International Journal of Chemical, Materials and Biomolecular Sciences
ISSN: 2415-6620
Vol:9, No:12, 2015

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 biocatalyst which is applied commercially for the process in industries, such as bioenergy, food, and pharmaceutical industry. Nowadays, biocatalysts are preferred in industries because they work in mild condition, high specificity, and reduce energy consumption (high pressure and temperature). But, the usage of lipase for industry scale is limited by economic reason due to the high price of lipase and difficulty of the separation system. Immobilization of lipase is one of the solutions to maintain the activity of lipase and reduce separation system in the process. Therefore, we conduct a study about lipase immobilization with the adsorption-cross linking method using glutaraldehyde because this method produces high enzyme loading and stability. Lipase is immobilized on different kind of resin with the various functional group. Highest enzyme loading (76.69%) was achieved by lipase immobilized on anion macroporous 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 (NH_2) and anion (OH^-) functional group. In addition, it also success to produce biodiesel until reach yield 50,6% through interesterification reaction and after 4 cycles stable 63.9% relative with initial yield. While for Aspergillus, niger lipase immobilized on anion macroporous-kitosan have unit activity 22,84 U/g resin and yield biodiesel higher than commercial lipase (69,1%) and after 4 cycles stable reach 70.6% relative from initial yield. This shows that optimum functional group on support for immobilization with adsorption-cross linking is the support that contains amino (NH_2) and anion (OH^-) functional group because they can react with glutaraldehyde and binding with enzyme prevent desorption of lipase from support through binding lipase with a functional group on support.

***Keywords*—**Adsorption-Cross linking, lipase, resin, immobilization.

I. Introduction

ENZYME is a promising biocatalyst for the organic reaction which is applied commercially for the process in industries, such as food, detergent, bioenergy, and pharmaceutical industries [1]. Reference [2] show that demand of enzyme is increasing every year, especially for biofuel production. Biodiesel is one of biofuel that has attracted the attention all over the world recent years because of biodegradability and eco-friendly. The conventional biodiesel technology is usually catalyzed by inorganic base and acid, which makes the separation of catalyst from products be difficult, high-energy consumption, and consequently, increases the cost of production [1]-[2]. Lipase is an important enzyme that can be used for biodiesel production and usually also participate in reactions such as hydrolysis, esterification, transesterification, and interesterification. Biodiesel production starts to using biocatalyst because they work in mild condition, high specificity, and reduce energy consumption for the process (high pressure and temperature) than chemical catalyst (acid and base) [3]. But, the usage of lipase for industry scale is limited by economic reason due to the high price of lipase and difficulty of the separation system.

Immobilization of lipase is one of the solutions to maintain the activity of lipase and reduce separation system in the process. One of immobilization method is adsorption-cross linking between support and enzyme. Cross-linking between lipase and support will maintain the activity and stability of enzyme during the reaction. Enzyme immobilization on resin with adsorption- cross-linking method has been done only on mesoporous resin [2]. Previous studies show that lipase immobilization on NW-ZT2 with adsorption-cross linking method also give high enzyme loading and high stability [4]. Because of that, this research will use various resin with the various functional group as support on immobilization using adsorption-cross linking with glutaraldehyde as cross-linker. Anion and Amino functional group are focussed on this research. Objectives of this research are to determine the best support for immobilization with the adsorption-cross linking method and the best support will be used for immobilization of Lipase *A.niger*.

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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 by *Aspergillus niger* strain from LIPI. Chitosan, acetic acid, KH_2PO_4, $K_2HPO_4.3H_2O$, and 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 the amino functional group was prepared

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

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

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

D. Immobilization Lipase with Adsorption-Cross Linking

Candida Rugosa Lipase or *Aspergillus niger* lipase powder 0.1 g was dissolved in 10 ml phosphate buffer solution (PBS pH 7, 0.1 M) and stirred until homogeny. Then lipase solution and support (10 ml and 0.75 g) were shaken at 30°C, 150 rpm for 4 h. Then, 0.5% glutaraldehyde solution was added to the system and reacted for 20 min. The immobilized lipase was separated and stored at 4°C until use.

