,24]. Nevertheless, the effective treatment before discharge into the environment of POME is needed since it contains a high chemical oxygen demand (COD), biological oxygen demand (BOD) and highly polluting properties [25]. The high organic compound in POME have indicated that it can be a good resource to generate methane gas via anaerobic system [26].

Gas chromatography-mass spectroscopy (GC-MS) results show that POME contains many free fatty acids from which palmitic acid and oleic acid) are the main compounds for biofuel production. POME also contains some mineral contents which can be used as nutrients for the microbe, followed by nitrogen (N), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), sulfur (S), silicon (Si), sodium (Na), aluminum (Al), and iron (Fe). [27–29], so its direct discharge to the environment could inflict undesirable eutrophication. Besides, POME conceives heavy metal, such as lead (Pb), cobalt (Co),

cadmium (Cd), zinc (Zn), mercury (Hg), tin (Sn), manganese (Mn), and chromium (Cr), but it is not the poisonous one [30].

Table 2. POME properties [22]

(A) General properties		(B) Fatty acid contents		(C) Mineral contents	
Parameter	Value[a]	Parameter	Value[a]	Parameter	Value[a]
pH	3.4-5.5	Caprylic acid	835-878	B	0.25-2.00
Temperature	44-90	Capric acid	1518-1583	Na	3.19-87.92
Density	876-985	Lauric acid	1142-1185	Mg	29.51-1144
Viscosity	4.17-4.69	Myristic acid	4533-461	Al	6.30-344
Turbidity	65,590-69,410	Pentadecanoic acid	784-813	Si	55-125
Oil & grease	2,234-27,166	Palmitic acid	743-8795	P	74.50-2370
BOD	10,250-43,750	Heptadecanoic acid	491-513	S	60-400
COD	15,000-100,000	10-heptadecanoic acid	394-415	K	314.32--5533
Total solids	11,500-79,000	Stearic acid	3567-3958	Ca	53.85-607
Total suspended solids	5,000-71,275	Oleic acid	4804-5708	V	0-0.01
Total dissolved solids	20,554-41,098	Linoleic acid	3011-3878	Cr	0.13-0.16
Total volatile solids	9,000-72,000	Linolenic acid	1511-1901	Mn	1.27-1.53
Total protein	460-1,580	Arachidic acid	1265-1308	Fe	0.32-0.48
Total organic carbon	9,584-38,616	Eicosatrienoic acid	708-766	Co	0.07-0.10
Total nitrogen	204-1,708	Eicosatetraenoic acid	394-415	Ni	0.04-0.06
NH_3-N	17-254	Eicosapentaenoic acid.	108-151	Cu	0.35-0.43
Hemicellulose	2,530-8,433			Zn	0.56-0.71
Cellulose	3,976-14,300			Se	0.40-0.49
Lignin	9,044-15,182			As	0.31-0.35
				Mo	0.22-0.25
				Cd	0.01-0.02
				Sn	0.07-0.09
				Pb	0.17-0.21

[a] Unit of all parameters is ppm (mg/L), except pH, Temperature (^oC), ρ (kg/m^3), μ (mPa·s), and turbidity (NTU)

I.4. Challenge for POME Utilization

Various studies regarding POME treatment and utilization have been attempted. POME has been redefined and changed from being a waste to a valuable resource [31]. Table 3 showed some current technology and applications for POME utilization. Bioremediation is the process that uses microbes to degrade POME and to remove the pollutant such as metal compounds, BOD, and COD [32]. In recent studies, bacterial, molds, fungi, and yeast have been shown to be able degrade POME [32–35]. Some researchers combined bioremediation and fermentation process to synthesize lipids since

some microbes can excrete different types of enzymes such as lipase and cellulase [28,33,36]. On the other hand, POME could be utilized for bioenergy production such as biogas, biohydrogen, and biodiesel since POME contains a high amount of BOD, COD, oil, and grease [37]. POME could generate biogas by anaerobic open pound system, pretreatment of POME, and POME co-digestions with other biomasses [38–40]. Biohydrogen production results from the fermentation of POME, where the simple sugars that consist in POME are converted to hydrogen [41–43]. Garritano *et al*. [44] stated that POME can produce biohydrogen via dark fermentation due to the presence of carbohydrates, lipids, and proteins. The residual oil of POME can be utilized for biodiesel synthesis via biological or chemical transesterification [45–48]. Lastly, POME can also be used as a substrate for cultivating microorganisms such as microalgae [27,29,49], yeast, fungi, and mold which can produce lipid [28,33,36,50], and lipase [51–53].

Table 3. Current utilization and technology for POME

POME Utilization	Method	Experimental condition	Reference
Biogas	Anaerobic digestion	Anaerobic digester system at mesophilic temperature (34.29 ± 0.8 °C)	[38]
	Anaerobic co-digestion (ACoD) process by using a solar assisted bioreactor (SABr)	The reactor was run at 308 K for 24 days ar 60 rpm	[54]
	Anaerobic co-digestion process with sewage biological sludge (SBS) and sewage chemical sludge (SCS)	The C:N ratio of POME:SBS and POME: SCS set up at 99:1, 95:5, 90:10, 85:15 and 80:20 (%v/w) under mesophilic temperature (35 °C)	[40]
Biohydrogen	Hungate tubes dark fermentation by bacterial consortia, *Clostridium beijerinckii* (ATCC 8260)	*Clostridium beijerinckii* was cultivated at 30 °C in the hydrolyzed POME (P003), containing 7.5 g/L of sucrose, during 8 days of fermentation and 20 % of the inoculum	[41]

	Dark fermentation by two stage anaerobic sequencing batch reactor system using enriched mixed culture	80 mL of raw POME diluted with water to obtain 20.0 g/L total carbohydrate content using 10 % (v/v) of enriched mixed culture as the inoculum was set at pH 6.0	[42]
	Dark fermentation by non-commercial enzyme	A total volume of 45mL of medium (composed of raw POME and sludge) was placed into penicillin flasks in pH 6.5. The flasks were purged with N^2 to ensure an anaerobic environment and were incubated under 35 °C and 150 rpm.	[55]
Biodiesel	Transesterification using lipase immobilized in PVA-alginate-sulfate beads	The experiment was carried out at agitation speed (300 rpm), oil/ methanol molar ratio (1:6), incubation period (5 h) and weight of the immobilized beads weight (2 g)	[45]
	Enzymatic transesterification using lipase from Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas	The optimum conditions for biodiesel production include enzyme loading of 40 kUnit, a methanol/oil molar ratio of 6:1, a water content of 3%, a stirring speed of 250 rpm, a reaction temperature of 40 °C and a reaction time of 12 h	[46]
	Transesterification with Local Zeolite	The mixture of zeolite and methanol was heating at 60 °C, 500 rpm. The methanol/oil molar ratio of 7:1 and 3 % catalyst were added into the reaction mixture with 4 h reaction time.	[48]
Lipid	Fermentation of microalga *Chlorella sorokiniana* CY-1 biomass using Novel-designed photobioreactor	The PBR was operated at 25°C, supplied with 2.5% and 0.1 vvm CO_2 aeration.	[49]
	Nannochloropsis sp cultivation diluted with seawater	Cultivation was performed for 14 days in a culture room with an ambient temperature of 23 ± 10.5 °C, pH 7.0 under a light intensity of 100 μmol m^{-2} s^{-1}, photoperiod 24 h	[36]
	Fermentation of *Chaetoceros affinis* under	Cultures (600 mL) were grown in 1 L Erlenmeyer flasks at 26 ± 2 °C under	[50]

	continuous light with aeration	continuous light (2000 lx) for up to 18 days.	
Lipase	Submerged fermentation of *Pseudomonas aeruginosa*	POME, olive oil (0.2 %, v/v), peptone (0.5 %, w/v), $CaCl_2$. $2H_2O$ (10 mM), Tween 80 (0.7 %, v/v) was incubated in a water shaker bath at 30 °C for 108 h	[52]
	Fermentation of *Aspergillus terreus* immobilized on *Luffa* sponge	90 mL diluted or undiluted POME was incubated at 30 °C for 7 days on a rotary shaker maintained at 210 rpm	[53]

However, some applications need to be improved because they are still insufficient or costly to be implemented. Nevertheless, our research aims to effectively utilize POME, especially the residual oil as the main source, and integrate it with other waste produced in the palm oil industry as presented in Fig. 4. In chapter II, this work demonstrated the effective utilization of POME for biodiesel production via a conversion route with diluted ethanol. POME, the leading liquid waste from the palm oil industries, could be used as an alternative sustainable feedstock for biodiesel production. This approach could also curb the environmental menace of POME. POME is, however, characterized by high water content and free fatty acids, which render the use of conventional chemical methods ineffective. The diluted ethanol was employed as the acyl acceptor for the transesterification to make this even more sustainable. The successful utilization of diluted ethanol in this process demonstrates the possibility of using bioethanol, which can be produced from EFB, another waste from the palm industry, for on-site biodiesel production, thus, relieving logistical bottle-necks.

Furthermore, this study combined the effective utilization of POME for both immobilizations of *Aspergillus oryzae* expressing various lipases as a carbon source and the production of biodiesel via a conversion route with diluted ethanol. To maintain

production cost-effectiveness in industrial applications, the ability to reuse whole-cell biocatalysts for an extended period was also successfully evaluated for producing biodiesel in a continuous batch. This research has been explored in chapter III.

Usually, the main focus of POME utilization with oleaginous yeast was for the bioremediation of POME. However, this work demonstrated the value-added bioconversion of POME, which consists of carbon sources to produce the single cell oil (SCO) even in inhibitory chemical compounds by oleaginous yeast is discussed in chapter IV.

Finally, we demonstrated an integrative utilization of several palm oil wastes to produce biofuel explored in chapter V. The biorefinery concept that integrates the utilization of palm waste to produce biodiesel was conducted. POME was used as the feedstock, and a distilled bioethanol solution of artificial palm sap served as an acyl acceptor for ethanolysis catalyzed by immobilized lipase with charcoal activated from coconut shells serving as a matrix for immobilization. This concept could solve the environmental problem around the palm industry and increase the value of palm oil waste. The kinetics, activation energy, and Gibbs free energy are investigated to determine the esterification process spontaneity, which consists of a high amount of water, and elucidate the case of free fatty acid (FFA) reacts faster and triacylglycerol (TAG) for producing FAEE. Ultimately, this study should help establish the sustainability of an integrated approach to biorefinery operations.

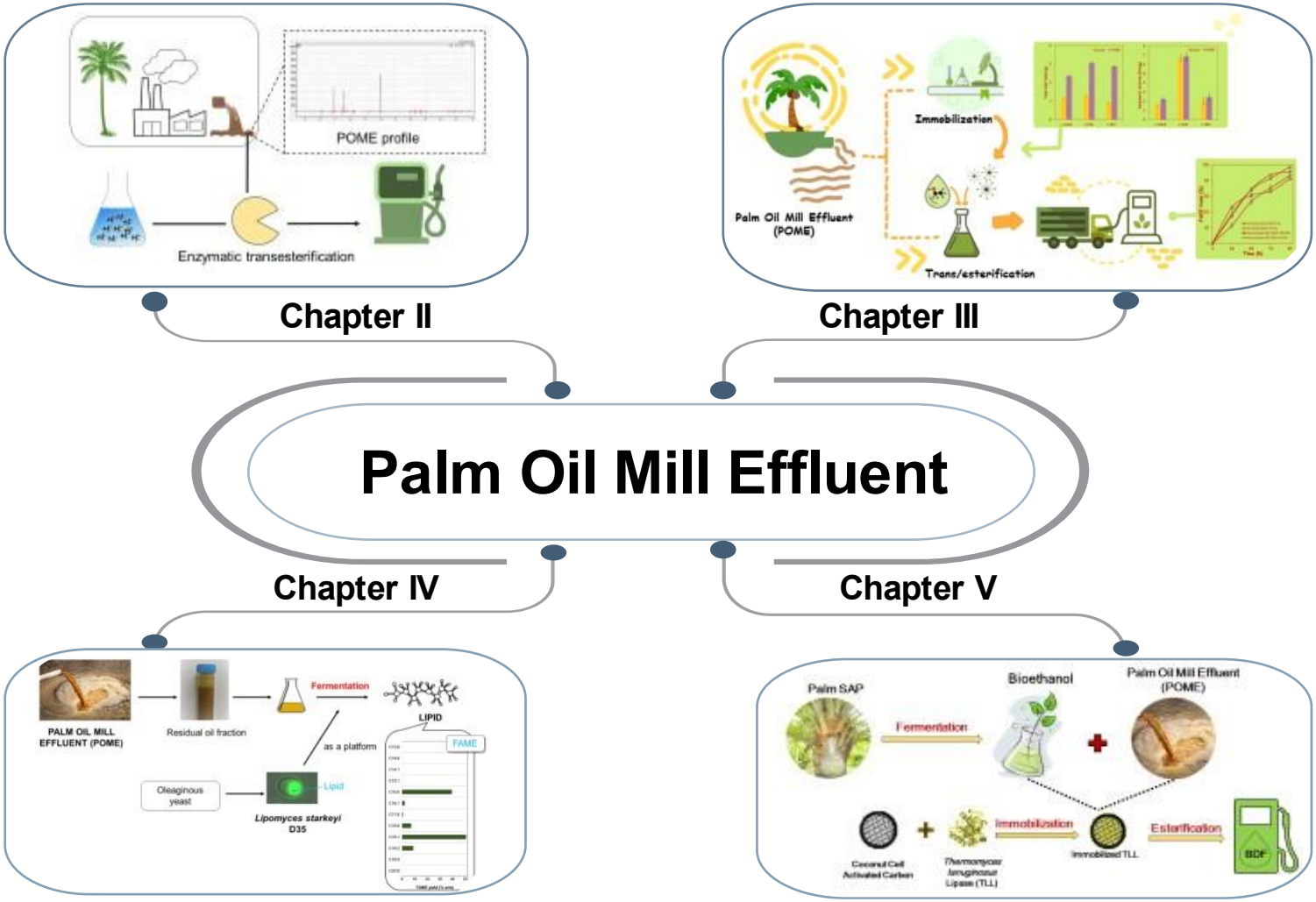

Fig. 4. The utilization of palm oil mill effluent for sustainable chemical and bioenergy productions.

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CHAPTER II
Lipase-catalyzed ethanolysis for biodiesel production of untreated palm oil mill effluent (POME)

II.1. 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 (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] .

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*. [16]
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. [18].
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

This study investigates the use of liquid lipase in the transesterification reaction between 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 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 (Fig. 1).

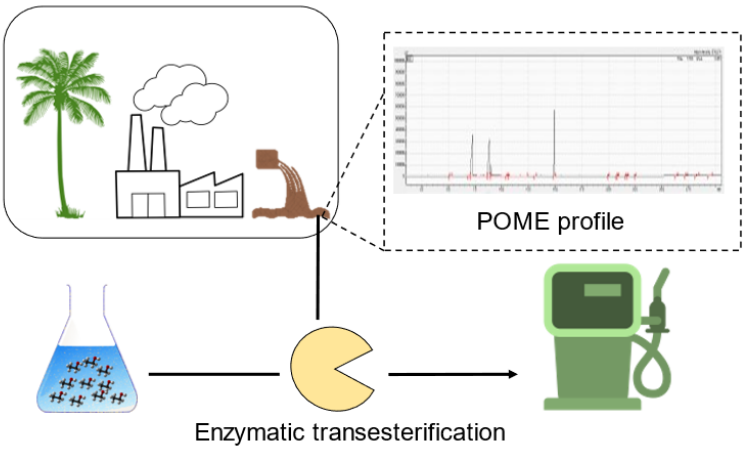

Fig. 1. The scheme of POME utilization by enzymatic transesterification using low ethanol concentration.

II.2. Materials and Method

II.2.1. 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).

II.2.2. 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.

II.2.3. 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 $^{\circ}$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 ^{o}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 ^{o}C for 2 min, increased to 350 ^{o}C at a gradient of 10 ^{o}C/min, then 370 ^{o}C at 7 ^{o}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; [19]

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

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

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.

II.2.4. 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 $p \leq 0.05$.

II.3. Results and Discussion

II.3.1. 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 2 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 [20]. 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 [21].

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.[22] 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).[23] 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.

Table 2. Analyzed parameters for characterization of POME

Parameters	Unit	Content[s, 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

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

[b] $p < .05$.

II.3.2. 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 2. 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 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.

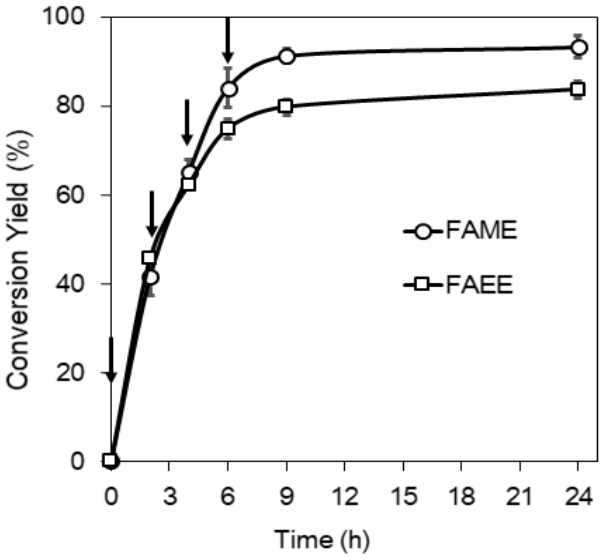

Fig. 2. Comparison of methanolysis and ethanolysis towards biodiesel synthesis from POME. Reaction conditions: lipase loading (2100 U), water (5 % v/w), total reaction time (24 h), oil to alcohol ratio (1:4), temperature (40 °C) and stirring speed (500 rpm).

