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Enzymatic biodiesel production from sludge palm oil (SPO) using locally produced *Candida cylindracea* lipase

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Biodiesel is a non-toxic, renewable and environmental friendly fuel. This study involved the production of biodiesel from sludge palm oil (SPO), a low-cost waste oil via enzymatic catalysis. The enzyme catalyst was a *Candida cylindracea* lipase, locally-produced using palm oil mill effluent as the low cost based medium. The results in solvent system for biodiesel production showed that ethanol gave higher yield of biodiesel as compared to methanol. One-factor-at-a time (OFAT) method was applied to investigate several factors for enzymatic biodiesel production. The optimum levels of ethanol-to-SPO molar ratio, enzyme loading, reaction temperature, mixing speed and reaction time were 4:1, 10 U, 40°C, 250 rpm and 24 h, respectively with maximum yield of biodiesel of 62.3% (w/w SPO). The SPO had a promising potential for enzymatic biodiesel production using locally-produced lipase.

Key words: Biodiesel, sludge palm oil, lipase, free fatty acid, fatty acid alkyl ester.

INTRODUCTION

Increasing energy crisis and environmental concerns by fossil fuel and its depletion reserve have led to a very intense research on sustainable and renewable sources of energy such as biodiesel (Röttig et al., 2010; Ganesan et al., 2009). Biodiesel which comprises of monoalkyl esters of long chain fatty acids has advantages as a renewable, biodegradable, clean, and non-toxic fuel (Patel et al., 2013; Moradi et al., 2013; Zheng et al., 2012; Maceiras et al., 2011; De Paola et al., 2009; Ganesan et al., 2009). Compared to fossil fuels, biodiesel produces less air pollutants, has lower sulphur content (0-24 ppm sulphur), lower aromatic content which reduces the smell of diesel exhaust and higher heat content which is about 88% of no.2 diesel fuel (Maceiras et al., 2011; Ghaly et al., 2010; Yee and Lee, 2008). Besides, it can be used neatly or by mixing it with diesel fuel in all conventional engines without modification (Moradi et al., 2013; Ganesan et al., 2009).

In general, the main challenge of biodiesel production is related to its feedstock and production method. The edible feedstock contributes to almost 80% of the overall cost of biodiesel production (Li et al., 2012). Therefore, the use of prevalent feedstocks of biodiesel which are edible oils cause the higher price of biodiesel (0.70 to 1.28 USD/L) (Yan et al., 2012). The edible oils such as sunflower, soybean, rapeseed, corn, and palm oils create competition between biodiesel and food producers thus it rises the concern on food security. The high cost of edible oils also becomes a major impulse for the search of low-cost alternative feedstock for biodiesel production.

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Abbreviations: **FFA**, Free fatty acids; **OFAT**, One-factor-at-a time; **POME**, palm oil mill effluent; **SPO**, sludge palm oil.

Many researchers are now interested in investigating the potential of waste oils. Various waste oils have been studied such as beef tallow (Liu et al., 2011), waste cooking oil (Bezergianni et al., 2010; Knothe and Steidley, 2009; De Paola et al., 2009; Anastopoulos et al., 2009), waste animal fats (Encinar et al., 2011), and grease (Yan et al., 2012; Li et al., 2012).

Malaysia, the major player in the world palm oil industry (Idris et al., 2012; Wafti et al., 2012), generates an abundance of low cost waste oil which is sludge palm oil (SPO). Hayyan et al. (2010) reported that about 40 million tons of SPO is produced annually. The SPO is rich with free fatty acids (FFA) (40 to 80% by weight) that can be converted into biodiesel (Wafti et al., 2012). However, this property is unfavorable for common method of biodiesel synthesis, homogeneous alkaline-transesterification because the limit of FFA content for that method is 1% (Hayyan et al., 2010) or 2.5% (Ghaly et al., 2010). The catalyst can easily promote soap formation, thus reduces the yield of biodiesel and complicates the separation process (Patel et al., 2013; Ghaly et al., 2010). Acid catalyst has been used to reduce the FFA content of SPO via esterification (Hayyan et al., 2010) but this method leads to two-step processes (acid-catalyzed esterification and alkaline-catalyzed transesterification) for biodiesel production, thus consumes more energy and effort for separation process. Besides, acid catalyst is corrosive and toxic (Patel et al., 2013; Yan et al., 2012).

