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SOLID STATE FERMENTATION USING AGROINDUSTRIAL WASTES TO PRODUCE ASPERGILLUS NIGER LIPASE AS A BIOCATALYST IMMOBILIZED BY AN ADSORPTION-CROSSLINKING METHOD FOR BIODIESEL SYNTHESIS

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ABSTRACT

Although technological advances have fueled the rising demand for lipase as a biocatalyst, commercial availability remains limited and costs prohibitive. To meet this need, an extracellular lipase enzyme from *Aspergillus niger* can be produced through solid state fermentation (SSF) using agroindustrial wastes including tofu dregs, coconut dregs, and corn bran. These agroindustrial residues still contain nutrients, especially lipids/triglycerides, making them a potential fermentation medium to produce lipase. Lipase with the highest activity level (8.48 U/mL) was obtained using a tofu dreg substrate, 4% inducer concentration, and 9-day fermentation period. This crude lipase extract was then dried with a spray drier and immobilized in a macroporous anion resin using the adsorption-crosslinking method. The immobilized lipase's activity was assayed by a biodiesel synthesis reaction; it showed 48.3% yield. The immobilized enzyme's stability was also tested through four cycles of biodiesel synthesis; in the fourth cycle, the enzyme maintained 84% of its initial activity.

Keywords: Adsorption–crosslinking; Agroindustrial waste; Lipase immobilization; Resin; Solid State Fermentation (SSF)

1. INTRODUCTION

Lipase is an enzyme with significant commercial applications in the food, medical, chemical, and pharmaceutical industries. In Indonesia, the demand for lipase is quite high, however, the commercially available lipase in the market is expensive and mostly imported. *Aspergillus niger* fungi are a potential lipase source because they can secrete extracellular lipase and produce higher quantities than other microbes (Koda et al., 2005). One method of extracellular lipase production is solid state fermentation (SSF), which uses solid substrate as a medium for mold growth. The SSF process is very economical, especially for countries like Indonesia that are rich in agroindustrial biomass and can use it as a cheap raw material (Castilho et al., 2000). Several agroindustrial residues have been used as substrates in microorganism cultivation to produce various enzymes (Mishra & Leatham, 1990; Kuhad & Singh, 1993; Muniswaran et al., 1994; Duenas et al., 1995; Hermansyah et al., 2015); the dominant one being wheat bran.

Some studies have shown that lipase produced by fermenting *Aspergillus niger* with SSF showed higher activity than those produced using submerged fermentation (Mohanasrinivasan et al., 2009; Farahbakhsh et al., 2013; Sarkar & Laha, 2013). In SSF, factors affecting the

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process include the substrate variety, time of fermentation, and the inducer concentration added to the environment. The variety of nutrients in the substrate will result in different quantities of lipase activity.

When used as a catalyst in an enzymatic reaction, lipase is unstable. It is also soluble in the reaction medium, which makes it hard to separate from the product and unable to be reused. This problem can be solved by immobilizing the enzyme in a certain support. Enzyme immobilization physically places the enzyme in a certain “space” to that its catalytic activity can be retained and, therefore, reused (Chibata, 1978). This "space" is a solid matrix called a "support." With immobilization, separating the enzyme from the product solution is easy and, therefore, drastically reduces the operational cost of the separation process. Resin is a porous, organic, synthetic support; it has a larger surface area than inorganic supports. Resin is also widely used as a support because of its retrievable nature. Enzyme immobilization can be achieved through various methods including adsorption-crosslinking, which results in immobilized enzymes that are more stable and show higher activity levels than enzymes obtained from other crosslinking methods (CLEA and CLEC). The principle of crosslinking is the formation of a three-dimensional network between the enzyme, support, and reactants. With the enzyme-enzyme and enzyme-support bonds, the stability of immobilized increases because few enzyme molecules fall off from the support.

In this study, extracellular lipase from *Aspergillus niger* with SSF was produced using three kinds of industrial wastes (tofu dregs, coconut dregs, and corn bran) as the fermentation substrate. The different nutritional contents of the substrates were observed, and the substrate producing the lipase with highest enzymatic activity was selected as the most compatible medium to produce lipase. Variations in the fermentation time and inducer concentration were also observed to determine the optimum conditions. Next, the conditions and substrate producing optimum lipase activity were used to produce lipase on a larger scale; the dry lipase extract (powder) was generated using a spray drying method. Then the lipase enzyme was immobilized in a macroporous anion resin and used in a biodiesel synthesis. The immobilized enzyme’s stability was observed by re-using the enzyme in four cycles of biodiesel synthesis.

