SCREENING AND CHARACTERIZATION OF L-GLUTAMINASE PRODUCED BY BACTERIA ISOLATED FROM SANGIHE TALAUD SEA

Penapisan dan Karakterisasi L-Glutaminase yang Diproduksi oleh Bakteri dari Perairan Sangihe Talaud

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ABSTRACT

L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) is a very important enzyme due to its role as flavor enhancer and antileukemic agent. Salt-tolerant L-glutaminase produced by marine bacteria is favorable in food industries. This study describes the screening of L-glutaminase producing marine bacteria from Sangihe-Talaud Sea, North Sulawesi, Indonesia. Screening of L-glutaminase was performed using a liquid medium and identification of selected isolate was performed using molecular-based 16S rDNA. Results showed that there were 7 isolates produced positive results of L-glutaminase, and one of them (II.1 isolate) produced the highest activity, i.e 147.99 U/L, equivalent to the specific activity of 62.32 U/mg. The isolate then selected for further study. Bacterial identification based on 16S rRNA sequencing has revealed that the isolate was 96% similar to *Pseudomonas aeruginosa* strain CG-T8. Characterization of extracellular L-glutaminase from the II.1 isolate showed that the enzyme worked optimally at temperature of 37-45 °C and pH 7. The enzyme was stable when NaCl solution was added up to 8% and began to decrease on addition of NaCl solution of 16% and 20% with relative activity of 79% and 74%, respectively. The effect of metal ions, *e.g* Mn^{2+}, Mg^{2+}, and Co^{2+} in the form of chloride salt, were able to increase enzyme activity, whereas the addition of other metal ions (Zn^{2+}, Fe^{3+}, and Ca^{2+}) decreased the activity. The molecular weights of L-glutaminase was estimated around 42 kDa and 145 kDa.

Keywords: L-glutaminase, marine bacteria, 16S rRNA, screening, characterization

ABSTRAK

L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) merupakan enzim yang sangat penting karena perannya sebagai penghasil *flavor* dan anti leukimia. L-glutaminase yang tahan garam yang diproduksi oleh bakteri laut sangat diharapkan oleh industri pangan. Penelitian ini bertujuan untuk melakukan penapisan bakteri laut penghasil enzim dari perairan Sangihe-Talaud, Sulawesi Utara, Indonesia. Penapisan dilakukan dengan menggunakan medium cair, dan identifikasi bakteri penghasil enzim dilakukan secara molekuler menggunakan 16S rDNA. Hasil penelitian memperlihatkan bahwa dari 7 isolat yang positif L-glutaminase, 1 isolat (isolat II.1) menghasilkan aktivitas enzim tertinggi yaitu 147,99 U/L, setara dengan aktifitas spesifik 62,32 U/mg. Isolat ini selanjutnya dipelajari lebih lanjut. Berdasarkan identifikasi molekuler, isolat ini memiliki kemiripan 96% dengan *Pseudomonas aeruginosa* strain CG-T8. Hasil karakterisasi enzim menunjukkan bahwa enzim ini bekerja optimal pada suhu 37-45 °C and pH 7. Enzim stabil ketika dilakukan penambahan NaCl sampai dengan 8% dan mulai berkurang ketika penambahan mencapai 16 dan 20%. Penambahan ion logam Mn^{2+}, Mg^{2+}, and Co^{2+} dalam bentuk garam klorida mampu meningkatkan kinerja enzim sementara penambahan ion Zn^{2+}, Fe^{3+}, and Ca^{2+} mengurangi aktivitas enzim. Enzim ini diperkirakan memiliki berat molekul 42 kDa dan 145 kDa

Kata kunci: L-glutaminase, bakteri laut, 16S rRNA, penapisan, karakterisasi

INTRODUCTION

L-glutaminase (L-glutamine amidohydrolase, EC.3.5.1.2) is an enzyme that catalyzes the hydrolysis of L-glutamine into L-glutamic acid and ammonia. This enzyme is getting popular due to its important role and its applications in both pharmaceuticals and food industries (Nandakumara et al., 2003). L-glutaminase is proposed as an enzyme therapy for cancer, especially for acute lymphocytic leukemia, in combination or as an alternative to L-asparaginase (Siddalingeshwara et al., 2010; Abdallah et al., 2012). The mechanism of L-glutaminase as anticarcinogenic correlates with glutamine depletion due to inability of the lymphatic tumor cell to synthesize glutamine as the normal cells. Glutaminase is also taking an important role that controls the delicious taste of fermented foods such as soy sauce and in general food products by increasing the glutamic acid content therefore, this enzyme has attracted a great attention in food industries (Moriguchi et al., 1994; Alexandra et al., 2003).

