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The Immobilization of Lipase From *Mucor Miehei* on Zeolite Matrix in Hydrolysis of Palm Oil Producing Free Fatty Acids with Solvent Free System

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Abstract

The enzymatic hydrolysis of palm oil can be conducted by using lipase produced from Mucor miehei to produce free fatty acid. This study aimed to compare the usage of lipase as free enzyme and as immobilized enzyme on zeolite matrix in the hydrolysis of palm oil as triglyceride producing free fatty acids which highly needed in various industrial sectors. Immobilization is an alternative hydrolysis reaction due to its usage on repetitive reaction, makes lipase reuseable, hence the whole process becomes efficient, and with moderate operational conditions. Solvent free reaction is applied, because the produced free fatty acids can be used directly in food, health, and natural flavorings industry. The palm oil used in the hydrolysis contains 0.815% initial free fatty acids as palmitate, in which water then added to it in weight ratio 1:3. Each effect of free lipase and immobilized lipase addition is 4%, 5%, 6%, 7%, 8%, and time reaction is 30, 60, 90, 120, 150 minutes are used as index to determine the amount of free fatty acids produced. The results showed that Immobilized lipase has better ability than the free one in hydrolysis of triglyceride in palm oil producing free fatty acid with 8% lipase addition and time reaction of 120 minutes. Palm oil hydrolysis using free lipase produced the highest FFA of 1.9747% after the addition of 5% lipase concentrate, with time reaction of 60 minutes. Meanwhile, palm oil hydrolysis using immobilized lipase produced the highest FFA of 1.9747% after the addition of 8% lipase concentrate, with time reaction of 120 minutes.

**This is a revised and extended version of an article which had been presented at SNTK UNNES 2017, Semarang, Indonesia, September 20th, 2017.*

INTRODUCTION

Lipase (acylglycerol acyl hydrolase, EC 3.1.1.3) is one of biocatalysts widely used in reactions such as hydrolysis, aminolysis, esterification, and transesterification producing chemical products. Lipase is produced from various sources, such as *Mucor miehei, Bacillus substilis, Thermomyces lanuginosus, Candida antartica, Candida rugosa, Candida cylindracea, Aspergillus niger, Rhizopus oryzae, Penicilium camembertii, Penicilium roquefortii* (Gupta et al., 2013). The catalyctic ability produced from free lipase is very high, but unstable due to the effects of pH, temperature, and organic solvent related to its chemical stability. Therefore, the immobilization of lipase on the matrix became an alternative to keep the stability. Immobilization of lipase is a method where lipase is unable to move freely due to being held in place. Benefits of applying immobilization technique are (a) the enzyme could be used for repetative reactions (b) the convenience for separation and purifying process of chemical products, (c) increasing enzyme's stability to temperature and chemical substance, (d) economical, (e) improving the reaction control and product's quality (Gupta et al.,

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2013). Various organic and inorganic compounds are used as matrix for enzyme's immobilization, such as zeolite, polypropylene, or micro-pored silica (Bayramoglu et al., 2015). Recently, zeolite is used as matrix for immobilization of lipase because it's a micro-pored material with wide specifications and high adsorption ability, and has active center, as shown in Figure 1.

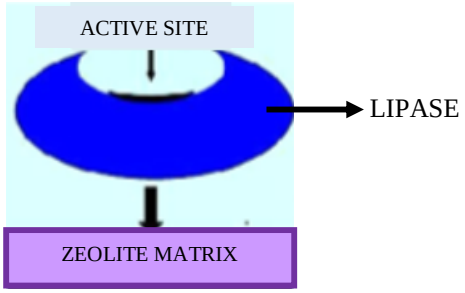

Figure 1. Method of Lipase Immobilization on Zeolite by adsorption (Gupta,2013)