The above problems can be avoided using enzyme catalyst such as lipase. Lipase is biodegradable, non-toxic, contributes to ease recovery of product and glycerol and requires moderate alcohol and mild reaction conditions (Zheng et al., 2012; Yan et al., 2012; Chen et al., 2011). It can also catalyze both esterification and transesterification (Yan et al., 2012), thus it is suitable for high FFA feedstocks like SPO. However, lipase-catalyzed biodiesel production is still less attractive for commercialization because commercial lipase is very expensive (Gog et al., 2012). Also, most lipase has poor stability in organic solvent (Zheng et al., 2012; Yan et al., 2012).

Therefore, in this study, biodiesel was produced from SPO using locally produced lipase as the catalyst. The lipase was produced from *Candida cylindracea* using low-cost based medium, palm oil mill effluent (POME). *Candida cylindracea* is a well known yeast that produces industrial lipase because of its non-pathogenic characteristic and it has been recognized as (GRAS) generally regarded as safe microorganism (Salihu et al., 2011). Besides, Nuylert and Hongpattarakere (2012) also stated that lipase secreted from *Candida cylindracea* has exhibited biosynthetic ability and high stability.

Several factors such as type of alcohol, alcohol-to-SPO molar ratio, enzyme loading, reaction temperature, mixing speed and reaction time that influence the enzymatic biodiesel production from SPO were investigated to increase the yield of biodiesel. This novel research can add in more information on the application of low-cost waste oil, SPO, especially in the enzymatic biodiesel production using low-cost locally-produced lipase. Furthermore, the enzymatic biodiesel production from SPO using the locally-produced POME based lipase would be competitive to the high cost of current practice of biodiesel feedstock and the environmental concern of these wastes disposal.

MATERIALS AND METHODS

Raw materials and chemicals

SPO was obtained from West Oil Mill of Sime Darby Plantation at Carey Island, Selangor, Malaysia. It was preserved in cold room at 4°C to avoid any decomposition, oxidation and changes of the FFA content. The locally-produced *Candida cylindracea* lipase was a contribution of an established research of Bioenvironmental Engineering Research Centre, International Islamic University Malaysia. The lipase production was done according to the methods of Salihu et al. (2011). All the chemicals such as organic solvents and reagents of laboratory and analytical grades with various brands (Merck, Sigma Aldrich, Supelco, Dr. Ehrenstorfer, HmBG, PC Laboratory, System and Bendosen) were purchased from local suppliers (Merck Sdn. Bhd, Teras Medik Sdn. Bhd and IT-Tech Research (M) Sdn. Bhd.)

Preparation and characterization of SPO

Prior to use, SPO was preheated at 60°C until it became homogeneous. The characteristics of the SPO such as acid and saponification values and FFA and moisture contents were determined according to Malaysian Palm Oil Board (MPOB) test methods (Ainie et al., 2005). The fatty acids composition of the SPO were analyzed using GC/FID (Perkin Elmer Clarus 500), split-splitless mode of injector, isotherm oven at 250°C using non-polar stationery phase BPX70 capillary column.

Production of lipase

The lipase production method was based on the study of Salihu et al. (2011). The yeast, *C. cylindracea*, ATCC 14830 was obtained from American Type culture collection while POME was obtained from West Oil Mill of Sime Darby Plantation at Carey Island, Selangor, Malaysia. The strain was grown on potato dextrose agar plates at 28°C for 4 days and sub-cultured every two weeks. It was maintained and preserved at 4°C. The PDA-plated culture of *C. cylindracea* was suspended in 10 mL of sterile distilled water and 1 mL of the suspension was used as the inoculums for pre-cultures. Lipase was produced in POME at 1% total suspended solid (TSS) as the basal medium containing nutrients (0.45% v/v peptone, and 0.65% v/v Tween-80) and 2.2% (v/v) inoculums. The initial pH was adjusted to pH 6.0 using 1 M NaOH. The flasks were incubated for 6 days at 28°C under orbital shaking at 150 rpm. The cell-free filtrate was used as a source of extracellular lipase.

Lipase activity and protein assays

Lipase activity was assayed according to spectrophotometric assay method reported by Gopinath et al. (2005) with a few modifications. The phosphate buffer in that method was replaced by phosphate-

Table 1. Fatty acids composition of SPO in this study.