Microbes i.e bacteria, fungi and yeast have been reported as potential L-glutaminase sources, and using that microbies as enzyme producers is more preferable due to their simple growth requirements, easy processing and handling as well as cheaper production cost. Reports showed that the majority of microbes producing L-glutaminase have been isolated from soil and aquatic (marine) environment (Nandakumara et al., 2003). Marine environment is expected as a unique L-glutaminase sources such as salt-tolerant and thermo-stable L- glutaminase which is needed by food industries (Desmond,1997; Prakash et al., 2009).

Screening of L-glutaminase from marine bacteria had been reported by Padma & Singhal (2009), from marine *Actinomycetes* by Balagurunathan et al. (2010), and marine fungi by Sabu (1999). However, information on the L-glutaminase from Indonesia Sea is very rare. The objective of this present study was to isolate, screen and identify marine isolates producing L-glutaminase from Sangihe Talaud Sea, Indonesia. Characterization of the enzyme (crude enzyme) was performed from the best isolate producing the highest activity of L-glutaminase.

MATERIALS AND METHODS

Qualitative Screening and Isolation of L-glutaminase Bacteria

Samples (seawater) were collected by water samplers attached to CTD from 4 m until 1500 m depth of Sangihe-Talaud Sea in North Sulawesi, Indonesia during INDEX SATAL 2010 Cruise on R/V Baruna Jaya IV. The sample was kept cool until reaching laboratory. In the laboratory, 1 ml of sample was taken from homogenized samples and initially added in to 20 ml liquid modified *Zobell's* media containing 1 g/L yeast extract and 5 g/L peptone dissolved in artificial seawater (ASW). For qualitative screening, 1 ml of enriched media containing the sample were grown in 100 ml flasks containing 20 ml media and put on water bath shaker at 100 rpm maintained at 30 °C for 48 h.The medium contained 5.0 g/L glucosa, 5.0 g/L glutamine, 6.0 g/L $Na_2HPO_4.2H_2O$, 3.0 g/L KH_2PO_4, 0.49 g/L $MgSO_4$, 0.05 g/L NaCl, 0.002 g/L $CaCl_2$ and 0.06 ml of 2.5% w/v ethanolic phenol red solution with pH adjusted to 7-8 (Padma & Singhal, 2009). The sample that showed positive result was indicated by the color changes of media, and was further taken up for quantitative screening.

Quantitative Screening for L-glutaminase

1 ml of enriched media containing the positive samples from qualitative screening were grown by streaking in media containing 5.0 g/L glucose, 5.0 g/L glutamine, 1,0 g/Lyeast extract, 6.0 g/L $Na_2HPO_4.2H_2O$, 3.0 g/L KH_2PO_4, 0.49 g/L $MgSO_4$, 0.05 g/L NaCl, and 0.002 g/L $CaCl_2$ with pH adjusted to 7-7.5 for 48 h at 100 rpm at 30 °C. The activity of extracellular L-glutaminase was estimated by spectrophotometer using Nessler reagent as described in analytical determination (Imada et al., 1973).

16S rRNA Sequencing Analysis of Selected Isolate

DNA extraction was performed using commercial extraction of DNA. DNA amplification by PCR was performed using F Primer (5'-CAGGCCT AACA CAGGCAAGTC-3') and R primer (5'-GGGCG GWG TGTACAAGGC-3'). Sequencing of amplification product of 16S rRNA gene was conducted at 1st base life science in Singapore. The sequence obtained was initially analyzed at National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (Blast) and phylogenetic tree was constructed to identify the isolate (Marchesi et al., 1998).

Growth Curve and Enzyme Production

Growth curve of the isolate producing the enzyme was determined by inoculating 1 loopfull of bacteria in L-glutaminase media, composing of 5.0 g/L glucosa, 1.0 g/L yeast extract, 6.0 g/L $Na_2HPO_4.2H_2O$, 3.0 g/L KH_2PO_4, 0.49 g/L $MgSO_4$, 0.05 g/L NaCl, 0.002 g/L $CaCl_2$. Absorbancy at 600 nm was recorded every hour during the first 4 h, followed by every 4 h. To obtain enzyme production profile, measurement of enzyme

activity was conducted following growth curve sampling. Preliminary study on the production of L-glutaminase was conducted by optimizing fermentation condition, i.e cultivation temperature of 25-35 °C, shaker speed of 80 and 100 rpm, and inoculum volume of 5% and 10%. The best result of the preliminary study was inoculum cultivation of 5%, and 10% then used to produce L-glutaminase (Padma & Singhal, 2009).