II.3.3. 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 3a. 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 statistical analysis showed that the yield of FAEE was significantly affected ($p < 0.05$) by the different concentration of lipase. Based on the results, 7000 U of lipase was optimal loading for the production of FAEE. However, high enzyme loading results in high production cost of biodiesel, therefore, the 2100 U lipase loading, which showed a close yield of 83.64 ± 1.93 % (w/w), was used for subsequent experiments.

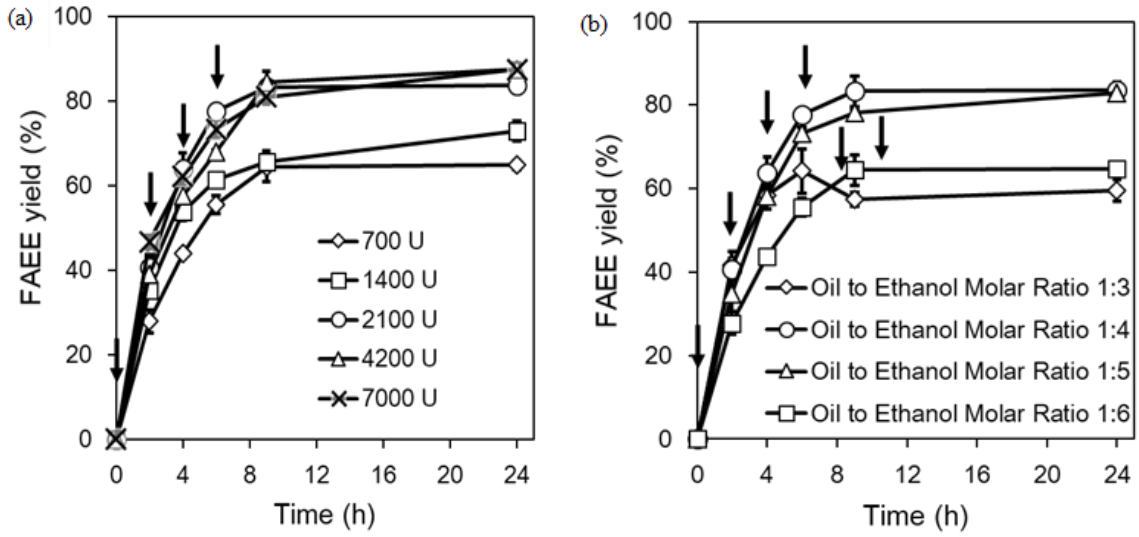

Fig. 3. FAEE production from POME with varying (a) lipase loading and (b) feedstock to ethanol molar ratio. Reaction conditions: excess water (5 % v/w), total reaction time (24 h), temperature (40 °C) and stirring speed (500 rpm).

II.3.4. Effect of Oil to Ethanol Molar Ratio on FAEE Production from POME

Experiments were performed to evaluate the synthesis of biodiesel by varying the molar ratio of oil to ethanol at four different levels, 1:3, 1:4, 1:5, and 1:6. Stoichiometrically, a 1:3 (TAG: ethanol) molar ratio is required for complete conversion to FAEE. As shown in Fig 3b., the FAEE conversion at 1:3 molar ratio was 59.61 ± 2.64 % (w/w). This was significantly lower than the theoretical yield. Enzymatic transesterification is known to be a reversible reaction, thus, as ester content increases, the equilibrium shifts to the dissociation of the products back to the reactants. An excess amount of ethanol is used to drive the equilibrium to the production of esters.[24] FAEE yield from POME improved significantly to 83.64 ± 1.93 % (w/w) by the addition of an extra molar equivalent of ethanol. The yield of FAEE was significantly affected ($p < 0.05$) by the different molar ratio between oil and ethanol.

However, according to the Ping-Pong Bi Bi mechanism which generally explains the enzymatic transesterification of oils, alcohol molecules can directly bind with enzyme and block the binding of substrate leading to a dead-end enzyme-alcohol complex in a competitive inhibition mechanism [25,26]. Short chain alcohols are also known to denaturize proteins, which are the main component of lipase. Consequently, 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.

II.3.5. 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. 4 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 (Fig. 5), leading to a much slower rate and low final yield.

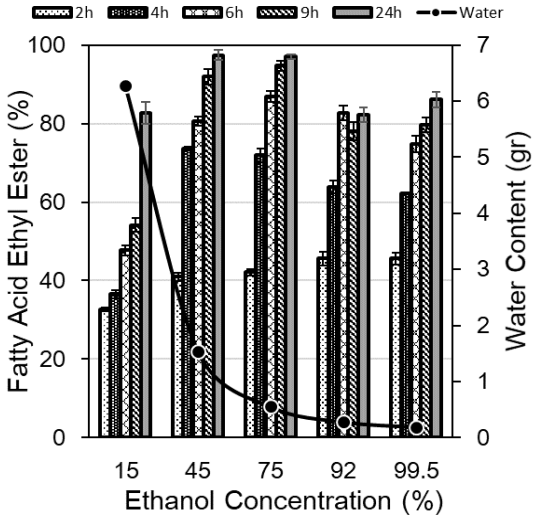

Fig. 4. Effect of ethanol dilution on FAEE production from POME. Reaction conditions: lipase loading (2100 U), excess water (5 % v/w), the total reaction time (24 h), temperature (40 °C) and stirring speed (500 rpm).

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 (Fig. 6). 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 4th molar equivalent after the 6th 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.

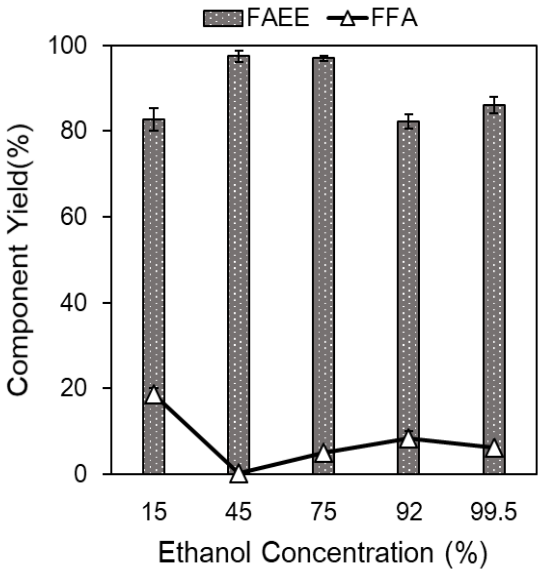

Fig. 5. FFA residue at 24 h from variation of ethanol dilution. Reaction conditions: POME to ethanol ratio (1:4), lipase loading (0.3 % v/w), water (5 % v/w), the total reaction time (24 h), temperature (40 °C) and stirring speed (500 rpm).

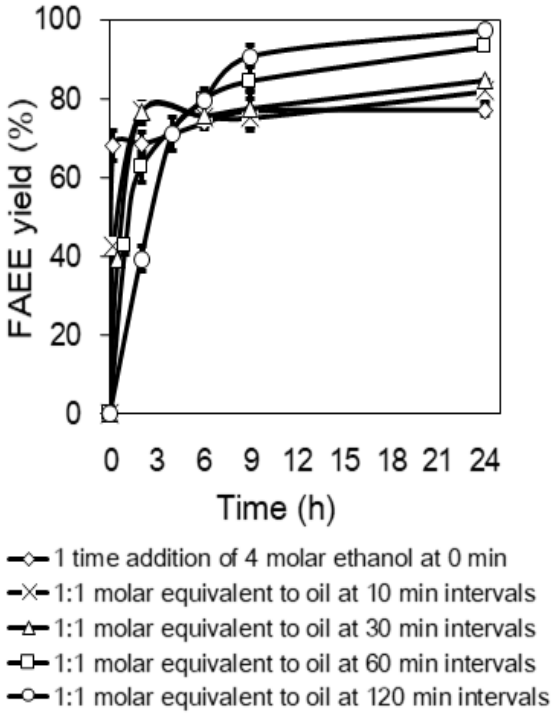

Fig. 6. Effect of ethanol addition rate under process parameter: ethanol concentration (45%), POME to ethanol molar ratio (1:4), lipase (0.3%), water addition on lipase (4.7%), the total reaction time (24 h), temperature (40 °C) and stirring speed (500 rpm).

II.3.6. 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 7. 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} [27].

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).

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 ºC	kg/m^3	D1298	868.29 ± 3.48	860 - 900
Viscosity at 40 ºC	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)

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

[b] $p < .05$.

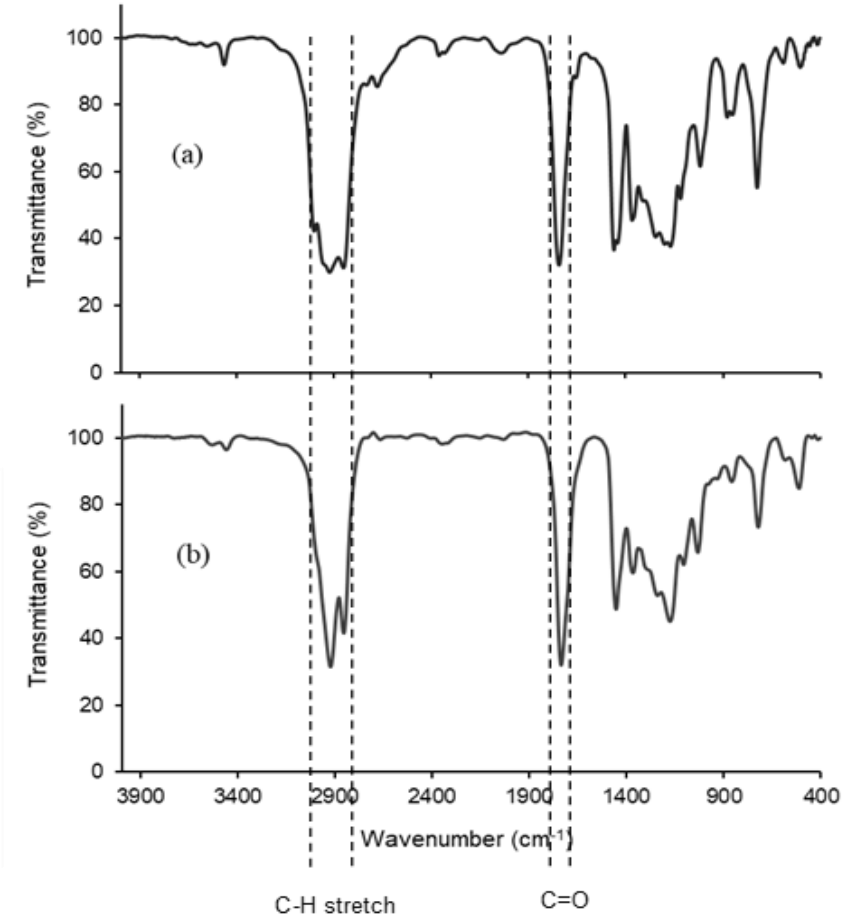

Fig 7. ATR-FTIR spectra from (a) commercial biodiesel and (b) biodiesel from POME.

II.4. 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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CHAPTER III
Utilizing palm oil mill effluent (POME) for the immobilization of *Aspergillus oryzae* whole-cell lipase strains for biodiesel synthesis

III.1. Introduction

Lipases (EC 3.1.1.3) are hydrolases that act on ester bonds in carboxylic esters [1–3]. Lipases play an important role in the breakdown of fats and oils with a subsequent release of long-chain triglycerides, diglycerides, monoglycerides, or free fatty acids [4–6]. Lipases react in a wide range of reversible conversion reactions, which is why they have been studied extensively for potential industrial applications such as biocatalysts in biodiesel synthesis, in oleochemicals, in pharmaceuticals, in biomedical assays, and for use in food additives [7–9]. Lipases are derived from nature and can be produced by microbial fermentation for different applications [10,11]. Lipases are easily cultivated, can catalyze a wide variety of hydrolytic and synthetic reactions, and offer a variety of catalytic activities [12,13].

Lipase B from *Candida antartica* (CALB) is a versatile enzyme that catalyzes esterification, and converts free fatty acids into alkyl esters [14,15]. CALB exhibits a high degree of substrate selectivity both concerning regioselectivity and enantioselectivity [16]. CALB also maintains stability under high temperature, and provides a high level of activity in water-lipid interfaces [17–19]. The best utilization of CALB as a biocatalyst is achieved either by using a solid carrier or by modification into solids for possible repeated uses, which is referred to as immobilization [2]. This technique can improve the catalytic stability and activity of lipase.

Nevertheless, a drawback to applying this process is the cost of the lipase immobilization medium. The development of efficient cultivation using the waste medium from agro-industrial waste is, therefore, necessary to reduce the cost. Palm oil is becoming a fast-growing and essential agro-industrial vegetable commodity in countries with tropical climates such as Indonesia, Malaysia, and Thailand. The palm oil industry is one of the most significant generators of agricultural waste, particularly palm oil mill effluent (POME) [20]. Each ton of crude palm oil (CPO) production will produce about 5 - 7.5 tons of POME [21]. Generally, POME refers to the liquid waste effluent resulting from various stages of palm oil processing including sterilization and digestion. POME is a thick, brownish fluid consisting of dirt, cellulosic materials and highly degraded oil [22]. POME also contains 18,000 mg/L of suspended solids and 40,500 mg/L of total dissolved solids [23,24].

Fig. 1. Bioprocess concept of utilizing palm oil mill effluent (POME) for the immobilization of *Aspergillus oryzae* whole-cell lipase strains for biodiesel synthesis

Treatment and disposal of POME have become serious environmental problems. The fat fraction of POME must be removed because it is a hazardous pollutant that is highly toxic to aquatic organisms due to high levels of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) [25,26]. In order to preserve the environment, an efficient and sustainable management system in the treatment and utilization of these by-products is needed. POME can be a reliable nutrient for microorganisms because it has a high organic content that includes carbohydrates, proteins, nitrogenous compounds, lipids, and minerals [27–29]. There have been many attempts to use POME as a basal medium for microbial production, but a use for the fat fraction of the wastewater has yet to be found (Table 1). Hermansyah *et al.* [12] utilized POME as a medium for cultivating *Pseudomonas aeruginosa* to produce lipase using submerged fermentation. In the present study, however, the fat fraction of POME was investigated as a basal medium that could be used for whole cell lipase immobilization for direct application as a biocatalyst in biodiesel production.

On the other hand, POME could also be utilized as a sustainable feedstock for biodiesel. In our previous research, the utilization of untreated POME as raw material for biodiesel was established [30,31]. Ultimately, the present study aimed not only to investigate the simultaneous cultivation and immobilization of *Aspergillus oryzae* expressing various lipases using POME as a carbon source but also to determine the utilization and valorization of untreated POME for biodiesel using this recommended system for whole-cell lipase-immobilization with low ethanol concentration (Fig. 1). In addition, we also evaluated the reusability of immobilized lipase in the presence of high levels of free fatty acids when using the residual oil of POME for biodiesel synthesis.

Table 1. Comparison study using POME as the nutrient in a basal medium

Strains	Basal Medium	Process	Use	Reference
Pseudomonas aeruginosa	POME, olive oil (0.2 %, v/v), peptone (0.5 %, w/v), $CaCl_2$. $2H_2O$ (10 mM), Tween 80 (0.7 %, v/v)	The submerged fermentation	Dry extract lipase	[12]
Aspergillus terreus (Fungi)	POME, peptone (0.05), KH_2PO_4 (0.14), $CaCl_2.2H_2O$ (0.0397), $MgSO_4.7H_2O$ (0.03), urea (0.01), CM-cellulose sodium salt (1.0), $FeSO_4.7H_2O$ (0.000915), $MnSO_4.H_2O$ (0.000179), $ZnSO_4.7H_2O$ (0.00025) and $CuSO_4$ (0.0014) in % w/v	Flask fermentation	Protease production	[27]
Bacillus cereus MF661883	POME (25 %, 50 %, 100 % v/v)	Flask fermentation	Lipid production	[28]
Anaerobic consortia and *Clostridium beijerinckii*	POME, supplemented with sucrose (7.5 g/L)	Hungate tubes dark fermentation	Biological hydrogen production	[32]
Chlorella sorokiniana CY-1 (Microalgae)	POME (30 % v/v), supplemented with 200 mg/ L of glucose, urea and glycerol	Novel-designated photobioreactor	Biomass and lipid production	[33]
Phaeodactylum tricornutum (Microalgae)	POME	Culture flask fermentation	Producing sulfated extracellular polysaccharide (eSPS)	[34]
Endophytic fungi	POME (25 – 75 %), yeast extract (0.125), peptone (0.125), potassium dihydrogen phosphate (0.03) in % w/v	Conical flask fermentation	Lipid production	[35]
Candida Antartica Lipase B *Fusarium heterosporum Bacillus thermocatenulatu*	POME (2.0), polypeptone (2.0), KH_2PO_4 (0.5), $NaNO_3$ (0.1), and $MgSO_4.7H_2O$ (0.05) in % w/v	Flask fermentation	Immobilized whole cell biocatalysts	This study

III.2. Materials and Method

III.2.1. Materials and Strains

The POME was obtained through collaborative research with PT. Agricinal Palm Oil Mills (Bengkulu, Indonesia). *A. oryzae* NS4 (*niaD*$^-$, sC$^-$) derived from the wild-type

strain RIB40, was used as the recipient of transformation. The construction of pSENSU-CALB for expressing *C. antartica* lipase B (r-CALB) was described in a previous paper.[14] Polyurethane foam BSPs (Bridgestone Corp., Osaka, Japan) measuring 6 x 3 x 3 mm cuboids were used as the matrix for immobilization of *A. oryzae* expressing r-CALB. Biodiesel oil-based fuel-palm was used as a comparison fuel for this study and was procured from the Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and from Sigma-Aldrich (Tokyo, Japan).