Fatty acid	Structure	Composition (%)
Capric acid	C10:0	0.04 ± 0.05
Lauric acid	C12:0	0.62 ± 0.82
Myristic acid	C14:0	1.25 ± 0.24
Palmitic acid	C 16:0	42.12 ± 1.02
Palmitoleic acid	C16:1	0.15 ± 0.02
Stearic acid	C18:0	4.26 ± 0.07
Oleic acid	C18:1	40.31 ± 1.03
Linoleic acid	C18:2	10.49 ± 0.81
Alpha-Linoleic acid	C18:3	0.26 ± 0.16
Arachidic acid	C20:0	0.43 ± 0.44

Table 2. Chemical properties of SPO in this study.

Chemical Properties	Value
FFA (% as palmitic acid)	51.64 ± 0.59
Acid value (mg KOH/mg)	113.17 ± 1.29
Saponification value (mg KOH/g)	191.92 ± 2.88
Moisture Content (%)	1.00 ± 0.04

citrate buffer because the pH of the buffer can be varied from 2.0 to 9.0. The phosphate-citrate buffer was prepared according to the study of Pearse (1980). 4-Nitrophenyl palmitate (pNPP) was used as substrate. First, 10 mL isopropanol containing 30 mg pNPP was mixed with 9 mL phosphate-citrate buffer. A total amount of 2.4 mL freshly prepared substrate solution was mixed with three different volume of enzyme solution (2, 4 and 6 μl) and then incubated at 37°C. After 30 min of incubation, absorbance at 410 nm was measured against an enzyme-free control. One enzyme unit was defined as 1 μmol of 4-nitrophenol enzymatically released from the substrate in milliliters per minute (mL/min). Lipase concentration was determined using common method of protein assay which was universal Bradford method where bovine serum albumin was used as the standard. The lipase with known activity and concentration was prepared in aliquots of 40 mL and preserved in freezer (-20°C) to avoid rapid reduction of its activity.

Enzymatic biodiesel production from SPO

All experiments were done in a batch mode and free enzyme system in 250 mL screw-capped flask. A 25 g of SPO was added to the flask with 4:1 alcohol-to-SPO molar ratio and 1:10 lipase-to SPO volume ratio. The mixture was then incubated in incubation shaker at 40°C and 200 rpm agitation speed for 5 h. At the end of the reaction, the mixture was centrifuged to remove the lipase before separating the product in separating funnel. The product was washed with recently boiled distilled water to remove glycerol, excess alcohol and remaining lipase. The upper liquid as biodiesel in the separating funnel was recovered and dried in oven to remove the remaining water and alcohol. The product was analyzed using GC/MS and the yield was measured.

Investigation and optimization of influential factors

The investigation and optimization of influential factors of enzymatic biodiesel production from SPO was done using OFAT method. First, two different alcohols (methanol and ethanol) were studied. The selected alcohol was then used for the investigation of optimum alcohol-to-SPO molar ratio (2:1, 3:1, 4:1, 6:1, 6:1, and 8:1), enzyme loading (5, 10, 30, 50, 70 and 100U), reaction temperature (30, 40, 50 and 60°C), mixing speed (100, 150, 200, 250, 300, 350 and 400 rpm) and reaction time (5, 12, 24, 36, 48, 60 and 72 h).

Analytical analysis

The presence of esters in biodiesel was identified using GC/MS (Agilent Technologies 7890A gas chromatography equipped with 5975C mass spectrometer). The GC/MS was operated with split-splitless mode of injection; the capillary column was DB-wax with a length of 30 m, film thickness of 0.25 μm and internal diameter of 0.25 mm. Helium was used as carrier gas with a flow rate of 30 mL/min, measured at 50°C; the run time was 35 min. The temperature of the injector and detector were 250 and 280°C respectively. 15 mg sample of biodiesel was measured in GC vial. The sample was diluted with n-hexane (AR Grade) to 1 mL final volume prior to injection into GC/MS. The yield of biodiesel is defined as the weight percentage of final product relative to the weight of SPO at the beginning of the experiment.

RESULTS AND DISCUSSION

Characteristics of SPO

Fatty acids composition determines the type of esters in biodiesel (Ruan et al., 2012). In this study, the fatty acids composition of SPO analyzed by GC/FID is shown in Table 1. Based on the results, it can be seen that the highest amount of fatty acids in SPO is palmitic acid with 42.12 ± 1.02%, followed by oleic acid with 40.31 ± 1.03% and linoleic acid with 10.49 ± 0.81%. Hayyan et al. (2010) and Chow and Ho (2002) also reported that palmitic acid is the most abundant fatty acid in SPO with 42.84% and 51% respectively.