Measurement of L-glutaminase Activity

For estimation of L-glutaminase activity, a method developed by Imada et al. (1973) was used. The L-glutaminase produced in fermentation broth converts L-glutamine to L-glutamic acid and release ammonia. This ammonia then quantified by spectrophotometry using Nessler reagent. The reaction mixture contained 0.5 ml of crude enzyme extract, 0.5 ml of 0.04 M L-glutamine, 0.5 ml of 0.5 M Tris-Cl buffer pH 8.4, and 0.5 ml distilled water. The reaction, at 37 °C for 30 min, was terminated by adding 0.5 ml of 1.5 M TCA. Then, 0.2 ml Nessler reagent was added to 0.1 ml reaction mixture and made up to 4 ml using distilled water. The absorbance of the mixture was measured at 450 nm after 20 min incubation at room temperature. One unit of L-glutaminase activity was defined as enzyme required for releasing 1.0 µ mol ammonia per min (Imada et al., 1973). The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard.

Characterization of Crude Extracellular L-glutaminase

To determine the optimal temperature, the temperature ranges used to react the enzyme mixture was 20 to 60 °C. Optimal pH determination was carried out by using buffer solution ranging from 5 to 10, i.e 6-8 using phosphate buffer, 8-9 using Tris-HCl buffer, and 9-10 using carbonate-bicarbonate buffer. Effect of salt on L-glutaminase activity was performed using addition of 0% to 20% NaCl. The effect of metal ions on the L-glutaminase activity on crude extracellular L-glutaminase were measured by similar L-glutaminase assays.

SDS-PAGE

Determination of the molecular weight of L-glutaminase, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 12% polyacrylamide gel and stained with silver staining (Sabu,1999).

RESULTS AND DISCUSSION

Qualitative Screening and Isolation of L-glutaminase Producers

Four samples of water collected from 4 m to 1000 m depth gave positif result of L-glutaminase (Table 1) qualitatively, where as the 3 other samples collected from 1250-1500 m showed negative result. From the four samples, 13 isolates have been succesfully isolated and was taken into further screening quantitatively. The screening study showed that approximately 7 of the 13 isolates gave positive result of L-glutaminase. Among the 7 isolates, the isolate II.1 gave the highest L-glutaminase activity of 147.99 Unit/L or 62.32 Unit/mg of specific activity. This isolate which was taken from 400 m depth of Sangihe-Talaud Sea, was then selected for further study.

Table 1. Screening of L-glutaminase isolates from 4 different depths of Sangihe Talaud Sea

N.D : not detected

16S rRNA Sequencing Analysis of Selected Isolate

The sequence homology studies showed the isolate II.1 had 96.0% similar to *Pseudomonas aeruginosa* strain CG-T8 (*P. aeruginosa* GU 339295.1). By comparing differences in the nucleic acid sequences of homologous genes from different organisms, a molecular phylogenetic tree was then constructed. The distance between the two sequences is an indicative of their relatedness to each other. Figure 2 showed the phylogenetic tree showing that our isolate had the same distance of relatedness with *P. aeruginosa* strain CG-T8 (*P. aeruginosa* GU 339295.1) and *Pseudomonas* sp. HO 609591.1. Molecular identification based on 16S rDNA sequencing proposed the isolate as *P. aeruginosa* strain CT-G8 with 96% similarity. Genus *Pseudomonas sp.* has already known as a source of microbial glutaminase, along with other bacteria, fungi and yeast which are mostly isolated from soil, and it was produced extracellularly. Review on microbial L-glutaminase showed that Pseudomonas specifically *P. aeruginosa*, *P. aurantiaca*, *P. aureofaciens*, *P. boreoplois, P. fluorescens*, *P. ovalis*, *P. schuylkilliensis* and *Pseudomonas* sp. have secreted L-glutaminase (Nandakumar et al., 2003).

Growth Curve of the Isolate II.1 and Production of the Enzyme

Figure 3 showed growth curve of the isolate, showing that the isolate reach logaritmic phase after 3 h cultivation up to 10 h cultivation, following stationary phase up to 21 h. L-glutaminase enzyme was produced since 9 h cultivation and attained peak production at 22 h cultivation, which was in stationary phase of Isolate II.1 growth. From this data, this enzyme might be produced as secondary metabolites.