2. EXPERIMENTAL METHODS

2.1. Materials

The agroindustrial wastes used for the fermentation substrate were obtained from the tofu, coconut milk, and corn industries. The substrates compositions were examined by proximate analysis, which was performed at Balai Besar Industri Agro (BBIA) laboratory in Bogor, Indonesia.

The pure chemicals were obtained from Merck. The *Aspergillus niger* isolate was obtained from Lembaga Ilmu Pengetahuan Indonesia (Indonesian Institute of Sciences). The *Candida rugose* lipase was bought from Sigma Chemical Co., St. Louis, MO, USA. This commercial enzyme has an activity unit of ≥700 units/mg solid, where one unit is defined as the amount of enzyme needed to hydrolyze one microequivalent of fatty acid from triglycerides in one hour. A macroporous anion resin was bought from Fluka Analytical, with the trade name of Lewatit ® MP-64, which is an anion exchange macroporous resin consisting of a polystyrene matrix.

2.2. Solid State Fermentation

Before starting fermentation, the *Aspergillus niger* isolate was precultured in a petri dish with a potato dextrose agar (PDA) medium. The SSF substrate was prepared by dividing 20 grams of the sample into each Erlenmeyer flask. In this experiment, the concentration of the olive oil inducer varied at 2, 4, and 8 percent (g/g dss) for each substrate. The fermentation effects were observed at 5, 7, 9, and 11 days, for a total of 36 samples.

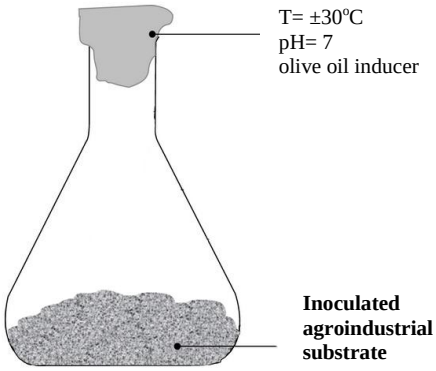

Figure 1 Solid state fermenter apparatus

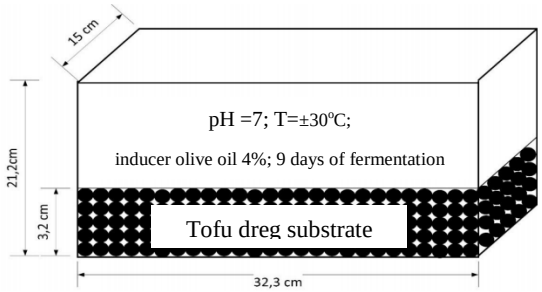

Figure 2 Upscaled SSF fermenter (Source: Hariyani, 2014)

Additional nutrients where added to the substrate-filled Erlenmeyer flasks in the following compositions: 65% distilled water, 0.34% NH_2CONH_2, 0.75% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.0375% $CaCl_2$, 1.8% NaH_2PO_4, and 0.045% $MgSO_4.7H_2O$ (g/g dss) (Falony et al., 2006). Then the samples were covered and sterilized in an autoclave for 15 minutes at 121°C.

After sterilization, inoculation of the *Aspergillus niger* was started. *A. niger* as much as 10% v/w of the dry substrate was prepared. The mold from the PDA medium was extracted using an ose needle, dissolved into 2 mL aqua dest, and then poured into the sample bottle/Erlenmeyer flask. Next, the sample was incubated at room temperature.

2.3. Lipase Extraction

The fermentation results were then dissolved with 400 mL distilled water and agitated with a magnetic stirrer for 30 minutes at room temperature. Next, the solution was filtered using a muslin cloth and centrifuged at 5000 rpm for 15 minutes at room temperature. The centrifuged solution was re-filtered with filtering paper, and the resulting crude lipase extract or supernatant was stored at 4°C (Qisthiya, 2013).

2.4. Lipase Activity Assay (Hydrolysis)

Lipase activity was analyzed by the titrimetric method using a polyvinyl alcohol-olive oil emulsion as substrate (Watanabe et al., 1977). A 30-mL sample of crude lipase extract was mixed with 0.3 g PVA and 5 mL olive oil and then allowed to react on a hotplate stirrer for 30 minutes.