Table 2 illustrates some chemical properties of SPO used in this study. Among all properties, acid and saponification values and FFA and moisture contents are the most important for the biodiesel production. According to the results, it was found that the FFA content of SPO was too high (51.64 ± 0.59%), thus it is unfavourable for the alkaline-catalyzed transesterification. The moisture content of SPO was 1.00 ± 0.04%. Water is the critical factor in enzymatic biodiesel production because it promotes the reversible reaction of esterification. Low moisture content of SPO is favourable especially when free lipase system was used for the locally-produced lipase which its solution was already has high amount of water. The saponification value of 191.92 mg KOH/g oil gave the average molecular weight of 877 g/mol.

Characteristics of locally-produced lipase

The lipase activity is highly dependent on the pH of the reaction mixture. Figure 1 shows the lipase activity at

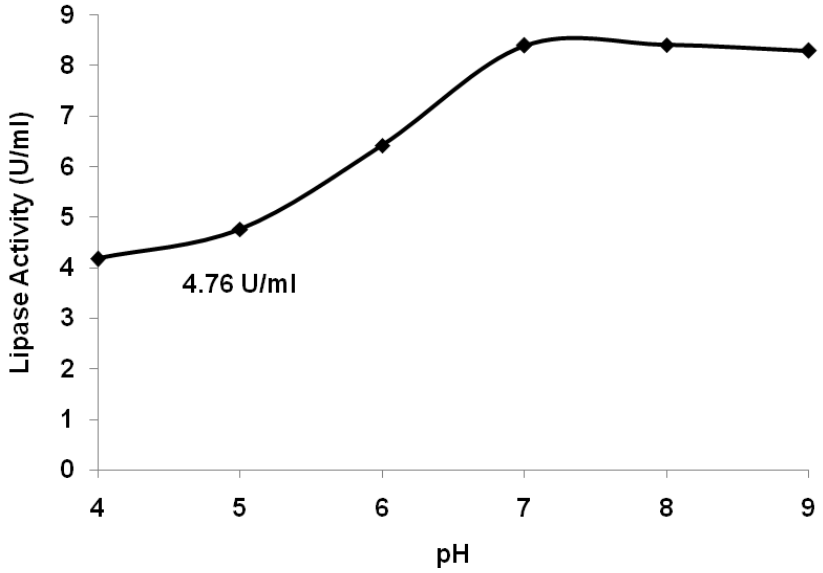

Figure 1. Lipase activity (U/ml) at different pH.

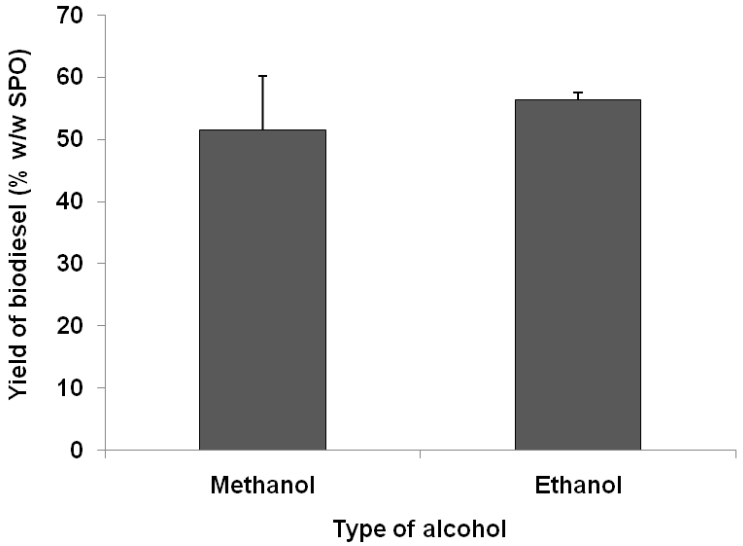

Figure 2. Effect of methanol and ethanol on biodiesel production from sludge palm oil (SPO) using locally produced *Candida cylindracea* lipase.

different pH. It can be seen from the result that the locally-produced lipase was an optimum activity at pH 7.0 to 8.0 similar with most lipases (Sivaramakrishnan and Muthukumar, 2012). However, the average pH of the reaction mixture containing SPO and alcohol was 5.0 ± 0.5. The activity of the lipase significantly decreases when the pH is less than pH 7.0. Unfortunately, the pH adjustment in the non-aqueous SPO and alcohol mixture is nearly impossible because the addition of any alkaline or base caused the soap formation. Thus, the lipase activity was taken at pH 5.0 which was 4.76 U/ml. The concentration of the lipase used in this study was 0.15 mg/ml.

