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Production and characterization of L-Asparaginase enzyme from symbiont bacteria of red algae *Eucheuma spinosum*

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Abstract

Red algae are one of the marine creatures which can produce a variety of both primary and secondary compounds. It is widely known that the existence of exploitation will cause species extinction if there is no cultivation effort. Due to this problem, the use of bacterial symbionts of algae is one of the solutions. This study aimed to obtain bacteria isolates that can produce the L – Asparaginase (L-ASNase) enzyme. The research design was explorative using experimental laboratory methods by using solid substrate fermentation techniques. The sample used was red algae *Eucheuma spinosum* obtained from Lae-Lae island in Spermonde archipelago of South Sulawesi, Indonesia. Analysis of the data displayed in the form of tables, graphs/images. Based on the results, two species of bacteria-derived L-Asparaginae enzyme were isolated. Protein content with the highest of the bacteria Enterobacter genus obtained from fractions 20-40% was equal to 1.780 mg/mL. The highest enzyme activity was in fractions of 20-40% with 32.011 IU/mL. The optimum temperature and pH of each was at 37 °C and pH 7.

***Keywords*:** Red algae, Bacteria, L–Asparaginase, Enzyme, *Eucheuma spinosum*.

1. Introduction

Red algae are one of the abundant marine creatures in the waters of Indonesia, including South Sulawesi. The marine lives have been exploited in the search target drugs due to a great potential to produce bioactive compounds [1]. The utilization of algae has been widely known that the exploitation without cultivation causes the species extinctions [2].

Some marine bacteria are reported to show bioactivities and have a symbiotic relationship with other organisms. The bacteria have capabilities similar to their host to produce bioactive compounds. The interaction of bioactive metabolites produced by marine bacteria related to the probable cause of bacteria-induced produced from the specific primary and secondary metabolites [3].

The bacteria growth is controlled, thus more suitable to use. It can be cultured and purified on the laboratory scale [4]. L-ASNase is one of the enzymes used in the medical world. This enzyme provides excellent benefits in cancer therapy, mainly in acute lymphoblastic leukemia [5]. Several microbes such as *E. coli, Erwinia cartova, Enterobacter aerogenes, Corynebacterium glutamicum,* and *Candida utilis* can produce the large amount of L-ASNase [6]. E-Moharram *et al.* (2010),

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published that the bacterium *Bacillus* sp, it can be used to produce L-ASNase [5]. *Enterobacter agglomerans* and *Staphylococcus aureus* were isolated from *red algae Eucheuma spinosum* originating from Lae-Lae Spermonde Islands, Makassar, Indonesia [7,8].

This research aimed to isolate and identify the bacteria from red algae *Eucheuma spinosum.* That can produce the L - ASNase enzyme, specify the optimum concentration of L - Asparagine and characterize L - ASNase bacteria.

2. Experimental

2.1 Location and Research Design

The experiment was conducted at the Laboratory of Microbiology, Hasanuddin University Teaching Hospital, and the Laboratory of Biochemistry, Faculty of Natural Science, Hasanuddin University in South Sulawesi, Indonesia. This study used explorative experimental techniques using solid substrate fermentation.

2.2 Sample Resourse

Samples for the source of isolated bacteria was taken from red algae *Eucheuma spinosum* which obtained from Lae-Lae island, Spermonde archipelago, South Sulawesi, Indonesia. The isolates used as inoculum in laboratory-scale fermentation were obtained from the isolation of the M-9 medium modified.

2.3 Sample Preparation

Samples of algae that have been collected were then cleaned from dirt and stored in a cool box. Further samples were refreshed in Nutrient Broth media.

2.4 Isolation of Bacterial Symbiont Macroalgae

Samples were taken in increments of 1 mL and dilution stratified to obtain the appropriate dilution. Furthermore, as many as 0.1 mL of dilution was spread into the agar medium and grown for 24 hours. Purification was carried out by growing colonies of bacteria in a petri dish containing an M-9 medium, selective for the growth of bacterial L - Asparaginase [9,10,11].

