Screening and Characterization of L-Asparaginase Free L-Glutamianse Produced by Marine Bacterial Isolates

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ABSTRACT

L-asparaginase (EC 3.5.1.1) is a potential pharmaceutical enzyme for ALL (Acute Lymphoblastic Leukemia) treatment. However, it can cause side effects due to the activity of enzyme L-glutaminase. Halophilic microorganisms might be potential source of the enzyme L- asparaginase free of L-glutaminase because of these microorganism are adapted to extreme environments which producing biocatalysts with different structures. The enzyme was screened from marine bacterial isolated from surface sea water and marine sediment. The enzyme was produced and characterized for optimum temperature, pH, and the effect of metal ions. The results showed that a total of 96 marine bacterial isolates, three isolates namely *Pseudomonas stutzeri*, *Marinobacter nitratireducens*, *Vibrio neocaledonicus* were detected by producing L-asparaginase free L-glutaminase. The highest activity was produced by *Marinobacter nitratireducens*, 0.887 U/ml. Enzyme production at the 60 hours showed the highest enzyme activity 1,625 U/ml and specific activity 1,700 U/mg. The maximum L-asparaginase activity occurs at temperature 40 °C and pH 8 of Tris-HCl buffer. The relative activity of enzymes decreases due to the presence of metal ion K^+ 5 mM, and Mg^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} 1 mM and 5 mM.

Keywords: L-asparaginase, marine bacteria, characterization

INTRODUCTION

L-asparaginase ec 3.5.1.1 is a tetramer protein that devalues Asn and Gln. L-Asparaginase (ASNase) inhibits the protein synthesis in T cells by catalyzing the conversion of l-asparagine to l- aspartate and ammonia, and this catalytic reaction is basically ireversible under the physiological

environment [1]. L-asparaginase is an enzyme used for therapy of Acute Lymphoblastic Leukemia. Acute Lymphoblastic Leukemia is caused by excess white blood cells in bone marrow [2]. L- astarase therapy has such effects as anaphylaxis, coaxial abnormalities, thrombosis, liver dysfunction, pancreatitis, hyperglycemia, and cerebral dysfunction. Side effects caused by the production of anti-asparaginase antibody or the l-glutaminase activity [3].

Halophilic microorganisms adapt to withstand the ecological niches with high concentrations of salt. These microorganisms produce unique biocatalyst, but there is less information about the potential of halophilic microbes to produce the pharmaceutical agent [4]. Marine microbes are a potential source of bio-active compounds that are essential for commercial. Marine bacteria as source of anti-biotic, secondary metabolites, and bio-active [5]. A Total of 27 bacterial colonies were isolated from sea sediment using standard isolation techniques. The L- asparaginase production capacity resulting from all isolates was tested using a minimum medium of M9 with L-asparagine as a single protein source and phenol red as a pH indicator. There are 10 isolates showing the production of a potential L-asparaginase in an assay plate which is selected for further study [6].

The work of an enzyme will be influenced by several factors, including the concentration of enzymes, substrate concentration, temperature conditions, the influence of pH and the influence of inhibitors [7]. In this study, screening of pharmaceuetical enzymes was conducted to select specific marine bacterial isolates resulting in a L-asparaginase of free L-glutaminase enzyme. This research includes the production of the enzyme which determine the optimum production time and characterization of enzymes which find out the temperature, optimum pH and influence of metal ions on the activity of the L-asparaginase of free L-glutaminase enzyme was produced by marine bacteria.

EXPERIMENTAL SECTION

Materials

96 isolates of the Marine bacteria InaCC collection, Artifcial Sea Water, yeast extract, pepton, agar, alcohol 70%, aquadest, $Na_2HPO_4.2H_2O$, KH_2PO_4, NaCl, L-asparagine/L-glutamine, , $MgSO_4.7H2O$, $CaCl_2.2H_2O$, glukosa, $NaNO_3$, BTB (Bhromtymol Blue), phenol red ammonium chloride, NH4Cl, Mercury (II) Iodide (HgI_2), Potassium Iodide (KI), NaOH, Na_2CO_3, $CuSO_4.5H2O$, sodium potassium tartrate, BSA (Bovine Serum Albumin), Folin reagent, citrate, phosphate, Tris- HCl, bicarbonate carbonate buffer, Trichloroacetic acid (TCA), $MgSO_4.7H_2O$, $K_2SO_4.2H_2O$, $ZnSO_4.7H_2O$, $NiCl_2.6H_2O$, $CuSO_4$.

