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PURIFICATION AND IMMOBILIZATION OF L-ASPARAGINASE ENZYME FROM THE THERMOPHILIC BACTERIA *Bacillus licheniformis* STRAIN HSA3-1a

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ABSTRACT

L-Asparaginase gives a great benefit in the cancer treatment, especially in acute lymphoblastic leukemia. L-Asparaginase is also proven to reduce the acrylamide content in the foods. The objective of this study was to perform immobilization and characterization L-Asparaginase produced from *Bacillus licheniformis* Strain HSA3-1a. The results showed that the free form L-Asparaginase from *B. licheniformis* HSA3-1a has optimum activity at pH 8 and 50^{o}C, with a specific activity of 616.26 IU/mg protein and stabilized at the optimum pH and temperature for 60 minutes. The immobilized L-Asparaginase with activated glutaraldehyde-carbon carrier has optimum activity at pH 7 and 60°C with a specific activity of 499.27 IU/mg protein and stabilized at the optimum pH and temperature for 60 minutes. The immobilized L-Asparaginase can retain its activity by 84.79% after 2 times repeated use.

KEYWORDS: ***L-Asparaginase, Bacillus licheniformis Strain HSA3-1a, immobilization, and specific activity***

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INTRODUCTION

L-Asparaginase has a great benefit in the cancer treatment, especially in acute lymphoblastic leukemia, given that the L-Asparaginase can break down L-Asparagine, one of the nutritional components of cancer cells, which is expected to inhibit the growth of these cells [1]. L-Asparaginase is also proven to reduce the acrylamide content in the foods. L-Asparaginase may prevent the formation of acrylamide by converting the amino acid of L-Asparagine as its precursor which is naturally present in the food into another form of amino acid, namely aspartic acid that is also commonly found in the food [2]. L-Asparaginase can be found in many animal tissues, bacteria, plants, and in the serum of mice, but is not found in humans. L-Asparaginase is produced in large quantities by some microorganisms; *E. coli, Erwiniacartova, Enterobacteraerogenes, Corynebacterium glutamicum, Candida utilities, Bacillus* sp., *Pisumsativum, and Streptomyces* sp. and these enzymes that exhibit antitumor activities [1,3,4]. Production of enzymes from thermophilic bacteria is increasingly becoming popular to be explored further to produce enzymes that have stability at high temperatures. Thermophile bacteria is often found in the extreme environment of hot springs at 60-80°C temperatures, such as *Thermus, Bacillus, Clostridium, Thermoanaerabacter, and Desulfomaculum* [5, 6, 7]. The bacteria *Bacillus sp* used in the production of the enzyme L-Asparaginase has been published by E-Moharam (2010). One of the thermophile *Bacillus* bacteria isolated from the hot springs in South Sulawesi, Indonesia was *B. licheniformis*[8]. The use of thermostable enzymes can save some amount of money because these enzymes have longer shelf lives and higher activity at high temperatures. Some advantages of the use of immobilized enzymes compared with the free enzyme are increase in enzyme stability, less amount of enzyme used, facilitation of separation and re-use of the enzyme and in some cases it can increase the enzyme activity. Seven media supporting the immobilization of L-Asparaginase from *Bacillus sp* have been investigated [1]. This supporting media is previously activated by glutaraldehyde which may react differently to the terminal amino residues in the enzyme protein. The immobilization of L-Asparaginase can be through a covalent cross linking between enzymes with different carriers such as activated carbon, chitin, carboxymethyl cellulose, silica gel, tricalcium phosphate, and chitosan. As reported the highest activity of immobilized L-Asparaginase is active carbon as its carrier[1].

MATERIALS AND METHODS

Purification of Bacteria B. licheniformis

Purification is performed by growing the bacteria *B. licheniformis* stock in the collection of Biochemistry and Biotechnology Laboratory Department of Chemistry, Hasanuddin University Makassar Indonesia, into the growth medium plate. The colonies that grow are then isolated, purified, and fertilized in a production medium culture of L-Asparaginase [8, 9, 10, 11].

Production of L-Asparaginase Enzyme

The production of L-Asparaginase is carried out by fertilizing the isolates into 45 mL inoculum in 100 mL Erlenmeyer flask. The Erlenmeyer is incubated at 50°C in shaker incubator for 24 hours with 200 rpm. Afterwards, 45 mL of inoculum is mixed with 500 mL of sterile production medium and then incubated for 48 hours at 50°C. The composition of the production medium are; 0.05% yeast extract, 0.01% peptone, 0.1% NaCl, KH_2PO_4 0.01%, 0.01% $MgSO_4.7H_2O$, 2.0 % L-Asparagine, 0.01% $CaCl_2$ and 0.7% ammonium sulphate [8, 9, 10, 11].