The component of zeolite is aluminium silicate crystals with basic structures that consist of tetrahedron combination of TO_4 (T = Si, Al), covalently bonded with oxygen atoms. The usage of lipase immobilization on zeolite matrix was chosen due to high specificity of the enzyme, requiring moderate condition, did not require high temperature for the reactions, so it lessened the required energy, lessened the by-product of side reactions, and more efficient to convert the substrate. Lipase could work on long-chain triglyceride hydrolysis, which was formed from ester and fatty acid. The zeolite being used as the matrix on lipase immobilization is inert; therefore, it won't damage the enzyme activities. The highest contribution of immobilized lipase's performance could be obtained by selecting the immobilization techniques and the proper matrix characteristics (Calgaroto et al., 2011). Zeolite and lipase present salient features in hydrophobic/hydrophilic roles, acid/base, mechanical/chemical resistance, crystal morphology and size, surface area, and pore diameter. Therefore, various compositions and sizes of zeolite molecules as matrix contribute significantly to its adsorption ability (Macario et al., 2007).

Based on the description above, the aim of this study is to assess the performance of immobilized lipase on zeolite matrix in the hydrolysis of palm oil to produce free fatty acid without addition of solvent, therefore it could be used directly as natural substrate on various food industry.

RESEARCH METHODOLOGY

Materials

The main materials used are coconut oil (local), palm oil (local), lemongrass oil (local), *Mucor miehei* (local), coconut pulp (local), ethanol 70%, NaF 0.5 M, FeSO4.7H20 (Merck), HCL (Merck), NaCl 1 M (Pa), $(NH_4)_2SO_4$ (Merck), pepton, NaOH and KOH 0.1 M, KOH alcoholic, K_2HPO_4, phenolphthalein, potato dextrose agar, aquadest.

Equipments

The equipments used in this study are: test tube, titration flask, 1 ml, 5 ml, and 10 ml measurement pipette, 25 ml pipette, watch glass, spatula, stirring rod, 100 ml and 250 ml laboratory flask, 100 ml graduated cylinder, pipette ball, 50 ml burette, 500 ml three-neck round bottom flask, condenser, inoculation loop, 100 ml beaker glass, autoclave, micropipette, centrifuge, vortex mixer, oven, laboratory furnace, incubator, analytical balance, heating jacket, hot plate.

Experiments

This study is carried out through several steps:

Producing Crude Lipase from Mucor miehei

Media sterilization that consists of 5% pepton, 1% KH_2PO_4, 0.001% $FeSO_4.7H_2O$, 10% olive oil, 10% palm oil, 20% dry coconut pulp, and aquadest using autoclave. After the temperature lowered, Mucor miehei suspension is added to the media, and incubated for five days at 40^0C using incubator shaker and rotational speed of 120 rpm. After incubating period, the biomass and the supernatant are separated (above) on 3,000 rpm for 30 minutes. The produced supernatant Is crude lipase which will be immobilized with zeolite.

Zeolite Activation

Zeolite with the size of 0.8 mm is washed with water at 70°C for one hour. Zeolite then is soaked in 1 M NaCl solution for 12 hours with two times replacement. Zeolite is heated at 300 °C for three hours in the oven. After the temperature lowered, immobilization can get started using crude lipase.