Instrumentation

Electrical pH, hotplate, magnetic stirrer, laminar air flow, rotary shaker, Thermo Scientific VARIOSKAN FLASH , incubator, waterbath, sentrifuge.

Procedure

Reviving the Marine Bacterial Isolates

A total of 96 marine bacterial isolates Ina CC (Indonesian Culture Collection)-LIPI collection was isolated from marine water and sediment of Karimun Kepri and Simeleu, Aceh. Isolates were being purified on the media Modified Sea Water Agar (3.8% ASW (Artificial Sea Water Marine Art SF-1), 0.1% yeast extract and 0.5% Pepton) [8]. 1.5% agar was added. The weighed material was then dissolved in 1000 ml of aquadest.

Prelimenary Test (Microplate Assay)

The Media for the preliminary test was Modified M-9 ($Na_2HPO_4.2H_2O$ 6.0 g, KH_2PO_4 3.0 g, NaCl 20.5 g, L-asparagine/L-glutamine 5.0 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 0.15 g, glucose 2.0 g dan agar 15.0 g in 1000 mL aquadest). 2.5 ml 3% (w/v) Bromothymol Blue stock solution was added as a pH indicator for screening L-Asparaginase (pH 7) [4], and 0.009% phenol red for control plate (pH 6.3). Control plate was supplemented with NaNO3 as a single source of nitrogen [9]. Samples were incubated at a temperature of 37 °C for 48 hours. The color indicator changes around the colony of bacteria to blue on BTB and becomes pink on phenol red as the resulting sign of L-asparaginase.

Screening L-asparaginase free L-glutaminase (Plate Assay)

The Media for the plate assay was Modified M-9 ($Na_2HPO_4.2H_2O$ 6.0 g, KH_2PO_4 3.0 g, NaCl 20.5 g, L-asparagin/L-glutamin 5.0 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 0.15 g, glucose 2.0 g dan agar 15.0 g in 1000 mL aquadest) [4], and 0.009% phenol red for control plate (pH 6.3). Production of L-asparaginase is characterized by the appereance of pink zones around the colony. Activity of the enzyme calculated semi-quantitatively with a relative ratio of zone diameter to colony diameter. Enzyme production is indicated by the enzyme zone index level [9]. Two isolates of screening results of L-asparaginase-free L-glutaminase enzyme with the highest zone index was continued to Nesslerization test for determining enzyme activity [9-10].

Bacterial Growth and Enzym Production

One isolate of the marine bacteria with the highest L-asparaginase activity inoculated into 10 ml of the Modified Sea Water Broth (3.8% ASW (Artificial Sea Water Marine Art SF-1), 0.1% yeast extract and 0.5% Pepton) [8]. Incubated during 18 hours with temperature 37 °C. 10 ml of starter inoculated into 90 ml of production media (Artificial Sea Water 3.8%, L-asparagine 0.5% as the main source of carbon and nitrogen, 90 ml aquadest) incubated for 72 hours with a temperature of 37 °C.

Measurement of marine bacteria growth and enzyme production is performed every 12 hours once during 72 hours. 1 ml sample was taken for measurement OD and 3 ml for enzyme activity test. The cell was eliminated by centrifugation 5000xg for 20 min [4]. Supernatan was used to test the extracellular activity of L-asparaginase [11]. Once the enzyme is produced, it is determined the activity of L-asparaginase enzyme and protein levels. The specific activity of the

enzyme L-asparaginase expressed in U/mg, or the result of a comparison of the activity of enzymes with protein levels [12]. L-asparaginase enzyme activity and specific activity are observed to determine optimum production time.

L-asparaginase activity is determined by measuring ammonia that is released during the reaction. The method used was Neslerisasi, then analyzed by spectrophotometer at 425 nm. The enzyme mixture consists of 900 µl of Susbtrat L-asparagine (50 mM) in phosphate buffers (pH 7) and 100 µl filtrate enzymes, incubated at a temperature of 37 ° C for 10 minutes. The reaction was discontinued with the addition of 100 µl 1.5 M trichloroacetate (TCA). Added 100 µl phosphate buffer (pH 7). The reaction mixture is centrifuged 10000 rpm for 5 min at 4 °c to remove deposits. The ammonia released on the the supernatant was determined colorimetric by adding 200-Nessler reagents to a sample of 200 µl of the supernatant and 1.6 ml of akuades. The mixture is Divortex and incubated at room temperature for 20 minutes. The absorbansi is measured at a wavelength of 425 nm (9-10]. Treat the control is done by replacing the enzyme the supernatant with the phosphate buffer pH 7. After the addition of TCA, added 100 µl supernatan. The Absorbansi obtained is determined by the mass of ammonia on the standard curve of ammonium chloride.