III.2.2. Immobilized *A. oryzae* Whole-Cell Biocatalyst Preparation

A recombinant *A. oryzae* strain was initially cultured on a Czapek-Dox (CD) agar plate at 30 ^{o}C for 6 – 7 days, and then the spores were harvested with 5 ml of distilled water.[36] The spore solution was aseptically inoculated into a 500 ml Sakaguchi flask containing approximately 200 pieces of BSP in 100 ml of medium. Each culture media composition mainly contained 2 g polypeptone, and other nutrients (0.5 g KH_2PO_4, 0.1 g $NaNO_3$, and 0.05 g $MgSO_4.7H_2O$). For the carbon source, whole POME without separation and glucose were used in the culture media by the same weight (2 g) as the criteria for the cultivation. In addition, palm and olive oil were also used as a substrate for comparing the growth of the cell in fatty acid media. The fungal strain was cultivated at 30 ^{o}C on a bioshaker (TAITEC GBR-300, TAITEC Corporation, Tokyo, Japan) at 150 oscillations per min for 96 h. After cultivation, cells of *A. oryzae* with membrane-bound lipases were naturally immobilized in the pores of the BSPs, and the immobilized whole cells were collected via simple filtration using a strainer, 100 µm pores, above a beaker glass to separate the immobilized cells and the liquid medium. Then, the immobilized

cells were washed with 50 ml distilled water twice at room temperature, and lyophilized for 48 h using a Benchtop Freeze Dryer (Labconco Corp., Kansas, USA). The immobilized whole cells expressing lipase were used as biocatalysts for esterification. The number of immobilized cells on the BSPs was calculated by the difference between the final weight of the immobilized BSPs and the initial weight of BSPs used. The specimen microphotographs of the *A. oryzae* expressing *C. antartica* lipase B in POME and glucose medium were recorded using a JEOL JSM-7100F field-emission scanning electron microscope (FE-SEM) (JEOL Ltd., Tokyo, Japan).

III.2.3. Catalytic Activity Assay of Immobilized Lipase

Hydrolysis was used to determine the catalytic activity of lipase.[37] The reaction mixture contained 2 g of olive oil, 9 ml of 0.1 M acetate buffer pH 5.6, and 1 ml of 0.05 M $CaCl_2$ in a 50 ml screw-cap bottle in a water bath system equipped with a Teflon-coated magnetic stirrer. The immobilized lipase was incubated at 40 ^{o}C under 400 rpm and was terminated by the addition of ethanol after 10 min. The obtained emulsion was titrated against 0.1 M NaOH until a pH of 10 was reached. One unit (U) of hydrolysis was defined as the amount of lipase (mg) that would liberate 1 µmol of free fatty acids per min from olive oil at pH 10.

III.2.4. Extracellular Protein Assay

A Pierce^{TM} bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Tokyo, Japan) is normally used to determine the protein concentration (unrecovered protein) that remains inside the culture filtrate following separation from the immobilized cells. The principle of this method relies on the ability of protein to reduce Cu^{+2} to Cu^{+1}

in an alkaline solution (the biuret reaction), which results in the formation of a purple color by BCA. The absorbance of the product was measured at A562 nm (PerkinElmer Plate Reader, Osaka, Japan) using the gradient curve of a standard BCA kit.

III.2.5. Lipase-Catalyzed Alcoholysis of POME

Alcoholysis of the POME using r-CALB and Novozym 435 was conducted at 40 ^{o}C in a thermo block rotator (NISSIN, Tokyo, Japan) at 35 rpm for 96 h. The alcoholysis was performed using 10 mL screw-capped cylindrical glass tubes. The reaction mixture consisted of 4 g of POME and 446 U of immobilized lipase used for r-CALB, and Novozym 435, to which 0.615 ml of a 45% v/v ethanol concentration or 0.278 ml of a 99.5 % v/v ethanol concentration were added stepwise at 0, 24, 48, and 72 h. To determine the amount of fatty acid ethyl ester throughout the reaction, 100 µl samples were taken every 24 h.

III.2.6. Reuse and Recycling of r-CALB for Biodiesel Production

To verify the reusability of the immobilized lipase, batch reactions were carried out using 10 mL screw-capped cylindrical glass tubes. Following the 96 h alcoholysis procedure, the synthesized biodiesel was poured and separated from the lipase. The immobilized whole-cell r-CALB was directly reused with no treatment and applied to 4g of fresh POME as feedstock, to which 0.56 g of 45% v/v ethanol concentration were added stepwise every 24 h. For 960 h of repeated batch reactions, biodiesel samples were taken at 24 h reaction intervals for chromatography analysis to determine the ethyl ester content.

III.2.7. Analytical and Statistical Methods

Samples taken at specified times were centrifuged at 12,000 x g for 5 min at 15 °C, and the compositional analysis of biodiesel produced during the reaction in the upper layer was performed via gas chromatography equipped with a Flame Ionization Detector (GC-FID) (GC-2010, Shimadzu, Kyoto, Japan) and a high-temperature capillary column (15m x 0.25 mm x 0.15 mm) (ZB-5HT, Phenomenex Inc., USA). During the analysis, Helium was employed as the carrier gas at a flow rate of 57.5 ml min^{-1}. The oven temperature of injector and detector were set at 320 and 370 °C, respectively. 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^{-1}, then 370 °C at 7 °C min^{-1} maintained at this temperature for 10 min. The retention times for fatty acids were identified using standard solutions of the respective fatty acid alkyl esters. The ethyl ester composition was reported as the percentage of ethyl ester in the sample using tricaprylin as an internal standard (Nacalai Tesque, Inc., Kyoto, Japan). The ethyl ester conversion was calculated using the gradient of the curves for the respective esters [38].

The total organic carbon and total carbon in the POME was analyzed using a total organic carbon analyzer (TOC-L, Shimadzu Corp., Tokyo, Japan). A functional group comparison between commercial biodiesel and POME biodiesel was characterized by Attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR) analysis using a Shimadzu AIM-900 Infrared Microscope equipped with an ITRraces-100 (Shimadzu Corp., Tokyo, Japan) [30].

The following parameters of POME (Table 2) were determined according to the methods of the American Oil Chemist's Society (AOCS Official Method) for the analysis

of oils and fats: the acid, saponification, and iodine values were performed following AOCS Method Cd 3a-63, Cd 3-35, and Cd 1-25, respectively.

All experiment values are presented as the average of triplicate readings to further verify the results. The values are expressed as the mean ± the standard deviation as calculated based on Eq. (1).

$$SD = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})} \quad [1]$$

In Eq. (1), SD represents the standard deviation, N, is the size of the sample dataset for the experiments, x_i, is the sample dataset, and $\bar{x}$ is the average value of the sample dataset

The data were subjected to one-way ANOVA using GraphPad Prism 9 (GraphPad Software, California, USA) to evaluate the significant differences.

III.3. Results and Discussion

III.3.1. Characteristics of POME for Immobilized Lipase Medium and Biodiesel Production

POME is a complex material with a composition that varies depending on processing of the palm oil. In most cases, POME contains high concentrations of organic matter such as free fatty acids (FFAs), proteins, carbohydrates, and minerals. This organic matter could be utilized during fermentation as a carbon source. POME samples were characterized (Table 2) mainly to determine the total organic carbon available. This particular POME contained 15.07 ± 0.01 g L^{-1} of total organic carbon (TOC) which is quite high compared to the other studies such as Ding *et al.*,[39] which reported that the initial TOC of POME for the cultivation of microalgae was 0.725 g L^{-1}, or Zaied *et al*.

[40] which reported that the initial TOC of POME to enhance bioenergy production by co-digestion was 9.68 g L^{-1}.

The presence of organic compound indicated the possibility that this POME could serve as a suitable substrate to nourish and grow the whole cells needed for immobilized fermentation. Many studies have tried to utilize POME as a nutrient for culture fermentation medium (Table 1). All studies have used the aqueous part of POME to produce microbes whereas the present study used the entire amount of untreated POME to immobilize recombinant lipase.

Table 2. Characterization of POME

Parameters	Unit	Content [a, b]
Acid value	mg KOH $(g\ oil)^{-1}$	169.72 ± 0.46
Saponification value	mg KOH $(g\ oil)^{-1}$	211.70 ± 8.51
Iodine value	g I_2 $(100\ g\ lipid)^{-1}$	53.54 ± 1.50
Total Carbon	g L^{-1}	15.82 ± 0.01
Total Organic Carbon	g L^{-1}	15.07 ± 0.01

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

[b.] $p < .05$.

III.3.2. Effect of Nutrient Supplementation on *A. oryzae* Expressing r-CALB Growth

In the present study, the immobilization efficiency was evaluated and is reported in terms of the total cell yield and the catalytic activity of the immobilized cells. The *A. oryzae* expressing r-CALB was able to grow in every nutrient medium even when other carbon sources were substituted for glucose. This suggests that this strain is capable of utilizing the organic compounds found naturally in oil as a source of nutrients as shown in Fig. 2. With the same 1 mL of inoculated spores in the cultivation medium, the highest

level of cell aggregation growth was achieved using a POME medium, 2.36 ± 0.02 g, as the carbon nutrient compared with using other mediums as a carbon source such as glucose (in accordance with the protocol culture), olive oil, and palm oil. This is based on the degradation of the organic compounds in POME that serves as a suitable substrate for the growth of cells. It showed that TOC decreased 26.60% w/w in POME media (Table 3). This TOC reduction could be attributed to the degradation of hydrocarbons by *A. oryzae* which facilitated increased the cell growth that could have resulted into higher substrate utilization.

Table 3. The TOC of culture media before and after immobilization

Carbon Source of Culture Media	TOC (g/L)		Consumption rate (%)
	Before Immobilization	After Immobilization	
POME	9.69 ± 0.06	7.12 ± 0.03	26.60
Glucose	12.43 ± 0.10	10.68 ± 0.06	14.12

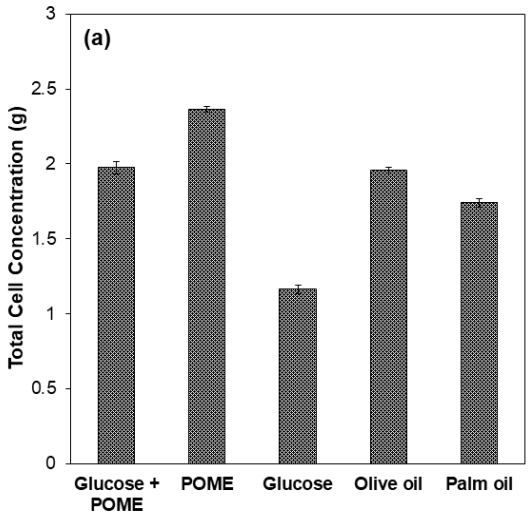

Fig. 2. Total cell yields of *A. oryzae* whole cells expressing r-CALB growth in culture media.

The hydrolytic activity of r-CALB was evaluated to test the functionality of the produced biocatalyst. The r-CALB cultivated in the mixing culture medium of POME and glucose (50 % w/v) offered the highest hydrolytic activity of 2.40 ± 0.03 U mg^{-1} (Fig. 3a). The POME culture medium achieved hydrolytic activity of 2.23 ± 0.02 U mg^{-1}, which was higher than that of the glucose culture medium, 1.61 ± 0.02 U mg^{-1} (*P*=.007).

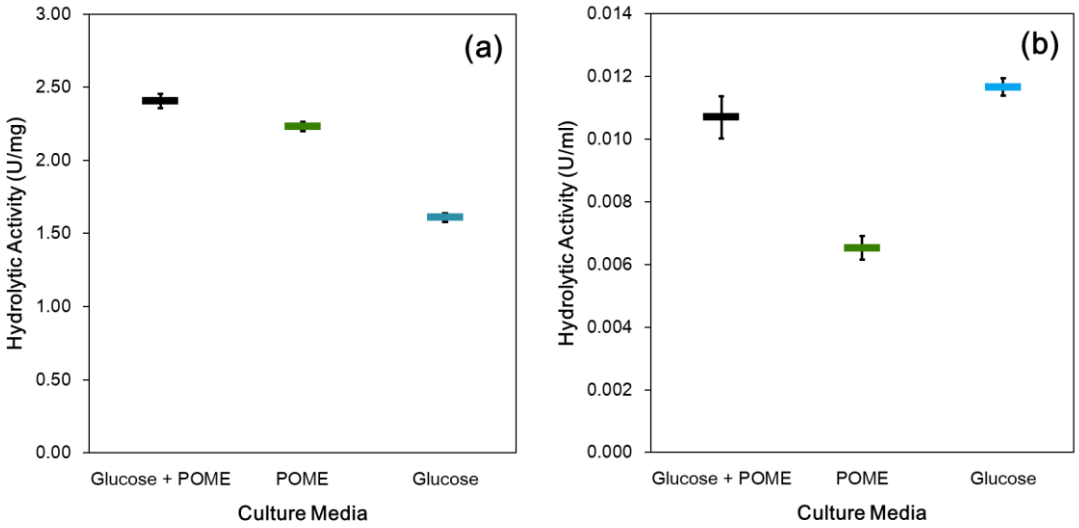

Fig 3. The hydrolytic activity of (a) immobilized r-CALB and (b) remaining medium of the immobilization

This indicated that the hydrolytic activity of the immobilized r-CALB were significantly affected by the kinds of carbon sources in the culture media. The high level of hydrolytic activity observed in whole cells immobilized in media containing POME indicates that substrate-related compounds intrinsic to POME may possess superior characteristics that induce lipase activity in immobilized whole cells. In addition, the hydrolytic activity in remaining POME media was lower than that in other media presented in Fig. 3b. This is in accordance with the results of protein that remained in the culture medium (Fig. 4.) where protein remained in POME culture medium was lower than that in glucose.

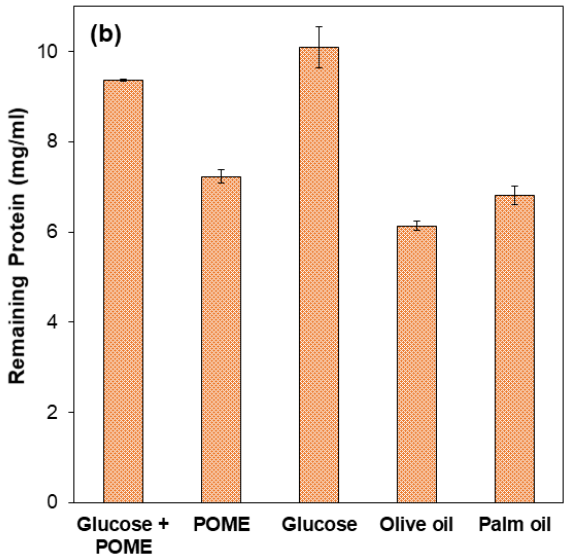

Fig. 4. Remaining protein of the *A. oryzae* expressing *C. antartica* lipase B (r-CALB) culture medium.

Scanning electron microscopy (SEM) was used to visualize the morphological features of the immobilized r-CALB to confirm success in a cell setting within a matrix, as presented in Fig. 5. It is apparent that the cells cultivated in POME (Fig. 5b) were adsorbed inside the polyurethane foam similar to cells cultivated in a glucose medium (Fig. 5c).

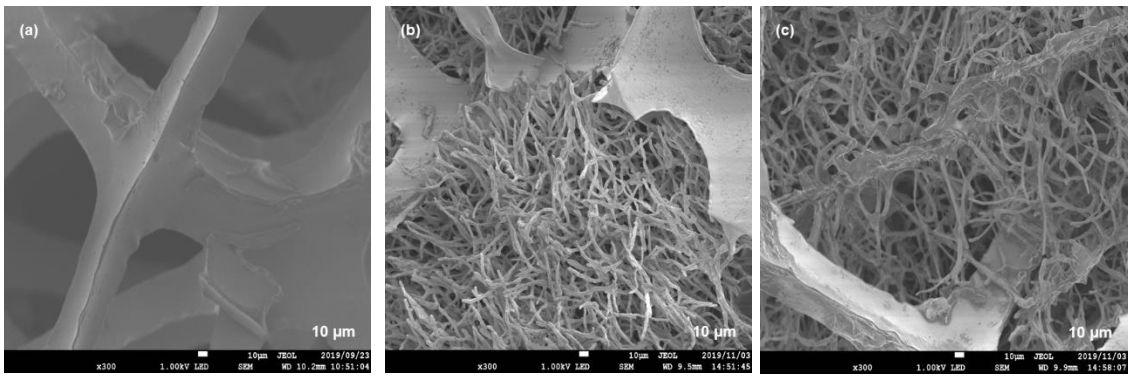

Fig. 5. SEM image of (a) empty polyurethane foams, (b) immobilization of *A. oryzae* r-CALB in glucose medium, (c) immobilization of *A. oryzae* r-CALB in POME medium.

