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Characterization of Immobilized Lipase from Fractionation Result of*Azospirillum Sp.* Prd1 Using Chitosan

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Abstract

Lipase is an enzyme that catalyses hydrolysis reaction to produce glycerol and fatty acid. The reaction efficiency of enzymes can be improved by immobilization. The purpose of this study was to characterize the immobilized lipase from Azospirillum sp. PRD1 bacteria using chitosan. The study was started by the production of lipase from *Azospirillum* sp. PRD1 bacteria followed by extraction by centrifugation. The obtained crude extract was fractionated using ammonium sulphate 60% (F60%). The F60% fraction was immobilized and was characterized. The characterization of the immobilized lipase consisted of the determination of optimum temperature, pH, substrateconcentration and incubation time. The lipase activity was tested using titrimetric method. The results showed that the optimum of activity of the immobilized lipase from Azospirillum sp. PRD1 bacteria using chitosan could be achieved at 40 °C, pH 7, incubation time at 45 minutes and 25% of substrateconcentration.

Keywords: immobilization, lipase**,** *Azospirillum sp*. PRD1.

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Introduction

Lipase is an important enzyme for the industry. Lipase is widely used in the processing of fats and oils, detergents and cleaning formulations, food processing, chemical synthesis and medicine, paper making, and in the production of cosmetics (Gupta, et al., 2004). Lipases are important biocatalysts in stereo-selective reactions. It plays an important role in the pharmaceutical industry especially in the enzymatic resolution of racemic drugs (Chaubey et al., 2006).

Many attempts have been made over the years to enhance the catalytic activity and operational stability of the industrial enzymes through the use of genetic engineering and immobilization. Immobilization of enzymes is the most common method used to provide the desired features of a conventional heterogeneous catalyst to biological catalysts. Immobilization of enzymes is not only improving its stability but also enhancing some advantages like reusability, and the easiness of product separation.

Lipase can be immobilized using physical adsorption, covalent binding, ionic interactions and entrapment (Vaidya, et al., 2008). Immobilization methods can be divided into two general classes; 1) chemical method, in which a covalent bond is formed by the enzyme and support 2). Physical methods in which weak interactions between support and enzyme are established (Chiou, *et al*., 2004). in this study, observations of trapping technique were chosen because of its simplicity in observation and economics value. Chitosan is used as a Matrix.

In this research the isolation of lipase from *Azospirillum* sp. PRD1 bacteria will be conducted followed by fractionation using ammonium sulphate 60% (F60%). The F60% was further immobilized using chitosan and to be characterized. The characterization was consist of the determination of optimum temperature, pH and substrateconcentration.

Methodology

The materials used in this study were pure isolates of Azospirillum sp. PRD1 collection of Unsoed Laboratory of Microbiology, Faculty of Biology, food grade chitosan, soy oil, NA (Nutrient Agar) medium, NB (Nutrient Broth) medium, distilled water, acetic acid buffer solution, Tris-HCl buffer pH 9, Arabic gum, phosphate buffer pH 7 and pH 8, acetone, ethanol, NaOH, phenolphthalein indicator, tri polyphosphate (TPP) and acetic acid.The tools used in this study were centrifuges, Memmert incubator shaker, autoclave, hot plate stirrer, separating funnel, instruments used were UV-Vis spectrophotometer, Shimadzu UV-1601, FT-IR 8201PC spectrophotometer

Production of lipase

Lipase production was performed referring to the previous research by Zusfahair et al., (2012). Isolates stock of Azospirillum sp. PRD1 was rejuvenated on NA media slant and was incubated for 2 x 24 hours at room temperature. *Azospirillum* sp. PRD1 bacteria rejuvenation results wasthen inoculatedinto NB medium and was incubated in a shaker incubator at a speed scale of 7 at room temperature for 10 hours. As much as 20% of the inoculum was transferred into the enzyme production medium (NB medium with 1% soybean oil inducer) and was incubated in a Kottermann incubator shaker at a speed scale 7 at room temperature for 14 hours. Liquid medium was then centrifuged using a cold centrifuge (6000 rpm, 4 °C, 10 min), to separate the cell debris and the supernatant. The supernatant obtained was the crude extract of lipase. Lipase crude extract was further fractionated using ammonium sulphate 60% (F60%). F60% fraction was immobilized and further characterized.

Measurement of lipase activity (Prazeres et al., 2006)

The activity of free lipase was measured using titrimetric method adapted from Prazeres et al., (2006). The substrate used was 25% soybean oil emulsion, 10% gum Arabic solution and phosphate buffer pH 7 at a ratio of 1: 2: 1 that was mixed until homogeneous. Enzyme reaction mixture consisted of 2.5 mL of soybean oil emulsion, which was pre-incubated in a shaker incubator with scale of 7, for 5 min at 35 °C, the mixture was further added with 0.1 mL of enzyme and the incubation was continued for 30 minutes. The reaction was stopped by the addition of 1.25 mL acetone: ethanol (1: 1) and were then titrated using 0.05 M NaOH. The Addition of enzymes as a control was carried out after the addition of acetone: ethanol (1: 1). Lipase activity was determined from the volume of NaOH required to titrate the sample reduced by the volume of NaOH required for titration control.