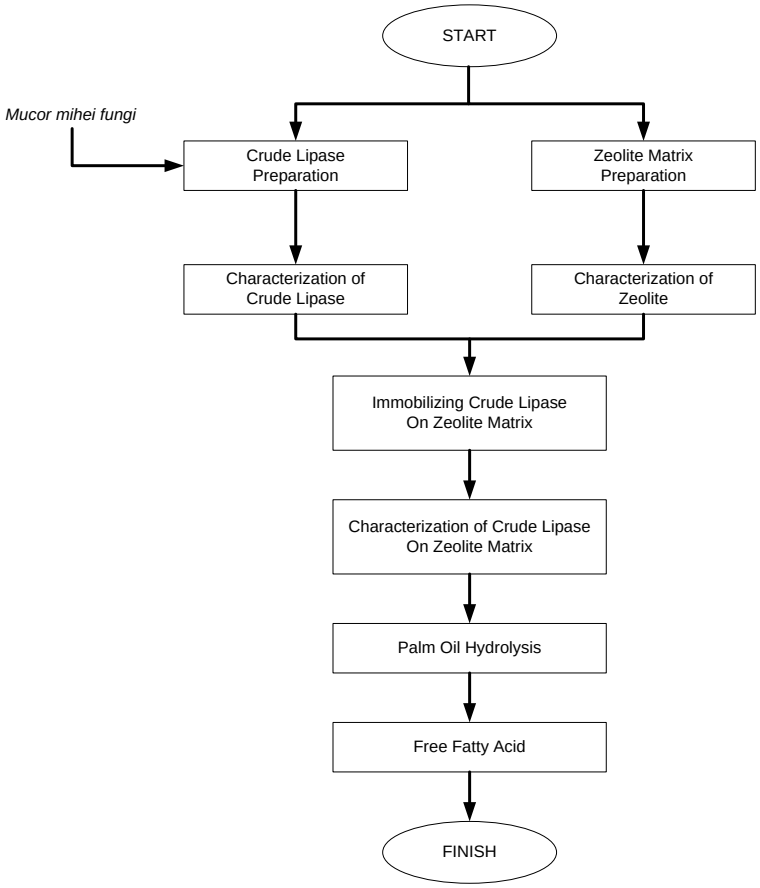

Figure 2. Work scheme of palm oil hydrolysis to Free Fatty Acid (FFA)

Lipase immobilization on Zeolite

One gram of lipase is added to one gram of buffer phosphate solution with pH 7 and the total volume is measured. Immobilization is carried out by mixing 30 ml crude lipase with 10 gram of activated zeolite and added with 0.5 ml NaF. The total is 0.5 M /10 ml crude lipase.

Hydrolysis

Hydrolysis is carried out by making reaction of coconut oil with water in 1:3 ratio (b/b). Next is the addition of free/immobilized enzyme with concentration of 4%, 5%, 6%, 7%, 8% and reaction time of 30, 60, 90, 120, and 150 minutes. The hydrolysis ability is shown by the increasing final %FFA compared to the initial %FFA.

Analyzing Crude Lipase

Two milliliters of olive oil in 100 ml titration flask, added with 1 ml of 0.05 M (pH 8) phosphate buffer solution, and 1 ml enzyme solution are incubated in shaker incubator with rotational speed of 120 rpm and at 40°C for ten minutes. The enzyme then is inactivated using 1 ml ethanol/acetone mixtures (1:1). Five drops of 1% phenolphthalein is added as indicator, and titrated using 0.005 N NaOH solutions.

$$Lipase\ activity\ \left(\frac{Unit}{ml}\right) = \frac{(ts - tb) \times MNaOH\ \times 1000}{Enzyme\ volume \times Minute} \quad (1)$$

Description: ts = sample titration
tb = blank titration

RESULTS AND DISCUSSION

In this study, palm oil is used as hydrolysis substrate which added with water and lipase as its biocatalysts. The weight ratio of oil and water is 1:3. Lipase is produced from fungus *Mucor miehei* using coconut pulp as the growth medium. For efficiency in lipase usage, immobilization is carried out with zeolite as matrix/carrier.

Enzyme production is carried out after the highest activities of crude lipase produced by *Mucor miehei* occurred. The result of this study is the best result obtained on day five. The obtained filtrate is crude extract of extracellular lipase enzyme or commonly called crude lipase, the result of solid state fermentation (SSF).