Protein content is determined by the Lowry method. The protein concentration is determined by the Lowry method, using Bovine Serum Albumin as the standard. 0.1 ml sample added 0.1 mL NaOH 2 N, hydrolyzed at 100 °c for 10 min on boiling waterbath. Then cooled at room temperature and add 1 mL Complex-forming reagent (solution A:B: C = 100:1:1). Furthermore, the solution is incubated at room temperature for 10 minutes. Added 0.1 mL of Folin reagent using vortex mixer, and incubated at room temperature for 30-60 minutes (not exceeding 60 minutes [13]. The absorbance value of the Lowry method is read at a wavelength of 750 nm [14].

Temperature Characterization

The optimum temperature determination of the L-asparaginase activity is analyzed by the mixture of reactions incubated at different temperatures ranging from 25 ℃ to 60 ℃ and the enzyme activity is determined by Nesslerisasi [15].]Temperature variations in the enzyme characterization of L-asaparaginase-free L-glutaminase are 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C. L-asparaginase enzyme activity is determined by neslerization method, then analyzed by spectrophotometer at 425 nm [9].

pH Characterization

The optimum pH determination is done with the the supernatant incubated in the 0.05 M buffer (El-Naggar et al., 2016) at the pH range of 5-10 (with optimum temperature). The buffers used are citrate (pH 5, 5.5, 6), phosphate (6, 6.5, 7, 7.5, 8), Tris-HCl (pH 8 and 9), and carbonate- bicarbonate (pH 9 and 10). L-asparaginase enzyme activity is determined by neslerization method, then analyzed by spectrophotometer at 425 nm [9].

Metal Ions Characterization

The effect of various metal ions on the enzyme activity is carried out by attaching enzymes to the reaction mixture with a total concentration of 1 mM [6]. The effect of metal ions on L-asparaginase activity can be determined by means of enzymes rereinkubated with a different metal solution at a total concentration of 5 mM (at optimum temperature and pH) [17]. The tested metal ions are Mg^{2+}, Ni^{2+}, Cu^{2+}, K^{+}, Zn^{2+}. Metal ions are added in the form of $MgSO_4.7H_2O$, $NiCl_2.6H_2O$, $CuSO_4$, $K_2SO_4.2H_2O$, $ZnSO_4.7H_2O$. A mixture of L-asparaginase enzymes without metal ions is used as a control. L-asparaginase enzyme activity is determined by neslerization method, then analyzed by spectrophotometer at 425 nm [9].

RESULTS AND DISCUSSION

Screening of L-asparaginase

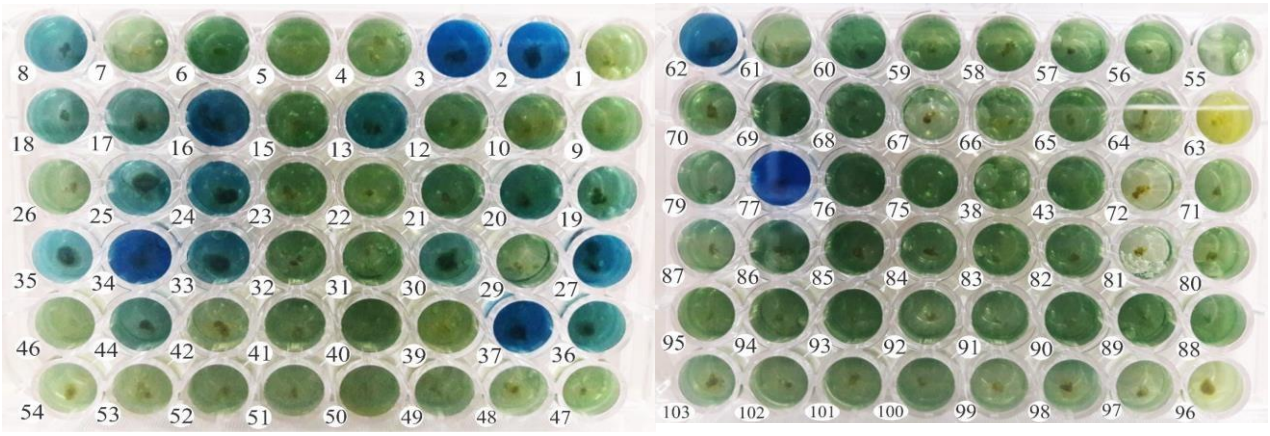

Figure 1. Screening of L-asparaginase (microplate). Blue color = L-asparaginase positive. Green color = L-asparaginase negative.