E. Enzyme Loading Assay

Enzyme concentrations were determined by the Lowry method using bovine serum albumin as the standard. Lowry and follin reagent was added to the sample and incubated. Then, the sample was analyzed by spectrophotometer UV-Vis 750 nm. The absorbance of spectrophotometer will be used for calculating enzyme concentration through the standard equation. Enzyme loading was calculated by:

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

F.Measurement of Lipase Unit Activity

The activity of lipase was assayed by titrating the fatty acid produced by the hydrolysis of olive oil. Olive oil emulsion was prepared by dissolved 0.3 g PVA in 5 ml water, then added 5 ml olive oil and stirred until homogeny. 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 were added to the 100 ml Erlenmeyer with ratio 1:12 mol. Then, 0.25 g lipase immobilized was added to the system. The reaction is conducted in 40°C, batch system, and sampling after 50 h [6]. Methyl ester (biodiesel) concentration was analyzed by HPLC instrument.

III. Results and Discussions

A. Result of Lipase Immobilization with Adsorption Cross-Linking Method

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

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

1. Effect of Functional Group on Enzyme Loading

Enzyme loading of lipase on all resin shown in Fig. 1. All enzyme loading after a cross-linking increase from enzyme loading after adsorption. This shows that the more lipase adsorbed on resin after the addition of glutaraldehyde

Enzyme loading after adsorption of lipase on resin with chitosan (MK, AGK, AMK) higher than enzyme loading after adsorption on resin without chitosan (M, AG, AM). This happens because the amino group on resin surface participated in making hydrophilic support, hydrophilic site of another amino group from lipase will be adsorbed and form hydrogen bonding, so the amino group on resin surface help increase loading lipase adsorption. The same results are reported by Bai, 2011 [7].

Enzyme loading difference between cross-linking and adsorption on anion resin higher than macroporous resin because anion (OH-) group on resin can react with glutaraldehyde and will bind more lipase to the resin. But, the enzyme loading difference on resin with chitosan (MK, AGK, and AMK) which contain amino (NH2) group lower than anion resin without chitosan. This happens because the amino group from chitosan may leaching to the system and detected

as protein when enzyme loading measurement.

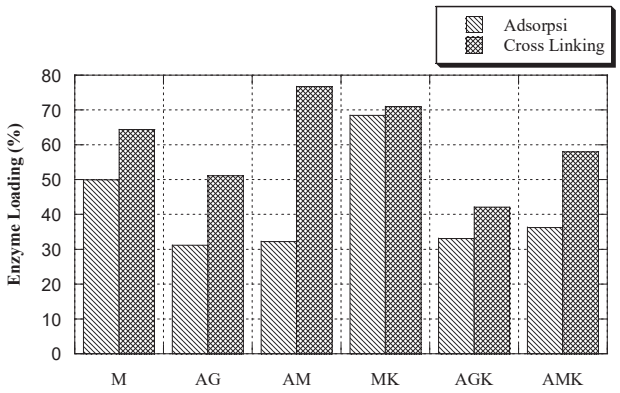

Fig. 1 Enzyme Loading Immobilization of *Candida rugosa* Lipase with Adsorption-Cross Linking

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 40ºC. The result of unit activity all of the lipase immobilized on resin with various functional group shown in Fig. 2. Unit activity of lipase immobilized on resin have anion group (15.3 and 20.4 U/g resin) higher than lipase immobilized on the macroporous resin which does not have a functional group (15.1 U/g resin). This happens because, on anion resin, glutaraldehyde reacts with anion group (OH^-), so glutaraldehyde will be reduced to react with adsorbed lipase which contains an amino group and prevent breakage the lipase catalytic site. On the contrary, glutaraldehyde will attack the adsorbed lipase on the macroporous resin which contains an amino group and caused damage in lipase catalytic site. Finally, the unit activity of lipase immobilized on anion resin higher than macroporous resin.