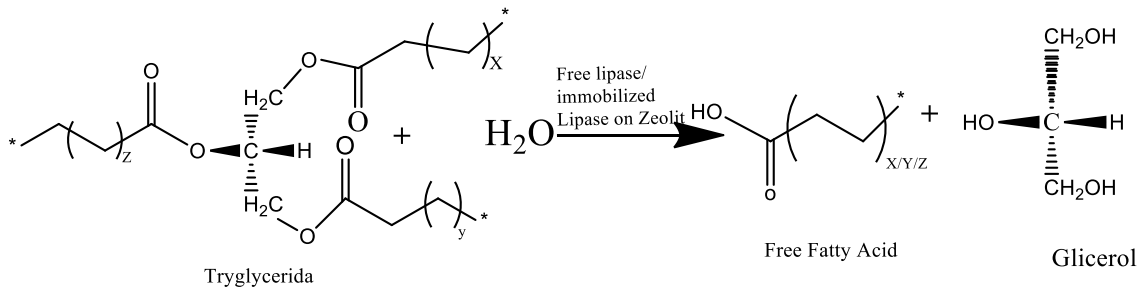

Figure 3. The Hydrolysis Reaction of Palm Oil Using Free Lipase / Immobilized (Gupta et al., 2013).

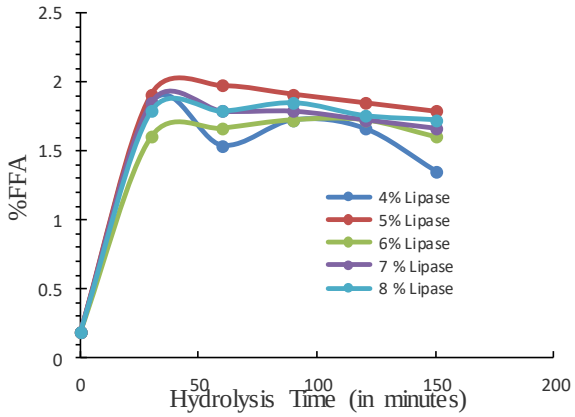

Figure 4. The Effect of Incubation Time on %FFA with Various concentration of Free Lipase

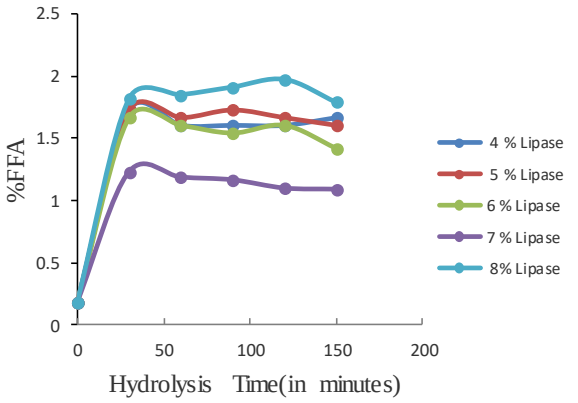

Figure 5. The Effect of Incubation Time on % FFA with Various concentration of Immobilized Lipase on Zeolite

Zeolite activity is carried out at temperature of 300°C using 1 M NaCl as activator. The heating process is to get rid of the water molecules trapped within zeolite crystal pores. On the other hand, activator NaCl takes role of cleansing the zeolite pore's surface and to get rid of contaminants, such as alkali metal. Activated zeolite then put into buffer solution containing lipase. The mixtures then stirred. During the stirring, adsorption process is expected to occur through the ion exchange on lipase and zeolite. In this study, the emulsifying agent is 0.5 M NaF. This gel emulsifying agent also prevents lipase from entering the zeolite pores because emulsifying agent gel (NaF) could form gel layer on zeolite pores' surface. The addition of NaF is expected to increase enzyme binding during immobilization by binding the enzyme stronger, especially on the stirring process and the final filtration.

Immobilization of lipase is carried out by adsorption method, where crude lipase is added with zeolite with ratio of 3:1 (v/b) at room temperature, and soaked for a while.

Hydrolysis process is carried out with the aim to determine the best reaction time and the addition of enzyme concentration (% lipase) to break down triglyceride into fatty acids within. Hydrolysis reaction could be observed on Figure 3.

On this process, the variables used are residence time during hydrolysis process and the addition of free and immobilized lipase concentration. Reaction time used here is 30, 60, 90, 120, and 150 minutes. Meanwhile, the addition of % crude lipase concentration here is 4%, 5%, 6%, 7%, and 8%. Hydrolysis process is performed at 40° C in water bath. The ratio of oil and water substrate here is 1:3 (b/b).