After 30 minutes, 15 mL of ethanol was added to act as a reaction stopper. A 2-mL sample of the solution was taken, 3 drops of phenolphthalein indicator were added, and then the solution was titrated to quantify the free fatty acid that had formed; NaOH 0.05 N was used as the titrant. The lipase activity was calculated using the following Equation 1 (Kavardi et al., 2012):

$$Lipase\ activity = \frac{N_{NaOH} \times V_{NaOH} \times 1000}{t \times V_{sampel}} \quad (1)$$

2.5. Drying of the Crude Lipase Extract

After the activity assay, the fermentation procedure was repeated using the operating conditions that yielded the highest lipase activity. The fermentation was upscaled to produce larger quantities of lipase supernatant (Figure 2). Then the lipase supernatant obtained from the upscaled fermentation was converted to a powder by spray drying. Spray drying was achieved by adding 0.5% w/v of skim milk and operating at the conditions of 130°C at the inlet and 70°C at the outlet (Hariyani, 2014).

2.6. Lipase Solution Preparation

One gram of *Aspergillus niger* lipase was dissolved in 10 mL of phosphate buffer (pH 7) to obtain a lipase solution of 0.01 g/mL. One mL of the solution was taken as a sample for the initial concentration in the enzyme loading analysis.

2.7. Adsorption-Crosslinking Immobilization

The *Aspergillus niger* lipase was adsorbed onto the resins and then reacted with glutaraldehyde as the crosslinking agent. The lipase solution (0.01 g/mL, 10 mL) and resin support (750 mg) were mixed in an Erlenmeyer flask, then agitated in a 30°C shaking water bath at 150 rpm for 4 hours. Then a 1 mL sample was extracted for enzyme loading analysis. A 0.5% glutaraldehyde concentration was added to the Erlenmeyer flask and then reacted in a shaking water bath under the same conditions for another 20 minutes. A 1 mL sample was taken for enzyme loading analysis after crosslinking.

2.8. Enzyme Loading Analysis

The enzyme loading was analysed using the Lowry method (Lowry et al., 1951). Enzyme loading determines the percentage of lipase successfully immobilized into the support resin. The initial concentration of lipase solution was 0.01 g/mL; the absorbance was then rechecked using a UV-Vis spectrophotometer to verify the initial concentration using a standard calibration curve. The remained lipase solution absorbed after immobilization was also checked to determine the final lipase concentration. Thereafter, the enzyme loading was calculated using the following formula:

$$X_L = \frac{C_E}{C_0} \times 100\% \quad (2)$$

$$C_E = C_0 - C_t \quad (3)$$

2.9. Immobilized *Aspergillus niger* Lipase Activity and Stability Assay

The immobilized lipase was then used as a biocatalyst in a non-alcohol route biodiesel synthesis. The reaction took place in a 100 mL Erlenmeyer flask, which functioned as a batch reactor. Palm oil and methyl acetate were put into the Erlenmeyer flask with a molar ratio of 1:12 and left to react in a 40^{o}C shaking water bath for 50 hours (Marno, 2008). One immobilized biocatalyst reaction batch used as much as 4% wt of the triglyceride substrate (Hermansyah et al., 2009).

A sample from the reaction was analysed using High-Performance Liquid Chromatography (HPLC) to calculate how much methyl esters (biodiesel) was formed. Enzyme stability analysis was accomplished by using the same biocatalyst for four consecutive reaction cycles. The lipase activity parameter was the biodiesel yield and was calculated using the formula below.

$$Yield = \frac{C_t}{3 \times C_0} \times 100\% \quad (4)$$

3. RESULTS AND DISCUSSION

3.1. Effect of Fermentation Substrate on Lipase Activity

Every fermentation substrate's lipase activity was assayed by titrimetric analysis. When considering substrate variation, other factors such as fermentation temperature, pH, and humidity were considered fixed variables. The highest activity level (6.07 U/mL) with a 2% inducer concentration was obtained with tofu dregs on the 9th day (Figure 4). Also on the 9th day, the coconut dregs produced lipase with an activity unit of 5.02 U/mL, and the corn bran substrate produced 3.92 U/mL. On the 5th and 7th days of fermentation, the lipase activity from the tofu dregs was the lowest of the substrates. On the 11th day of fermentation, however, while all lipase activity decreased, lipase from the tofu dregs maintained the highest activity level at

3.73 U/mL; the corn bran and coconut dreg showed activity unit levels of 1.77 U/mL and 1.43 U/mL, respectively.

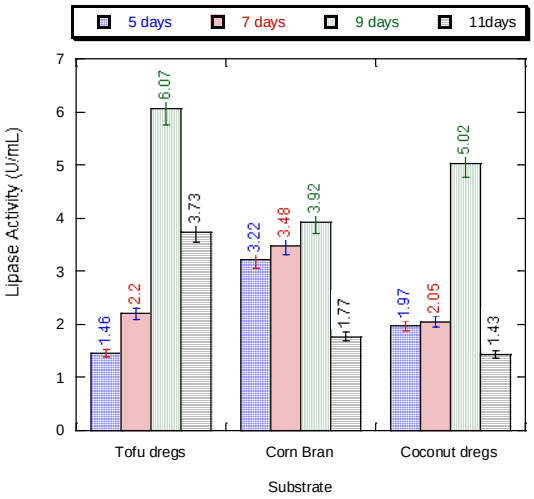

Figure 3 Effect of fermentation substrate and time (olive oil inducer 2% wt, T= ±30°C, pH=7)