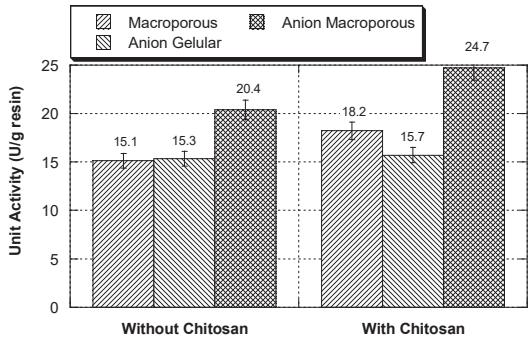

Fig. 2 Unit activity of *Candida rugosa* lipase immobilized with adsorption-cross linking

Unit activity of lipase immobilized on anion macroporous (20.4 U/g resin) higher than the unit activity of lipase immobilized on macroporous resin with chitosan (18.2 U/g resin) which have an amino group (NH_2) on the resin surface. This happened because of enzyme loading on anion group higher than an amino group of chitosan because this amino group may leach from resin surface. If glutaraldehyde reacts with anion group, the catalytic site of lipase will not damage because too much react with glutaraldehyde and as consequences unit activity of lipase immobilized on anion macroporous higher than lipase immobilized on macroporous resin with chitosan.

Then, the unit activity of lipase immobilized on resin with chitosan (MK, AGK, AMK) higher than the unit activity of lipase immobilized on resin without chitosan (M, AG, AM). This happens because lipase adsorbed on a support which contains amino group through the hydrophilic site, so the hydrophobic site which is the catalytic site of lipase will be easy to reach his substrate oil which also hydrophobic. In addition, there is a much functional group on anion macroporous resin with chitosan which has anion group and amino group, so there is much lipase will binding with anion and the amino group of support. Therefore, the unit activity of lipase immobilized on resin with chitosan higher than lipase immobilized on resin without chitosan. Highest unit activity obtained by lipase immobilized on anion resin with chitosan because there are many anions and an amino group on the support which prevents glutaraldehyde attack catalytic site of lipase.

3. Effect of Functional Group on Biodiesel Yield and Stability

Only two immobilized lipases that used for biodiesel synthesis, there is lipase immobilized on anion macroporous resin (AM) and lipase immobilized on anion macroporous with chitosan (AMK). This immobilized lipase was used because have high unit activity than other immobilized lipase. Both of lipase are then tested their activity on biodiesel synthesis, and we will compare the hydrolytic activity and interesterification activity of immobilized lipase. The result of biodiesel yield of lipase immobilized on AM and AMK shown in Fig. 3.

Lipase immobilized on anion macroporous with chitosan AMK produce biodiesel yield (50.6%) higher than lipase immobilized on anion macroporous AM (27.2%). The activity of lipase to catalyze interesterification reaction proportional to the hydrolytic activity of lipase. This shows that catalysis activity of lipase immobilized on anion macroporous with chitosan better than lipase immobilized on anion macroporous. Then, the lipase immobilized on anion macroporous with chitosan are used again until 4 cycles of biodiesel synthesis to testing the stability of immobilized lipase. Biodiesel yield every cycle shown in Fig 4.

After 2nd cycles of biodiesel synthesis, lipase immobilized on anion macroporous with chitosan stable reach 99.6% relative from initial biodiesel yield. Then, after 3rd and 4th cycles, biodiesel yield is decreasing become 75.7% and 63.9% relative from initial yield. This shows that after 4th cycles, lipase immobilized on anion macroporous with chitosan can maintain the activity for catalyzing interesterification reaction 63.9% relative with initial yield (from 50.6% become 32.4%). However, there is possibility biodiesel yield will continue to decline after 5th cycles and so on.

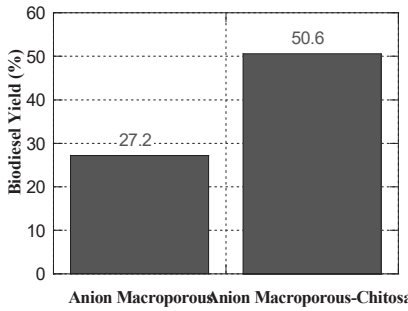

Fig. 3 Biodiesel Yield of *Candida rugosa* Lipase Immobilized with Adsorption-Cross Linking

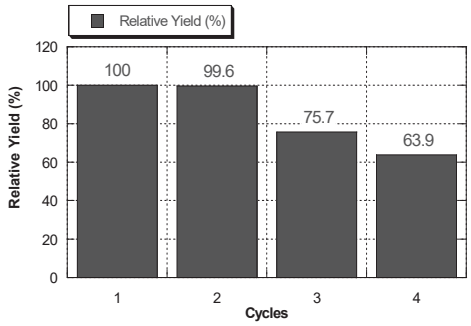

Fig. 4 Stability of *Candida rugosa* Lipase Immobilized with Adsorption-Cross Linking

