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Immobilization of Isolated Lipase From Moldy Copra (*Aspergillus Oryzae)*

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Abstract: Enzyme immobilization is a recovery technique that has been studied in several years, using support as a media to help enzyme dissolutions to the reaction substrate. Immobilization method used in this study was adsorption method, using specific lipase from *Aspergillus oryzae*. Lipase was partially purified from the culture supernatant of *Aspergillus oryzae*. Enzyme was immobilized by adsorbed on silica gel. Studies on free and immobilized lipase systems for determination of optimum pH, optimum temperature, thermal stability and reusability were carried out. The results showed that free lipase had optimum pH 8,2 and optimum temperature 35 ^{0}C while the immobilized lipase had optimum 8,2 and optimum temperature 45 ^{0}C. The thermal stability of the immobilized lipase, relative to that of the free lipase, was markedly increased. The immobilized lipase can be reused for at least six times.

Keywords: Llipase, Immobilization, *Aspergillus oryzae*, Silica gel

Introduction

Indonesia is one of the coconut producing countries in the world. Coconut is an industrial raw material for manufacturing plant oil. In its processing, first the coconut must be made into copra. The processing of coconut into copra results in moldy copra. The field survey shows that 1-15% of the coconut processed into copra is moldy, so it is potential to be waste. The fungi found in copra have been identified of genus *Aspergillus oryzae* and *penicillium.* Genus *Aspergillus* consists of *Aspergillus oryzae* and *Aspergillus niger* both produce lipase enzyme, whereas *penicillium* does not[1].

Lipase is a very important enzyme in modern biotechnology. Many industries use enzyme as biocatalysis. Lipase is well known to have high activity in hydrolytic reaction and chemical compound synthesis. Lipase can be a biocatalysis for hydrolytic reaction, esterification, alcoholysis, acidolysis, and aminolysis. The enzyme catalysis capacity for chemical reaction can be described through its activity. The catalytic reaction level by enzyme correlates indirectly to its enzyme activity. Several microbes can be used to produce lipase: *Candida*, *Aspergillus* and *Rhizopus*[2].

The commercial lipase is very expensive due to its difficult long production process. The use of dissolved lipase enzyme as a biocatalizator is not very economical compared to dissolved lipase enzyme (immobile lipase). Dissolved enzyme is relatively unstable and cannot be used again and again (reusable)[3]. The use of enzyme is limited to once use only so that each initial processing must use new enzyme. This is not efficient and costly. These defects can be overcome through enzyme immobility to increase its stability.

Immobile enzyme is an enzyme both physically and chemically are not free to move so that it can be controlled and managed when the enzyme must contact with substrate. Immobilization prevents enzyme diffusion into reaction mixture and it is easy to retrieve from product flow by separation of simple solid-liquid, so that the enzyme can be reusable[4].

In order to immobilize enzyme, observation to support material used is necessary. The supports commonly used are polystyrene latex and EP400 accurel[5], Hp-20[6], eupergit[7] and silica micro-particle[8]. In this study lipase immobilization was conducted by adsorption using silica gel as a support. The use of silica gel as a support material is due to its surface adsorption ability and good intra-molecule and has interlocking capacity to provide large surface for the media[9].

Experimental

The culture (isolated *Aspergillus oryzae*) was grown under optimal conditions for lipase production. The initial volume of the culture was 500 mL containing pepton 0.5 g/100 mL, KH_2PO_4 0.1 g/ 100 mL, $FeSO_47H_2O$ 0.001 g/100 mL and olive oil 1 mL/100 mL. Temperature and pH were controlled at 37 ^{0}C and 7.0. After incubation for 8 days, the culture broth was centrifuged at 3500 rpm for 30 min. The clear supernatant containing the lipase was used for future studies.