2.5 Characterization of Bacterial Symbiont Macroalgae.

Pure isolated bacteria producing L-Asparaginase enzyme bacteria were identified by standard test methods of Cappucino and Sherman with hints of Bergeys Manual of Determinative Bacteriology [12]. Microbiological tests were conducted by looking at the differences in the colony and cell morphology [13]. The biochemical tests were based on a variety of products of metabolites [14].

2.6 Determination of Substrate Concentration L - Asparagine and Optimum Incubation Time

Determination of the optimum concentration of L - Asparagine substrate and incubation time was carried out by inoculating the bacteria *Enterobacter cancerogenus* colonies into liquid medium inoculum L - Asparaginase in various concentrations of L - Asparagine substrates, 6, 8, 10, 12, and 14 g/L. Inoculum media was incubated on a shaker for 24 hours at 37 °C. Furthermore, the mixed media-inoculum was incubated in the medium of production, with some variation in the ratio between the concentration of the inoculum media production media is 1:10. Then the production medium was incubated for 3 x 24 hours at 37 °C. Sampling was done every 12 hours to measure the activity of L - Asparaginase on each variation of the concentration of L - Asparagine substrate and determination of the optimum incubation time in culture fermentation culture.

2.7 Production of L - Asparaginase Enzyme

Production of L - Asparaginase was conducted by culturing the isolates into 50 mL of inoculum medium in a 100 mL Erlenmeyer flask. Inoculum media was incubated at 37 °C in an incubator shaker at 200 rpm for 24 hours. Then, 100 mL of inoculum was mixed with 1,000 mL of sterile production medium and incubated for 2 x 24 hours at 37 ° C in an incubator shaker at 200 rpm. The composition of the production medium consisted of KH_2PO_4 0.75 g/L, $Na_2HPO_4.2H_2O$ 6 g/L, L-Asparagine 8 g/L, NaCl 0.5 g/L, MgSO4.7H2O 1.0 g/L, $CaCl_2.2H_2O$ 1, 0 g/L, glucose 3 g/L, to 20 g/L, and phenol red indicator 0.05 g/L [9,10,11].

2.8 Pre-Purification of L – Asparaginase

Liquid cell culture medium results fractionated with ammonium sulphate saturation of 20-40% [10,15]. Enzyme solution of ammonium sulphate fractionation results was further purified by dialysis

using a cellophane membrane (Sigma). After dialysis, the obtained enzyme was determined for its protein content and the activity of L - Asparaginase [16].

2.9 Determination of Protein Content

Determination of protein content was done by using a modified method of Lowry *et al*., using Bovine Serum Albumin as a standard solution [10,17].

2.10 Enzyme Activity Test of L – Asparaginase

Enzyme characterization was conducted by making a standard solution of ammonium chloride and ammonia measurement of enzyme activity with the Nessler method [10]

2.11 Characterization of L – Asparaginase Enzymes

2.11.1 The influence of temperature on the enzyme activity of L - Asparaginase.

Activity of L-Asparaginase was evaluated on various temperatures at an optimum pH. The enzyme was incubated at a temperature between 20 ºC - 60 ºC in an interval of 10 ºC. Incubation was performed for 60 minutes, and then the residual activity was measured [10,17].

2.11.2 Effect of pH on the enzyme activity of L – Asparaginase. Activity L - Asparaginase in evaluation at varying pH.

The enzyme was incubated in 0.05 M buffer of pH 4-10. In each condition, the amount of ammonia that was released then measured. The buffer used was potassium phosphate (pH 4.0 - 7.0) and Tris HCl (pH 8.0 - 10.0). Incubation was performed for 60 minutes, and then the residual activity was measured [10,17].

2.12 Data Collection and Analysis

Data obtained from observations tabulated and displayed in the form of tables and images/graphics. L-asparaginase activity was calculated using formula 1, as follow:

$$\text{Activity of Enzyme } \left(\frac{\text{IU}}{\text{mL}}\right) = \frac{(y-b)}{a} \times \frac{\text{V.Total}}{\text{V.Analyzed}} \times \frac{1}{\text{V.Enzyme}} \times \frac{1}{\text{t Incubation}} \quad (1)$$

where:

Y = Absorbance

a = Slope

b = intercept

V. Total = Volume of enzyme + substrate + buffer + TCA (2.5 mL)

V. Analysis = Total volume analyzed (0.5 mL)

V. Enzyme = Volume enzymes analyzed (0.1 mL)

t Incubation = Incubation time of enzyme reaction (10 minutes).