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

Dry extract of *Aspergillus niger* Lipase which we produce has unit activity 55.83 U/g lipase, higher than the unit activity of a wet extract of *Aspergillus niger* Lipase 13.82 U/ml. This happens because of dry extract is purer than wet extract which has high water contents [8]. Then, this lipase is immobilized on the best support (anion macroporous resin with chitosan). The result of immobilized dry extract *Aspergillus niger* lipase with adsorption-cross linking also evaluated through enzyme loading, unit activity, and biodiesel synthesis. Then, we compared the result with immobilization result of *Candida rugosa* Lipase.

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

		Immobilized *Candida rugosa* Lipase	Immobilized *Aspergillus niger* 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 almost same like *Candida rugosa* Lipase (Commercial), although enzyme loading total of *Aspergillus niger* lipase lower than *Candida rugosa* Lipase. In addition, the unit activity of immobilized *Aspergillus niger* lipase also almost same with immobilized *Candida rugosa* lipase despite the unit activity of free lipase have a high difference (55.83 U/g lipase and 702.22 U/g lipase respectively). This happens because of the catalytic efficiency of *Aspergillus niger* lipase 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 [9]. Therefore, although the unit activity of free lipase has a high difference, it possible unit activity of immobilized *Aspergillus niger* lipase also almost same with immobilized *Candida rugosa* lipase because of their catalytic efficiency.

TABLE III
CATALYTIC EFFICIENCY

	[9]	This Experiment, 2015
Immobilization	Adsorption	Adsorption-Cross Linking
Catalytic Efficiency		
- *Candida rugosa* Lipase	0.045	0.035
- *Aspergillus niger* lipase	0.370	0.410

Biodiesel yield of immobilized *Aspergillus niger* lipase higher than immobilized *Candida rugosa* lipase. This shows that *Aspergillus niger* lipase more active on interesterification reaction than hydrolysis reaction. In addition, immobilized *Aspergillus niger* lipase more stable than immobilized *Candida rugosa* lipase because after 4th cycles it maintains 70.6% relative from initial biodiesel yield. The stability of immobilized *Aspergillus niger* lipase compared with immobilized *Candida rugosa* lipase shown in Fig.5. This shows that immobilization lipase with adsorption-cross linking method prevents desorption of lipase from support through binding lipase with a functional group on support, as consequences the stability of activity immobilized lipase can be maintained.

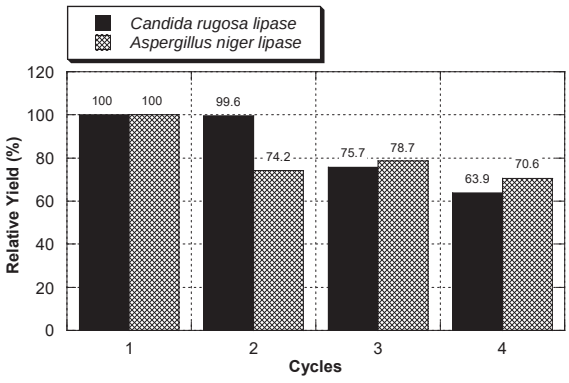

Fig. 5 Comparison Stability of Immobilized *Aspergillus niger* lipase and *Candida rugosa* Lipase with Adsorption-Cross Linking Method

IV. Conclusion

The presence of anion and an amino group on support give a good effect on immobilization of lipase with the adsorption-cross linking method. Lipase immobilized on anion macroporous with chitosan which contains amino (NH_2), and anion (OH-) group have highest unit activity (24.7 U/g resin), biodiesel yield (50.6%), and after 4th cycles can maintain the activity 63.9% relative with initial yield because glutaraldehyde react with anion and amino group on support and prevent leakage in catalytic site of lipase. In addition, *Aspergillus niger* lipase more active (69.1%) and stable on interesterification reaction (after 4th cycles can maintain the activity 70.6%) than hydrolysis reaction. This shows that immobilization lipase with adsorption-cross linking method prevents desorption of lipase from support through binding lipase with a functional group 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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