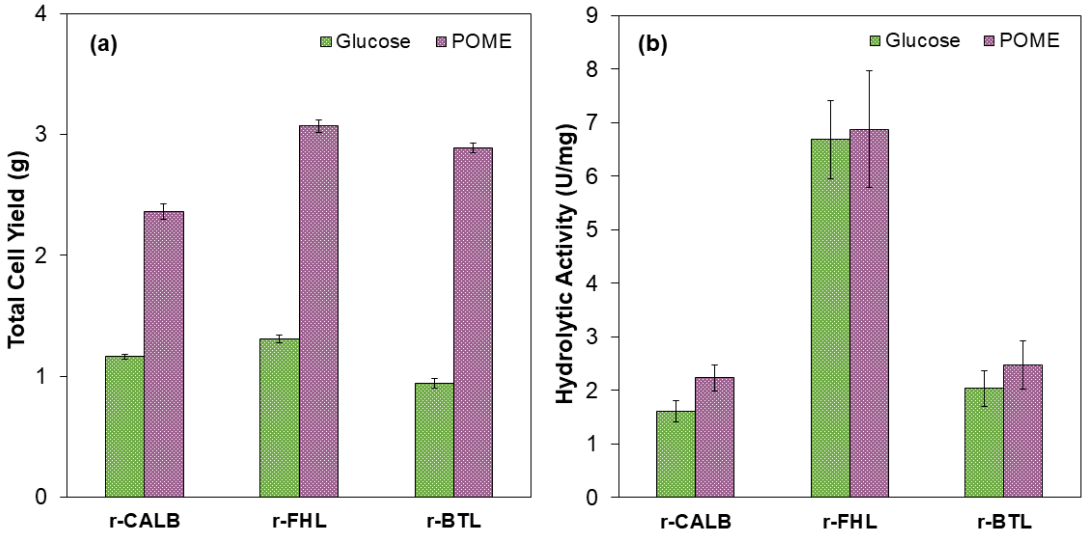

Fig. 6. Effect of POME culture medium with recombinant lipase by *A. oryzae* expressing *C. antartica* lipase B (r-CALB), *F. heterosporum* (r-FHL), *B. thermocatenulatus* (r-BTL) on total cell yield and hydrolytic activity.

The cell aggregation efficiency of r-CALB was compared with other *A. oryzae* expressing lipase strains cultivated in a POME medium such as *Fusarium heterosporum* lipase (FHL) and *Bacillus thermocatenulatus* lipase (BTL), as presented in Fig. 6. All strains showed a higher total cell yield (Fig. 6a) and higher levels of hydrolytic activity (Fig. 6b) compared with that of the glucose culture medium. Although there were no significant differences in terms of lipase activity (Fig. 6b), these results revealed that the recombinant filamentous fungi lipase strain cultivated in a POME medium could be more efficient for lipase immobilization.

III.3.3. Application of Immobilized r-CALB Cultivated in POME Medium for Biodiesel Production

POME containing 93.35 ± 0.25% FFA, 1.50 ± 0.07% MG, 1.43 ± 0.02% DG, and 3.38 ± 0.08% TG was subjected to esterification using r-CALB immobilized in a POME

medium. Although r-FHL indicated higher hydrolytic activity, r-CALB was more efficient at converting FFA in the POME to produce alkyl esters in the presence of high amounts of water at a better rate than other lipases such as r-FHL or r-BTL.[14,41] On the other hand, the esterification of POME requires a temperature as high as 40 ºC, at which r-FHL could lose its catalytic activity [38,42]. The comparison of transesterification reaction data between r-CALB and r-FHL was presented in Fig. 7. It showed that the reaction rate in transesterification using r-FHL was much lower than that using r-CALB. The high amount of residual ethanol and the high temperature in the reaction mixture may be responsible for the inactivation of r-FHL. Therefore, in the present study, r-CALB was used and compared with a commercial immobilized lipase (Novozym 435) with high catalytic activity, to evaluate the catalytic performance of r-CALB in esterification when using POME as a feedstock, as shown in Fig. 8.

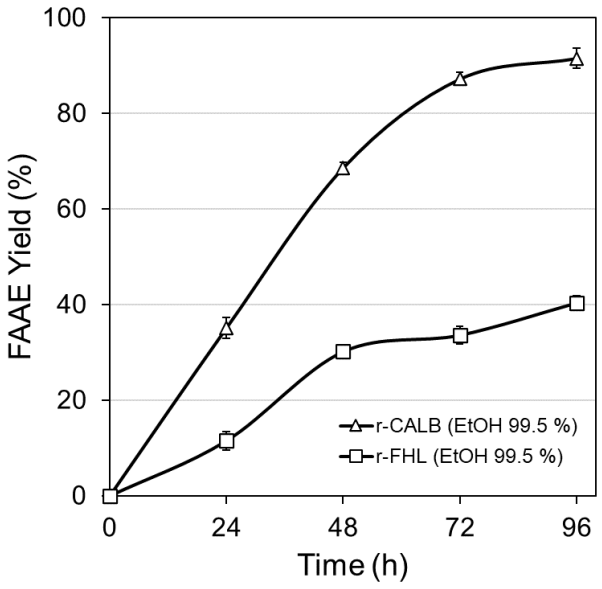

Fig. 7. Ethanolysis of POME using r-CALB and r-FHL cultured in POME medium. Reaction conditions: 4 g POME, 446 U of immobilized lipase, 40 ºC reaction temperature, 35 rpm agitation, and 1 to 4, oil to ethanol molar ratio added at 0, 24, 48, and 72 h.

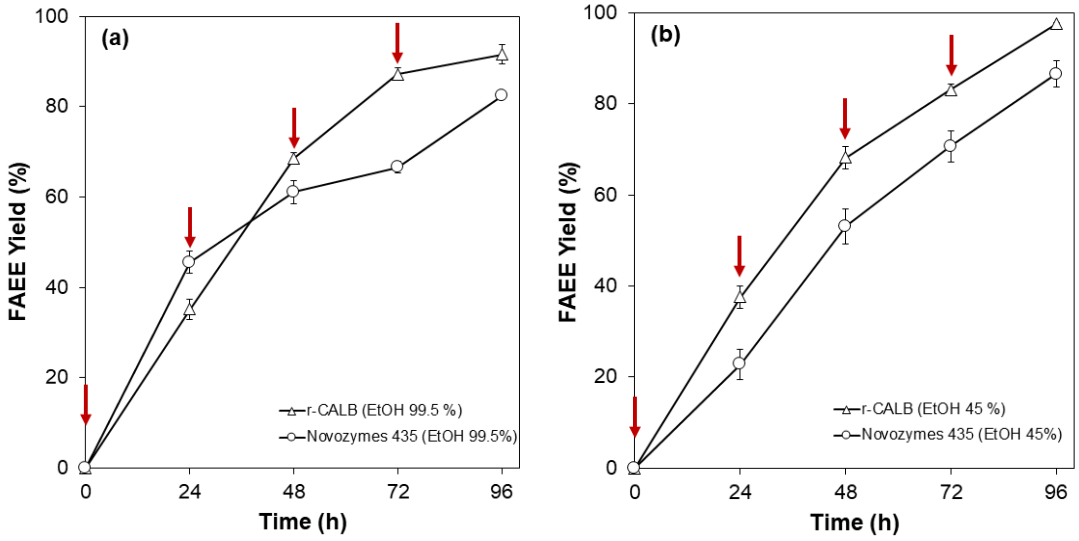

Fig. 8. Ethanolysis of POME using r-CALB cultured in POME medium and Novozym 435 in (a) 99.5% v/v ethanol concentration and (b) 45% v/v ethanol concentration. Reaction conditions: 4 g POME, 446 U of immobilized lipase, 40 ºC reaction temperature, 35 rpm agitation, and 1 to 4, oil to ethanol molar ratio added at 0, 24, 48, and 72 h. Arrows indicate ethanol addition time.

The r-CALB achieved 91.57 ± 2.17% w/w FAEE, which was higher than that obtained by Novozym 435 (Fig. 8a). The yield of this study was higher than that of other studies such as Adachi *et al.* [14] which obtained 90.5% (w/w) ME yields by using the r-CALB strain that cultured in protocol media as biocatalyst and hydrolysate palm oil as substrate. That result revealed the effectiveness of using r-CALB for transesterification of POME that contains high levels of FFA. Many studies have verified the usability of r-CALB as a biocatalyst [41]. This reaction, however, was far from meeting the FAAE standard for biodiesel synthesis, which is 96.5 % w/w. Optimization of biodiesel from POME was accomplished in our previous research using a lower concentration of ethanol

[30]. Based on the results of our previous research, r-CALB was used in a comparative study with Novozym 435 (a commercially available hydrophobic acrylic resin-immobilized CALB) to ascertain its effectiveness in catalyzing the ethanolysis of POME (Fig. 8b). The FAEE yield increased from 91.57 ± 2.17 to 97.52 ± 0.21% w/w. The results indicated that these processes could improve the FAEE rating via the dilution of ethanol, which suppresses the deactivation effect on lipase.

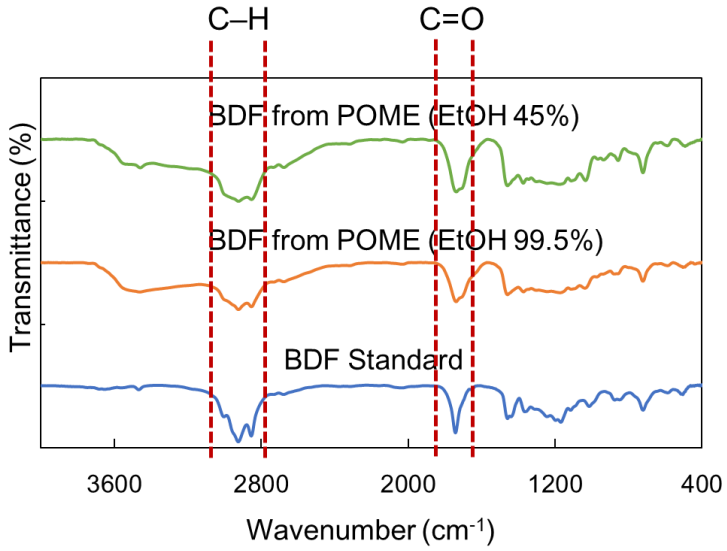

Fig. 9. FTIR spectra of *A. oryzae* r-CALB-catalyzed biodiesel in 99.5 and 45% v/v ethanol concentrations compared with the biodiesel standard.

FTIR was used to characterize the biodiesel produced in this study. Fig. 9 lists the results of the refined palm-oil biodiesel examination as a reference in the FTIR analysis. Mid-infrared spectral data (4000-400 cm^{-1}) were used to identify the functional groups of organic and inorganic bonding. The functional peaks in the sample indicate similar spectra features. The peaks indicated the symmetric and asymmetric stretching vibrations of -C-H alkane groups present in FAEE at 3,040 to 2,780 cm^{-1} [43]. A strong peak of -C=O stretching around 1,740 cm^{-1} in all samples was attributed to carbonyl ester groups in biodiesel synthesis. The presence of $-CH_3$ groups in biodiesel was indicated at 1,450

cm^{-1} [44]. The FTIR spectra for POME biodiesel showed characteristics similar to the reference biodiesel.

III.3.4. Reuse and Recycling of r-CALB for Biodiesel Production

In order to maintain production cost-effectiveness in industrial applications, the ability to reuse whole-cell biocatalysts for an extended period is extremely necessary. Ten cycles of the batch reaction were carried out using r-CALB grown in a POME medium. As a feedstock for esterification, POME produced a more than 95% w/w conversion to FAEE even after r-CALB was recycled ten times. As shown in Fig. 10, the final FAEE content in each cycle was slowly decreased to around 3% w/w, but an FAEE content of more than 95% w/w was maintained even after the 10^{th} batch cycle.

Even though, the hydrolytic activity of r-CALB decreased after 10 times reusability from 2.23 U/mg to 1.59 U/mg. The residual activity of lipase was 71.47 % inasmuch as the degradation of the hydrolytic activity was affected by the residual ethanol that induced the deactivation of the lipase. It indicated that a mild agitation could suppress attrition of the surface of r-CALB in the system which could maintain the stability during the long-term esterification. In addition, the presence of fatty acids in POME contributes to the high level of lipase stability in immobilized r-CALB during long-term esterification, which suggests that a continuous series of batches could be processed. Some studies have described the use of fatty acids to achieve higher levels of cell aggregation and lipase activity [37,38]. Hence, the process advantages of using POME either as a carbon source for a lipase-immobilized biocatalyst or as feedstock for biodiesel synthesis are maximized by using this continuous batch approach.

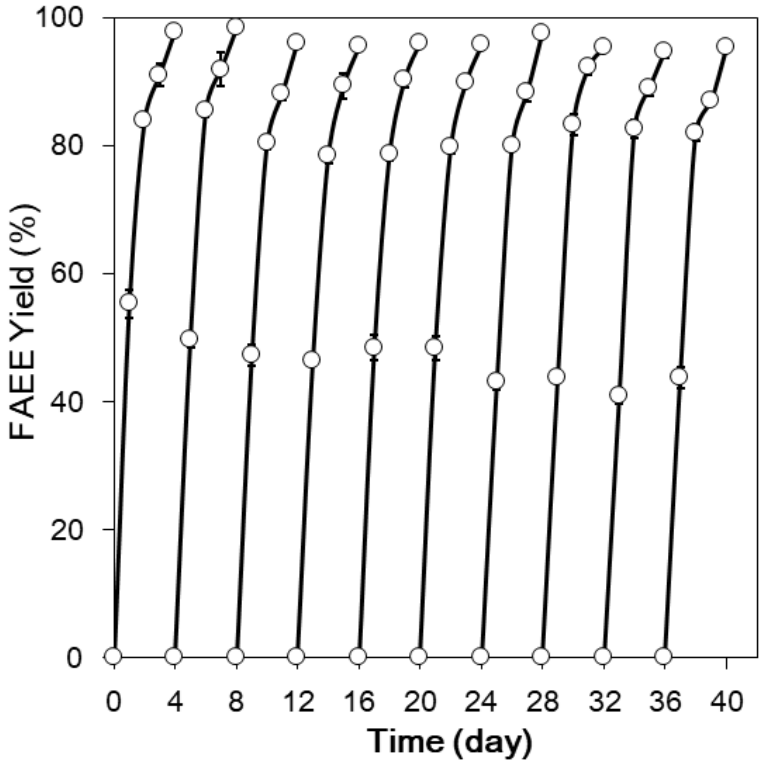

Fig. 10. Repeated fatty acid ethyl ester (FAEE) synthesis using POME as a feedstock with *A. oryzae* r-CALB cultured in a POME medium. Reaction conditions: 446 U of immobilized lipase (r-CALB), 4 g POME, 40 ºC reaction temperature, 35 rpm agitation, and 0.56 g ethanol (45 % v/v) added stepwise at a 24 h interval.

III.4. Conclusions

In this study, the potential of POME as a carbon source for immobilized recombinant lipase by *A. oryzae* was evaluated in terms of its biodiesel production. POME showed efficiency and significant enhancement of the cell growth of whole-cell lipases. These results suggest that POME is a suitable nutrient that can be used to cultivate *A. oryzae* expressing r-CALB. POME culture medium doubled the total amount of cell weight and improved the hydrolytic activity compared with the use of a glucose medium. Immobilized lipase r-CALB cultivated in a POME medium demonstrated excellent

potential as a biocatalyst for converting POME to biodiesel with a better rate of conversion than commercial Novozym 435 lipase. This biocatalyst preparation can be reused for more than 10 cycles, and produced an ethyl ester content of more than 95% even after the 10^{th} batch. Therefore, the utilization of POME both as a nutrient for cultivating lipase and as feedstock for producing biodiesel could increase the economic value of the industrial processes and provide additional incentive to apply this treatment that is inexpensive and sustainable.

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CHAPTER IV
Palm oil mill effluent (POME) as the supplemented carbon source for lipid production by *Lipomyces starkeyi* D35

IV.1. Introduction

In recent years, the utilization of single-cell oils (SCOs) has gained industrial interest due to their similar fatty acid structure and composition to vegetable oils, representing a source of other oleochemical products [1–3]. Oleaginous microorganisms, the third-generation feedstock, are currently under development since they can accumulate lipids more than 20 % of their dry weight, and genotypically in yeast by the presence of ATP-citrate lyase (ACL) [4–6]. Yeasts show high metabolic flexibility, faster growth rates, and easy to cultivate on large scales [7].

A number of oleaginous yeasts such as *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon,* and *Lipomyces* have been extensively studied for lipid accumulation [8]. In particular, *Lipomyces starkeyi* is a promising candidate to produce SCOs, and possesses high flexibility in carbon source utilization [9]. However, the production route indicates that 70% of the total medium cost for the fermentation has shown as the major factor hindering the SCOs production.

Palm oil mill effluent (POME) is a large volume of effluents with a low pH due to the organic and free fatty acids [10]. In addition, a high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) in POME creates environmental distress for the arable land [11,12]. Therefore, an effective method is required to utilize POME in other to preserve the environment. It has been presented in some recent reports that POME contains a significant amount of nutrients such as carbohydrates, proteins, and mineral

salts, which can stimulate the growth of SCOs [13]. This utilization could reduce the cost of the productions of microbial lipid because the major cost of lipid production is the cost of carbon source. In this study, POME is used as the carbon source to accumulate lipid under defined culture conditions with *L. starkeyi*. The lipid accumulation ability was analyzed by following its growth and lipid productivity with varying the type of medium. Moreover, the quality of SCOs were examined by evaluating their fatty acid profile.

IV.2. Method and Materials

IV.2.1. Materials

L. starkeyi D35 yeast strain (NBRC10381) was derived from the NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan. The yeast strain preserved in glycerol stock was revived by streaking onto a potato dextrose agar (PDA) plate. The cell was transferred on yeast extract-peptone-glucose (YPD) agar plate (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 15 g/L agar) for the short-term storage. YPD medium was prepared in a composition mixture of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose, and yeast extract-malt extract-peptone-glucose (YMG) was prepared in a composition mixture of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L glucose. POME was obtained from PT Agricinal Palm Oil Mills (Bengkulu, Indonesia). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and from Sigma-Aldrich (Tokyo, Japan).

IV.2.2. Lipid production

One colony from a YPD agar plate was inoculated into a 100 mL Erlenmeyer flask in 12 mL of medium. To evaluate the capability of POME as supplemented carbon source

for lipid production by *L. starkeyi* D35, 5 g/L POME was supplemented into media culture which was divided into 2 categories in each media cultures, with or without glucose. The fermentation was incubated in a bioshaker (Taitec GBR-300, Taitec Corp., Tokyo, Japan) at 190 oscillations per min for 96 h at 30 ºC. The samples were taken every 24 h to determine the dry cell weight (DCW) of biomass, lipid productivity, and fatty acid composition throughout the reaction.

