Int J Biol Med Res.2015;6(1):4798-4804

Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original Article

Pseudomonas putida E16 16 sRNA potential as enzyme-producing bacteria

Mashuri Masri[a*], Muh. Nasrum Massi[b], Ahyar Ahmad[c], Rosdiana Natzir[d]

[a]*Islamic State University of Alauddin Makassar, Department of Biology, Faculty of Science and Technology, Jl. Sultan Alauddin, Samata Campus, Gowa Regency, South Sulawesi, Indonesia*
[b]*Hasanuddin University, Department of Microbiology, Faculty of Medicine, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea Makassar, South Sulawesi, Indonesia*
[c]*Hasanuddin University, Department of Chemistry, Faculty of Mathematic and Natural Science, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea Makassar, South Sulawesi, Indonesia*
[d]*Hasanuddin University, Department of Biochemistry, Faculty of Medicine, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea Makassar, South Sulawesi, Indonesia*

ARTICLE INFO

Keywords:
L-Asparaginase
Production
Pseudomonas putida strain E16 16S
Purification

ABSTRACT

Pseudomonas putida strain E16 16S Ribosomal RNA is identified molecularly as bacterial symbiont spesies of macroalga *Sargassum polycystum* which produce L-Asparaginase (E.C.3.5.1.1), levels of optimum fermentation time and substrate concentration are 48 hours and 10g/L respectively, 60-80% Fraction is The Highest L-Asparaginase Activity (2,03 IU/ml). Gel filtration chromatography sefadeks G-100 has a purity level of 11.505 compared to the crude extract which continued to CM-Sephadex C50 ion-exchange chromatography, the pure L-Asparaginase of fraction 28 have purity level of 197.112 compared to crude extract.

Introduction

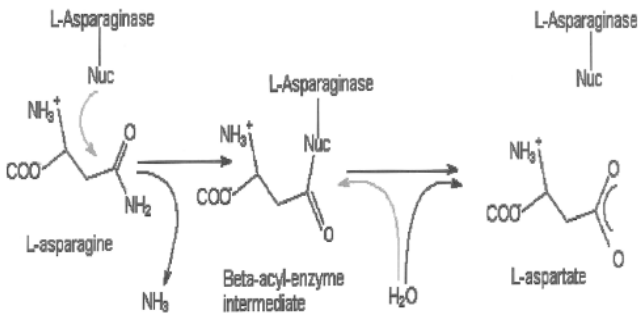
Macroalgae is one of the marine organisms that play a role in the food chain cycle as primary producers. To maintain themselves in their habitat, algae produce a variety of compounds consisting of primary compounds that are essential for the process of cell metabolism and secondary compounds that are not essential for the growth of organisms and it found in the unique form1. Secondary metabolites play a role as a host defense against pathogens, parasites, predators, competitors and epibiota and its production dependents on the condition of bio-geography[2]. The nature of secondary metabolites as a means of self defense marine organisms apparently has tremendous potential as a source of various diseases medicine[3]

The enzyme is largely biopolymer molecular form of the protein. Enzymes act as catalysts in biochemical reactions that occur within living cells or also called biokatalisator that lowers the activation energy of the substrate with a very specific outlines. Because of the nature and without any side effects, the enzyme has been widely used outside the body in an industrial scale. The resulting product is very specific so it can be calculated easily.

The enzyme L-Asparaginase is an enzyme that catalyzes the hydrolysis of L-asparagine to aspartate acid and ammonia4. L-asparagine is a nutrient for cancer cells5. L-Asparaginase can inhibit the growth of cancer cells[6].

L-Asparagine is one component of the nutrients to the cancer cells. Administration of L-Asparaginase on cancer cells can break down L-Asparagine, which is expected to inhibit the growth of these cells. L-Asparaginase of great benefit in the treatment of cancer[7]

Fig. 1. Schematic illustration of the reaction mechanism of L-Asparaginase[4].



L-Asparaginase can be found in many animal tissues, bacteria, plants, and in the serum of mice, but not found in humans. L-Asparaginase is produced in large quantities by several microorganisms including *E. coli, Erwinia cartova, Enterobacter aerogenes, Corynebacterium glutamicum, Candida utilities*, and *Pisum sativum*[8]

The purpose of this study is to obtain the enzyme L-asparaginase of macro algae Sargassum polycystum with higher purity gel filtration chromatography using sephadex G-100 sephadex continued in ion-exchange chromatography sephadex CMC50 and get sephadex fraction of data whose the highest specific activity.

* Corresponding Author : **Mashuri Masri**
Islamic State University of Alauddin Makassar,
Department of Biology, Faculty of Science and Technology,
Jl. Sultan Alauddin, Samata Campus, Gowa Regency,
South Sulawesi, Indonesia.

Materials and Methods

Materials M-9 medium : [KH_2PO_4 (pa), $MgSO_4.7H_2O$ (pa), $CaCl_2.2H_2O$, NaCl, glucose, L-Asparagine (pa), agar, phenol red indicator], sephadex G-100 , CM sephadex C50 were purchased from sigma-Aldrich St.Louis, Missouri, USA. all chemicals are used is pure standard analytic.

