

Effect of Anion and Amino Functional Group on Resin for Lipase Immobilization with Adsorption-Cross Linking Method

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Abstract—Lipase is one of biocatalysts which have been used commercially for processes in industries, such as bioenergy, food, and pharmaceutical industry. Nowadays, biocatalysts are preferred in industries because they work in mild condition, have high specificity, and reduce energy consumption (high pressure and temperature). But, the usage of lipase for industrial scale is limited by economic reason due to expensive price of lipase and difficulty of separation system. Immobilization of lipase is one of the solution to maintain activity of lipase and reduce separation system in process. Therefore, we conduct study about lipase immobilization with adsorption-cross linking method using glutaraldehyde because this method produce high enzyme loading and stability. Lipase is immobilized on different kind of resin with various functional group. Highest enzyme loading (76.69%) was achieved by lipase immobilized on anion macroporous resin which have anion functional group (OH⁻). However, highest activity (24.69 U/g support) through olive oil emulsion method was achieved by lipase immobilized on anion macroporous-chitosan which have amino (NH₂) and anion (OH⁻) functional group. In addition, it also succeeded to produce biodiesel with yield reaching 50.6% through interesterification reaction and after 4 cycles it still produced a yield of 63.9% relative to initial yield. Meanwhile, for *Aspergillus niger* lipase immobilized on anion macroporous chitosan is show unit activity of 22.84 U/g resin and biodiesel yield higher than commercial lipase (69.1%) and after 4 cycles it is still stable reach 70.6% relative from initial yield. This shows that optimum functional group on support for immobilization with adsorption-cross linking is support that contains amino (NH₂) and anion (OH⁻) functional groups because they can react with glutaraldehyde and bind with enzyme, therefore preventing the desorption of lipase from support through binding lipase with functional group on support.

Index Terms—Adsorption-cross linking, lipase, resin, immobilization.

I. INTRODUCTION

Enzyme is a promising biocatalyst for *organic* reaction which have been applied commercially for processes in industries, such as food, detergent, bioenergy, and pharmaceutical industries [1]. Based on Wang *et al.* [2] the demand of enzymes is increasing every year especially for biofuel production.

Biodiesel is one of biofuels that has attracted the attention from all over the world in recent years because biodegradability and eco-friendly nature. The conventional

biodiesel technology is usually catalyzed by inorganic base or acid, which makes the separation of catalyst from products have certain drawbacks such as more complex process and higher energy consumption, therefore consequently increases the cost of production [1], [2]. Lipase is an important enzyme that can be used not only for biodiesel production but also in reactions such as hydrolysis, esterification, *transesterification*, and interesterification. Biodiesel production starts to utilize biocatalyst because their ability to work in mild condition, have higher specificity, and reduces energy consumption for process (high pressure and temperature) than chemical catalyst (acid and base) [3]. But, the usage of lipase for industrial scale is limited by economic reason due to the expensive price of lipase and difficulty of separation system.

Immobilization of lipase is one of the solution to maintain activity of lipase and reduce separation system in process. Characteristics of support and immobilization methods are two important key in enzyme immobilization. One of the support that can be used for the immobilization of enzymes is resin. Resin is a synthetic organic support a wider surface than inorganic (metal) support because in general the resin has the pores so that it can adsorbed better than the inorganic support.

One of immobilization methods is adsorption-cross linking between support and enzyme. Cross linking between lipase and support will maintain the activity and stability of enzyme during reaction. Enzyme immobilization on resin with adsorption-cross linking method has been done on mesoporous resin [2]. Previous studies show that lipase immobilization on NW-ZT2 with adsorption-cross linking method gives high enzyme loading and high stability [4]. Because of that, this research will use resins with various functional groups as a support on immobilization by adsorption-cross linking method with glutaraldehyde as cross-linker. Anion and Amino functional group are focused in this research. Objectives of this research are to determine the best support for immobilization with adsorption-cross linking method and the best support will be used for immobilization of *A. niger* lipase.

II. MATERIALS AND METHODS

A. Materials

Candida rugosa Lipase, macroporous resin Amberlite, and anion macroporous resin Lewatit MP-64 was purchased from Sigma Aldrich. *Aspergillus niger* Lipase was produced independently with *Aspergillus niger* strain from LIPI. Chitosan, acetic acid, KH₂PO₄, K₂HPO₄·3H₂O, and

Manuscript received May 30, 2016; revised December 17, 2016.