Effect of methanol and ethanol

Short-chain alcohols are usually used as the acyl-acceptor in biodiesel synthesis. The effects of methanol and ethanol are illustrated in Figure 2. Based on the results, it was found that the ethanol gave higher yield of biodiesel (56.5% w/w SPO) than the methanol (51.6%

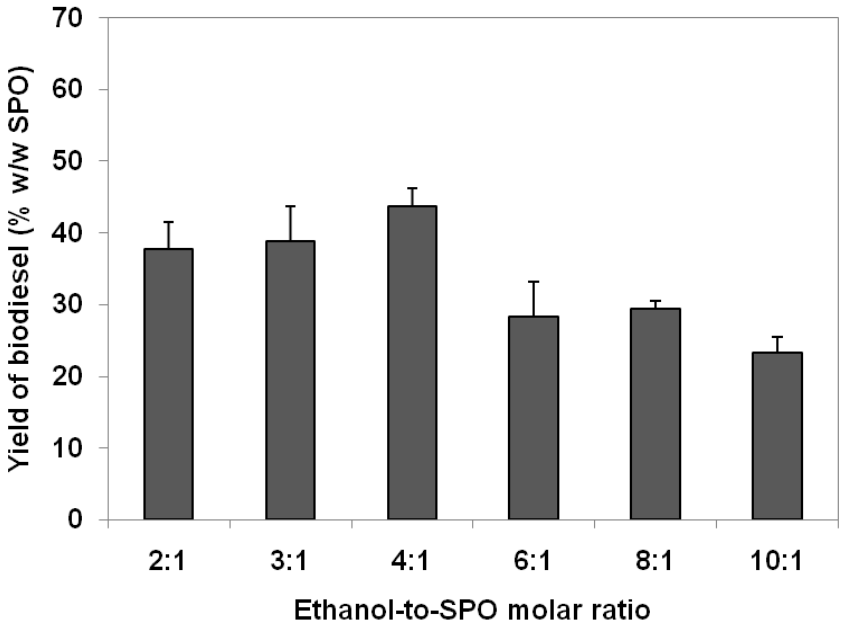

Figure 3. Effect of ethanol-to-SPO molar ratio on biodiesel production from sludge palm oil (SPO) using locally produced *Candida cylindracea* lipase.

w/w SPO). Although, the difference between the yield of ethanol and methanol was small, but in terms of cost and economic benefit, ethanol can be more attractive as compared to methanol because ethanol can now be produced from the renewable and low cost agricultural biomass (Ghaly et al., 2010). Thus, ethanol biodiesel appears as a 100%-renewable alternative (Rosa et al., 2009). Besides, methanol is a polar solvent which can trip water molecules from the enzyme surface thus affecting its native conformation and reduces its activity (Sivaramakrisnan and Muthukumar, 2012). Ethanol was found to give less inhibition effect to the activity of lipase because it is less hydrophilic than methanol. Nuylert and Hongpattarakere (2012) also stated that most lipases studied so far including the lipase secreted by *C. cylindracea* were very sensitive to methanol.

Effect of ethanol-to-SPO molar ratio

Ethanol solvent system was used for further investigations. The effect of ethanol-to-SPO molar ratio is illustrated in Figure 3. Based on the results, it was found that the maximum yield of biodiesel (43.8% w/w SPO) was achieved at 4:1 ethanol-to-SPO molar ratio. Even though, the stoichiometry requires only 3 moles of alcohol to convert 1 mol of triacylglyceride into 3 moles of esters, however the transesterification reaction is a reversible or an equilibrium-limited reaction (Röttig et al., 2010; Patel et al., 2013). Therefore, excess alcohol is used to favor the forward reaction which produces more esters (Patel et al., 2013). Similarly, the esterification is also a reversible or an equilibrium-limited reaction. More alcohol is needed to shift the reaction forward. Unless the high amount of water (by-product of esterification) will cause the reversible reaction, thus lowering the yield of biodiesel.