Methods

1.Sampel Preparation

The surface of algae

A total of 15 grams of algae samples were rinsed with 30 ml of physiological NaCl. Furthermore water rinsing results were put into 30 mL of nutrient broth medium and then shaken using a shaker at room temperature for 24-48 hours.

The Inside of algae

A total of 15 grams of algae samples were rinsed with 30 ml of physiological NaCl. Furthermore, algae that has been rinsed mashed in a blender and added physiological NaCl. The suspension is then put into 30 mL of nutrient broth medium and then shaken using a shaker at room temperature for 24-48 hours.

Isolation of Bacterial Symbiont macroalgae

1 mL of samples were taken and dilution stratified to obtain the appropriate dilution. Furthermore, 0.1 mL of dilution is spread into the agar medium and grown for 24 hours9. Purification is done by growing bacterial colonies of the bacteria prior to culture in a petri dish containing selective medium M-9, which has been modified by the composition of KH_2PO_4 0.75 g/L, $Na_2HPO_4.2H_2O$ 6 g/L, L - Asparagine 10 g/L, NaCl 0.5 g/L, MgSO4.7H2O 1.0 g/L, $CaCl_2.2H_2O$ 1.0 g/L, gukosa 3 g/L, to 20 g/L, and phenol red indicator 0.05 g/L10,11,12,13 were incubated for 24 hours at 37oC. Phenol red stock solution was made 2.5% in ethanol at pH 7 and then added to the media. In this medium L-Asparagine is used as a nitrogen source. Production of L- Asparaginase by bacteria will release ammonia increases the pH of the culture medium. Changes in pH to form a pink color around the colonies which produce L-Asparaginase[11].

2. Molecular Identification

DNA Ekstraction

DNA extraction is done in accordance with the procedures of QIAamp® DNA kit (QIAGEN) A total of 140 mL of bacterial swab (sample) mixed into 1.5 ml micro-tubes containing 560 mL of buffer AVL then vortex for 1 minute and incubated at room temperature for 10 minutes. Tubes were centrifuged for a while to reduce fluid tube attached to the lid, then added 560 mL of absolute ethanol into the tube and vortex 1 minute. A total of 630 mL mixture inserted into the column and then centrifuged at 8000 rpm for 1 minute in orther the solution out of the column and into the reservoir tube and then discarded, while the DNA remained in the column. Column washed with wash solution containing ethanol that is AW1 and AW2 respectively by adding 500 mL and centrifuge at 8000 rpm for 1 minute. To remove excess wash solution that may remain in the column, centrifuge at 12,000 rpm for 1 minute. Reservoir tube was then replaced with 1.5 ml sterile eppendorf tube. To release the DNA trapped in the column added 60 mL elution solution AVE (AVE buffer) and then incubated at 50 º C for 1 minute. Centrifugation at 12,000 rpm for 1 minute, liquid containing the DNA deposited in a sterile tube[14].

DNA Amplification

A master mix containing 1 mL U1, U2 1 mL, 5 mL 10X buffer, 2 mL 25 mM MgCl2, 1 mL of 10 mM dNTPs, 0.25 mL of hot start, 29.75 mL H2O and 10 mL of DNA. Dissolving 10 mL of supernatant and 40 mL master mix so that the total volume of 50 uL sample then put in a PCR machine. PCR cycle stages, namely denaturation, annealing and extension, carried out at a temperature of 94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 1 minute 30 seconds for the second stage (stage 35 cycles to 30 cycles for the first and second stage). PCR cycle begins with a pre-denaturation step at 94ºC for 5 minutes and ended with a final extension step at 72ºC for 5 minutes[14].

Gel Agarose Electroforesis

a 2% agarose was used in this study. A total of 5 mL ethidium bromide solution (10 mg / mL) was added to the agarose gel,

TAE solution was then added to the electrophoresis tank to cover the upper surface of the gel. Into each well, put 5 mL of PCR product was mixed with 1 mL after loading dye solution (40% sucrose, 0.25% bromophenolblue). In one of the wells included 5 mL (0.5 mg) of DNA molecular weight marker XIV (100 bp) as a DNA size standard. Electrophoresis device is run by giving power to the voltage to 80 volts (V) for 30 minutes. Once the process is complete agarose gel electrophoresis, the gel was entered in a Gel Doc XR (Biorad, USA)14.

Sequencing

RNA forward samples were sent to MacroGen Korea for sequencing. Nucleotides were inserted into the BLAST program.

1. Determination of the optimum concentration of substrate L - Asparagine, optimum incubation time and optimum growth rate of bacterial symbionts macroalgae.

Production of L - Asparaginase done by culturing the isolates into 45 ml inokulum medium in a 250 mL Erlenmeyer flask. Flasks were incubated at 37 °C in an incubator shaker at 200 rpm for 24 hours. Inoculum medium was incubated is mixed with medium production. The composition of the production medium is : Na2HPO4.2H2O 6 g/L, KH2PO4 3 g/L, NaCl 0.5 g/L, L - Asparagine, MgSO4.7H2O 2 g/L, CaCl2 0.1 M 1 mL, 10 mL glucose 20% (pH 7.0). Production of L-Asparagine made in the production medium with various concentrations of the substrate L-Asparagine 6 g/L, 8 g/L, 10 g/L, 12 g/L, and 14 g/L. Then incubated for 3 x 24 hours at 37oC. Every 12 hours of sampling to measure the activity of L-Asparaginase on each variation of the substrate L-Asparagine concentration and incubation time. The rate of bacterial growth was measured by optical density at a wavelength of 646 nm[10,11,15].