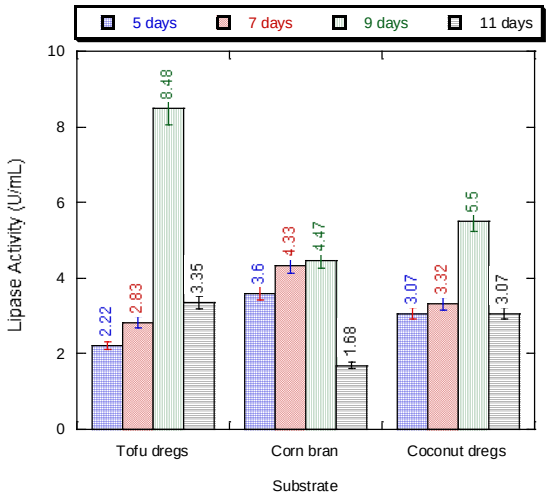

Figure 4 Effect of fermentation substrate and time (olive oil inducer 4% wt, T= ±30°C, pH=7)

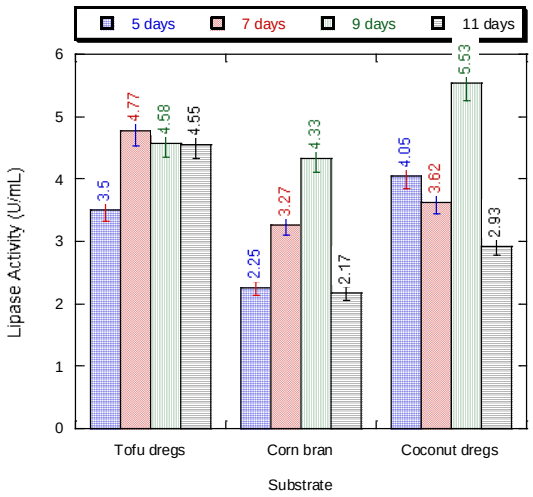

Figure 5 Effect of fermentation substrate and time (olive oil inducer 8% wt, T= ±30°C, pH=7)

Similar phenomena were observed under the same conditions with the 4% inducer concentration (Figure 5). The highest activity (8.48 U/mL) was obtained from the tofu dreg substrate on the 9th day of fermentation, followed by the coconut dreg (5.53 U/mL) and corn bran (4.47 U/mL) substrates. On the 5th and 7th days of fermentation, the highest activity was obtained from the corn bran and the lowest from the tofu dreg. But on the 11th day, the lipase from the tofu dreg maintained the highest activity (3.35 U/mL); the lipase from coconut dreg also maintained an activity unit of 3.07 U/mL, while the corn bran only produced 1.68 U/mL.

The patterns observed in the 2% and 4% inducer concentrations changed in the 8% concentration. In the 8% inducer (Figure 6) concentration, the highest activity was obtained from the coconut dreg substrate on the 9th day of fermentation (5.53 U/mL), while on the same day, the tofu dreg and corn bran substrates showed 4.58 U/mL and 4.33 U/mL, respectively. On the 5th day, the highest activity was also obtained from coconut dreg substrate (4.05 U/mL), followed by the tofu dreg (3.5 U/mL) and corn bran (2.25 U/mL) substrates. On the 7th day, the tofu dreg substrate showed the highest activity (4.77 U/mL), followed by the coconut dreg (3.62

U/mL) and corn bran (3.27 U/mL) substrates. On the 11th day, however, the tofu dreg substrate showed the highest lipase activity (4.55 U/mL), followed by the coconut dreg (2.93 U/mL) and corn bran (2.17 U/mL) substrates.

Several important points can be drawn from these observations. First, in the 2% and 4% inducer concentrations, the tofu dreg substrate showed the highest activity level. Second, in general, the highest lipase activity was observed on the 9th day of fermentation. Third, the coconut dreg substrate showed the highest lipase activity in the 8% inducer concentration. The causes of these observations can be deduced by examining the composition of the respective substrates. Samples from each fermentation substrate were analysed using proximate analysis. The results are summarized in Table 1.