Preliminary study on optimizing the cultivation condition of L-glutaminase production was conducted by modified cultivation method of Padma *et al*. (2009). Figure 4 (A,B,C,D) shows the optimization results demonstrating that the extracellular L-glutaminase from the II.1 isolate was maximally produced when the isolate cultivated at 30 °C, in media having pH 6, using volume inoculum of 5% and at shaking speed of 100 rpm. Based on the growth curve (Figure 3), the inoculum used was the 8-12 h old isolate having 0,65-0,75 Optical Density (OD) at 600 nm which was equal to $59x10^7$–$69x10^7$CFU/ml of the inoculum used.

Characteristics of L-glutaminase Enzyme

The crude extracellular L-glutaminase produced by *Pseudomonas aeruginosa* strain CG-T8- II.1. performed optimally at pH 7,0 and stable at 37-45 °C Figure 5. A,B). The enzyme could tolerate NaCl concentration up to 16% and 20%, and loosing the activity by 21% and 25.88%, respectively. The effect of metal ions Mg^{2+}, Co^{2+} and Mn^{2+}as Cl_2 salt increased the activity, while addition of Ca^{2+}, Fe^{3+}, and Zn^{2+}reduced the activity (Figure 6. A,B).

Al Hammed & Jassim (2011) reported that the activity of L-glutaminase from *Serratia plymuthica* was

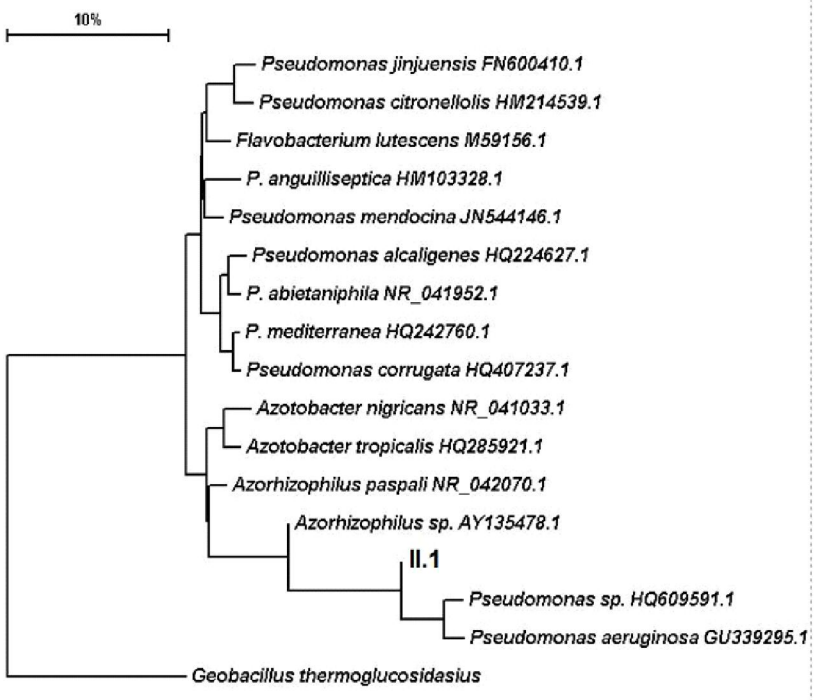

Figure 2. Phylogenetic tree of Isolate II.1

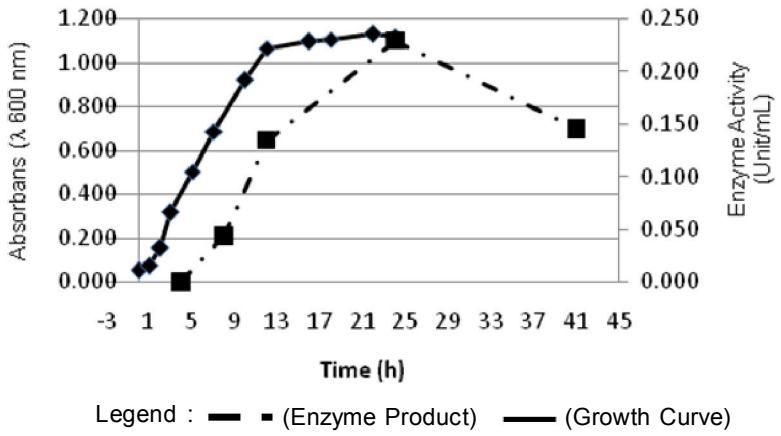

Figure 3. Growth curve of II.1 isolate and it's enzyme production

raised by addition of tetracycline and chloramphenicol with increasing specific activity of glutaminase up to 10.8 U/mg and 15.31 U/mg respectively, in comparison with the control of 3.1 U/mg.