However, too excessive alcohol can cause the conformational change of the lipase protein structure thus inhibits its activity and reduces the yield of biodiesel. This effect can be seen when the ethanol-to-SPO molar ratio increased to 6:1. Therefore, 4:1 was the optimum ethanol-to-SPO molar ratio for the enzymatic biodiesel production from SPO. The optimum level of 4:1 alcohol-to-oil molar ratio was also reported by Li et al. (2012). The moderate amount of alcohol is favorable in biodiesel production because it reduces the cost of raw materials and effort for removing the excess alcohol.

Effect of enzyme loading

Enzyme loading is the limiting factor for the water content in this enzymatic biodiesel production because the lipase solution was in liquid form. Whenever, the enzyme loading increases, the water content also increases. Higher amount of enzyme is favourable for the biodiesel synthesis but the higher amount of water is unfavourable for the biodiesel synthesis. The variation of enzyme loading and water content are shown in Table 3.

The effect of enzyme loading in this study is illustrated in Figure 4. Based on the results, it was found that the

Table 3. Variation of enzyme loading and water content.

Enzyme loading (U)	Water volume (mL)	Water content (% in SPO)
5	1.050	3.4
10	2.100	6.5
30	6.299	17.4
50	10.498	25.9
70	14.697	32.9
100	20.995	41.2

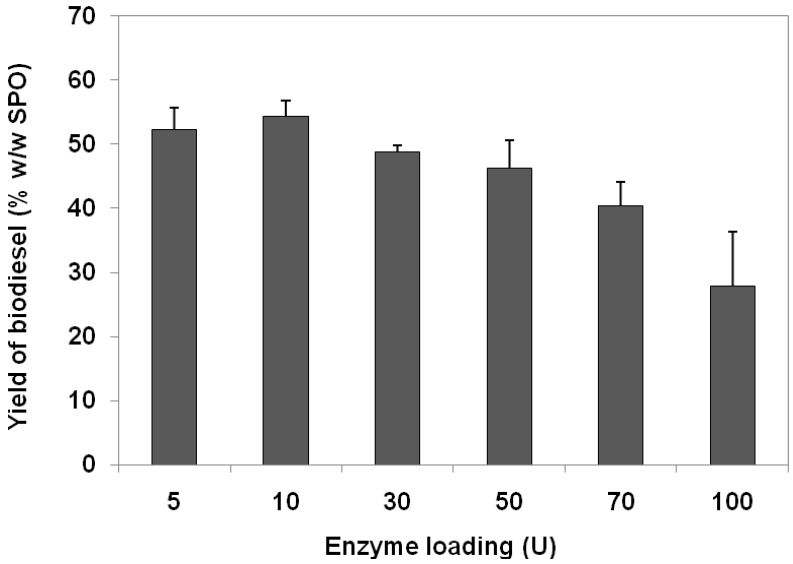

Figure 4. Effect of enzyme loading on biodiesel production from sludge palm oil (SPO) using locally produced *Candida cylindracea* lipase.

highest yield of biodiesel (54.4% w/w SPO) was achieved at 10 U. The decreasing of biodiesel yield when enzyme loading is more than 10 U might be due to the excess amount of water that promotes reversible esterification reaction (Patel et al., 2013). All the FFA might not be fully converted into biodiesel within the 5 h reaction and remained in the product. The remaining non-converted FFA was solidified and was removed at the end of biodiesel separation process, thus gave the lower yield of biodiesel. Besides, the excessive amount of water also affects the transesterification of TAG to be slower because of the competition between the alcohol and water molecules in the catalytic site of the lipase (Patel et al., 2013).