Table 1 Proximate Analysis Results

Parameter	Composition (% wt)		
	Tofu dregs	Corn bran	Coconut dregs
Water	10.9	8.49	9.72
Ash	2.9	0.76	6.68
Protein	22.2	9.73	4.64
Lipid	10.7	2.12	5.94
Carbohydrates	53.3	78.9	73.00

Overall, the highest lipase activity was obtained from fermentation using tofu dregs as the substrate. Tofu dregs have the highest level of protein, which can be used as a nitrogen source for enzyme formation, and the highest level of lipids, which can be used as a carbon source that induces *Aspergillus niger* to produce more lipase. Carbon is needed to grow mold. Nitrogen's effects on enzyme activity can be observed in Dayanandan et al.'s study (2013), which showed that adding peptone as a nitrogen source increased lipase production.

Lipase from fermentation using corn bran as the substrate showed relatively low activity because even though the protein concentration is high (which supports enzyme production), the lipid concentration is low; thus, lipase production is low. Because corn bran is high in carbohydrates, fermentation using corn bran as the substrate produces more saccharides-degrading enzymes such as amylase, and maltose. Coconut dregs are desirable for lipase production because the carbon and lipid levels are significant, but because those levels are lower than tofu dregs, the enzymatic activity produced is also lower.

3.2. Effect of Inducer Concentration on Lipase Activity

Figure 7 shows the effect of the inducer concentration on the tofu dreg substrate's lipase activity. On the 5th and 7th days of fermentation, the increase in inducer concentration from 2% to 8% was directly proportional to the increase in lipase activity. But on the 9th day, the increase in inducer concentration was proportional to increased lipase activity only up to the 4% concentration; it decreased at the 8% inducer concentration. On the 11th day, while lipase activity decreased as the inducer concentration increased from 2% to 4%, it increased when the inducer concentration increased to 8%.

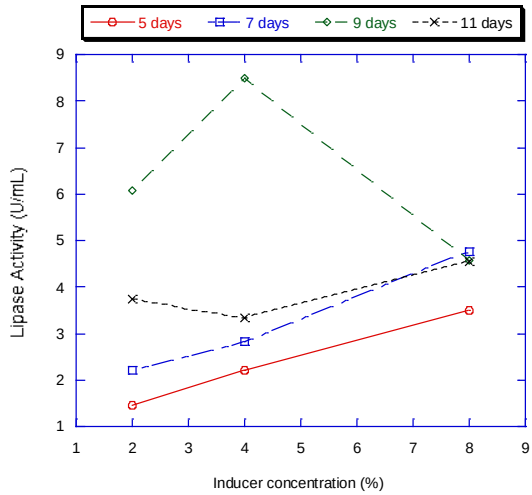

Figure 6 Effect of inducer concentration and fermentation time (tofu dreg substrate, T= ±30°C, pH=7)

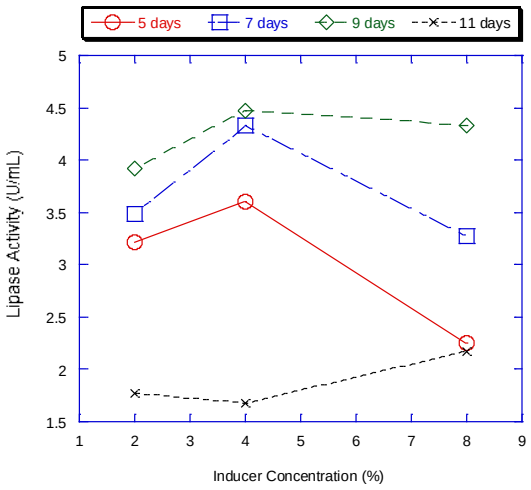

Figure 7 Effect of inducer concentration and fermentation time (corn bran substrate, T= ±30°C, pH=7)

Figure 9 shows the effect of the inducer concentration on the corn bran substrate's lipase activity. On 5th and 9th days of fermentation, increasing the inducer concentration from 2% to 4% was directly proportional to the increase in lipase activity. When the inducer concentration was increased to 8%, however, lipase activity decreased. Contrastingly, on the 11th day of fermentation, activity decreased when the inducer concentration increased from 2% to 4%, and activity increased when the inducer concentration was increased to 8%.

The effect of the inducer concentration on the coconut dreg substrate's lipase activity is depicted in Figure 9. On the 5th to 9th days of fermentation, lipase activity was directly proportional to inducer concentration; adding inducer resulted in higher lipase activity. But on the 11th day of fermentation, lipase activity increased as the inducer concentration was increased from 2% to 4%, but decreased when the inducer concentration reached 8%.