In this hydrolysis, water breaks the alkyl group in the oil's triglyceride into free fatty acid and glycerol. At the end of hydrolysis process, two layers will be formed, which consist of organic phase containing half free fatty acid and water phase containing glycerol. Meanwhile, lipase is on the layer between the organic and water phase (Al-Zuhair et al., 2003). The upper layer acts as fatty acid and the lower layer acts as glycerol. Several factors that affect the hydrolysis are temperature, catalyst, comparison between reactants, reaction time and stirring.

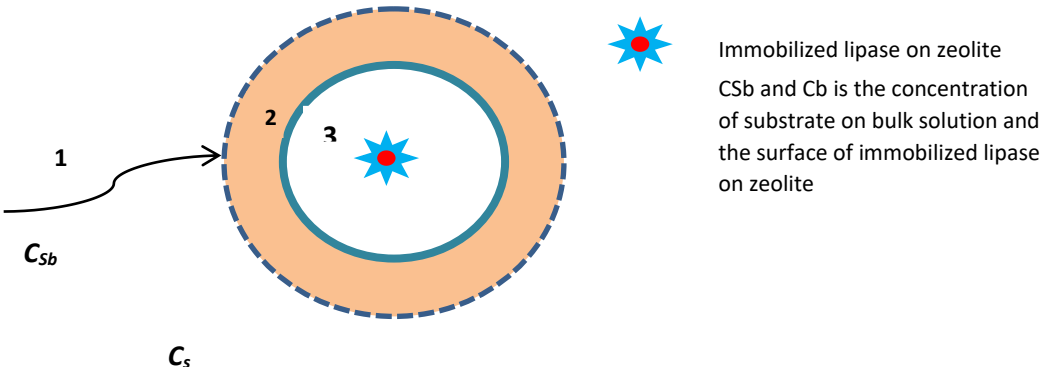

Figure 6. Stages of Immobilization Reaction

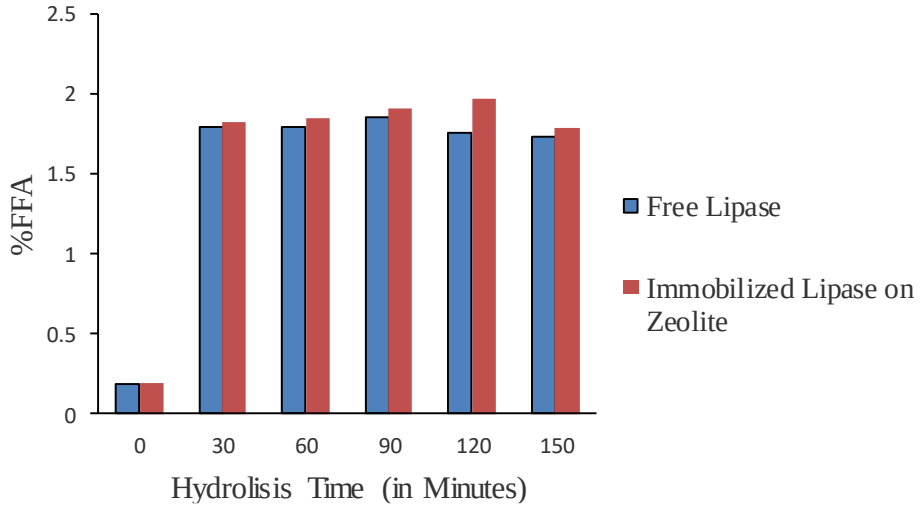

Figure 7. The Effect of Incubation Time on % FFA with 8% Free Lipase and Immobilized Lipase on Zeolite.

The analysis aims to assess the formation of produced free fatty acid, using acid number, or % Free Fatty Acid (FFA). Acid number is the mass of potassium hydroxide (KOH) in milligrams needed to neutralize 1 gram of oil. To assess whether the palm oil has broken down into its fatty acids, therefore the initial % FFA and after hydrolysis in palm oil substrate are analyzed. The concentration of FFA in palm oil before hydrolysis was 0.185%.