Effect of reaction temperature

Reaction temperature is very important for enzymatic biodiesel production. Lipase activity is significantly dependent on the reaction temperature. In general, for the 10°C increment of temperature, enzyme activity can increase up to 50 to 100% until it reaches the optimum temperature. In this study, the effect of reaction temperature is shown in Figure 5. Accordingly, the results show that the highest yield of biodiesel (51.8% w/w SPO) was achieved at optimum temperature of 40°C. This optimum reaction temperature is within the range of optimum temperature (30 to 40°C) of *C. cylindracea* lipase that was reported by Park et al. (2008). Further increasing the temperature caused the reduction in the yield of biodiesel because enzymes are protein and they become denatured when heated beyond their optimum temperature (Sivaramakrishnan and Muthukumar, 2012). Besides, the rate of reaction of lipase in enzymatic biodiesel production from SPO was higher at higher reaction temperature because at higher temperature, SPO can melt homogenously and has lower viscosity, thus allowing higher diffusion rate of substrate into the lipase's active site. Meanwhile, at lower temperature, the

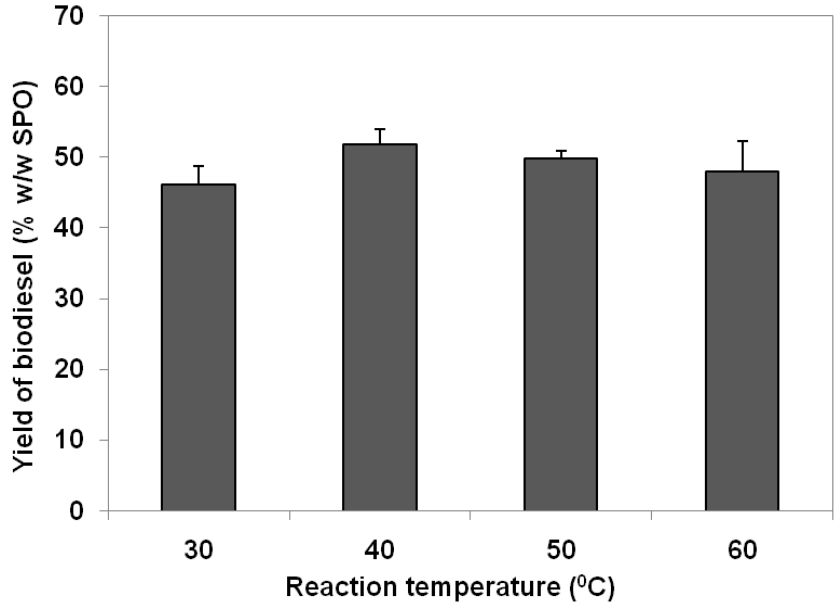

Figure 5. Effect of reaction temperature on biodiesel production from sludge palm oil (SPO) using locally produced *Candida cylindracea* lipase.

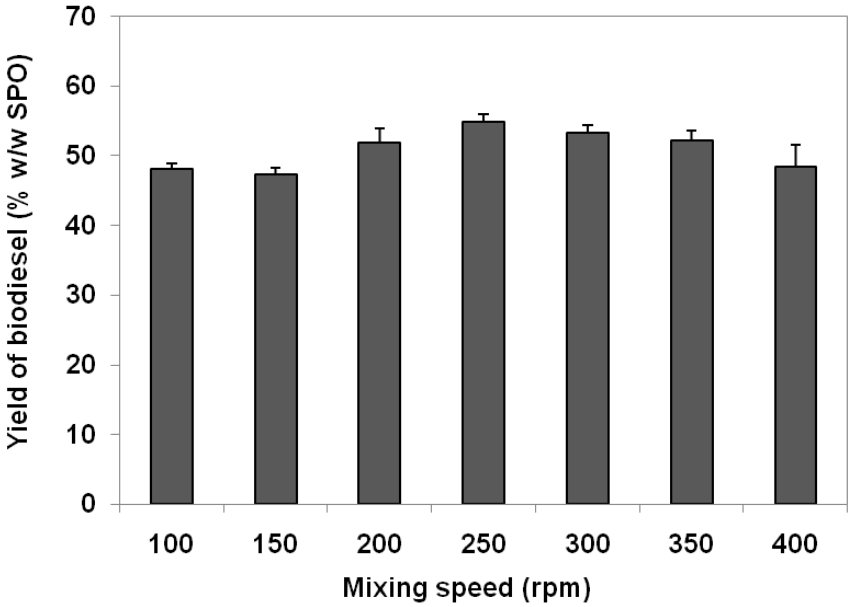

Figure 6. Effect of mixing speed on biodiesel production from sludge palm oil (SPO) using locally produced *Candida cylindracea* lipase.

viscosity of SPO was higher, thus causing poor diffusion between the phases and slower reaction rate.