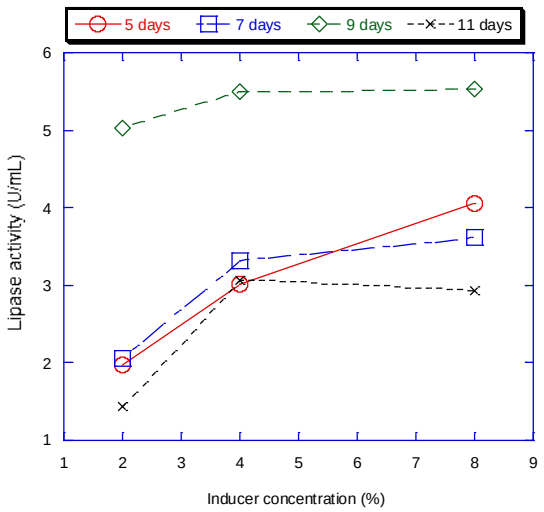

Figure 8 Effect of inducer concentration (coconut dreg substrate, T= ±30°C, pH=7)

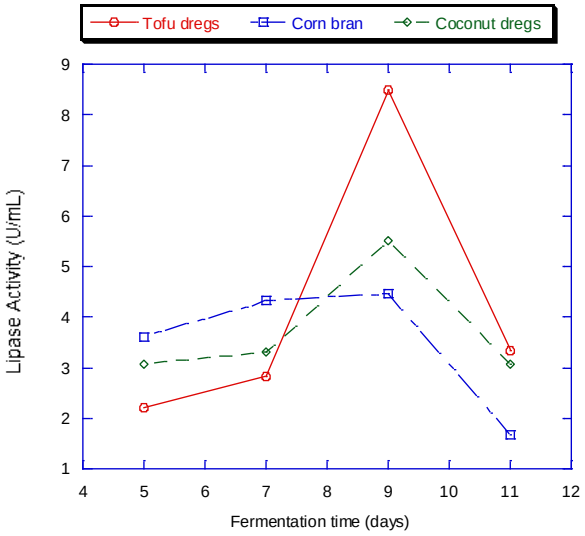

Figure 9 Effect of time and substrate (4% inducer, T= ±30°C, pH=7)

Analysis of the lipase activity reveals that in the tofu dreg and corn bran substrates, the 4% inducer concentration yielded the highest activity. The fermentation using coconut dreg

substrate, however, showed the highest activity with 8% inducer concentration. Generally, lipase activity will increase related to lipid concentration in the environment. However, if the lipid concentration is too high, it will affect the lipase produced by mold; excessive lipid levels can be cytotoxic for the mold and kill it. High lipid concentration can also cause a biphasic system that will inhibited the oxygen transfer and nutrition absorption from the substrate to the microorganism, in this case, the *Aspergillus niger* (Zarevucka, 2012). This explains why the highest lipase activity from the tofu dreg substrate was obtained with the 4% inducer concentration and why activity decreased with the 8% inducer concentration. Thus, the inducer concentration added to the fermentation environment needs to be adjusted to the substrate composition so that the lipid contents will not be excessive or deficient.

3.3. Effect of Fermentation Time on Lipase Activity

Fermentation time is an important variable because it directly affects mold growth and the concentration or amount of lipase that is secreted by the mold at certain times. Analysis of the activity unit for each fermentation substrate shows that the highest lipase activity was obtained on the 9^{th} day of fermentation. On the 11^{th} day, lipase activity significantly decreased, as depicted in Figure 10. This activity unit trend can be explained by comparing it to microorganism growth patterns. Mold growth follows the level of nutrition present in the environment and is modified by other factors. Usually one fungi unit will grow exponentially in the beginning. This exponential phase is followed by a plateau or stationary phase, where the organic nutrition in the environment has decreased, and concludes with a death phase. This life cycle is typical in every fungal-growing system that has a fixed amount of nutrition.

In this experiment, on the 5^{th}, 7^{th}, and 9^{th} days of fermentation, the growth of the *Aspergillus niger* mold was in the initial logarithmic phase; the amount of fungal biomass kept increasing because of the abundance of nutrition in the environment. Enzyme activity in the beginning was low and increased with fermentation time in a manner directly proportional to the growing biomass. But on the 11^{th} day, the nutrition in the environment was depleted; the enzyme-producing microorganism entered the death phase, and activity drastically decreased. This result matches Sa'adah's study that showed a decrease in activity on the 10^{th} day of fermentation (Sa'adah & Noviana, 2010). Therefore, the optimum time to extract extracellular lipase from a fermentation system is 9 days.