Enzyme activity assay

Lipase enzyme activity is determined by using Vorderwulbecke *et al.*[10] modified with the following procedures: As much as 0.1 mL of lipase enzyme solution added with 0.89 mL borate buffer with concentration 0.05 M (pH 8.2). The reaction began by adding 0.01 mL substrate *p*-nitrophenilbutirate 0.1 M (dissolver dimetilsulfoxide) and rapidly shaken and then the reaction mixture was incubated for 10 minutes at 45 ^{0}C temperature, then the reaction mixture was measured its adsorption at the wave length 410 n m. The lipase enzyme activity was measured based on *p*-nitrophenol formed from lipase enzyme hydrolysis result to substrate *p*-nitrophenilbutirate.

Partial purification of enzyme

To 2180 mL of the culture supernatant, ammonium sulphate was added (70% saturation) at 4 ^{0}C over night. The precipitate was collected by centrifugation at 10.000 rpm at 4 ^{0}C for 20 min and dissolved in 3 mL 0.05 M borate buffer (pH 8.2). The lipase activity and the protein concentration were determined[11].

Ion-exchange chromatography

Three (3 mL) sample was put into the column that has been filled in with matrix Q sepharosa FF (column length 14.5 cm and diameter 1 cm) which has been balanced previously with borate buffer with concentration 0.05 M pH 8.2 for one night. After all samples were put into the matrix, gradient-mixer and fraction collector were operated. The gradient-mixer contained borate buffer with concentration 0.05 M, pH 8.2 and NaCl (0-0.4 M) as smoothing solution. The volume of each fraction 3.0 mL and each fraction was measured for its protein adsorption and enzyme activity. Active fraction with high enzyme activity was gathered and used for gel filtration column chromatography[12].

Gel-filtration chromatography

The active fraction obtained from column chromatography of ion substitute was put into a column with sephadex G-75 matrix (column length 35 cm and diameter 1 cm) has been balanced previously with borate buffer at concentration 0.05 M pH 8.2 for one night. Then it was smoothened with the same buffer. The volume of each fraction was 3.0 mL and each fraction was measured for its protein adsorption and enzyme activity. The active fraction with the highest enzyme activity was used further.

Immobilization

As much as 2.5 mL purified lipase enzyme was added with 0.5 g silica gel made with eight variations of which the mixture was shaken for certain time from 0-120 minutes at room temperature. For every 15 minutes the mixture was taken and then centrifuged for 5 minutes at speed[6] 1000 rpm. The obtained supernatant was tested for its enzyme activity and protein level to find out the immobilization optimum time. The immobile enzyme was calculated by the formula:

$$C_e = C_o - C_t$$

In which: C_e = total immobilized enzyme (U/mL)

C_o = total enzyme before immobilization (U/mL)

C_t = total enzyme at t time (U/mL)

Characterization of immobilized lipase

Characterization of immobile lipase enzyme comprises: determination of pH, optimum temperature, thermal stability and operational.

Determination of pH and optimum temperature of immobile lipase

The determination of optimum pH to lipase activity and observation to the impact of pH using immobilized lipase. The determination of optimum pH was done by varying pH during immobilization (pH: 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8 and 9.0). The arrangement of pH variation was done by regulating the pH borate buffer.

The determination of optimum temperature to lipase activity was done by making variation of temperatures (20, 25, 30, 35, 40, 45, 50 and 55) ^{o}C. The regulation of temperature was done by incubating the solution in the shaker incubator.

Determination of thermal stability of immobile lipase

Testing the thermal stability was done by exposure of free lipase enzyme to optimum temperature for 120 minutes. Free lipase enzyme was tested its enzymatic activity at its optimum condition. The same thing was done to immobile lipase enzyme.

Determination of operational stability of immobile lipase

Testing the stability of immobile lipase was done based on Sigurgisladittor *et al.*[13] method as follows. As much as 1 mL mixed reaction containing substrate *p*-nitrophenibutrate (based on determination of lipase enzyme activity) was added into immobile enzyme then in the shaker for 90 minutes at 45 ^{o}C temperature (optimum temperature of immobile lipase enzyme). Then it was centrifuged with speed 1000 rpm for 5 minutes. The supernatant produced was tested for its enzyme activity. The pellet was washed with borate buffer and then was used to determine the next lipase enzyme activity.