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glutaraldehyde as other immobilization materials. Olive oil, distilled water, and PVA for unit activity testing. Palm oil and methyl acetate for biodiesel synthesis

B. Preparation of Amino Functional Group on Resin

Resin with amino functional group was prepared according to the methodology described by Kai Liu [5] with slight modification. Chitosan solution was prepared by adding 0.2 g chitosan into 25 ml 5% v/v acetic acid. Then 2 g of macroporous resin was added and shaken in water bath 150 rpm, 30°C for 8 h. Macroporous-chitosan resin was then filtered by filter paper and stored in 4°C. Anion-Chitosan resin was obtained from same method by replacing macroporous resin with anion resin.

C. Production of Dry Extract (Powder) *Aspergillus niger* Lipase

Aspergillus niger lipase was produced by fermentation solid state for 5 days using rice bran medium and 2% olive oil inducer. Then, the result of fermentation was extracted by mixing, filtration, and centrifugation. Skim milk powder (12%) was added in to the supernatant and then dried using spray dryer.

D. Immobilization of Lipase with Adsorption-Cross Linking

0.1 g of *Candida rugosa* Lipase and *Aspergillus niger* lipase powder was dissolved in 10 ml phosphate buffer solution (PBS pH 7, 0.1 M) and stirred until it is fully. Lipase adsorption was done in shaking water bath with operating condition of 30°C and 150 rpm for 4 hours. Then, 0.5% glutaraldehyde solution was added to the system and reacted for 20 min. The immobilized lipase was then separated and stored at 4°C until usage.

E. Enzyme Loading Assay

Enzyme concentrations were determined by the Lowry method using bovine serum albumin as the standard. Then, the sample was analyzed by UV-Vis spectrophotometer at 750 nm. Absorbance of spectrophotometer will be used for calculate enzyme concentration using standard equation. Enzyme loading was calculated by :

$$X_L = (C_0 - C_t / C_0) \times 100\% \quad (1)$$

F. Measurement of Lipase Unit Activity

The activity of lipase was assayed by titrating the fatty acid produced in the hydrolysis of olive oil. Olive oil emulsion was prepared by dissolving 0.3 g PVA in 5 ml water, and adding 5 ml olive oil and stirred until fully dissolved. Olive oil emulsion and 4% lipase immobilized was reacted at 40°C for 30 min for hydrolysis and ethanol was added to terminate the reaction [SNI]. The produced fatty acid was titrated using 0.05 M NaOH. One unit of activity was defined as the amount of enzyme required to release 1 µmol of free fatty acid per min under the assay conditions.

G. Enzymatic Production of Biodiesel

Biodiesel synthesis was carried out by interesterification reaction. Palm oil and methyl acetate was added into the 100 ml erlenmeyer with molar ratio 1:12. Then, 0.25 g of immobilized lipase was added into the system. The reaction were conducted at 40°C, batch system, and sampling after 50 h [6]. Methyl ester (biodiesel) concentration was analyzed by HPLC instrument.

II. RESULTS AND DISCUSSIONS

A. Result of Lipase Immobilization with Adsorption Cross Linking Method

Lipase which was used in this step is *Candida rugosa* lipase. Objective of this step is to determine the best support for immobilization with adsorption-cross linking method. Results of lipase immobilization with adsorption-cross linking (Table I) were evaluated through enzyme loading, unit activity, and biodiesel synthesis.

1) Effect of functional group on enzyme loading

Enzyme loading of lipase on all resin are shown in Fig. 1. All enzyme loading after cross linking increased from enzyme loading after adsorption. This shows that the amount of lipase adsorbed on resin increased after the addition of glutaraldehyde.

TABLE I : SUMMARY OF *CANDIDA RUGOSA* LIPASE IMMOBILIZATION RESULT WITH ADSORPTION-CROSS LINKING METHOD

Code	Resin	Porosity	Chitosan Addition	Functional Group	% Enzyme Loading (Adsorpsi ; Cross Linking)	Unit Activity (U/g resin)
M	Macroporous	Yes	Without Chitosan	None	(49.25 ; 63.56)	15.1
MK		Yes	With Chitosan	Amino (-NH ₂)	(62.88 ; 70.00)	18.2
AG	Anion Gelular	No	Without Chitosan	Anion (OH ⁻)	(30.69 ; 50.18)	15.3
AGK		No	With Chitosan	Anion (OH ⁻) Amino (-NH ₂)	(32.72 ; 41.63)	15.7
AM	Anion Macroporous	Yes	Without Chitosan	Anion (OH ⁻)	(31.75 ; 75.60)	20.4
AMK		Yes	With Chitosan	Anion (OH ⁻) Amino (-NH ₂)	(35.83 ; 57.24)	24.7

Enzyme loading after adsorption of lipase on resin with chitosan (MK, AGK, AMK) is higher than enzyme loading after adsorption without chitosan (M, AG, AM). This happened because the amino group on resin surface participated in making a hydrophilic support, where hydrophilic site of other amino group from lipase will be

adsorbed and form hydrogen bonding and therefore helps increasing lipase adsorption [7]-[9].