IV.2.3. Dry cell weight determination

1 mL of sample taken at specified times was used to gravimetrically determine the DCW of the biomass. The cells were separated by centrifugation at 12,000 xg for 5 min at 20 ºC, washed with 1 mL distilled water twice at room temperature, and dried at -80 ºC. The frozen cells were lyophilized for 24 h using a Benchtop Freeze Dryer (Labconco Corp., Kansas, USA).

IV.2.4. Lipid determination from *L. starkeyi* D35

Total lipid accumulations were measured by Gravimetric analysis by Folch method. The 10 mg freeze-dried cells were transferred to 2.0 mL microvial with an O-ring sealed cap (Watson, Japan) containing 0.6 mm zirconia beads. Lipids were extracted by 1.5 mL Folch solvent (2 to 1 of chloroform to methanol, v/v). The cells were pulverized using a Shake Neo ver. 1.0 BMS-M10N21 (BMS, Tokyo, Japan) at 1500 rpm for 15 min. Cells were subsequently centrifuged at 12,000 x *g* for 5 min, the supernatant was removed, and the cells were washed with 1.5 mL distilled water. The cells were pulverized for the second time, and the filtrate was removed after centrifugation. The cells were dried up to

80 ºC to the constant weight. The number of lipids was determined by the differences between initial and final weight of the cells.

IV.2.5. Catalytic activity assay

The catalytic activity in the filtrate were evaluated using p-nitrophenyl butyrate (pNPB) as a chromogenic substrate. The composition mixture of 5 µL pNPB and 250 µL ethanol was dissolved into 50 mL distilled water. The hydrolytic activities were carried out at 37 ºC by incubating for 10 min in a Bioshaker (Taitec, Saitama, Japan). After incubation, 50 µL trichloroacetate (5 % w/v) was added to terminate the reaction. The absorbance of the product was measured using UV-Vis spectrophotometer (PerkinElmer Plate Reader, Osaka, Japan) at A400 nm using the gradient curve of p-nitrophenol (pNP) standard. 1 unit (U) of the catalytic activity was defined as the amount of lipase that liberates 1 µmol of pNP from pNPB per minute.

IV.2.6. Microscopic fluorescence visualization

The staining method using boron dipyrro-methene (BODIPY) was prepared to visualize the SCO production. 100 µL culture broth of *L. starkeyi* D35 was centrifuged at 12,000 x *g* for 5 min. The cells were washed with 0.9 % w/w phosphate saline buffer (PBS) pH 7.0 two times. After washing, the cells were suspended in 50 µL of 0.9 % w/w PBS pH 7.0. 2 µL BODIPY stock solution was added and mixed well, and the cells were kept in the dark place for 1 h. Afterwards, 10 µL cell suspension was placed on a digital inverted fluorescence microscope (Keyence Biorevo BZ-9000, Keyence, Osaka, Japan) to capture the fluorescence images.

IV.2.7. Fatty acid methyl esters analysis

To specify the fatty acid methyl esters (FAMEs) composition, transesterification was performed by following the protocol from fatty acid methylation kit (Nacalai Tesque, Inc., Kyoto, Japan). The extracted FAMEs were analyzed with a gas chromatography-mass spectroscopy (GC-MS QP 2010 Ultra, Shimadzu) instrument, equipped with a DB-23 capillary column 0.25 mm x 30 m (Agilent J&W Scientific, Santa Clara, CA, USA). During the analysis, helium was employed as the carrier gas at a flow rate 0.8 mL/min with 1:5 split ratios. The oven temperature of injector was set at 250 °C, and the column was configured at 50 °C for 1 min, increased to 190 °C at a gradient of 25 °C/min, then 235 °C at 5 °C/min maintained at this temperature for 4 min. FAMEs were identified and quantified by comparison of their retention times and peak areas with a Supelco 37 component FAME mixture standard from Sigma-Aldrich (Tokyo, Japan). Caprylic acid (C8:0) was used in each sample as the internal standard.

IV.3. Results and Discussion

IV.3.1. Effect of POME as supplemented carbon sources for biomass growth

The ability of *L. starkeyi* D35 to efficiently produce lipid by using POME as supplemented carbon source was evaluated in this study. Fig. 1 presents the fermentation of *L. starkeyi* in a complex medium w/o the presence of glucose and POME as an alternative substrate. YPD and YMG were used as the control media to evaluate and investigate the efficiency of *L. starkeyi* to accumulate fatty acids. The difference is YPD medium contained higher nutrients than YMG. Usually, glucose was used as the carbon source for the fermentation process. However, it showed that the cells only evolved but not produced lipid in the glucose medium although all the sugar has been consumed.

Therefore, these mediums usually proceeded as the pre-culture stage before the fermentation process if they used glucose as the carbon source.

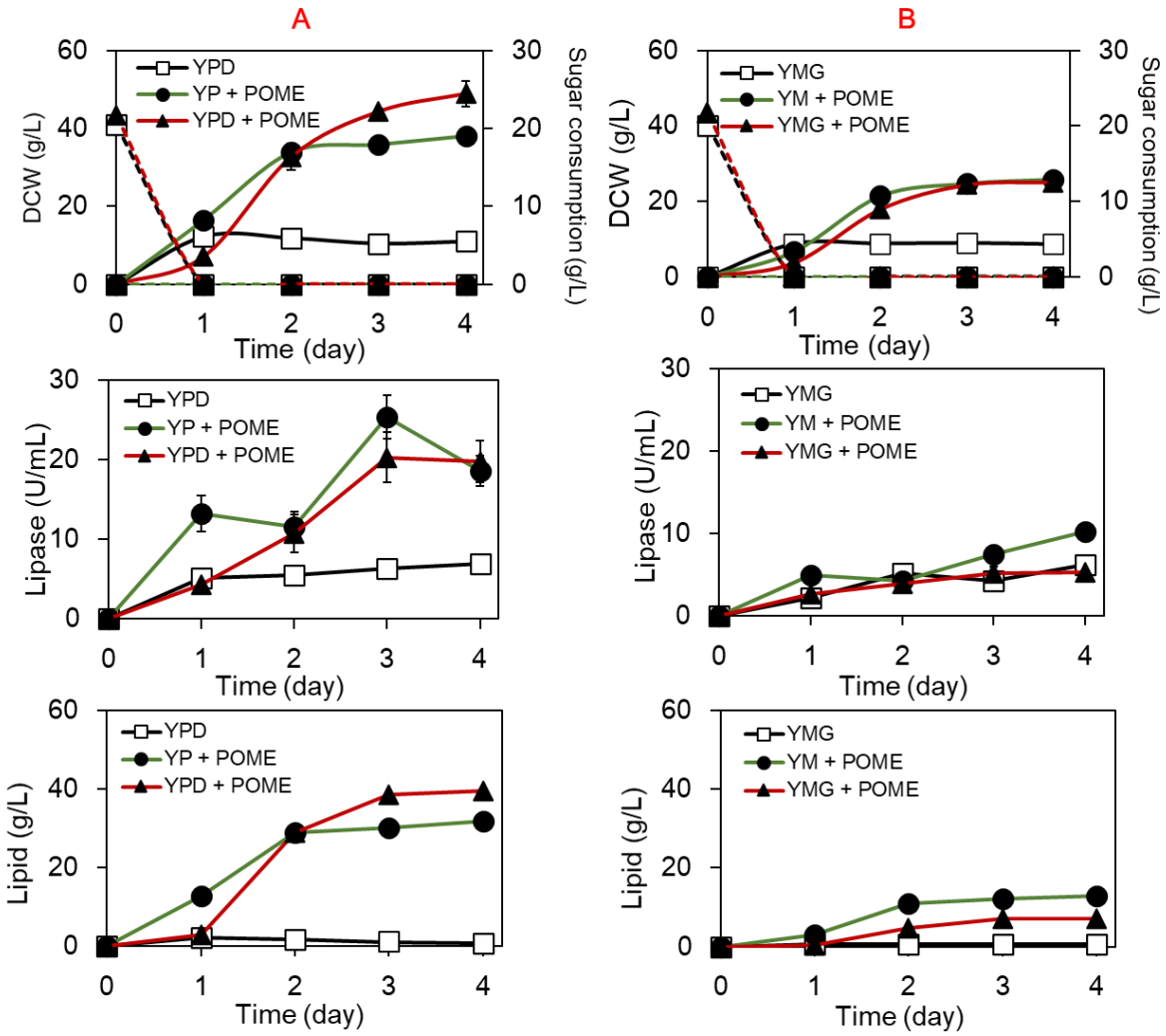

Fig. 1. Fermentation of *L. starkeyi* in complex medium w/o the presence of glucose and POME as alternative substrate (A) rich nutrient and (B) low nutrient.

In this study, 5 % w/w POME was used as a supplemented or substitute for carbon sources in these culture media. Both media increased the growth of cells when POME was added to the medium. However, YPD media is more effective than YMG media for *L. starkeyi* D35 to grow. The yeast produced approximately 48.89 g/L within 4 days by adding 5 % w/w POME into YPD culture media. This biomass yield (48.89 g/L) is higher

than that (30 g/L) of our previous study in which sap medium was used as the culture media [9]. This result could be attributed that POME has sufficient nutrients to support *L. starkeyi* D35. This high DCW also occurs because using POME as a substrate can excrete lipase during fermentation to aid the lipid accumulation by *L. starkeyi*.

IV.3.2. Lipid productivity by *L. starkeyi* D35

The lipid contents of *L. Starkeyi* D35 using POME as carbon sources are reported in Table 1. The highest lipid yield of 39.45 g/L was achieved by the mixture of YPD and 5 % w/w POME in culture media followed by YP and 5 % w/w POME with the lipid yield of 31.81 g/L. In general, the supplementation of the culture media with POME could provide beneficial nutrition and a suitable carbon source for lipid accumulation by *L. Starkeyi* D35 although the lipid yield in yeast and malt extracts (YM) is lower than in yeast extract (Y). This SCOs production results indicated that *L. Starkeyi* D35 can thrive and accumulate fatty acids in POME into intracellular lipid. This also might be beneficial in valorizing the value of POME from waste to valuable resource [14].

Table 1. Lipid production by *L. Starkeyi* D35

Culture Media	Dry cell weight (g/L)	Lipid content (% w/w)	Lipid productivity (g/L)
YPD	10.99 ± 0.56	8.36 ± 1.16	0.92
YP + POME	37.99 ± 2.07	83.72 ± 5.16	31.81
YPD + POME	48.89 ± 3.30	80.69 ± 4.68	39.45
YMG	8.61 ± 0.39	6.79 ± 0.33	0.59
YM + POME	25.78 ± 1.64	49.82 ± 1.72	12.84
YMG + POME	25.13 ± 0.96	27.93 ± 0.85	7.02

Fig. 2 showed how the lipid had been accumulated in the cell of *L. starkeyi*. These stain results indicated that using POME as the carbon source not only can enhance the DCW but also can produce the SCOs in one fermentation process.

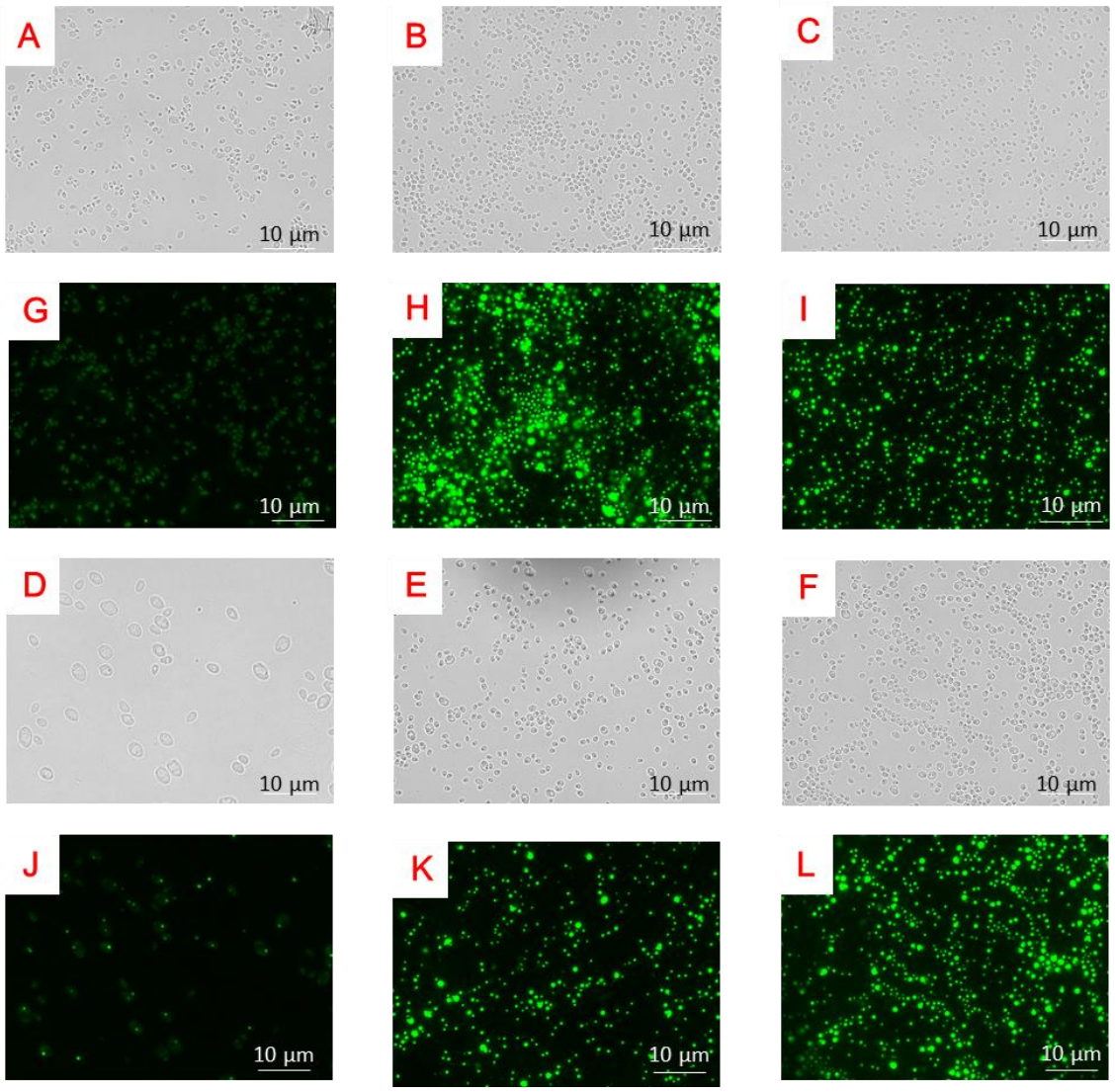

Fig. 2. Fluorescence microscopy of the single cell oil produced by *L. starkeyi* D35 in several culture media. (A-F) The microscopic images of *L. starkeyi* D35, and the microscopic fluorescence image stained by BODIPY (493/505) after cultivated in (A, G) YPD, (B, H) YP + POME, (C, I) YPD + POME, (D, J) YMG, (E, K) YM + POME, and (F, L) YMG + POME. (G-L)

IV.3.3. FAME profile

A summary of the FAME profiles is shown in Fig. 2. On average, the profile of the accumulated lipids is mainly long chain fatty acids with 16 and 18 carbon atoms for both YM and YP medium. Oleic acid (C18:1) was the predominant fatty acids followed by palmitic acid (C16:0), linoleate acid (C18:2), and linolenic acid (C18:3). These results are similar to fatty acid profiles obtained by previous research [9,13,15,16] although in different culture conditions [17]. Generally, the fatty acids profile of *L. Starkeyi* D35 was remarkably similar to that of POME itself which means *L. Starkeyi* D35 could effectively accumulated the fatty acids from POME to be intracellular lipids. Therefore, the residual of POME which is usually used for the feedstock of biodiesel could be utilized as nutrient for SCOs production.