Isolation and Purification of L-Asparaginase Enzyme

The medium liquid of cell culture results is separated from the cell mass by filtration. The filtrate containing ammonium sulphate enzyme is then added while stirring by the degree of saturation of ammonium sulphate 20-40%. After overnight settling, the precipitate formed is separated by centrifugation at 10,000 rpm for 30 min with temperature of 4°C. The precipitate obtained is dissolved in 10 mL of each buffer 0.1 M Tris-HCl pH 8. The enzyme solution of

ammonium sulphate fractionation results is put in a cellophane bag and immersed into a vessel containing enough distilled water. The dialysis vessel is placed over a magnetic stirrer fitted with a low speed. Dialysis performed for 48 hours by replacing the solution several times with 0.01 M Tris-HCl buffer solution of pH 8 to show no more ammonia in buffer solution of dialysis results. The experiment is carried out with the addition of Nessler reagent that will react to form brown color if there is ammonia in the buffer solution.

Determination of Protein Concentration

The determination of protein concentration is carried out by using a modified method by Lowry; and using Bovine Serum Albumin (BSA) as standard solution.

Assay of L-Asparaginase Enzyme Activity

L-Asparaginase activity is determined by the colorimetric method[12] at 50°C using UV-Visible Spectrophotometer (Shimadzu, UV-2600) by measuring the amount of ammonia produced from L-Asparaginase catalysis using Nessler reagent. The reaction mixture consisting of 0.1 mL enzyme, 0.2 mL Tris-HCl buffer 0.05 M pH 8.6 and 1.7 mL of 0.01 M L-asparagine is incubated at optimum temperature for 10 minutes. The reaction is stopped by addition of 0.5 mL of 1.5 M solution of trichloroacetic acid (TCA) after the centrifugation at 10,000 rpm for 5 minutes, and 0.5 mL of the supernatant is diluted with 8.5 mL aquadest and then added with 1 mL Nessler reagent. The ammonia released by the hydrolysis of L-Asparaginase enzyme reacts to the Nessler reagent. Afterwards, its level is determined using the standard ammonium chloride. One unit of L-Asparaginase (IU) is defined as the amount of the L-Asparaginase enzyme that catalyzes the release of one µmol of ammonia per minute at the test conditions.

Immobilization of L-Asparaginase Enzyme

Immobilization enzyme was carried out using the medium supporting active carbon. 200 mg of the medium supporting active carbon is mixed with 2 mL of Tris-HCl buffer 0.01 mol of pH 8 which contains 2.5% glutaraldehyde at a room temperature of 4°C for 2 hours. A carrier that formed is then filtered and washed with distilled water to remove unbound glutaraldehyde. The carrier that formed is incubated with 5 mL of 0.05 M Tris-HCl buffer pH 8 containing 1 mL enzyme. After 4 hours of shaking in temperature of 4°C, unbound enzyme is released by washing with 0.05 M Tris-HCl buffer pH 8. No activity of the proteins is found in rinse buffer.

Characterization of L-Asparaginase Enzyme

Enzyme characterization is performed on the L-Asparaginase in free and immobilized forms.

a. Effect of pH on activity of L-Asparaginase

L-Asparaginase activity is evaluated in several pH condition. Free and immobilized enzymes are each incubated in 0.05 M buffer of pH 6-10. In each condition, the amount of ammonia that is released is measured. Buffer that is used is potassium phosphate (pH 6.0 to 7.0), Tris-HCl (pH 8.0 to 9.0) and Glycine-NaOH (pH 10).

b. Effect of temperature on activity of L-Asparaginase

The L-Asparaginase activities are evaluated with various temperatures at optimum pH. Free and immobilized enzymes are respectively incubated at temperatures between 30-70°C with intervals of 10°C.

c. Determination of the pH and Temperature Stability on enzyme activity

The stability of pH on enzyme activity is determined by pre-incubation of enzyme and buffer at optimum pH. Pre-incubation is performed for 2 hours and activity of L-Asparaginase is tested at 30 minutes intervals at the optimum temperature. Determination of temperature stability is performed by pre-incubation of enzyme and buffer of optimum pH at optimum temperature for 2 hours and its activity tested at 30 minutes intervals [1,8].

d. Determination of Stability of Immobilized L-Asparaginase Enzyme Operation

The operational stability of the immobilized L-Asparaginase enzyme activity is performed with a test of the activity of L-

Asparaginase to enzyme solution repeatedly up to 5 repetitions at optimum pH and temperature.