Enzyme loading difference between cross linking and adsorption on anion resin is higher than macroporous resin because the anion (OH⁻) group on resin is able to react with glutaraldehyde and bind more lipase to the resin. But, the

enzyme loading difference on resin with chitosan (MK, AGK, and AMK) which contains amino (NH_2) group is lower than anion resin without chitosan [10]. This happened because the amino group from chitosan might have leached off into the system and was detected as protein at enzyme loading measurement.

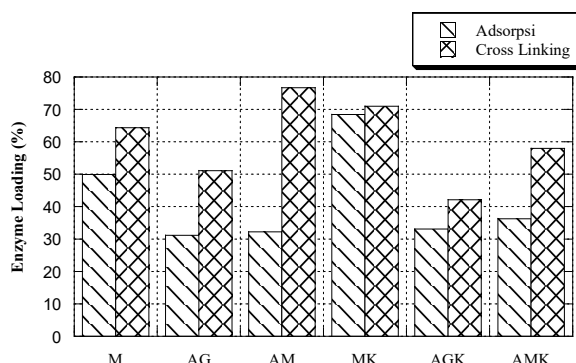


Fig. 1. Enzyme Loading Immobilization of *Candida rugosa* Lipase with Adsorption-Cross Linking.

2) Effect of functional group on unit activity

Lipase unit activity (U) is defined as lipase ability to release 1 μmol of free fatty acid per minute under 40°C . Activity assay of lipase immobilized on resin with various functional groups is shown in Fig. 2. Unit activity of lipase immobilized on resin-with anion groups are (15.3 and 20.4 U/g resin) higher than lipase immobilized on macroporous resin which doesn't have functional group (15.1 U/g resin). This happened because on anion resin, glutaraldehyde reacts with anion group (OH^-), and the reaction between glutaraldehyde and the adsorbed lipase which contain amino group is reduced, therefore preventing breakage of the catalytic site of lipase. On the contrary, in macroporous resin which contains amino group, glutaraldehyde will attack the adsorbed lipase and causes damage in the lipase's catalytic site. Finally, the activity unit of lipase immobilized on anionic resin is higher than macroporous resin.

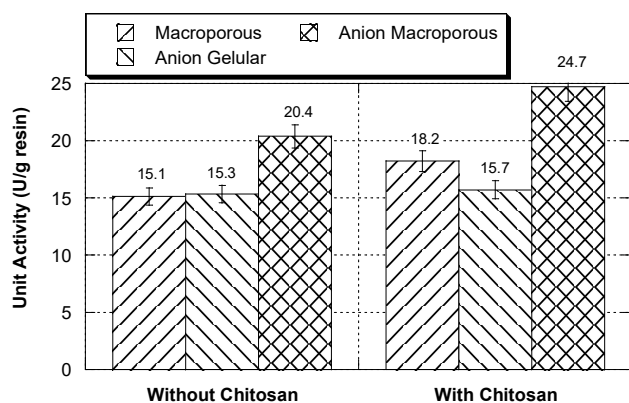


Fig. 2. Unit Activity of *Candida rugosa* Lipase Immobilized with Adsorption-Cross Linking.

Activity unit of lipase immobilized on anion macroporous (20.4 U/g resin) is higher than that of lipase immobilized on macroporous resin with chitosan (18.2 U/g resin) which have amino group (NH_2) on the resin's surface. This happened because enzyme loading on anionic resin is higher than resin with amino group from chitosan because the amino group

might leach from the resin's surface. If glutaraldehyde reacts with the anion group, the catalytic site of lipase will not be damaged and as consequences activity of lipase immobilized on anion macroporous resin is higher than lipase immobilized on macroporous resin with chitosan.

The activity of lipase immobilized on resin with chitosan (MK, AGK, AMK) are higher than those of lipase immobilized on resin without chitosan (M, AG, AM). This happened because lipase was adsorbed on a support that contains amino group through hydrophilic site, so it will be easier for the hydrophobic site which is the catalytic site of lipase to reach its substrate oil which is also hydrophobic. In addition, there are many functional groups on anion macroporous resin with chitosan which have anion and amino groups, so there are more lipase binded with anion and amino group of support. Therefore, the activity of lipase immobilized on resin with chitosan is higher than lipase immobilized on resin without chitosan. Highest activity was obtained by lipase immobilized on anion resin with chitosan because The existence of anion and amino groups on the support prevents glutaraldehyde from attacking the catalytic site of lipase.