Based on the preliminary screening test results of L-asparaginase on the Modified M-9 that incubated 48 hours there are 21 isolates detected producing L-asparaginase (Figure 1). The production of L-asparaginase in the isolates was characterized by the alteration of the media colour containing the BTB (Bromthymol Blue) indicator at pH 6.8 (green) to blue. The enzyme L- asparaginase is able to degrades L-asparagine in the media into aspartic acid and ammonia. The accumulation of ammonia in the media causes a change in the media colour indicating the alkaline condition, which is blue. The BTB color indicator provides a green color at a neutral pH (7.0) and a dark blue at a higher pH (8.0-9.0), indicating that the microorganisms could potentially produce L- asparaginase [3].

Screening of L-asparaginase free L-glutaminase

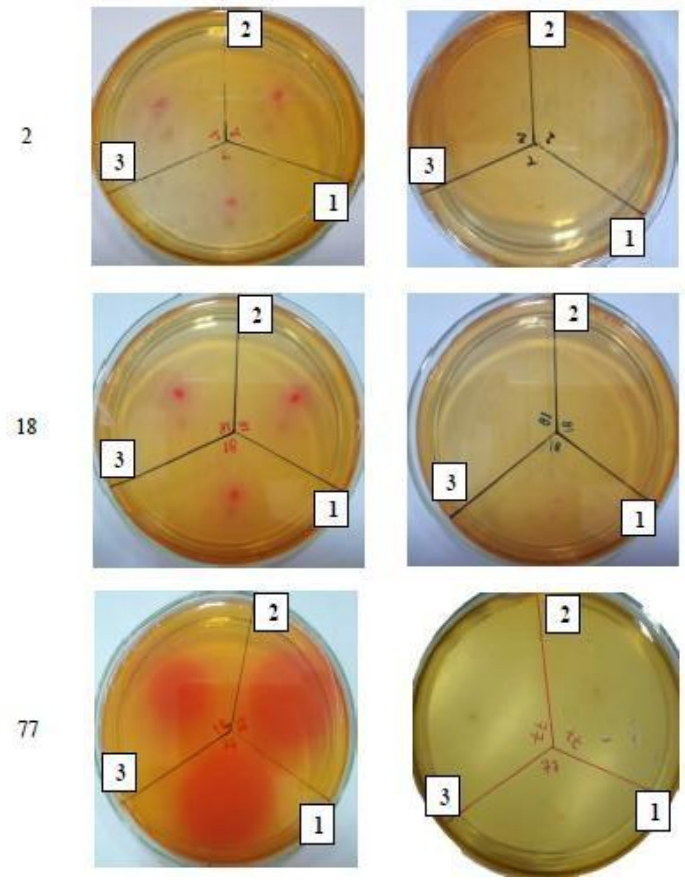

Figure 2. Screening of L-asparaginase bebas L-glutaminase (plate). 1 = First replay; 2 = Second replay; 3 = Third Replay. There is a red zone = Positive L-ASP (left); Positive L-GLS (right). No red zone = Negative L-ASP (left); Negative L-GLS (right)

According to Figure 2., three isolates have passed the free L-asparaginase enzyme L- glutaminase, characterized by the presence of a pink zone around the colony. The change of media color with the phenol red indicator indicates a pH increase due to the accumulation of ammonia in the media. The color indicator shows a yellow color on the acidic condition and turns pink in alkaline conditions [11]. The three isolates of marine bacteria number 2, 18, and 77 are able to produce L-asparaginase-free L-glutaminase enzymes are *Pseudomonas stutzeri*, *Marinobacter nitratireducens*, *Vibrio neocaledonicus*. The L-asparaginase enzyme reportedly was produced by the genus *Bacillus*, *Vibrio*, *Marinobacter*, and *Pseudomonas* [18]. In his research reported that L-Asparaginase was produced by *Pseudomonas Stutzeri* MB-405. The enzyme is very specific hydrolyzed L-asparagine, but does not hydrolyze L-glutamine [19]. Isolated *Marinobacter arcticus* of the brown *algae Sargassum* sp was able to produce the L-asparaginase free L-glutaminase enzyme [20]. The enzyme L-asparaginase-free L-Gutaminase was produced also by *Vibrio cholerae* [21] and *Vibrio succinogenes* [22].