Salt tolerance L-glutaminase was important for its application, especially for food industry needs. It was reported that marine isolates *M. luteus* K-3 and *B. subtilis* were shown to be highly salt-tolerant up to 16 and 25% of NaCl, respectively. Isolate II.1 produced quite stable L-glutaminase. However, the relative activity of the enzyme decreased by 21% when added with 16% NaCl solution.

Result of SDS-PAGE of the enzyme showed the occurence of two (2) protein bands of 42 kDa and 145 kDa in all samples, *i.e* crude enzyme, ultrafiltration fraction and amonium sulphate fraction. It was assumed that the bands represent the sub unit and native enzyme from glutaminase class A. As mentioned

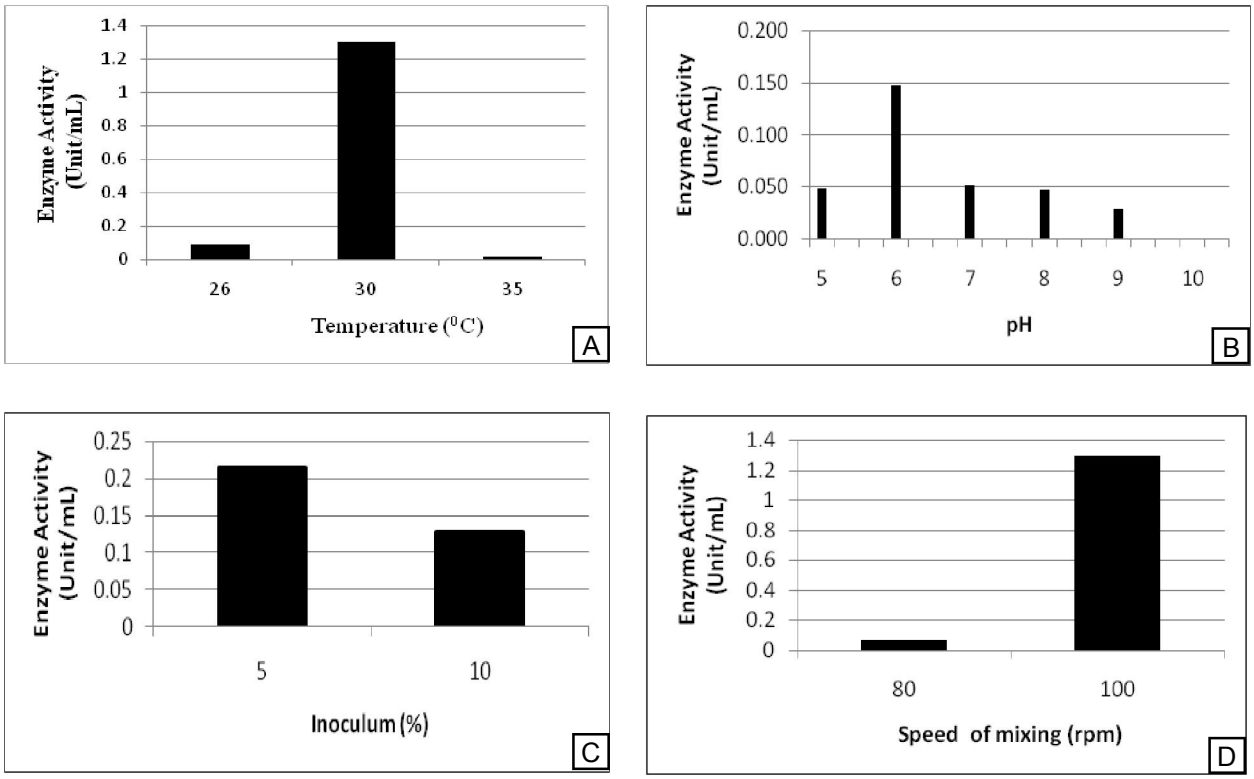

Figure 4. Production of the enzyme at different treatment: (A) incubation temperature; (B) pH media; (C) 2 (two) different concentration of inoculum; (D) mixing speed.