Immobilization of lipase using chitosan (Neau, 2002)

a. determination of lipase activity: optimal chitosan concentration on immobilized lipase beads

Preparation of chitosan beads was performed on a comparison of enzyme F60%: chitosan (1: 4) with a fixed 10 volume. The concentration of 2% chitosan means that there were 2 grams in a 100 mL of solution, where the solvent was 1% acetic acid. The preparation of beads was performed by taking the solution (a mixture of enzyme-chitosan) with a syringe dropped slowly into a solution of 20 mL of 2% TPP (2 grams in 100 mL of solution, where the solvent is aquadest) which was allowed to stand for 120 minutes contact time. The formed Beads were then filtered and were added with a 0.05 M phosphate buffer solution and were stored in the refrigerator, ready to be used for the hydrolysis reaction.The Activity of the immobilized beads was measured by titrimetric method (Prazeres et al., 2006). Immobilized lipases were then characterized.

Characterization of Immobilized Lipase

a. determination of optimum temperature on the activity of the immobilized lipase

Determination of the optimum temperature on the activity of the immobilized lipase in hydrolysing soybean oil hydrolysis was carried out at temperature variation of 30°C, 35°C, 40°C and 45°C. The enzyme reaction mixture was adjusted to pH 7 and was incubated corresponding to the temperature variations that were determined.

b. determination of optimum PH on the activity of the immobilized lipase

Optimum PH determination on the activity of the immobilized lipase in hydrolysing soybean oil was performed at pH variation of 5, 6, 7 and 8 the enzyme reaction mixture was incubated at the optimum temperature.

c. determination of the optimum substrateconcentration on the activity of the immobilized lipase

Determination of substrateconcentration on the activity of the immobilized lipase on hydrolysing soybean oil was performedon the substrate variation of 15, 20, 25 and 30%. Enzyme reaction mixture was incubated at the optimum temperature and pH.

d. determination of the optimum time on the activity of immobilized lipase

The activity of the immobilized lipase in hydrolysing soybean oil was performed on the time variation of 15, 30, 45 and 60 minutes. Enzyme reaction mixture was incubated at optimum temperature, pH and substrate.

Results and discussion

Characterization of F60% Immobilized Lipase.

Characterization of fraction 60% (F60%) immobilized lipase involves determination of the temperature, pH, substrateconcentration and time of incubation of both the immobilized and free lipase activity, and the stability of the immobilized lipase. The Characterization of the enzymes was performed usingimmobilized beads made at the optimum conditions with 2% chitosan concentration, volume ratio enzyme: chitosan = 1: 4, 120-minute contact time and 2% concentration of TPP.

a. determination of Optimum Temperature for the Immobilized Lipase

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Determination of the optimum temperature of the lipase immobilized F60% was performed using temperature variation of 30, 35, 40, 45 and 50 °C using free lipase as a control. The influence of temperature on the activity of lipase immobilized F60% can be seen in Figure 1. Figure 1 shows that the hydrolysis activity of both free and immobilized lipase was increasing along with the increase in temperature. This is presumably because the interaction of the enzyme and substrate is also increasing along with the temperature resulting in increasing product. The optimum hydrolysis activity of both of the immobilized and free lipase occurs at a temperature of 35 °C and 40 °C, respectively. The difference in the optimum temperature of the immobilized lipase and free enzyme is because the immobilized enzyme on beads that will hinder contact between the enzyme and the substrate, therefore, it requires a higher temperature to obtain the optimum activity than the free enzyme. Wuryanti (2009) reported that after the immobilization of a certain enzyme, there will be a change in the character of the enzyme. The study of papain immobilization with carrageenan derived from seaweed changed the character of the free enzyme. Papain was initially optimum at the temperature of 35 °C but after immobilization the optimum temperature conditions reached 41 °C.

The immobilized lipase activity decrease as the temperature increases. This is due the conformation changing of the enzyme at high temperature resulting in lower enzyme activity.

b. Determination of Optimum pH of the F60% immobilized Lipase

Determination of the optimum pH of the F60% immobilized Lipase was performed at the optimum temperature with substrate pH variation of 5, 6, 7, 8 and 9 with free lipase used as the control. Effect of pH on the activity can be seen in Figure 2. Figure 2 shows that the hydrolysis activity of both free and immobilized lipase is low at pH 5. This condition is due to the protonated enzyme that loses its negative charge so that the enzyme activity is decreased. The hydrolysis activity of both free and immobilized lipase continue to increase with the increasing pH and reach optimum at pH 7. At the optimum pH, the enzyme structure will meet the three-dimensional shape that is suitable to form the enzyme substrate complex, resulting in optimum activity. The hydrolysis activity of Lipase is decreasing with the increase in pH, because at high pH the substrate is ionized so that the positive charge lost so that the enzyme activity is inhibited.