The highest extracellular enzyme zone index is demonstrated by the sea bacteria Marinobacter nitratireducens, which is 2.54 whereas. Testing of enzyme activity with nesslerization

method is conducted on isolate of the production of marine intracellular enzymes *Marinobacter Nitratireducens* and the extracellular *Vibrio neocaledonicus*. Isolate bacteria *Marinobacter nitratireducens* have a higher enzyme activity of 0887 U/ml, compared with the isolation of bacteria *Pseudomonas stutzeri* with enzyme activity of 0.697 U/ml. Isolate bacteria *Marinobacter Nitratireducens* selected For the production of enzymes and characterization of enzymes because the resulting L-asparaginase is extracellular and has the highest enzyme activity. Extracellular L- asparaginase is more profitable than an intracellular type due to the accumulation of enzymes in the broth culture higher under normal conditions, easy to extract and downstream processing [23]. ***Bacterial Growth and Enzyme Production***

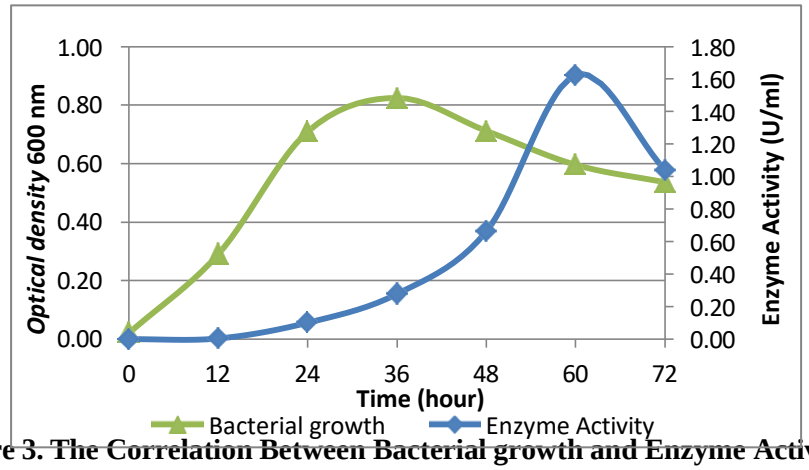

Figure 3. The Correlation Between Bacterial growth and Enzyme Activity.

Table 1. pH of Media Production

Time production	pH media
0	7
12	7-8
24	7-8
36	7-8
42	8-9
60	8-9
72	9

Activity of L-asparaginase enzyme maximum at to 60 hours time production with pH media 8-9. Oligotrof is a microorganism that can live in a nutrient-poor environment, one of which is on sediment and seawater [24]. These microorganisms grow slowly and have a low metabolic rate, so they can cause low population density. *Marinobacter Nitratireducens* grew at a pH of 6-8, with an optimum pH of 7 [25[.