Results and Discussion

Characteristics of free lipase

The microbe produced lipase isolated from moldy copra, namely *Aspergillus oryzae* was grown in production medium containing olive oil as inducer and produced maximally on day eight through fermentation process at 37 ^{o}C temperature. The lipase was partially purified by ammonium sulphate precipitation followed by Q sepharose FF column chromatography and sephadex G-75 column chromatography. This partially purified enzyme was used for immobilization.

Lipase immobilization

Determination of immobilization time

Optimum immobilization time was determined by varying 8 different times for immobilization process. Figure 1 show that time has an effect on immobilization process of lipase enzyme at silica gel matrix. Within 15-90 minutes the amount of immobile lipase enzyme increased with the increase of immobilization time. After 90 minutes the amount of immobile lipase enzyme as relatively constant or there was an insignificant increase until 120 minutes. At this condition it can be said that beginning from the 90th minute the matrix active site has been saturated by lipase enzyme and has reached immobilization balance.

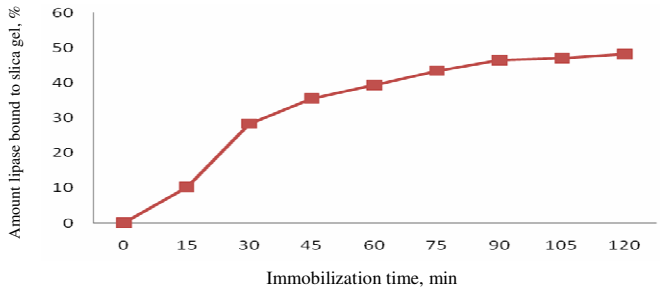

Figure 1. Effect of time to immobile lipase on silica gel

Characterization of immobile lipase

Characterization of immobilized lipase enzyme comprise the determination of optimum pH and optimum temperature. Figure 2 shows that the activity of free lipase enzyme at pH 7.0 is low then there is an increase to pH 8.2. At pH 8.2 the activity of free lipase enzyme provides maximum activity in which at this condition the activity of free lipase enzyme reaches 100%. At pH 8.4 the activity of lipase enzyme begins to decrease up to pH 9.0. The same thing happens to immobile lipase enzyme so that both free lipase enzyme and immobile lipase enzyme have similar optimum pH that is 8.2. The activity of free lipase enzyme is a little bit bigger than the immobile one due to the immobile lipase enzyme is bound to silica gel matrix so that the speed for contact with substrate is smaller.

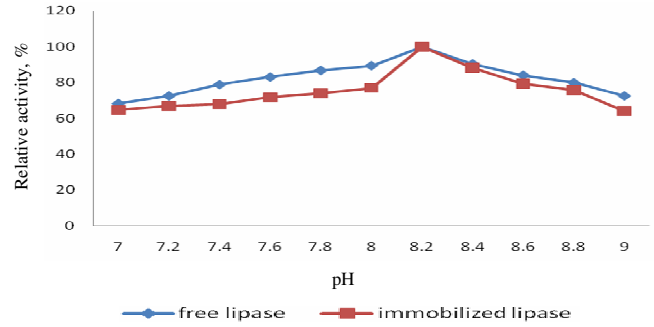

Figure 2. Effect of pH on free and immobile lipase

Figure 3 shows that the activity of free lipase enzyme increases with the increase of temperature from 20 ^{0}C to 55 ^{0}C. At the temperature of more than 35 ^{0}C, the activity of free lipase enzyme decreases. The highest activity occurs at the 35 ^{0}C temperature with relative activity 100%. The increase of temperature up to the optimum temperature will increase the flow of enzymatic reaction, but the increase of temperature above the optimum temperature will decrease the flow of enzymatic reaction. The test of immobile lipase enzyme activity has optimum temperature 45 ^{0}C whereas the free lipase enzyme 35 ^{0}C. This shows that the silica gel matrix is able to protect the immobile lipase enzyme from heat so that it is able to exist at higher temperature compared to free lipase enzyme. The study by Dosanjh and Kaur[14] indicates that immobile lipase enzyme is at HP-20 matrix, optimum pH 8.0 and optimum temperature 54 ^{0}C.