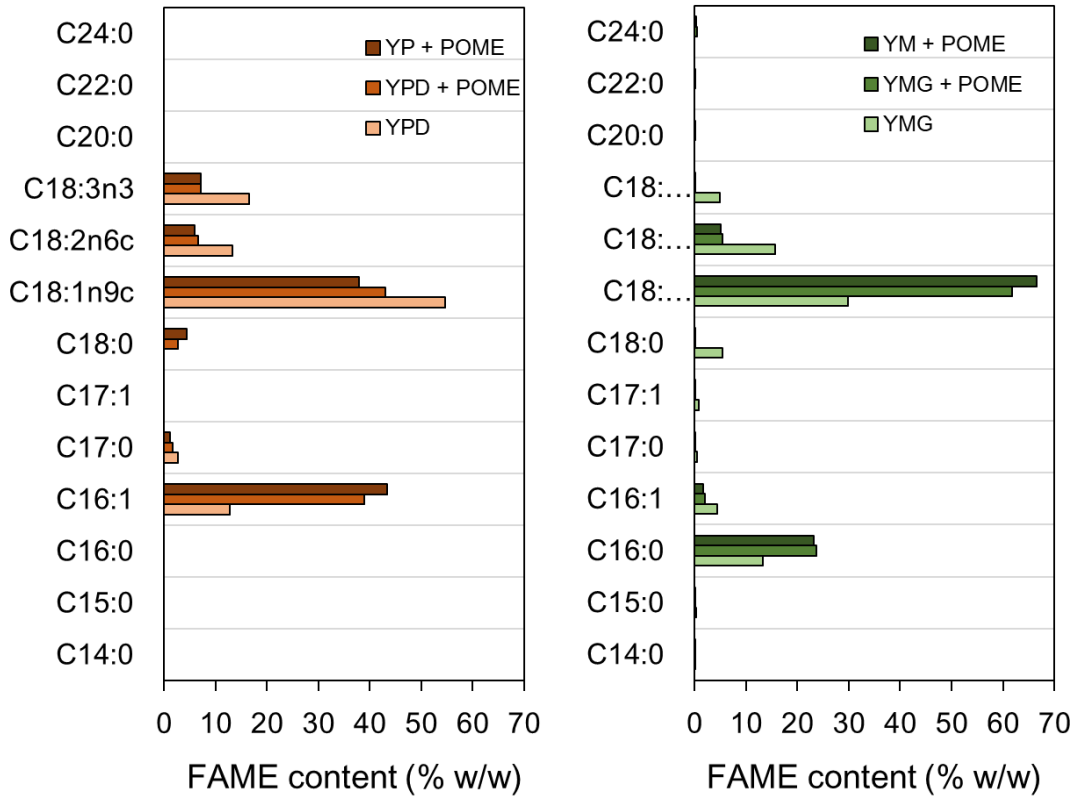

Fig. 2. FAME's profile of the lipid produced by *L. starkeyi* D35 in different culture media

IV.4. Conclusions

This study has demonstrated that POME could be utilized as a substitute nutrient of carbon source, especially glucose in culture media, and *L. starkeyi* D35 efficiently accumulate lipids in this medium. High lipid yield of 39.45 g/L was achieved in 4 d in the mixture of YPD and POME, even 31.81 g/L lipid in the mixture medium without glucose, just POME. Currently, the fatty acid profiles of *L. starkeyi* D35 intracellular lipids dominates C16 and C18, which indicated that the quality of lipid by *L. starkeyi* D35 strain was considered as a material of biodiesel. Therefore, additional metabolic engineering design, research and optimization studies could enhance the longer chain carbons so it could produce the high-valued fatty acid productions

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CHAPTER V
An integrative bioconversion process of biodiesel production from waste fractions of the palm oil industry

V.1. Introduction

The development of renewable resources plays an essential role in the future of mankind because of the depletion of fossil fuels and the seemingly ever-increasing rate of greenhouse emissions [1,2]. Biomass is one of the most promising candidates for energy diversification, particularly in the form of biodiesel [3]. Biodiesel can be expanded to fulfill the energy demand, minimize fuel combustion's impact on the greenhouse effect, and alleviate climate change issues [4–6]. Biodiesel, known as fatty acid alkyl esters (FAAE), is derived from the reaction between oil/fats and short-chain alcohols in the presence of a catalyst [7,8]. Edible vegetable oils such as soybean oil, palm oil, and rapeseed oil are usually used to produce biodiesel. However, the high cost of raw materials, the risk of transferring agricultural land or crops, the needing more arable land, and losses in food supplies have been the main obstacles to biodiesel production. Investigating alternative raw materials for biodiesel production is needed to resolve this problem. Currently, there is a shift towards biodiesel production using low-cost feedstocks such as used cooking oil, animal fats, or grease oil [9], and utilizing the palm oil industry's waste as the feedstocks could be an alternative choice to alleviate the problems for producing biodiesel.

Palm oil (*Elaeis guineensis*) is almost ubiquitous in daily life as it provides both edible products and inedible products such as soaps, butter, detergents, cooking oils, or oleochemical products. Indonesia is the world's largest producer and exporter of palm oil

[10–12]. Total palm oil production in Indonesia has achieved more than 51.4 million tons in a 14.7 Ha cultivated area [13]. More than 70 % of the fresh fruit bunches are generated from the palm oil processing stage as wastes. Palm oil mill effluent (POME) leads to the most significant portion of all waste generated in the palm oil mill. Hambali *et al.* [14] assumed that around 58% of POME would be generated after processing. Therefore, 118.40 million tons of POME were generated from palm oil mills to produce 42.87 million tons of CPO in 2019 [13]. POME consists of 95 – 96% water, 0.6 – 0.7% oil, and 4 – 5% total solids made up of cellulosic material, fat, oil, and grease [15]. POME possesses high COD of 15,000 - 100,000 ppm and BOD of 10,250 - 43,750 ppm, which become a severe threat since its biodegradation could induce aquatic hypoxia [16,17]. In addition, the pH of POME is low, about 3.4 - 5.5, due to the organic compounds and grease from partial degradation of palm fruits before processing [18,19]. This immediate release of waste contributes to severe environmental damage [20,21], biodiversity losses [22], and the proliferation of hazardous pollutants for aquatic organisms [23,24]; therefore, effective treatment before discharge into the environment of POME issue is necessary for the continuity of all living things [25].

Many studies have attempted to utilize and valorize the waste of palm oil, but most of those studies focused on investigating only one type of waste. Some previous studies have used POME as a feedstock for biodiesel production [26–32]. Those studies focused on exploring POME via transesterification with methanol catalyzed by either alkali or lipase. The use of methanol should be transformed into ethanol due to its high environmental toxicity, flammability, and limited resources. In addition, ethanol can easily be obtained from the fermentation of palm oil sap [33–35] or empty fruit bunches [36–38]. This type of bioethanol production could be an alternative short-chain alcohol

for the reaction of biodiesel production, which would be an appropriate step toward green and sustainable production. Although bioethanol comprises high amounts of water, our previous study showed that the residual oil from POME can directly be utilized for biodiesel production via enzymatic trans/esterification with a low ethanol concentration [26]. Thus, this study integrates several wastes that are resulted in the palm oil industry as not only feedstock but also green acyl acceptor to produce biodiesel.

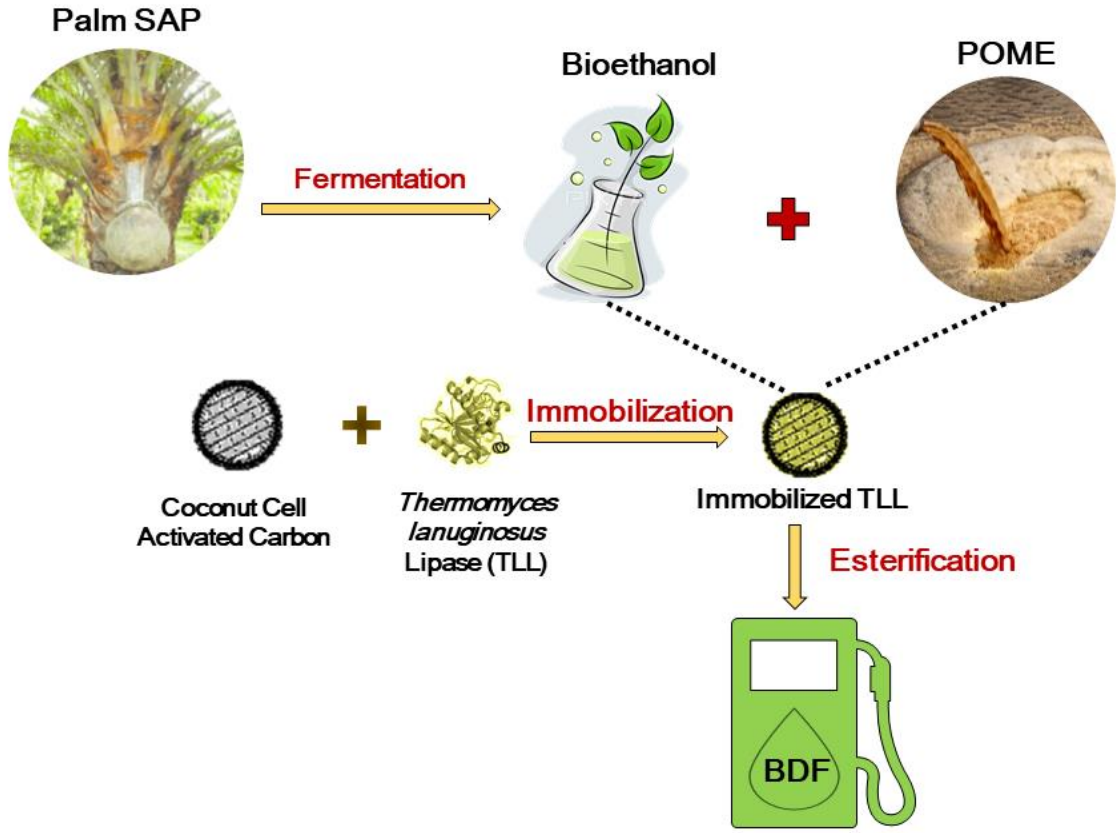

Fig. 1. An integrative bioconversion process scheme for biodiesel production from palm oil waste.

The palm oil waste was utilized in the present study presented in Fig. 1. The concept integrates the POME and SAP approach using streams of palm oil waste with activated carbon from coconut shells to produce FAEE via a reaction mediated with

Thermomyces lanuginosus lipase (TLL). This concept could solve many of the environmental issues of the palm industry and increase the value of palm oil waste. In the present study, kinetics, activation energy, and Gibbs free energy are also investigated to determine the spontaneity of the esterification process of POME using an immobilized TLL. The major concern of this investigation is to elucidate how free fatty acids (FFA) react faster than triacylglycerol (TAG) to produce FAEE in water-containing systems. Ultimately, this study should help establish the sustainability of an integrated approach to biorefinery operations from the palm oil industry.

V.2. Materials and Method

V.2.1. Materials

Activated charcoal with a 30 – 60 mesh from coconut shells was obtained from Nacalai Tesque Inc (Kyoto, Japan). POME was acquired through collaborative research with PT Agricinal (Bengkulu, Indonesia). A liquid formulation of *Thermomyces lanuginosus* lipase (TLL), Callera Trans L (CalT), was purchased from Novozymes (Bagsverd, Denmark). All other reagents were purchased from Nacalai Tesque, Inc., (Kyoto, Japan) and Sigma-Aldrich (Tokyo, Japan).

V.2.2. Immobilization of TLL on activated carbon from coconut shells

The lipase immobilization was carried out in a test tube. Each culture media was composed of 1 mL TLL, 1 or 3 g of activated charcoal from coconut shells, 9 ml of buffer solution. The buffer media was initiated in three kinds of buffer; 0.02 mol L^{-1} phosphate buffer (pH 7.0), 0.005 mol L^{-1} of tris buffer containing 0.01 mol L^{-1} of $CaCl_2$ (pH 7.0), or 0.005 mol L^{-1} of bicarbonate buffer (pH 9.0). The lipase was cultivated at 25 ºC for 6 h

using a bio shaker (BR-23FP, Taitec Corp., Saitama, Japan) at 200 oscillations per min. The effect of the adsorption time was evaluated by sampling 200 µL of the supernatants at 0, 1, 3, and 6 h. After cultivation, the supernatant was removed. The immobilized lipases were washed twice with 1 mL deionized water and lyophilized in a freeze dryer (Labconco Corp., Kansas, USA) for use in biodiesel production.

V.2.3. Preparation of bioethanol fermentation and distillation for biodiesel production

A single colony of the *Saccharomyces cerevisiae* F118 strain was initially cultured in yeast-media agar plates and aseptically inoculated into 12 mL of yeast extract peptone dextrose (YPD) media in 50 mL Erlenmeyer flasks in a composition mixture of 10 g L^{-1} bacto yeast extract, 20 g L^{-1} bacto peptone, and 50 g L^{-1} glucose. This strain was incubated at 30 °C and 150 rpm for 12 h in an orbital shaker (BioShaker G: BR-200, TAITEC, Corp., Saitama, Japan).

After pre-cultivation, the cell solution was transferred into a 200 mL Erlenmeyer flask containing 108 mL of artificial palm SAP prepared based on a fermentation procedure established by Kosugi *et al.* (2010) [30]. The artificial palm SAP contained 8.90 g L^{-1} sucrose, 49.10 g L^{-1} glucose, 1.30 g L^{-1} xylose, and 1.30 g L^{-1} galactose. The total sugar concentration of the artificial palm SAP was 60.60 g L^{-1}. The fermentation was performed at 30 °C and 150 rpm for 48 h. At 0, 6, 12, 24, and 48 h, 300 µL samples were taken and centrifuged at 15 °C, 6300 xg, for 1 min to determine the ethanol concentration. The bioethanol that resulted from the fermentation was subjected to distillation using a SRE-M3 rotary evaporator (Shibata, Tokyo, Japan).

V.2.4. Ethanolysis of POME using a distilled bioethanol solution from artificial palm SAP

The ILs of TLL in activated carbon from coconut shells was used to catalyze the ethanolysis of POME using distilled bioethanol as an acyl acceptor. The reaction mixture contained 4 g POME, 500 mg ILs, and 0.20 g distilled water, to which 0.436 mL of distilled ethanol was added stepwise at 0, 6, 12, and 24 h leading to a total molar ratio of 1 to 4. For comparison, 99.5, 70, and 45% v/v ethanol concentrations were used for the reaction process under the same conditions as that for bioethanol. Then, 100 µL samples were taken at specified times to determine the amount of fatty acid ethyl ester (FAEE) throughout the reaction.

V.2.5. Reuse of the ILs for the transesterification of POME

A reaction that used either recovered ILs after 48 hours of reaction or the remainder of the aqueous phase and ILs for the following esterification reaction was carried out to evaluate the reusability of ILs. The ILs were reused directly without treatment and applied to 4 g fresh POME, and 0.436 mL distilled ethanol solution was added stepwise at specified times.

V.2.6. Kinetic study

The esterification reaction was carried out using four different temperatures (30, 35, 40, and 45 °C) at 35 rpm. The reaction mixture consisted of 4 g POME, 0.22 g ethanol, and 1,500 U of TLL. Samples were withdrawn at 0, 1, 3, and 6 h to evaluate the rate constants and the reaction's activation energy.

V.2.7. Kinetic model

The following were combined to study the kinetics of POME with a TLL biocatalyst at a 1:1 ethanol-to-POME ratio. The scheme of the esterification reaction of free fatty acid (FFA) to FAEE is given by Eq. (1).

$$FFA + EtOH \rightleftharpoons FAEE + H_2O \quad (1)$$

In Eq. (1), the following abbreviations apply:

FFA = Free fatty acid

EtOH = Ethanol

FAEE = Fatty acid ethyl ester

H_2O = Water

The equilibrium K constant was calculated by measuring the Gibbs energy by using a Spartan'18 Parallel Suite (Wave function, Inc., Tokyo, Japan). The equilibrium geometry was studied at ground state in water using the density functional (B3LYP/6-311G**). Based on this calculation, the K constant was high; therefore, the irreverse reaction is denoted in this equation and described by Eq. (2).

$$[FFA]\,[EtOH] \xrightarrow{k} [FAEE][H_2O] \quad (2)$$

In Eq. (2), k = the forward reaction rate constant. The rate constants of the reaction sequences were determined by establishing differential equations using the rate law in Eq. (2), as shown by Eq. (3).

$$r_{FFA} = -\frac{d[FFA]}{dt} = k[FFA][EtOH] \quad (3)$$

The triglyceride molar ratio of the initial ethanol was 1 to 3. Therefore, in this equation, the ethanol is represented by Eqs. (4) and (5).

$$[EtOH]_0 = \frac{1}{3}[FFA]_0 \quad (4)$$

$$[\mathrm{EtOH}] = [\mathrm{FFA}] - \frac{2}{3}[\mathrm{FFA}]_0 \quad (5)$$

When Eq. (5) is substituted for Eq. (3), the result is Eqs. (6) and (7).

$$-\frac{\mathrm{d}[\mathrm{FFA}]}{\mathrm{dt}} = \mathrm{k}[\mathrm{FFA}]\left([\mathrm{FFA}] - \frac{2}{3}[\mathrm{FFA}]_0\right) \quad (6)$$

$$\frac{\mathrm{d}[\mathrm{FFA}]}{[\mathrm{FFA}]\left([\mathrm{FFA}] - \frac{2}{3}[\mathrm{FFA}]_0\right)} = -\mathrm{k}\,\mathrm{dt} \quad (7)$$

Finally, the k constant equation is described by Eq. (8).

$$\frac{3}{2[\mathrm{FFA}]_0}\ln\left(3 - \frac{2[\mathrm{FFA}]_0}{[\mathrm{FFA}]}\right) = -\mathrm{k}\,\mathrm{t} \quad (8)$$

V.2.8. Activation energy determination

The activation energy was determined using the Arrhenius equation, which is described by Eq. (9).

$$\mathrm{k} = \mathrm{A}e^{-\frac{\mathrm{E_a}}{\mathrm{RT}}} \quad (9)$$

In Eq. (9), k represents the rate constant, E_a is the activation energy, R is the gas constant (8.314 J mol^{-1} K^{-1}), T is the temperature in Kelvin, and A is the Arrhenius factor. By the plot of the temperature inverse and the natural logarithm of the kinetic constants, the activation energy can be calculated using Eq. (10).

$$\ln \mathrm{k} = \ln \mathrm{A} - \frac{\mathrm{E_a}}{\mathrm{RT}} \quad (10)$$

Taking the natural log, which leads to the linearization of both sides, forms y = mx + b, in which y is ln k and x is 1/T with a y-intercept of ln A and the slope of $-E_a/R$.

V.2.9. Analytical method

The ethanol concentration was analyzed using high-performance liquid chromatography (HPLC) (LC-20AB, Shimadzu, Kyoto, Japan) equipped with a Coregel-87H3 column (7.8 mmLD x 300 mm Transgenomic Inc., Omaha, NE) and a refractive

index detector (RID-10A) for peak identification. Samples (300 µL) were centrifuged and filtered (0.45 µm) before injection (10 µL). The column was configured at a temperature program starting from 80 ºC, and 0.005 mol L^{-1} H_2SO_4 with a flow rate of 0.60 mL min^{-1} as a mobile phase.