3. Result

3.1 Characteristics of the sample

Eucheuma spinosum is red algae obtained from Lae-lae Island. Two species of bacteria were retrieved from microsymbiont brown algae which yields L-ASNase enzyme. Table 1 shows the morphological characteristics of the isolated bacteria. The identification showed that there were two groups of bacteria based on morphological characteristics of stem cells, namely Gram-positive and Gram-negative rods. The characters and the identification of isolated bacteria producing L - ASNase enzyme can be seen in Table 2.

Table 1. The Morphological Characteristics of the Isolated Bacteria from symbiont of red algae *Eucheuma spinosum*

No.	Code of Isolate	Characterization of colony				Morphological cell	
		Form	Margin	Elevation	Colour	Gram reaction	Shape
1.	2.2.1	Circular	Entire	Convex	Slimy white.	Positive (+)	Rods
2.	2.2.8	Circular	Entire	Convex	Slimy white.	Negative (-)	Rods
3	Control	-	-	-	-	-	-

Description
2.2.1 : Isolate 1 part in *Eucheuma spinosum*
2.2.8 : Isolate 8 part in *Eucheuma spinosum*

Table 2. The characters and the identification of isolated bacteria producing enzyme L – Asparaginase

No.	Code of Isolate	Characters morphological and biochemical	Result Identification
1.	2.2.1	Colony circular, entire, convex, slimy beige. Cells rod-shaped, gram-positive, catalase-positive, urea negative, positive motility, ferment glucose and sucrose, but not ferment lactose and mannitol, test IMVIC (-,+,-,-)	Species: *Bacillus cereus*
2.	2.2.8	Colony circular, entire, convex, slimy white. Cells rod-shaped, gram-negative, catalase-positive, urea positive, negative motility, ferment mannitol, glucose, and sucrose, but not ferment lactose, test IMVIC (-,-,+,+), H_2S and gas at negative in TSI	Species: *Enterobacter cancerogenous*

3.2 Determination of Substrate Concentration L - Asparagine and Optimum Incubation Time in Fermentation Media.

Measurement of enzyme activity results in some L - Asparagine concentration can be seen in Figure 1. Data from the study showed that substrate L - Asparagine concentration was 8 g/L, with the highest activity of the L - ASNase enzyme equal to 51.98 IU/mL. While the optimum time of incubation to produce the L - ASNase enzyme activities was 48 hours and the highest activity was 25.21 IU/mL, the concentration of the substrate L - Asparagine optimum fermentation medium was 8 g/L. As shown in Figure 2, research data showed that the bacteria growth on the substrate concentration of L - Asparagine optimum at 8 g/L, obtained the highest value of $OD_{660\ nm}$ of 0.95 with 48 hours incubation time.

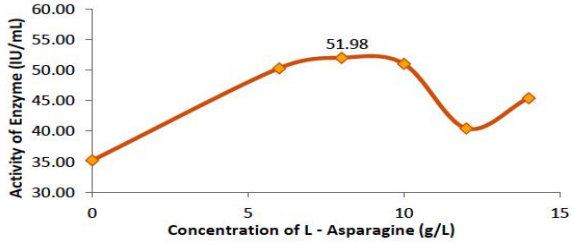

Fig. 1. Effect of concentration of L - Asparagine substrate on the enzyme activity at 37 °C

3.3 Determination of Protein Content

Each fraction has the protein content namely: ammonium sulphate saturation levels 0-20% (fraction 1), 20-40% (fraction 2), 40-60% (fraction 3), 60-80% (fraction 4), and 80-100% (fraction 5) and crude extract protein (fraction 0) in a row was 1.067 mg/mL, 1.780 mg/mL, 0.442 mg/mL, 0.432 mg/mL, 0.363 mg/mL, and 0.393 mg/mL, respectively.