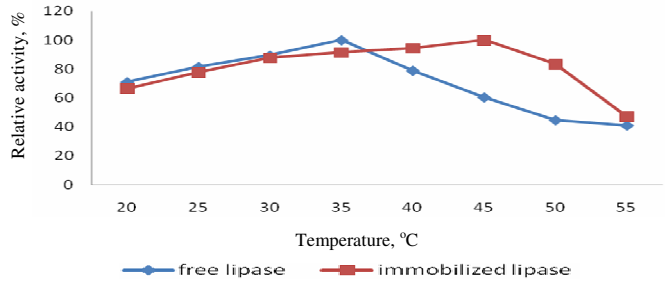

Figure 3. Effect of temperature on free and immobile lipase

Thermal stability of immobile lipase

The results of free lipase enzyme and immobile lipase enzyme stability test are shown in Figure 4.

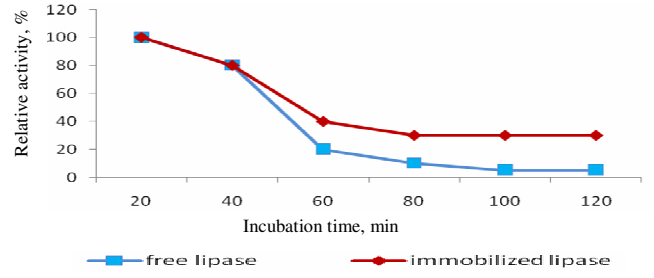

Figure 4. Determination of thermal stability of free and immobile lipase

Free lipase enzyme at optimum temperature 35 ^{o}C can only exist up to 60 minutes heat with relative activity remains 20%. The decrease of activity begins at the heat for 40 minutes up to 100 minutes in which the activity relatively remains 5%. Immobile lipase enzyme at optimum temperature 45 ^{o}C is able to exist up to 80 minutes heat and the remaining activity is relatively constant up to 120 minutes heat. The decrease of activity begins at incubation time 40 minutes up to 80 minutes in which relative activity remains 38%. If it is compared to free lipase enzyme, the thermal stability of immobile lipase enzyme is better.

Operational stability of immobile lipase

Repeated use of immobile lipase enzyme at silica gel adsorption decreases the activity of lipase enzyme. This can be seen in Figure 5.

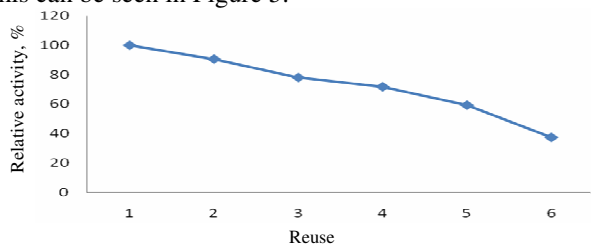

Figure 5. Reuse immobile lipase.

Figure 5 shows that immobile lipase enzyme can be used again and again. The use of the lipase enzyme activity again and again will decrease its activity. Immobile lipase enzyme can be used six times. In its sixth use the catalytic activity is about 37.50%. Free lipase enzyme can only be used once due to its mix with reaction product so that destruction process must be done to separate the lipase enzyme from reaction product. In the application this is not very economical knowing that lipase enzyme is expensive. The decrease of activity after using it again and again is due to the weakness of binding between lipase enzyme and support adsorption since it is supported by Van der Waals binding, hydrogen binding and hydrophobic interaction. If disorder occurs due to repeated use, this binding can be damaged causing the enzyme is released from the adsorbent.

Conclusion

Immobile lipase of *Aspergillus oryzae* at moldy copra has optimum pH 8.2 and optimum temperature 45 ^{o}C. Free lipase has optimum pH similar to immobile lipase that is 8.2 and optimum temperature 35 ^{o}C. The stability of immobile lipase thermal is better than free lipase. Immobile lipase can be used (reusable) six times

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