RESULTS AND DISCUSSION

Characterization of L-Asparaginase Enzyme

Some characteristics of the L-Asparaginase enzyme in free and immobilized form studied in this research include: the influence of pH, temperature, pH stability, temperature stability, and operational stability of immobilized L-Asparaginase enzymes.

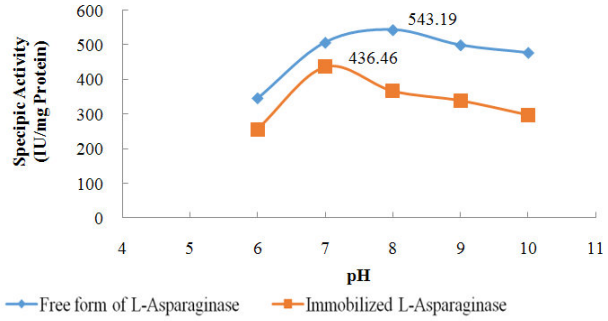

Figure 1
Determination the Optimum pH of free and Immobilized L-Asparaginase

a. Effect of pH on L-Asparaginase Activity

Figure 1 shows the optimum pH of free L-Asparaginase is pH 8; while that of the immobilized L-Asparaginase enzyme is pH 7. This difference is caused by the molecules capacity of enzyme protein and its matrix carrier, i.e. active carbon that leads to a decrease in optimum pH. Specific activity of the free L-Asparaginase enzyme increases in line with the increase of pH 7 up to pH 8. Specific activity of free L-Asparaginase at pH 6 is 345.8 IU/mg protein, 506.64 IU/mg protein at pH 7 and optimum at pH 8 with a specific activity of 543.19 IU/mg protein which decreases at pH 9 and 10. Mean while, the specific activity of the immobilized L-Asparaginase enzyme increases up to pH 7. Specific activity of immobilized L-Asparaginase at pH 6 is 255.19 IU/mg protein and optimum at pH 7 with a specific activity of 436.46 IU/mg protein, the activity decreases at pH 8, 9, and 10.

b. Effect of Temperature on L-Asparaginase activity

Figure 2 shows the activity of L-Asparaginase to the temperature changes. L-Asparaginase activity is directly proportional to the increase in temperature from 30°C up to optimum temperature of 50°C and then decreased at a temperature of 60°C and 70°C. Specific activity of free L-Asparaginase is 616.26 IU/mg protein.

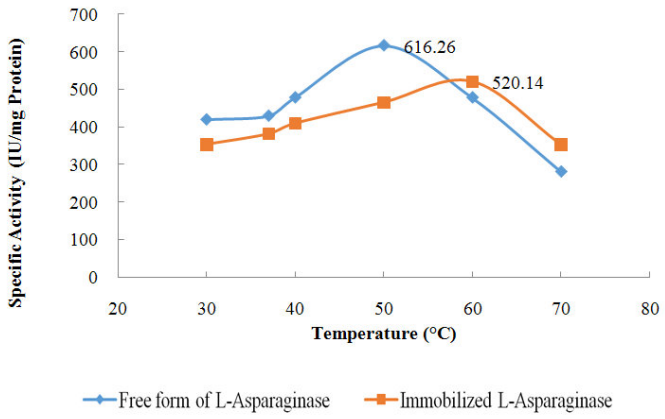

Figure 2
Determination the Optimum Temperature of Free and Immobilized L-Asparaginase

Specific activity of the immobilized L-Asparaginase enzyme increases to a temperature of 60°C. Specific activity of immobilized L-Asparaginase at 50°C is 464.36 IU/mg protein and the optimum temperature of 60°C with a specific activity of 520.14 IU/mg protein.

c. *Stability of L-Asparaginase on pH and Temperature*

L-Asparaginase in free form is stable in conditions of optimum pH (pH 8), it can be stable for 60 minutes with an 89.1% relative activity then decreases again to 68.6% at pre-incubation of 90 minutes and 44.8% remaining for its relative activity at 120 min pre-incubation. This suggests that L-Asparaginase in free form can retain the active enzymes and are able to work well even after storage for 1 hour at optimum temperature as shown in Figure 3. The stability of the immobilized L-Asparaginase enzyme form to the optimum pH (pH 7) as shown in Figure 4 has a relative activity at 30 min (85.35%), 60 minutes (78.02%), 90 minutes (59.71%), and 120 min (45.05 %) of incubation.