According to (Ketaren, 1986) the most saturated fatty acid in palm oil is palmitic acid. Figure 4 displays the palm oil hydrolysis using 5% free lipase with reaction time of 60 minutes produced the highest FFA, which is 1.9747%. On Figure 5, with immobilized lipase and the same reaction time, the FFA produced is 1.6662%. This is caused by the limited mass transfer on immobilized lipase with micropored zeolite, from liquid substrate to the lipase's active site through several steps as shown in Figure 6.

The stages are; 1) mass transfer from bulk liquid to the layer surrounding the immobilized lipase on zeolite; 2) diffusion through liquid layer; 3) diffusion from particles to active site of enzyme on matrix (Lee, 2002). Therefore, for the same reaction time of 60 minutes, free lipase produced FFA quicker, hence the FFA concentration is higher. It is could be seen on Figure 4 showing palm oil hydrolysis with immobilized lipase produced the highest FFA concentration, which is 1.9747%, with the addition of 8% lipase and time reaction of 120 minutes. The same concentration of % FFA also obtained on hydrolysis with 5% free lipase and shorter reaction time.

Afterwards, 8% concentration is used as comparison between the immobilized lipase and free lipase, because it's more stable and more reusable. If with usage of 5% immobilized lipase, the FFA is stable at 40^{o}C with reaction time of 75 minutes and three times reuse in continued reactions, resulting enzyme relative activity of 20%. Meanwhile, with usage of 8% immobilized lipase, stable at 40^{o}C for 120 minutes and six times reuse in continued reactions, resulting enzyme activity of 51%. On Figure 7, with the addition of 8% lipase, the immobilized lipase has better ability than the

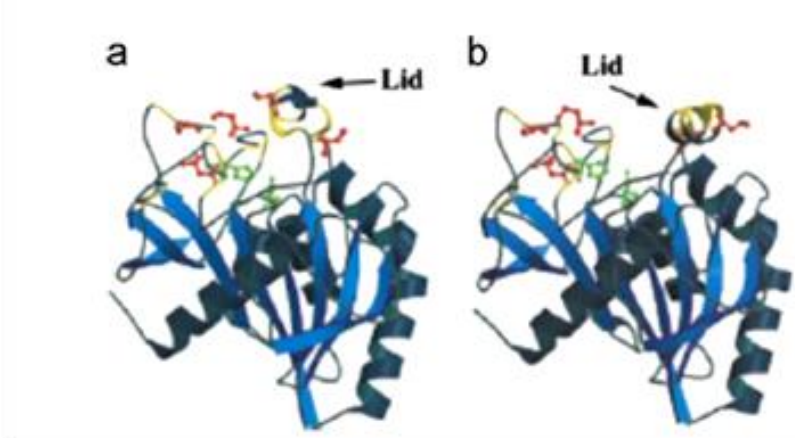

Figure 8. Three dimensional structure of lipase (a) closed Lid, (b) opened Lid (Gupta et al., 2013)

free lipase to hydrolyze triglyceride in palm oil to FFA.

It is caused by the "lid" structure of lipase. After the enzyme is immobilized, the "lid" structure that triggers configuration changing will undergo restructuring, just as shown on Figure 8. Configuration changing happened due to the contact between enzyme with its interfacial domain that induces hydrophobic (enzyme/substrate) pulled out, meanwhile hydrophilic (enzyme/substrate) pulled in (snapping it open) hence the contact between enzyme and substrate is intensified (Patel et al., 2015).

According to the research of Macario et al. (2007) adsorbed lipase on hydrophobic matrix shown more significant hyperactive compared to free lipase.

CONCLUSION

Palm oil hydrolysis using free lipase produced the highest FFA of 1.9747% after the addition of 5% lipase concentrate, with time reaction of 60 minutes. Meanwhile, palm oil hydrolysis using immobilized lipase produced the highest FFA of 1.9747% after the addition of 8% lipase concentrate, with time reaction of 120 minutes. After the addition of 8% lipase, immobilized lipase has better ability than free lipase on triglyceride hydrolysis of palm oil to be FFA.

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