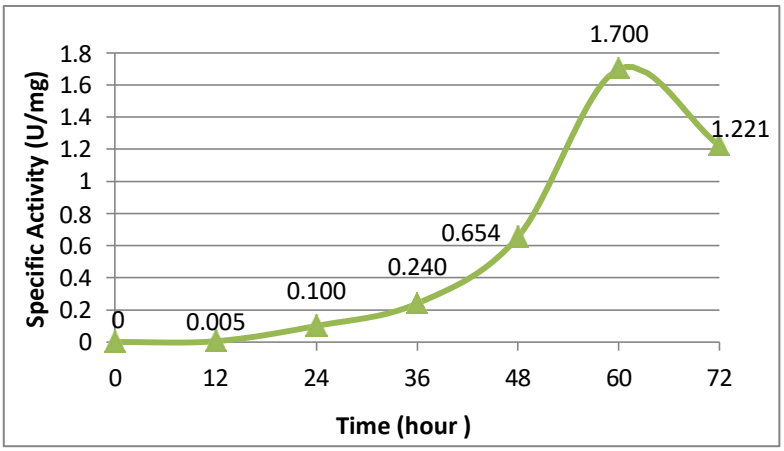

Figure 4. Specific Activity of L-asparaginase free l-glutaminase

The specific enzyme activity is maximal at 60 hours. It signifies that the highest degree of enzyme purity is at the production of 60 hours. Specific activity (unit/MG) is a measure of the purity of the enzyme, whose value increases when the purity of the enzyme increases because the number of contaminated proteins usually decreases [26]**.** The optimum production time of the L- asparaginase-free enzyme l-glutaminase produced by *Marinobacter Nitratireducens* at 60 hours time production because it has a maximum specific activity enzyme activity.

Optimum Temperature Determination

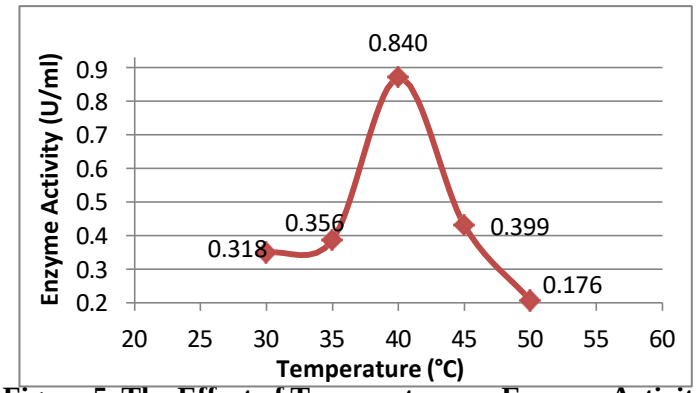

Figure 5. The Effect of Temperature on Enzyme Activity

The maximum enzyme activity of 0.840 U/ml is shown by the peak of the curve at a temperature treat of 40 °C, where the enzyme is able to catalyse the substrate optimally in biochemical reactions. The optimum temperature of the L-Asparaginase produced Halophil bacteria in the genus *Vibrio* is 37 º C [18]. While bacteria Bacillus SP has a maximum activity of L- asparaginase at temperatures 38 º C [27]. Temperature is one of the most important parameters in bioprocesses. The optimum temperature for the production of L-asparaginase enzymes is largely marine organisms ranging from 26 to 37 °C. Some exceptions are the *Enterobacter hormaechei* and *Streptomyces* sp. SS7 bacteria which have an optimal temperature of 40 °C and 45 °C respectively. Therefore, these marine microorganisms are very likely to produce a thermostabilized enzyme that can be applied in the food industry [28].

Optimum pH Determination

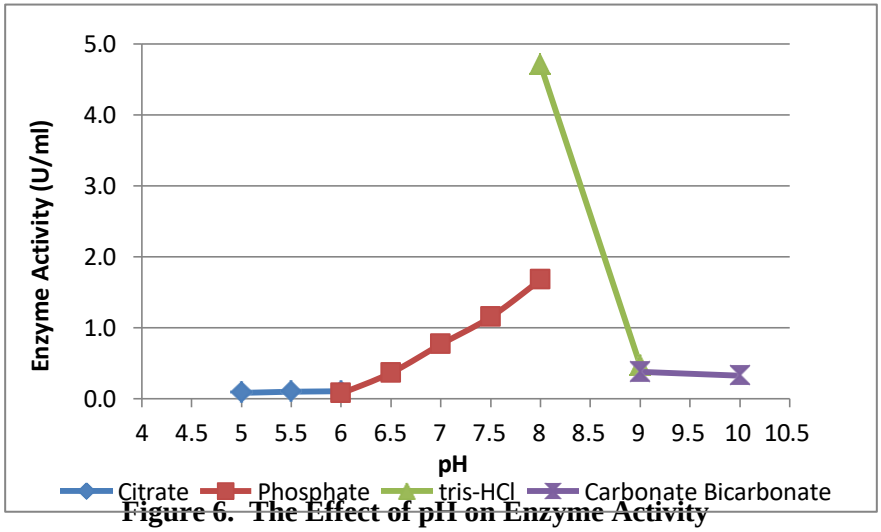

Figure 6. The Effect of pH on Enzyme Activity

The activity of the maximum L-asparaginase enzyme at pH 8 the Tris-HCl buffer. The activity of the enzyme L-asparaginase marine bacteria *Streptomyces noursei* MTCC 10469 has maximum activity at pH 8 with a buffer Tris-HCl [29]. The pH test in the L-asparaginase activity manufactured by *Streptomyces ginsengisoli* was carried out in the range of 6 to 10, and maximum activity occurred in the pH 8 treatment with a Tris-HCl buffer [30]. Ion affects the activity of enzymes either by their ionic strength and nature. Different enzyme activity can be very different when tested in two different buffers, even at the same pH and concentration. This can happen because the buffer components such as mono or divalent metal ions directly affect the catalytic process, which is required as an essential cofactor [31].