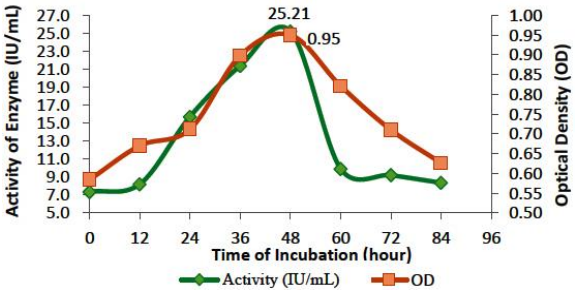

Fig. 2. The effect of incubation time on enzyme activity and growth of bacteria at the substrate concentration of L – Asparagine 8 g/L and temperature of 37 °C

3.4 Enzyme Activity L - ASNase Test

L - ASNase activity of each level of saturation ammonium sulphate fraction of 0-20% (fraction 1), 20-40% (fraction 2), 40-60% (fraction 3), 60-80% (fraction 4), and 80-100 % (fraction 5) and crude extract (fraction 0) respectively was 25.779 IU/mL, 32.011 IU/mL, 18.414 IU/mL, 18.839 IU/mL, 17.280 IU/mL, and 22.521 IU/mL, respectively.

3.5 Characterization of Enzymes

The temperature effect on L – ASNase enzyme as the results of activity test L - ASNase to temperature changes, it indicates that L - ASNase activity increases with the increase of temperature from 20 °C to an optimum at 37 °C and then decline at the temperature of 40 °C to 60 °C. The optimum temperature of the enzyme activity of L - ASNase was 29.178 IU/mL, as shown in Figure 3.

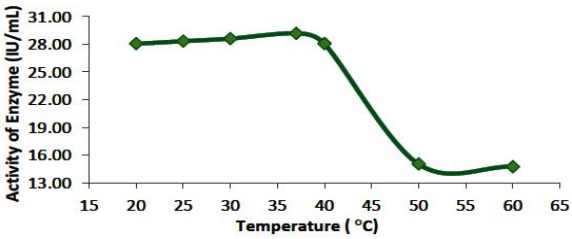

Fig. 3. Effect of temperature on the enzyme activity of L – Asparaginase at pH 8.

3.6 Effect of pH on the enzyme activity of L - Asparaginase

The activity of L - ASNase increases with pH increasing up to optimum level at pH 7. Figure 4 shows that the enzyme activity increased with 19.830 IU/mL activity and then started to decrease at pH 8 with the activity of 18.414 IU/mL.

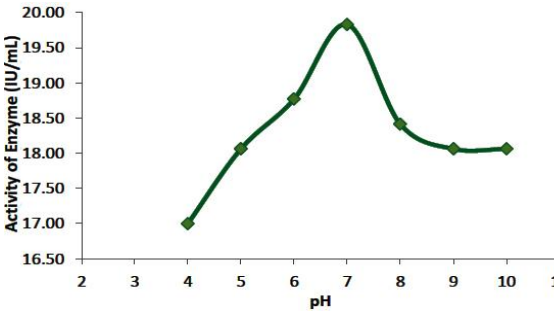

Fig. 4. Effect of pH on the enzyme activity of L – Asparaginase at 37 °C.

4. Discussion

Bacterial isolates producing enzyme L - ASNase showed the colour change in the media could determine positive results in the growth medium. The formation of the pink/red indicates the formation of ammonia. As a result, L - Asparagine substrate hydrolysis into aspartic acid and ammonia molecules were released [10].

Based on the M-9 medium observation obtained, two isolates were adequate for producing enzyme L - ASNase. Bacterial isolates were then identified in microbiological and biochemical tests. The characteristics of isolated bacteria producing L - ASNase enzyme showed that the isolates on qualitative observations could grow satisfactorily after 24 hours of incubation. The 2.2.8 isolates code were identified as species *Enterobacter cancerogenus*. Furthermore, based on the results of molecular testing should be done by using specific primers gene with complementary region encoding L-ASNase enzyme or using universal primer pairs to analyze 16 S RNA and constructed the phylogenetic tree.