3) Effect of functional group on biodiesel yield and stability

Only two immobilized lipase that were used for biodiesel synthesis, they are lipase immobilized on anion macroporous resin (AM) and lipase immobilized on anion macroporous with chitosan (AMK). These immobilized lipase were used because they showed higher unit activity than the other immobilized lipase. The activity of both lipase were then tested by biodiesel synthesis and we will compare the hydrolytic and interesterification activity of immobilized lipase. The result of biodiesel yield from lipase immobilized on AM and AMK are shown in Fig. 3.

Lipase immobilized on anionic macroporous resin with chitosan AMK was able to produce biodiesel yield (50.6%) higher than lipase immobilized on anion macroporous resin AM (27.2%). Activity of lipase catalyzing interesterification reaction is proportional with the hydrolytic activity. This shows that catalysis activity of lipase immobilized on anion macroporous resin with chitosan is better than lipase immobilized on anion macroporous resin without chitosan. The lipase immobilized on anion macroporous resin with chitosan were reused in 4 cycles of biodiesel synthesis to test the stability of immobilized lipase. Biodiesel yield in every cycle is shown in Fig. 4.

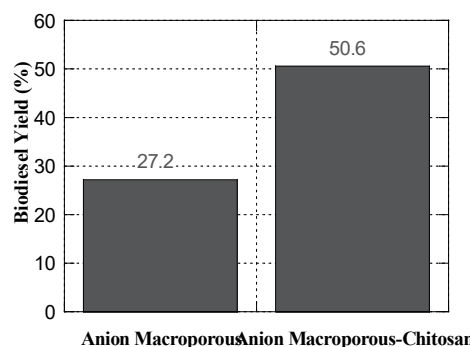


Fig. 3. Biodiesel Yield of *Candida rugosa* Lipase Immobilized with Adsorption-Cross Linking.

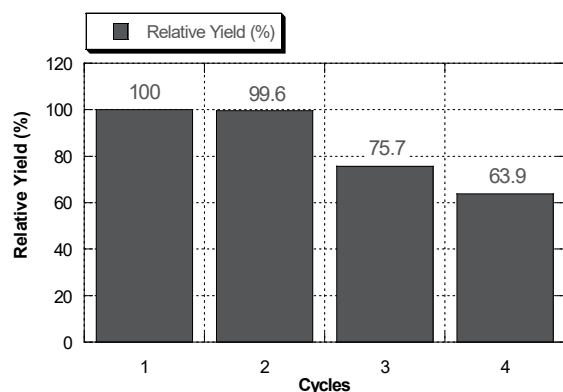


Fig. 4. Stability of *Candida rugosa* Lipase Immobilized with Adsorption-Cross Linking.

After 2nd cycle of biodiesel synthesis, lipase immobilized on anion macroporous with chitosan is still stable with biodiesel yield reaching 99.6% relative to the initial yield. Then, after 3rd and 4th cycles, biodiesel yield decreased to 75.7% and 63.9% relative from initial yield. This shows that after four reaction cycles, lipase immobilized on anion macroporous resin with chitosan is able to maintain the activity to catalyze interesterification reaction with biodiesel field of 63.9% relative to the initial yield (from 50.6% become 32.4%). However, it is predicted that the biodiesel yield will continue to decline after 5th cycles and so on.

B. Immobilization of *Aspergillus niger* Lipase with Adsorption-Cross Linking

The dry extract of *Aspergillus niger* Lipase that we produced have unit activity of 55.83 U/g lipase, which is higher than that of crude extract / supernatant of *Aspergillus niger* Lipase 13.82 U/ml. This happens because the dry extract is more pure than the crude extract that had high water contents [11]. Then, this lipase was immobilized on the best support (anion macroporous resin with chitosan). Result of immobilized dry extract of *Aspergillus niger* lipase with adsorption-cross Linkin was also evaluated through enzyme loading, unit activity, and usage in biodiesel synthesis. Then, we compared the result with immobilization result of *Candida rugosa* lipase in Table II.