Effect of mixing speed

Mixing is another important factor for the enzymatic biodiesel production because the activity of lipase can only occur at the water-oil interface. Mixing can generate more water-oil interfaces so that more lipase can react on oil for enzymatic transesterification and esterification. The effect of mixing speed is illustrated in Figure 6. Based on the results, it was found that the highest yield of biodiesel (54.9% w/w SPO) was achieved at the optimum mixing

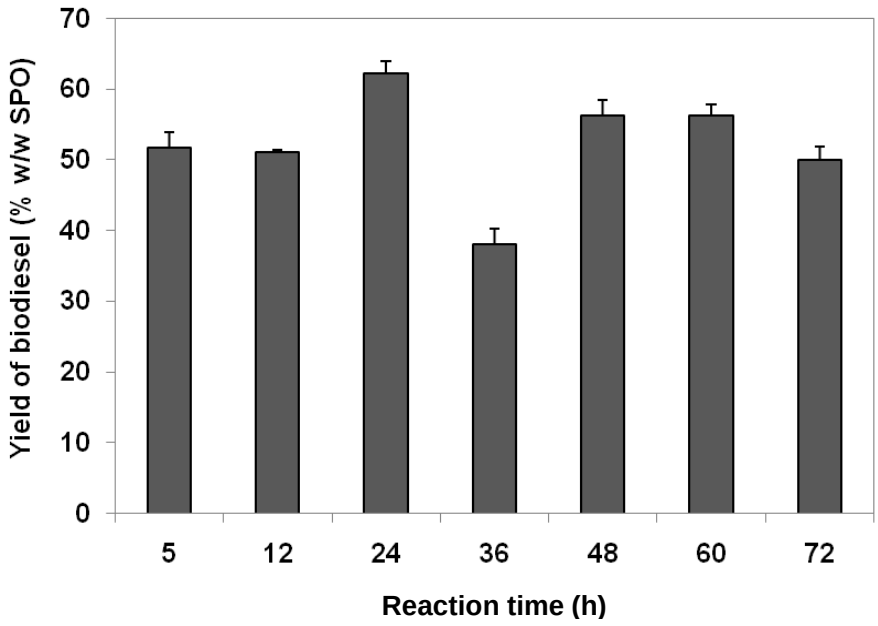

Figure 7. Effect of reaction time on biodiesel production from sludge palm oil (SPO) using locally produced *Candida cylindracea* lipase.

speed of 250 rpm. Although higher mixing speed can increase the contact between the substrate and the catalytic site of lipase, but too high of mixing speed can destroy the structure of enzyme (Jung et al., 2010). Meanwhile, lowering the mixing speed can significantly reduces the contact surface between the enzyme and the substrate due to the enzyme clustering thus gave lower reaction rate (De Paola et al., 2009).

Effect of reaction time

In general, the reaction time for the enzymatic biodiesel production is always longer than the biodiesel production using chemical catalyst. The reason might be due to the moderate alcohol-to- oil molar ratio, temperature and mixing speed that cause the forward reaction of transesterification and esterification to be slower. In this study, the effect of reaction time is shown in Figure 7. Based on the results, it was found that the highest yield of biodiesel (62.3% w/w SPO) was achieved at the optimum reaction time of 24 h. Mander et al. (2012) also reported that the 24 h is the optimum reaction time of enzymatic biodiesel production from olive oil using non-immobilized *Streptomyces* sp. CS268 lipase. Shorter reaction times were found to be not adequate to establish the kinetics of the enzyme activity due to the partially reversible character of the ethanolysis process (Verdugo et al., 2010). Meanwhile, longer reaction time leads to the reduction of end product (biodiesel) due to the reversible reaction (hydrolysis) thus resulting in the loss of esters (Mathiyazhagan and Ganapathi, 2011).

Conclusions

The present study shows that different alcohol, alcohol-to-oil molar ratio, enzyme loading, reaction temperature, mixing speed and reaction time are influential factors for enzymatic biodiesel production from SPO. At this stage, the highest yield of biodiesel was 62.3% w/w SPO with the optimum conditions (4:1 ethanol-to-SPO molar ratio, 40°C temperature, 250 rpm mixing speed, and 24 hours reaction time). However, the yield of biodiesel can be further improved by immobilizing the lipase thus reducing the amount of water that affect the reversible reaction significantly. The utilization of SPO and the locally produced POME based lipase shows a promising potential to reduce the cost of biodiesel production and to reduce the environmental pollutions that are caused by these palm-oil industrial wastes.

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