A study showed that the L – ASNase enzyme could be produced from several microbes such as *E. coli, Erwinia caratovora, Enterobacter aerogenes, Staphylococcus aureus* [6]. Several studies have also reported that various kinds of bacteria are known to live as symbionts produce bioactive compounds that is similar with their hosts, such as *Bacillus* sp., [5,9], *Enterobacter agglomerans* [7], and *Staphylococcus aureus* [8].

Enterobacter sp. is able to produce bioactive compounds that can inhibit pathogenic bacteria [18]. Another study found bacterial symbionts of group *Enterobacter* sp. and cyanobacteria on green algae, microalgae, and sponges that produce bioactive compounds especially antibacterial compounds [19,20,21].

The results of enzyme activity measurements at a wavelength of 450 nm on several variations of concentration, as seen in Figure 1, showed that L - Asparagine substrate achieved an optimum concentration of 8 g/L with the activity of 51.99 IU/mL. Once the optimum activity was reached, the resulting activity declined despite the increase in L - Asparagine concentration.

The addition of substrate concentration can increase the value of enzyme activity. When the substrate concentration is at its optimum, the enzyme is in the saturated state. When the substrate concentration is increased, there will be a decrease in enzyme activity. The optimum incubation time was determined by measuring the enzyme activity at a wavelength of 450 nm. As can be seen in Figure 2, that the optimum incubation time required to produce the enzyme activities L - Asparaginase was 48 hours with the highest value of the activity of 25.21 IU/mL. An enzyme secreted by bacteria showed an increase over the length of time of fermentation.

The highest concentration of protein found in fractions 20-40% was equal to 1.780 mg/mL. From these data, it is suspected that the fraction of 20-40% is a type of protein with low solubility in water.
The results of enzyme activity measurements have shown that the crude extract's enzyme activity was 22.521 IU/mL. The enzyme activity results from the fractionation of various salts of ammonium sulphate saturation levels indicate that the enzyme activity in each fraction was different. The highest enzyme activity was obtained at the saturation level of 20-40%, with the value of the activity of 32.011 IU/mL.

Figure 4 shows that the activity of the enzyme L - ASNase has increased to a temperature of 37 °C (optimum temperature) with the activity of 29.178 IU/mL. After passing through the optimum temperature, a decrease occurred in enzyme activity at 40, 50, and 60°C, with 28.045 IU/mL, 15.014 IU/mL, and 14.731 IU/mL, respectively. The chemical reaction would take place slowly at low temperatures, whereas at higher temperatures the reaction is faster. The temperature rise before the denaturation process can increase the reaction rate.

Research conducted by El-Bessoumy *et al.* (2004), demonstrated that the L - ASNase enzyme from *Pseudomonas aeruginosa* 50071 obtained optimum temperature at 37 °C and suggested that the bacterium *Escherichia coli* producing L - ASNase

activity decreased despite an increase after reaching the optimum temperature at 37 °C [6].

Figure 4 shows that the enzyme activity increased up to pH 7 (pH optimum) with the activity of 19.830 IU/mL and then began to decrease at pH 8 with the activity of 18.414 IU/mL. The result was consistent with the study of Ahmad *et al.* (2013), which reported that the enzyme activity of free and immobilized thermophilic bacterium *Bacillus licheniformis* strain HSA3-1a increases with increasing pH up to pH 8, not only on the free enzyme but also on immobilized enzyme [10]. E-Moharram *et al.* (2010) were also reported that L - ASNase free form and immobilization from *Bacillus* sp. R36 has a pH optimum at pH 7 [5].

5. Conclusions

This study concludes that the identification of bacterial symbionts obtained two species of red algae species of bacteria, namely *Bacillus cereus* and *Enterobacter cancerogenus*. The highest protein content of L - ASNase of the bacteria *Enterobacter cancerogenus* obtained from fractions 20-40% was equal to 1.780 mg/mL. The highest enzyme activity was in fractions 20-40% in the amount of 32.011 IU/mL. The optimum temperature and pH of each were 37 °C and pH 7. The results of molecular testing should be done by using specific primers gene with complementary region encoding L-ASNase enzyme or using universal primer pairs to analyze 16 S RNA and construct the phylogenetic tree.

6. Conflicts of interest

The authors declare that there are no conflicts of interest

7. Research Fund Source

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