TABLE II: COMPARISON IMMOBILIZED CANDIDA RUGOSA LIPASE AND ASPERGILLUS NIGER LIPASE ON ANION MACROPOROUS RESIN WITH CHITOSAN (ADSORPTION-CROSS LINKING METHOD)

	Immobilized <i>Candida rugosa</i> Lipase	Immobilized <i>Aspergillus niger</i> Lipase
1 Enzyme Loading		
- Adsorption	35.83 %	35.12 %
- Cross Linking	57.24%	48.86%
2 Unit Activity		
- Free	702.2 U/g lipase	55.83 U/g lipase
- Immobilized	24.69 U/ g resin	22.84 U/g resin
3 Biodiesel Yield	50.6 %	69.1 %

Enzyme loading of *Aspergillus niger* lipase is almost the same like *Candida rugosa* Lipase (Commercial), although the total enzyme loading of *Aspergillus niger* lipase was lower than *Candida rugosa* Lipase. In addition, the unit activity of immobilized *Aspergillus niger* lipase is also at a similar value with immobilized *Candida rugosa* lipase despite the free lipase activity showing high differences (55.83 U/g lipase and

702.22 U/g lipase respectively). This happens because catalytic efficiency of *Aspergillus niger* lipase is higher than *Candida rugosa* lipase (Table III). Catalytic efficiency, defined as the ratio between activity and loading, gives the hint of the adaptation level of the immobilized lipase. The higher the ratio the lower the amount of lipase inactivated by the immobilization process [12]. Therefore, although unit activity of free lipase have high difference, it is possible that the activity of immobilized *Aspergillus niger* lipase is almost the same with immobilized *Candida rugosa* lipase because of their catalytic efficiency difference.

TABLE III: CATALYTIC EFFICIENCY

	Salis, 2008	This Experiment, 2015
Immobilization	Adsorption	Adsorption-Cross Linking
Catalytic Efficiency		
- <i>Candida rugosa</i> Lipase	0.045	0.035
- <i>Aspergillus niger</i> lipase	0.370	0.410

Biodiesel yield of immobilized *Aspergillus niger* lipase is higher than immobilized *Candida rugosa* lipase. This shows that *Aspergillus niger* lipase is more active on interesterification reaction than hydrolysis reaction. In addition, immobilized *Aspergillus niger* lipase is more stable than immobilized *Candida rugosa* lipase because after 4th reaction cycle it maintained 70.6% of its initial activity. The stability of immobilized *Aspergillus niger* lipase is compared with immobilized *Candida rugosa* lipase and is shown in Fig. 5. This shows that enzyme immobilization of lipase with adsorption-cross linking method was able to prevent desorption of lipase from support because of the bond created between lipase and functional groups on support, and therefore the enzymatic activity stability of immobilized lipase can be maintained.

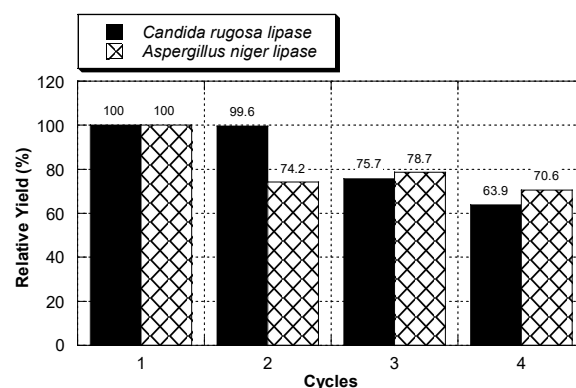


Fig. 5. Comparison Stability of Immobilized *Aspergillus niger* lipase and *Candida rugosa* Lipase with Adsorption-Cross Linking Method.

III. CONCLUSION

The presence of anion and amino groups on support gives a good effect on immobilization of lipase with adsorption-cross linking method. Lipase immobilized on anion macroporous with chitosan which contains amino (NH₂) and anion (OH⁻) groups has the highest unit activity (24.7 U/g resin), biodiesel yield (50.6%), and after 4th reaction cycle it was able to maintain 63.9% of its initial activity because glutaraldehyde

reacts with the anion and amino groups on support and prevents damage in catalytic site of lipase. In addition, *Aspergillus niger* lipase is more active (69.1%) and stable on interesterification reaction (after 4 reaction cycles it was able to maintain 70.6% of its initial activity) than hydrolysis reaction. This shows that immobilization of lipase with adsorption-cross linking method prevented desorption of lipase from support through binding lipase with functional groups on support.

ACKNOWLEDGMENT

This work was supported by grants from DP2M, Direktorat Jenderal Pendidikan Tinggi Indonesia (DIKTI) and Direktorat Riset dan Pengabdian Masyarakat (DRPM) Universitas Indonesia.

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