FAEE was measured using gas chromatography equipped with a flame ionization detector (GC-FID) (GC-2010, Shimadzu, Kyoto, Japan). Samples were separated using a ZB-5HT Interfeno capillary column 15 m x 0.25 mm x 0.15 mm (Phenomenex Inc., Torrance, CA) at a temperature program starting at 130 ºC for 2 min with an increase to 350 °C at a gradient of 10 °C min^{-1} that was followed by an increase to 370 °C at 7 °C min^{-1}, which then was maintained for 10 min. Tricaprylin was used as an internal standard for measuring the ethyl ester composition.

The formation of biodiesel in the reaction process was confirmed by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) studies using a Shimadzu AIM-900 Infrared Microscope equipped with an ITRraces-100 (Shimadzu Corp., Tokyo, Japan).

V.3. Results and Discussion

V.3.1. Immobilization of *Thermomyces lanuginosus* lipase on activated charcoal from coconut shells

The functional activity efficiency of activated charcoal from coconut shells was measured via lipase adsorption (Supplementary Fig. S1) and hydrolytic activity (Fig. 2). The protein that remained in the buffer media was measured to investigate the lipase's rate of absorption into the matrix. Supplementary Fig. S1 shows how variations in the buffer media and in the weight of the charcoal did not alter the lipase adsorption rate,

which ranged from 4.77 mg ml^{-1} to 0.60 mg ml^{-1} or around 90% of the protein. These results indicate that the activated charcoal offered an effective surface void for lipase absorption. However, significant differences were apparent in the hydrolytic activity of the lipase immobilization, as presented in Fig. 2. The hydrolytic activity of the catalysts was measured to examine the effectiveness of enzyme functionality in the reaction process. Fig. 2 shows that the hydrolytic activity in 1 g charcoal was higher than it was in 3 g, which demonstrated how the absorption rate of activated charcoal decreases the hydrolytic activity per mg of immobilized lipase in proportion to the larger surface area and to the voids that affect the absorption of lipase. Lipase immobilization in Tris-HCl buffer showed the highest level of hydrolytic activity (3.40 U/mg). This result indicates that Tris-HCl buffer induces lipase activity more effectively than others due to buffers' type and ionic strength that influence the enzymatic activity in aqueous reaction systems [39]. Therefore, the immobilized lipase cultivated in Tris-HCl buffer media was applied as the biocatalyst for esterification reaction during the entire experiment.

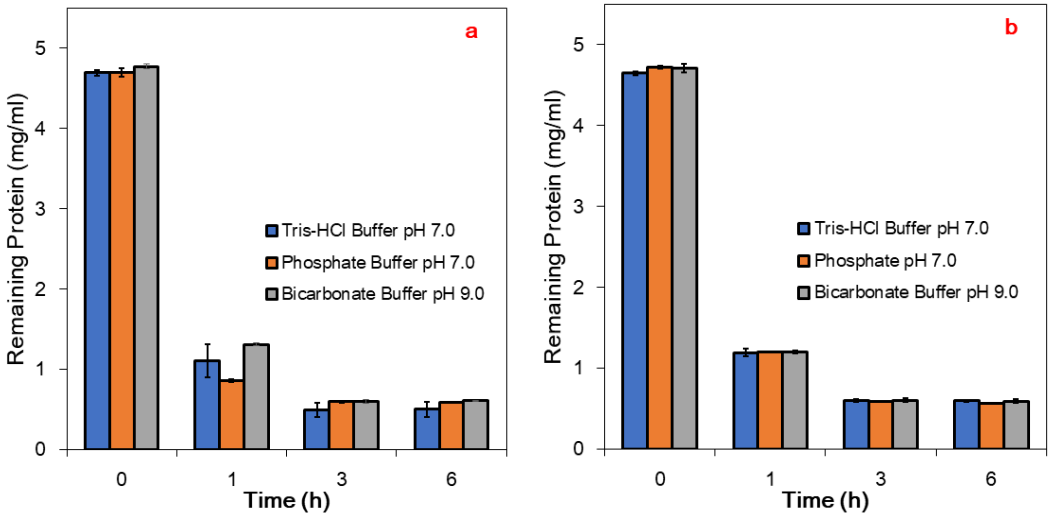

Fig. 2. Remaining protein of *Thermomyces lanuginosus* lipase (TLL) using different buffer media in (a) 1 g and (b) 3 g of charcoal activated from coconut shell as matrix

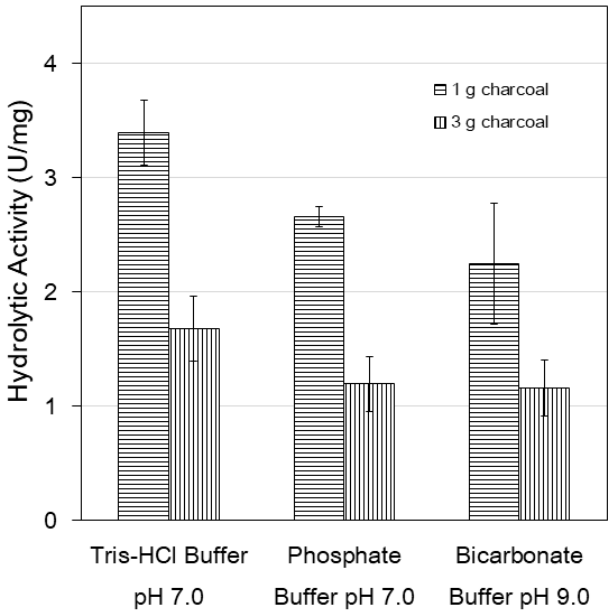

Fig. 3. The hydrolytic activity of immobilized TLL using different buffer media

V.3.2. Bioethanol fermentation and distillation for biodiesel production

Bioethanol could be an alternative short-chain alcohol for the reaction of biodiesel synthesis, which would be an appropriate step toward green and sustainable production. The ethanol fermentation of the artificial palm sap was conducted using a yeast strain (*S. cerevisiae* F118) to produce bioethanol. After fermentation, the fermented sap contained around 27 g L^{-1} ethanol, as shown in Supplementary Fig. S2. In general, the bioethanol production rate rapidly increased for 24 h and reached an optimum value at 48 h of fermentation. In the present study, the sugar content was exhausted after 24 h, which indicated a rapid sugar consumption by the yeast during the fermentation process. The bioethanol contained 3% v/v ethanol and was purified via simple distillation to increase the ethanol concentration. As shown in Supplementary Fig. S3, the first stage of distillation improved the ethanol concentration from 27 to 84 g L^{-1}, which is equal to 10%

v/v ethanol concentration. That amount was still low, which complicated its use as an acyl acceptor in the esterification process due to the vast amount of water in bioethanol.

Nevertheless, the second stage of distillation needed to perform to obtain at least a yield of 45% v/v ethanol concentration based on results reported by Rachmadona *et al.* [26], who showed that esterification using POME as a feedstock had gained a higher yield of FAEE more than 96.5 % w/w. Supplementary Fig. S3 illustrates the second stage result of the enhancement for the ethanol concentration from 84 g L^{-1} (10 % v/v ethanol concentration) to 502.5 g L^{-1} (63 % v/v ethanol concentration). This result indicated that bioethanol after purified two times by simple distillation is suitable to serve as acyl acceptor for the esterification. This result indicated that the application of bioethanol after the second stage of distillation would be sufficient for subsequent experiments.

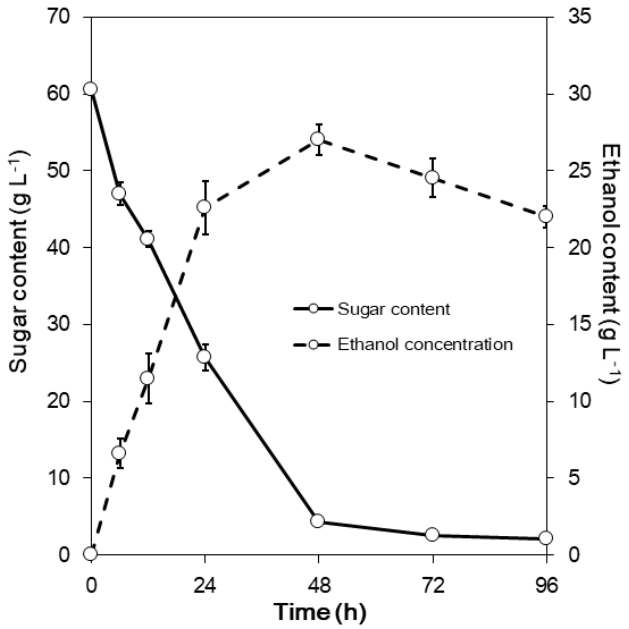

Fig. 4. Bioethanol production and sugar consumption rate from artificial palm sap using F118.

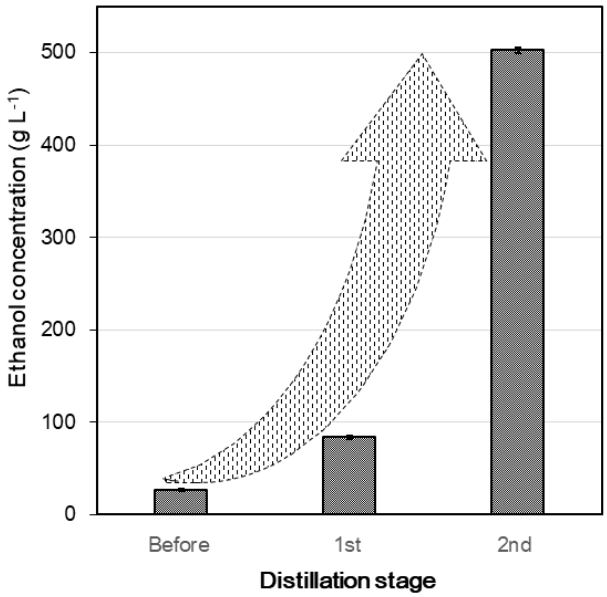

Fig. 5. Ethanol concentration after distillation process.

V.3.3. Lipase-ethanolysis using POME and a distilled bioethanol solution

POME is a potentially promising feedstock for biodiesel production [26,28–30]. Previous studies of biodiesel production have used POME as feedstock with a low ethanol concentration [26]. Fig. 3 shows that the effective utilization of POME for biodiesel production by the variation of ethanol concentration. The highest FAEE yield was obtained, 98.56% w/w, using 45% v/v ethanol since the water could suppress the deactivation effect of lipase in the reaction. However, this concept could not be applied to transesterification using feedstock that contained a high amount of TAG due to the resultant hydrolysis reaction, as presented in Table 1. That result indicated how the reaction rate of transesterification was slower than that of esterification when using a low ethanol concentration due to a competitive reaction with hydrolysis. As a result, the FFA reaction rate was increased near the end when using raw materials that contained greater amounts of TAG. Also, the FAEE yield was meager, which further established that the

use of ethanol dilution is ineffective for transesterification. In a similar manner, Fig. 4 shows the change in Gibbs free energy (ΔG) for a transesterification process using high amounts of water to determine the reaction's spontaneity. That process revealed that when using ethanol containing high amounts of water the ΔG for the reaction conversion of TAG to either fatty acid ethyl ester (FAEE) or free fatty acid (FFA) was lower than 0 (ΔG < 0). That result demonstrated how the process is exergonic and will proceed spontaneously in the forward direction to form products. Nevertheless, hydrolysis simultaneously yielded FFA along with FAEE via esterification. Contrary to TAG, the conversion of FFA to FAEE was not disturbed by hydrolysis. Fig. 3 shows the reaction of POME, feedstock containing high amount of FFA, with 10% v/v bio-ethanol could obtain higher FAEE yield than the conversion of the feedstock containing high amount of TAG, as shown in Table 1.

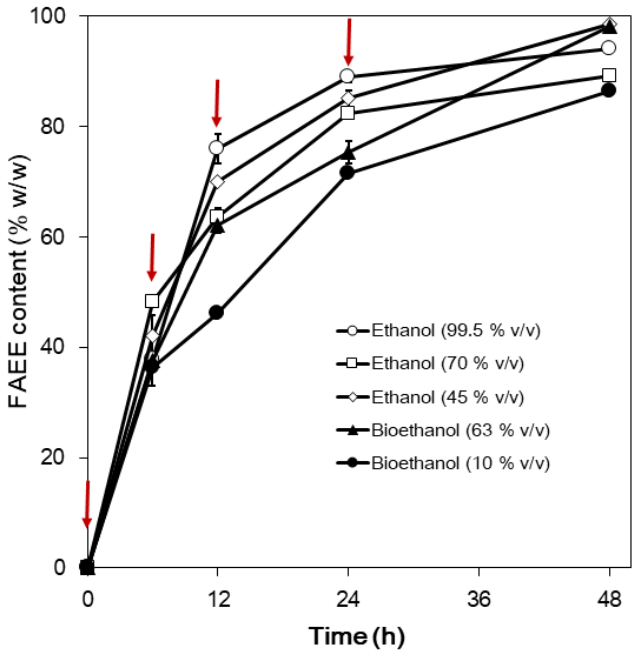

Fig. 3. Ethanolysis of POME using, (○) 99.5 % v/v ethanol, (□) 70 % v/v ethanol, (◊) 45 % v/v ethanol, (▲) 63 % v/v bioethanol (●), and 10 % v/v bioethanol catalyzed by TLL.

Table 1. Time-dependent changes of component yield by ethanolysis using a low ethanol concentration.

Feedstock	Time (h)	Component yield (% w/w)				
		FAEE	FFA	MAG	DAG	TAG
Refined palm oil	0	0	0	0	4.7	95.95
	6	9.27	6.05	2.46	16.07	55.43
	12	25.6	8.32	7.05	19.78	22.7
	24	42.87	10.76	6.62	16.28	12.91
	48	53.2	9.16	5.03	11.21	8.35
Crude palm oil	0	0	5.1	0	5.28	87.69
	6	14.78	7.08	3.12	15.86	47.11
	12	31.19	8.86	6.72	19.79	19.87
	24	43.71	10.82	6.02	13.75	10.67
	48	55.13	9.29	4.64	10.15	7.51
Rapeseed oil	0	0	0	0	0	99.02
	6	3.91	2.43	1.17	8.66	68.73
	12	7.72	4.84	2.59	15.09	57.86
	24	14.85	8.24	4.85	18.59	44.52
	48	31.27	7.57	6.42	18.26	20.88
Rice bran oil	0	0	0	0	0	98.42
	6	7.9	4.83	2.35	17.06	63.62
	12	18.35	8.22	5.33	23.62	33.47
	24	31.56	9.61	7.58	25.6	18.27
	48	55.24	5.99	5.51	13.45	7.45
Soybean oil	0	0	0	0	0	99.54
	6	5.51	3.46	1.38	7.16	65.24
	12	20.19	9.18	6.4	21.68	38.34
	24	32.63	7.03	8.68	22.25	27.67
	48	48.68	7.48	9.46	13.21	16.01
Palm oil mill effluent	0	0	93.35	1.5	1.43	3.72
	6	42.11	53.45	2.32	1.29	0.84
	12	70.1	26.93	1.36	0.84	0.77
	24	85.12	12.91	1.13	0.52	0.32
	48	98.56	1.02	0.2	0.1	0.12

Afterward, the biorefinery concept that integrates the utilization of palm waste to produce biodiesel was conducted. POME was used as the feedstock. The second stage of distilled bioethanol solution (63 % v/v ethanol concentration) of artificial palm sap served

as an acyl acceptor for ethanolysis catalyzed by immobilized lipase with charcoal activated from coconut shells served as a matrix for immobilization. This integration achieved an FAEE content of 98.11% w/w after 48 h, as shown in Fig. 3. This result suggests that although the ethanol concentration was low, the FAEE yield was sufficiently high to fulfill the standard requirement, EN 14214, for alkyl ester content.

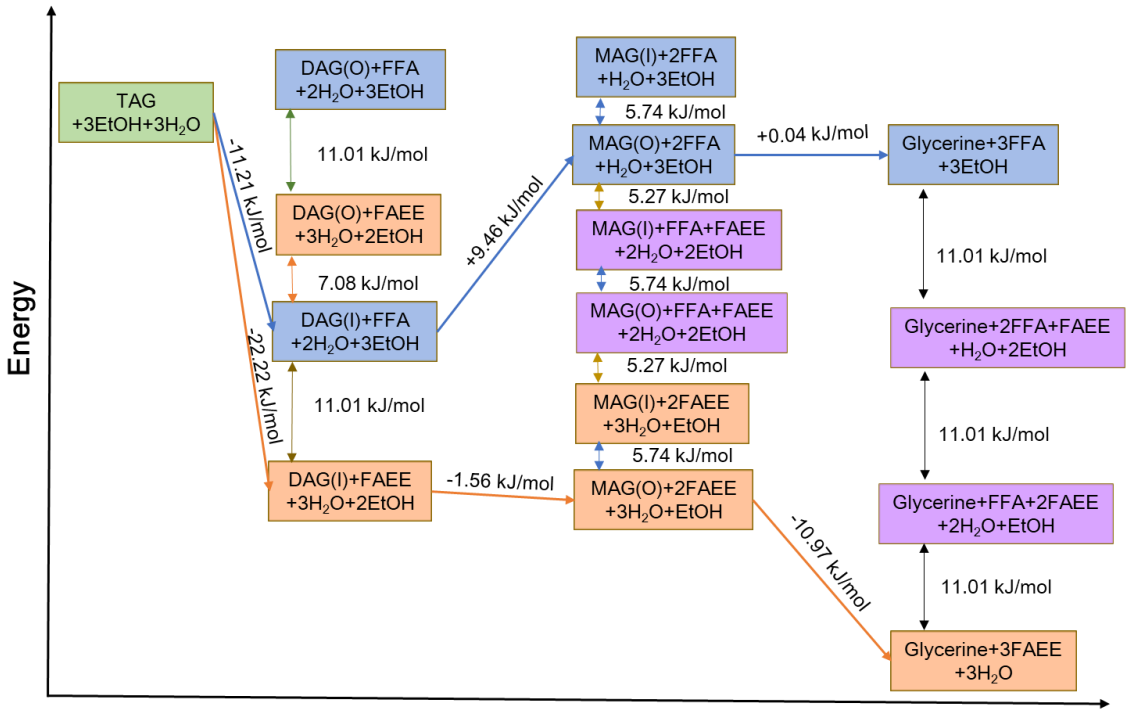

Fig. 7. Gibb's energy diagram of transesterification from triacylglycerol (TAG).