3.4. Dry Lipase Powder Production Results

Supernatant from the fermentation was spray dried, resulting in a dry lipase powder with a fine, white appearance. This dry lipase has a longer shelf life than crude lipase (supernatant). The activity of dry lipase powder was then measured using an olive oil emulsion titration; it showed 8.1 U/mL of activity. Compared to the crude extract (8.48 U/mL), the activity decreased. This decrease is caused by enzyme denaturation in the high-temperature spray drying process. But the decrease was not very significant because using skim milk as the additive protects the enzyme molecules. The comparison between commercial *Candida rugose* lipase and *Aspergillus niger* dry lipase from the tofu dreg substrate is illustrated in Figure 11.

Thus, the enzyme activity from this experiment amounts to 11% of commercial enzyme activity, which is 81 U/g, the commercial *Candida rugose* lipase reached 708.2 U/g. The differences of both lipase activity because of the purity of those lipases. The experiment lipase was not yet purified (crude extract) which is the *Candida rugosa* lipase was already purified.

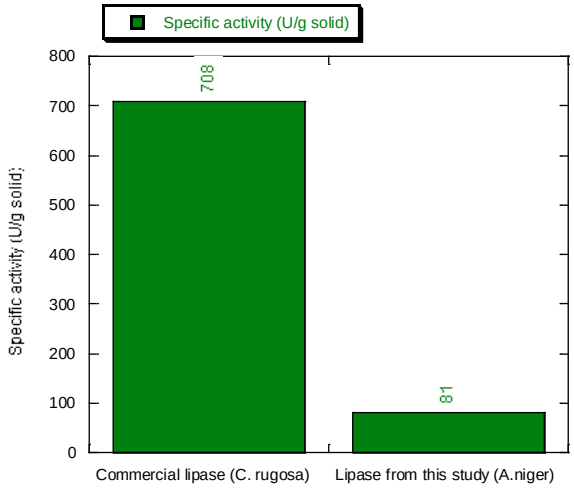

Figure 10 Comparison of *Aspergillus niger* lipase activity from tofu dreg substrate and commercial lipase (olive oil emulsion hydrolysis, T=40^{o}C, t=30 mins)

3.5. *Aspergillus niger* Lipase Immobilization Results

Three samples of lipase solution were taken from immobilization process for analyzing enzyme loading. They were the initial concentration (C_0), concentration after adsorption (Ct_a), and concentration after crosslinking (Ct_C). Enzyme loading analysis was done following the Lowry method, and the absorbance was measured with a UV-Vis spectrophotometer at 750 nm wavelength. The enzyme concentration was obtained from a standard calibration curve, which was made using a standard solution for protein analysis, Bovine Serum Albumin (BSA).

From the absorbance data, the enzyme loading at adsorption was calculated to be 7.96% of the initial enzyme concentration. This value is relatively low mainly because in adsorption the bonds are weak, so it is possible that the enzyme molecules were desorbed. Compared to a previous study by Kurnia and Albels (2015), enzyme loading at this stage was low; in that study, adsorption enzyme loading reached 31.75% and 34.82%.

After the addition of a crosslinking agent, enzyme loading increased to 53.9%. This increase is because glutaraldehyde is a reactive bifunctional component that can react with the OH group on the support surface and the amino groups in the enzymes allowing more enzyme molecules to be bound to the support (Chen et al., 2005).

The activity of immobilized lipase enzyme was analysed. The results showed that the activity of the immobilize lipase was 82.67 U or 459 U/g support, which is slightly higher than the free lipase activity (81 U). This outcome is possible because the temperature stability of immobilized lipase is greater than that of free lipase, as showed in da Silva et al.'s study (2008). This result also corresponds with previous research demonstrating that immobilized lipase activity is more thermally stable than free form lipase activity.

3.6. Biodiesel Synthesis using Immobilized Lipase Results

The product produced by the biodiesel synthesis reaction was analysed by HPLC, which showed the concentrations of the triglyceride, diglyceride, monoglyceride, and methyl ester components. These calculations show that the biodiesel yield produced was relatively high, 49.2% in the first cycle (Figure 12). The biodiesel yields in the second to fourth cycles show the stability of the immobilized enzyme in repeated use. The calculations show that, even after four cycles, the yield decrease was not very significant, approximately 83% of initial activity. These results show that the lipase molecules remained immobilized inside the support and a minimal number of molecules were desorpted. The immobilized enzyme in a study by Albels only

maintained 64% of its initial activity in the fourth cycle (Figure 12). Thus, the immobilized lipase in this experiment demonstrated strong stability (Albels, 2015).