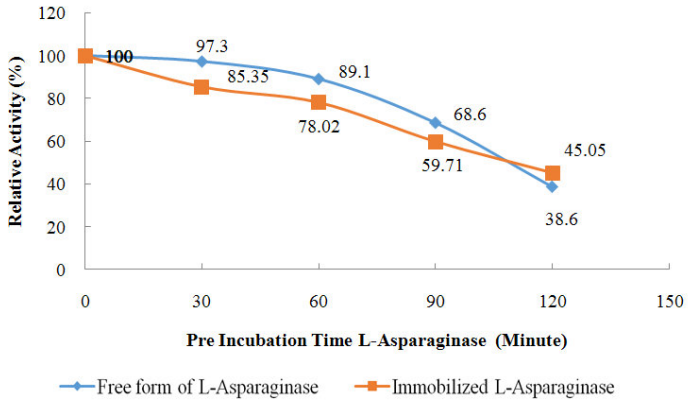

Figure 3
Determination of Stability at Optimum pH of Free (pH 8) and Immobilized L-Asparaginase (pH 8)

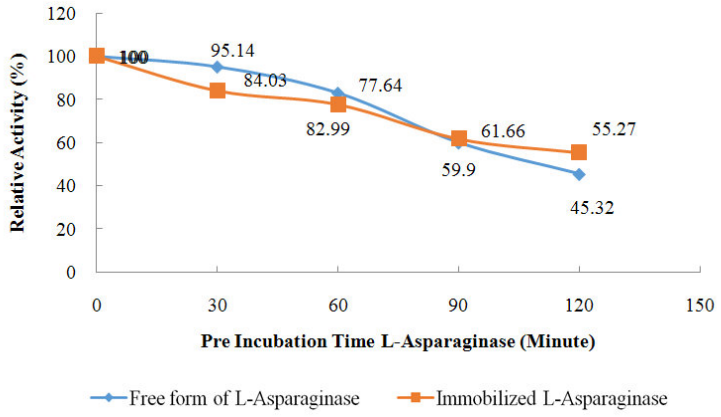

Figure 4
Determination of Stability at Optimum Temperature of Free (50°C) and Immobilized L-Asparaginase (60°C)

Figure 4 shows the stability of the free L-Asparaginase to pre-incubation at optimum temperature (50°C). Pre-incubation up to 60 minutes displays has 82.9% relative activity. Its relative activity declines at the 90 minutes and 120 minutes pre-incubation with relative activity of 59.9% and 45.32%, respectively. The stability of immobilized L-Asparaginase form to the pre-incubation at optimum temperature (60°C) shows the relative activity of 77.64% at the 60 minute pre-incubation, and a decline in relative activity at the 90 minutes and 120 minutes pre-incubation with relative activity of 61.66 and 55.27%, respectively.

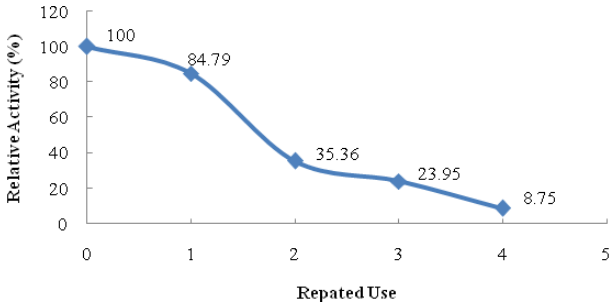

Figure 5
Operational Stability (Repeated Use) Immobilized L-Asparaginase

The operational stability of the immobilized L-Asparaginase enzyme activity is performed with a test of the activity of L-Asparaginase to enzyme solution repeatedly up to 5 repetitions at optimum pH and temperature. The immobilized L-Asparaginase enzyme can be used again as shown in Figure 5. The immobilized L-Asparaginase can be used twice with relative activity of 84.79%.

CONCLUSION

L-Asparaginase from *B. licheniformis* HSA3-1a in a free form has optimum activity at pH 8 and temperature of 50°C with a specific activity of 616.26 IU/mg protein and is stable at the optimum pH and temperature for 60 minutes. The immobilized L-Asparaginase with active glutaraldehyde-carbon carrier has optimum activity at pH 7 at 60°C with a specific activity of 499.27 IU/mg protein and is stable at the optimum pH and temperature for 60 minutes. The Immobilized L-Asparaginase can retain its activity by 84.79% after 2 times repeated use.

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