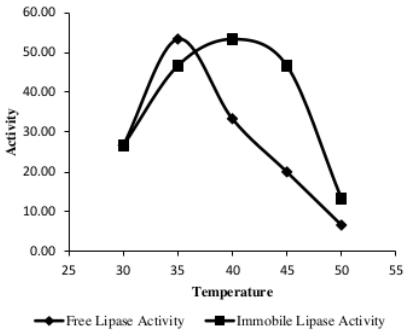

Figure 1. Effect of temperature variation on the activity of the F60% immobilized lipase

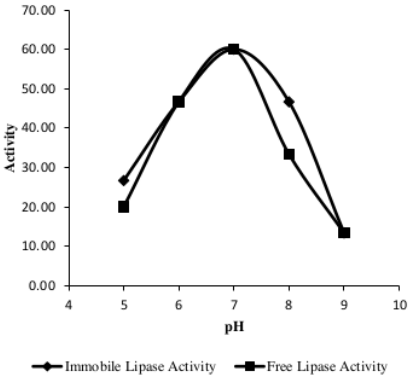

Figure 2. Effect of pH on lipase activity

c. Determination of Optimum Concentration of the F60% Immobilized Lipase

Determination of the optimum substrateconcentration of free and immobilized lipase was performed with variation of substrateconcentration of 15, 20, 25, 30, and 35%.

Figure 3 shows that the increase in the concentration of the substrate will increase the activity of both free and the immobilized lipase, vice versa. This condition is related to the enzyme active site that is only slightly in contact with the substrate. The higher substrateconcentration will create more contact between the enzyme and the substrate to form an enzyme substrate complex that will increase its activity

The Activity of the free and immobilized lipase is optimum at substrateconcentration of 25% with free and immobilized lipase activity of 66.7 U/mL and 60 U/mL, respectively. The low activity of the immobilized lipase compared to free lipase at the optimum substrateconcentration is because the enzymes are

protected by matrix of chitosan resulting in the decrease in the interaction of the enzyme with the substrate (Smith, 1990 in Su'i, et al., 2007).

The activity of the Immobilized lipase was relatively stable after reaching the optimum substrateconcentration. This is presumably because lipase is protected by chitosan matrix so that the influence of the environment can be minimized. The activity of free lipase drop significantly after reaching the optimum concentration. This is due to the feedback control in which the excess of the resulting product becomes a barrier for the action of the enzyme.

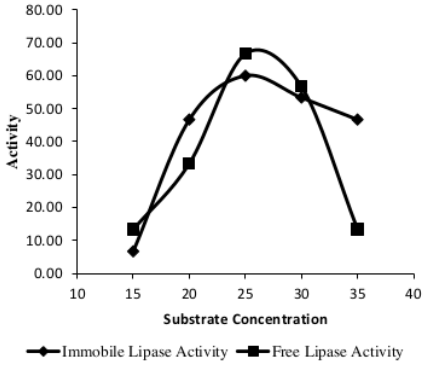

Figure 3. The effect of substrateconcentration on the F60% Immobilized Lipase

d. Determination of Optimum Incubation Time of the F60% Lipase Immobilized

Determination of the optimum incubation time was performed on time variation of 15, 30, 45, 60 and 75 minutes. The determination of the optimum incubation time can be seen in Figure 4. Figure 4 shows that the longer the contact time, the activity of both free and immobilized lipase increase. This is because the contact between the enzyme and the substrate to form an enzyme-substrate complex is influenced by the length of incubation time. A short incubation time will result in less enzyme substrate complex formed so that the enzyme activity is low. The Increase in incubation time will increase the enzyme substrate complex at which the contact occurs between the enzyme and the substrate ost longer that lead to increase in enzyme activity. The activity of both free and immobilized lipase achieve optimum at incubation time of 45 minutes. The enzyme activity decreased after that because the a lengthy shaking during incubation will cause denaturation (Pandey, 1999).

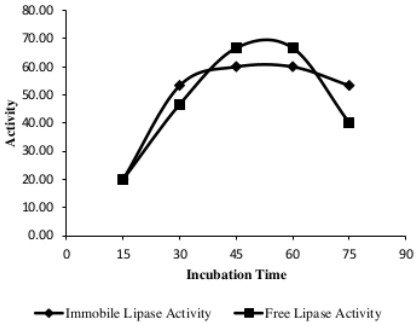

Figure 4. Effect of variation of the incubation time on the activity of the F60% immobilized lipase

Conclusions

The results showed that the optimum of activity of the immobilized lipase from bacteria Azospirillum sp. PRD1 using chitosan could be achieved at 40 °C, pH 7, incubation time at 45 minutes and 25% of substrateconcentration.

Acknowledgment

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