The Effect of Metal Ions

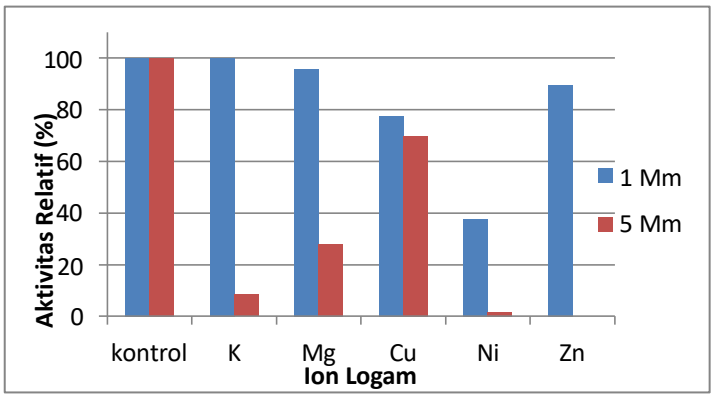

Figure 7. The Effect of Metal Ions on Enzyme Activity

The addition of metal ions K^+ concentrations of 5 mM, and metal ions Mg^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} concentrations of 1 mM and 5 mM are able to decrease the relative activity of enzymes. The addition of metal ions K^+ mM concentration does not decrease the enzyme relative activity. Metal ions K^+ mM were only able to increase 4.6% relative activity of the enzyme L-asparaginase produced *Paenibacillus barengoltzi* [32]. Metal ion K^+ does not provide a significant effect of L-

asparaginase derived from *Streptomyces brollosae* NEAE-115 [30]. The addition of metal ion metal ions divalent Mg^{2+}, Zn^{2+}, and Cu^{2+} with a concentration of 1 mM has a minor effect because it is only able to inhibit the relative activity of the enzyme L-asparaginase less than 10%. While the metal ions Ni^{2+} inhibit vigorous until its relative activity decreases to 22% [16]. The effects of metal ions Mg^{2+}, Zn^{2+}, and Cu^{2+} at a concentration of 1 mM caused a decrease in relative activity [32].

A post-hoc test as an advanced test of the ANOVA test is done on a variable metal ion concentration because the results of ANOVA indicate a noticeable difference. The Duncan test showed that there was a significant difference in the activity of the L-asparaginase enzyme on the concentration of 5 mM metal ions on a 1 mM metal ion concentration and control treatment. But there is no significant difference between the concentration of 1 mM metal ions with control. The higher the concentration of metal ions Mg^{2+}, Cu^{2+}, K^{+}, Ni^{2+}, Zn^{2+} then the more impede to the relative activity of the enzyme L-asparaginase, characterized by the decline in the relative activity of enzymes. The higher the concentration of metal ions the higher the decline in its relative activity. The L-asparaginase enzyme produced by *Streptomyces* sp, inhibited its activity by the addition of metal ions Cu^{2+} and Zn^{2+} with concentrations of 1 mM, 3 mM, and 10 mM [34].

CONCLUSION

Based on the research done, it can be concluded that screening 96 marine bacterial isolates resulted in three isolates. The specific produce enzyme L-asparaginase-free L-glutaminase, namely *Pseudomonas stutzeri*, *Marinobacter nitratireducens*, *Vibrio neocaledonicus*. The number 18 isolates of Marinobacter nitratireducens are able to produce the highest enzyme activity. The enzyme L-asparaginase of marine bacteria *Marinobacter nitratireducens* has the optimum production time of the hour to 60, the optimum temperature of 40 °c and the optimum pH of 8- buffer Tris-HCl. Addition of metal ions K^{+} 5 mM concentration, and metal ions Mg^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} concentrations of 1 mM and 5 mM Able to decrease the relative activity of enzymes.

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