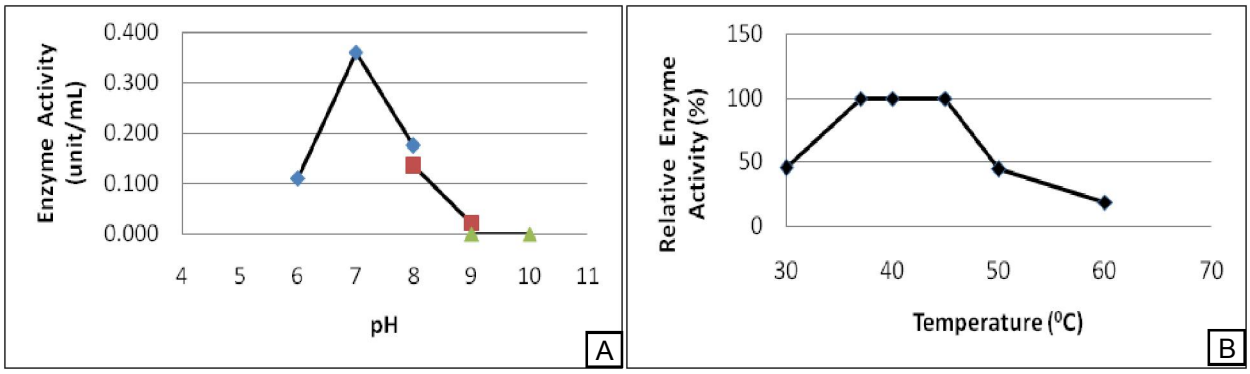

Figure 5. (A) pH and (B) Optimum temperature of L-glutaminase produced by *Pseudomonas aeruginosa* strain CG-T8- II.1.

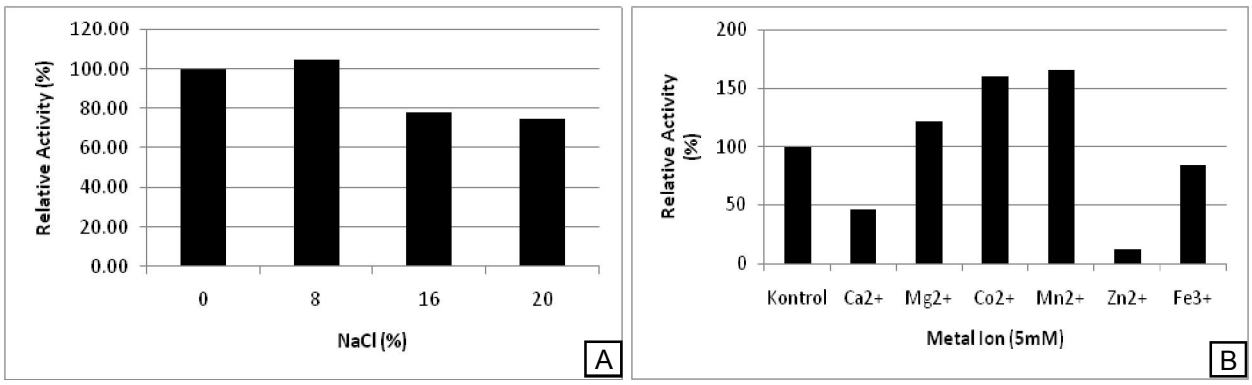

Figure 6. (A) Effect of NaCl and (B) metal ion as chloride salt on the enzyme's activity.

in the literature glutaminase class A having molecular weight around 35 kDa and 137 kDa (Nandakumara et al., 2003).

CONCLUSION

L-glutaminase producing marine bacteria was isolated from Sangihe-Talaud Sea, North Sulawesi-Indonesia from the depth 400 m with the activity of 147.99 Unit/L or 62.32 Unit/mg. Identification using 16S rRNA sequencing revealed the isolate has 96% similarity to *Pseudomonas aerginosa* strain CT-G8. L-glutaminase has been produced maximally in fermentation condition of 30 °C, 100 rpm, pH of media 6,0, and with starter inoculum of 5%. The crude enzyme can perform optimally at 37- 45 °C, pH 7.0 and retain >50% relative activity when added with NaCl up to 20%. Addition of metal ions Mg^{2+}, Co^{2+}, and Mn^{2+} in the form of Cl^{2+} increased the enzyme activity, while addition of other metal ions (Ca^{2+}, Fe^{3+}, and Zn^{2+}) decreased the enzyme activity. Result of SDS-PAGE revealed that the L-glutaminase might approximately had molecular mass of 42 and 145 kDa.

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