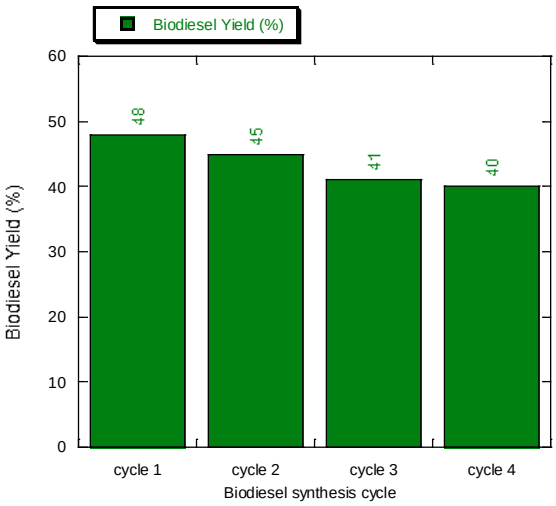

Figure 11 Biodiesel yield percentage (interesterification, T=40^{o}C, t=50 jam, 4%wt immobilized A. *niger* lipase from tofu dreg substrate)

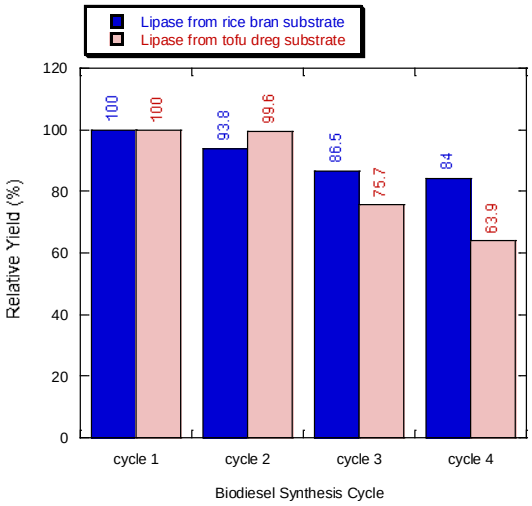

Figure 12 Relative yield comparison of lipase from tofu dreg substrate and lipase from rice bran substrate (Albels, 2015) (interesterification, T=40^{o}C, t=50 jam, 4%wt immobilized A. *niger* lipase from tofu dreg substrate)

4. CONCLUSION

Considering lipase activity, tofu dregs functioned as the best solid state fermentation substrate/medium to obtain extracellular *Aspergillus niger* lipase. Drying the lipase supernatant into a fine powder resulted in an activity unit of 810 U/g. The *Aspergillus niger* lipase from the tofu dreg substrate was successfully immobilized onto a macroporous anion resin support using an adsorption-crosslinking method; the activity level was 827 U/g. When the immobilized *Aspergillus niger* lipase was used in a biodiesel synthesis reaction, a 48.3% biodiesel yield was obtained; this lipase was stable enough to maintain 84% of its initial activity through four cycles. Based on these results, the use of agroindustrial waste as a medium to produce lipase is a process that is eco-friendly and has strong potential to be used on an industrial scale. To capitalize on this potential, future research should conduct studies on other types of agroindustrial waste and explore the best way to implement this process on an industrial scale. Therefore, decreasing and reusing agroindustrial waste can open new opportunities.

5. ACKNOWLEDGEMENT

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Nomenclature

U	Amount of enzyme that frees 1 µmol of fatty acid per minute per 1 ml of samples ($\mu mol\ menit^{-1}\ ml^{-1}$)
U/g solid	Enzyme activity unit specific to solid enzyme mass (µmol $menit^{-1}$ g^{-1})
U/g support	Enzyme activity unit specific to immobilized enzyme mass (µmol $menit^{-1}$ mL^{-1})
$\mathbf{N_{NaOH}}$	Normality of NaOH titrant (mol mL^{-1})
$\mathbf{V_{NaOH}}$	Volume of titrant needed to reach equilibrium (mL)
$\mathbf{V_{sample}}$	Volume of sample solution titrated (mL)
C_E	Immobilized enzyme concentration (g mL^{-1})
C_0	Enzyme concentration before immobilization (mL^{-1})
C_t	Enzyme concentration at period t (mL^{-1})
X_L	Enzim loading (%)
Yield	Ratio of product (biodiesel) to initial concentration of reactant (triglycerides) (%)
g/g dss	gram/gram dry substrate solid

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