To characterize the functional groups of carbon bonding in the biodiesel produced in this study, ATR-FTIR was performed using mid-infrared spectral data (4,000-400 cm^{-1}) presented in Fig. 5. It compares the biodiesel production using POME with ethanol (45% v/v) using bioethanol (63% v/v). Commercial biodiesel from palm oil was examined as a standard reference for the analysis. The samples of all models showed similar spectra features. The peaks at 3,075 to 2,760 cm^{-1} indicated the stretching vibrations characteristic of the symmetric and asymmetric −C−H alkane groups in FAEE. A substantial rise in the −C=O in all samples stretched around 1,810 to 1,600 cm^{-1} referred to the function of

carbonyl compounds. POME showed a vast peak at 3,500-2,500 cm^{-1} due to the dimer of carbonic acids, but this peak wasn't present in the other specters. Moreover, when the areas between 1,500 and 400 cm^{-1} were compared, all specters fitted entirely with the exception of that for POME. Based on these results, POME was shifted to biodiesel through an esterification reaction.

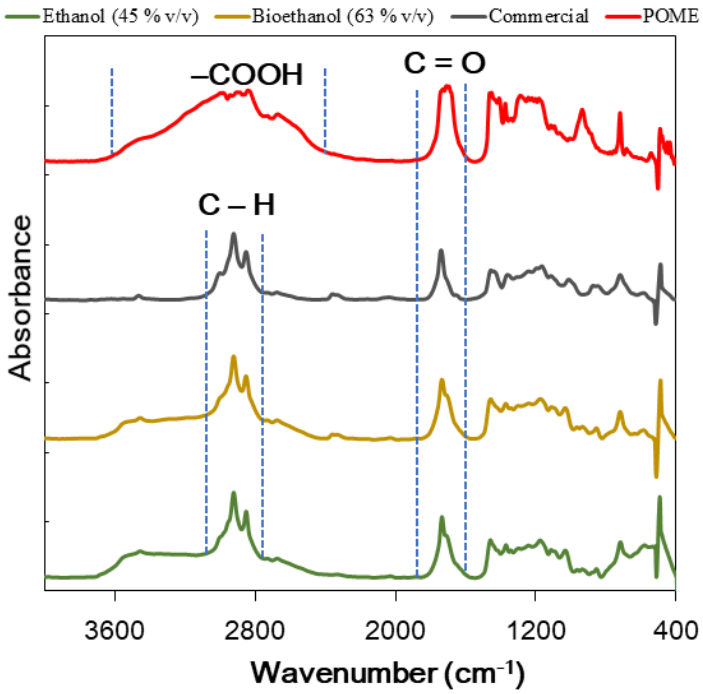

Fig. 8. Fourier transform infrared spectroscopy spectra of TLL-catalyzed biodiesel in 45% v/v ethanol and bio-ethanol rather than the biodiesel standard and POME before the reaction.

V.3.4. Kinetic study of the spontaneity of esterification when abundant water was present

Kinetics of the esterification were evaluated using immobilized TLL under optimal conditions at 30, 35, 40, and 45 ºC. Table 2 indicates the value of k was increased to 4.71 x 10^{-3} h^{-1} at 40 ºC and 5.16 x 10^{-3} h^{-1} at 45 ºC, the latter of which was found to be

the maximum rate for high consumption of FFA. However, although the value of k in 45 ^{o}C is the highest value, it will be difficult for TLL to proceed in the reaction due to the denaturation of lipase by the addition of ethanol. In this reaction, the ΔG was also examined by density functional theory (DFT) calculation (B3LYP/6-311G**) to reveal the spontaneous reaction between reactants and products. The negative value of ΔG in the reaction system indicates that the esterification reaction proceeded spontaneously for producing biodiesel from POME.

Table 2. Kinetic parameters of the esterification reaction catalyzed by lipase

Lipase	**Temperature (^{o}C)**	**k constant [a]**	**Ea (kJ mol^{-1})[a]**
TLL	30	-0.002	58.88
	35	-0.003	
	40	-0.005	
	45	-0.005	
CAL-B	30	-0.041	10.71
	35	-0.044	
	40	-0.048	
	45	-0.041	

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

The activation energy (E_a) and Arrhenius factor A were examined using the Arrhenius plot of the logarithms of various rate constants with the plot of ln (k) versus (1/T). E_a was used to examine the minimum amount of extra energy required to convert the products. The values of *k* presented in Table 2 were then used to determine the activation energy plotted in Supplementary Fig. S4. The activation energy for the esterification of POME using immobilized TLL was 58.88 kJ mol^{-1}, which differs slightly

from the 39.7 kJ mol^{-1} reported by Zhou et al. [40] for the methanolysis of phoenix seed oil. This result indicated that immobilized TLL could lower the E_a and increase the reaction rate of POME and ethanol.

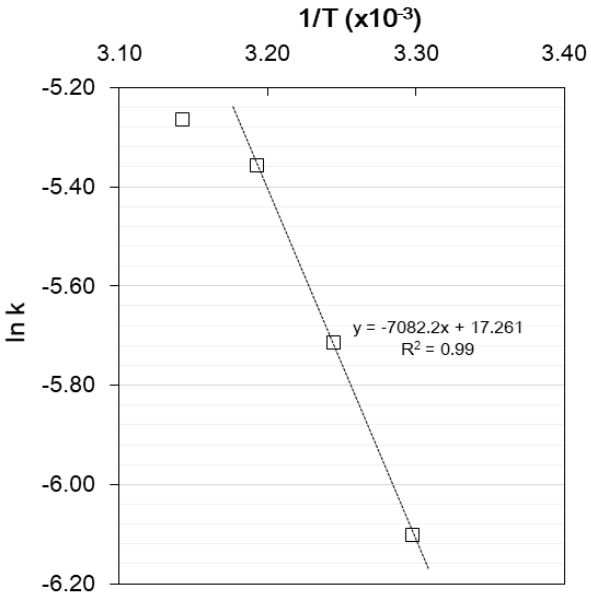

Fig. 9. Arrhenius plot of the esterification reaction catalyzed by TLL for estimation of activation energy.

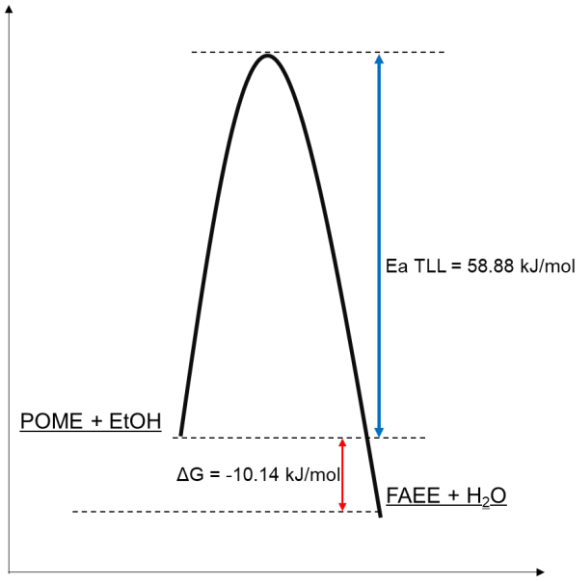

Fig. 10. The relationship of Gibbs energy and activation energy in the esterification reaction.

V.3.5. Recycling of TLL for ethanolysis using POME and bioethanol as feedstock

The direct recycling of TLL during five batch cycles of ethanolysis using POME and bioethanol as a feedstock and an acyl acceptor was investigated to observe the biocatalysts' effectiveness. FAEE content remained at more than 70% w/w after the fifth batch using the 2nd stage of distilled bioethanol solution presented in Fig. 6a by directly using the immobilized lipases only for the esterification reaction. This FAEE content gradually decreased in each batch due to the lipase denaturation affected by the release of the lipase in the water phase. However, if the water phase after the reaction remained in conjunction with the immobilized lipases (Supplementary Fig. S5) for direct use in the next batch, there was a significant improvement in FAEE content from 70 to 90% w/w after the fifth batch (Fig. 6b).

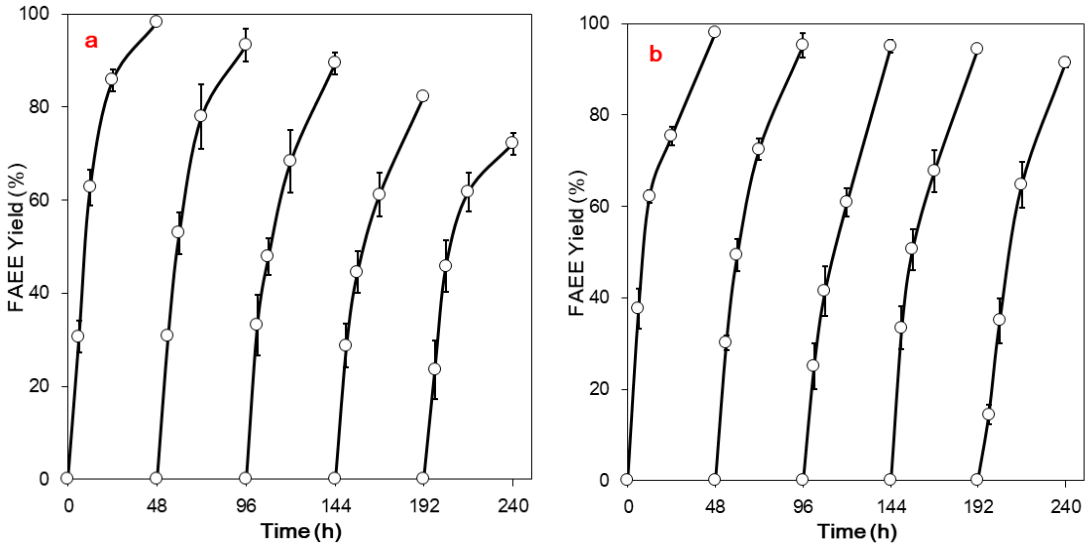

Fig. 11. Recycling of TLL during five cycles of batch ethanolysis using POME and bioethanol as feedstock on a thermo block rotator by (a) only ILs (b) ILs and the remaining water phase.

This result reveals that lipase is released into the water phase so that maintaining the water phase for the following batch reaction could help preserve the activity of the lipase for FAEE conversions. This study also obtained a higher yield than that in the previous research [41], which used hydrolysate soybean oil as a feedstock, and yielded just more than 50% w/w fatty acid methyl ester (FAME) after five batches. These results also reveal the effectiveness of using ethanol as an acyl acceptor rather than methanol for biodiesel conversions [41]. Given these results, biorefinery utilization of waste from the palm oil industry seems to be a desirable integration for biodiesel production.

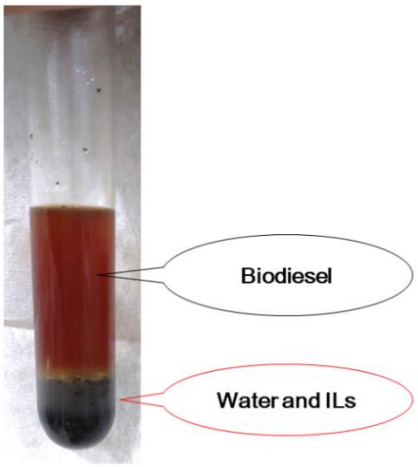

Fig. 12. The image of biodiesel product from POME and bioethanol.

V.4. Conclusions

These studies successfully integrated the utilization of palm oil waste for biodiesel production. An integrative approach used POME and SAP from streams of palm oil waste combined with activated carbon from coconut shells to produce FAEE through a TLL-mediated reaction. This biorefinery concept produced a high FAEE yield (98.11% w/w) due to a high bioethanol concentration after two sessions of the distillation process.

Moreover, the ILs maintained a high FAEE content of more than 90% w/w after the fifth batch. Hence, this study has established an integrative conversion system for biodiesel synthesis from palm industry waste.

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CHAPTER VI
General Conclusion and Future Research

Palm oil mill effluent (POME) is the waste from the palm oil industry, which must be treated to a standard requirement before its discharge to the environment. Many applications have been used to develop POME from being a waste to a value-added product. This leads to added advantages for the palm oil industry. This study was aimed to utilize and investigate the residual oil of POME for a low-cost feedstock, nutrients, or substrates in biotechnological applications.

POME presents as a low-cost feedstock for biodiesel production without interfering with the food supply. The direct use of POME with esterification reaction is effective due to a large amount of free fatty acid (FFA) consisted in POME. However, the use of conventional chemical methods is ineffective because the FFA can result in soap formation, reduce the yield of biodiesel, and complicate the separation process. Lipase-catalyzed process had been used to convert POME into biodiesel. This process also reveals that POME can be effectively converted to biodiesel even in a low ethanol concentration.

Besides the utilization of POME as feedstock for biodiesel production, the potential of POME as a nutrient for microorganism cultivation was investigated. This study explores the ability of POME as a carbon source for immobilizing recombinant lipase by *A. oryzae*. POME showed efficiency and significant enhancement of the cell growth of whole-cell lipases. These results suggest that POME is a suitable nutrient that can be used to cultivate *A. oryzae* expressing r-CALB. POME culture medium doubled

the total amount of cell weight and improved the hydrolytic activity compared with the use of a glucose medium.

To further explore the POME's ability as carbon source for microorganism, an oleaginous yeast, *Lipomyces starkeyi*, which can accumulate lipid was prepared. This oleaginous yeast is evaluated by the fermentation in complex medium w/o the presence of glucose and POME as alternative substrate. High lipid yield of 39.45 g/L was achieved in 4 d in the mixture of yeast extract-peptone-glucose with 5 % w/w POME. The fatty acid profiles of *L. starkeyi* D35 intracellular lipids dominates C16 and C18, which indicated that the quality of lipid by *L. starkeyi* D35 strain was considered as a material of biodiesel. In this work, further exploration in additional metabolic engineering design, research and optimization studies is needed to enhance the longer chain fatty acids so it could produce the high-valued oleochemicals.

In the other hand, the integrative utilization of several palm waste with POME was evaluated in this study to establish the sustainability of an integrated approach to biorefinery operations. This study's concept integrates the POME and SAP approach using streams of palm oil waste with activated carbon from coconut shells to produce fatty acid ethyl ester via a reaction mediated with *Thermomyces lanuginosus* lipase (TLL). This biorefinery concept produced a high FAEE yield (98.11% w/w) due to a high bioethanol concentration after two sessions of the distillation process. Moreover, the ILs maintained a high FAEE content of more than 90% w/w after the fifth batch. Further work is required to develop the utilization of POME in a large scale, especially for commercialization in palm oil industry.

LIST OF PUBLICATIONS

Chapter II

N. Rachmadona, J. Amoah, E. Quayson, S. Hama, A. Yoshida, A. Kondo, C. Ogino, Lipase-catalyzed ethanolysis for biodiesel production of untreated palm oil mill effluent, Sustain. Energy Fuels. 4 (2020) 1105–1111. https://doi.org/10.1039/c9se00457b.

Chapter III

N. Rachmadona, E. Quayson, J. Amoah, D.A. Alfaro-Sayes, S. Hama, M. Aznury, A. Kondo, C. Ogino, Utilizing palm oil mill effluent (POME) for the immobilization of *Aspergillus oryzae* whole-cell lipase strains for biodiesel synthesis, Biofuels, Bioprod. Biorefining. (2021) 1–11. https://doi.org/10.1002/bbb.2202.

Chapter IV

N. Rachmadona, Y. Harada, J. Amoah, E. Quayson, S. Hama, A. Kondo, C. Ogino, Palm oil mill effluent (POME) as supplemented carbon source for lipid production by *Lipomyces starkeyi* D35. (Submitted to Chemistry Sustainability Energy Materials)

Chapter IV

N. Rachmadona, Y. Harada, J. Amoah, E. Quayson, S. Hama, A. Kondo, C. Ogino, An integrative bioconversion process of biodiesel production from waste fractions of the palm oil industry. (Under review in Renewable Energy).

Co-authored Publications

I. E. Quayson, J. Amoah, N. Rachmadona, S. Hama, A. Yoshida, A. Kondo, C. Ogino, Biodiesel-mediated biodiesel production: A recombinant *Fusarium heterosporum* lipase-catalyzed transesterification of crude plant oils, Fuel Process. Technol. 199 (2020). https://doi.org/10.1016/j.fuproc.2019.106278.

II. E. Quayson, J. Amoah, N. Rachmadona, K. Morita, L. Darkwah, S. Hama, A. Yoshida, A. Kondo, C. Ogino, Valorization of palm biomass waste into carbon matrices for the immobilization of recombinant Fusarium heterosporum lipase towards palm biodiesel synthesis, Biomass and Bioenergy. 142 (2020) 105768. https://doi.org/10.1016/j.biombioe.2020.105768.

III. H. Wijaya, K. Sasaki, P. Kahar, E. Quayson, N. Rachmadona, J. Amoah, S. Hama, C. Ogino, A. Kondo, Concentration of lipase from Aspergillus oryzae expressing Fusarium heterosporum by nanofiltration to enhance transesterification, Processes. 8 (2020). https://doi.org/10.3390/PR8040450.

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