4. Ammonium sulfate fractionation

Centrifugation at 3000 rpm for 30 minute 4 ° C to separate the liquid medium with a mass cell culture results. The filtrate containing the enzyme with ammonium sulfate is then added gradually while stirring until saturation of 0-20%, and then allowed to stand overnight at 4 ° C. The precipitation that formed was separated by centrifugation at 10,000 rpm for 30 minutes. The same thing is done at 20-40% saturation, 40-60%, 60-80% and 80-100%. The precipitation which formed was dissolved in Tris-HCl buffer pH 8.6[16,17].

5. Dialysis

The dialyzed ammonium sulfate fraction was put in a cellophane bag and then dipped into a vessel containing distilled water to taste. Dialysis vessel placed over a magnetic stirrer fitted with a low speed. Dialysis performed for 48 hours by replacing the solution several times with tris-HCl buffer solution of pH 8.618.

6. Sephadex G-100 gel filtration

Matrix was washed with buffer B pH 8.3. Slowly sephadeks matrix G-100 column chromatography was poured into 1.5 cm diameter and 30 cm high column. Further samples (fraction 60-80%) is poured into the column slowly. After all the samples poured into the matrix, 45 ml of Tris HCl, 45 ml of Tris HCl + NaCl, 45 ml of Tris HCl + NaCl + CaCl2, and then determined the eluent flow rate of 0.3 mL/minute until 45 fractions, measurement of protein by UV spectrophotometer Vish with a wavelength of 280 nm and measurement of enzyme activity with Nessler method17.

7. CM-Sephadex C50 ion-exchange chromatography

Matrix was washed with buffer B pH 8.3. Slowly sefadeks matrix G-100 column chromatography was poured into a diameter of 1.5 cm and height 36.5 cm. Furthermore, F9-F14 sample (a mixture of active fractions from the gel filtration fraction) was poured into the column slowly. After all the samples contained in the matrix, add 45 ml of Tris HCl, 45 ml of Tris HCl + NaCl, 45 ml of Tris HCl + NaCl + CaCl2, then determined the eluent flow rate of 0.3 mL/minute to 45 fractions, measurement of protein by UV spectrophotometer Vish with a wavelength of 280 nm and measurement of enzyme activity with Nessler method17.

8. Determination of protein concentration

Determination of protein content is done by using a modified method of Lowry et al., Using Bovine Serum Albumin standard solution17.

RESULTS AND DISCUSSION

L-Asparaginase enzyme derived from a variety of bacteria, but the nature of biochemical, enzyme activity, substrate specificity, molecular weight, glutaminase and antitumor activity are different from each bacterial species depends on genetic diversity and the natural conditions in which bacterial symbionts grow 19,20, which in this study came from Sargassum polycystum at Barrang Lompo Island, South Sulawesi, Indonesia.

Growth comes from the inside of the macroalgae Sargassum polycystum has the highest activity compared to isolates originating from algae surface. This is in accordance with Abubakar et al., (2011) which states that the population abundance and specific bacterial symbionts originating from the inside of macroalgae because it directly interacts with bioactive compounds produced by macroalgae. Bacteria enter and interact with macroalgae bioactive compounds when its cell wall was damage.

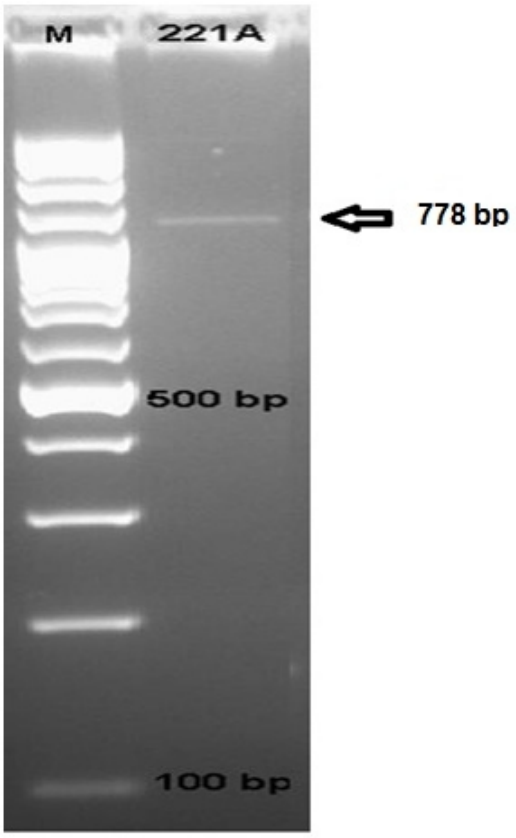
1. Molecular Identification of bacterial symbiont spesies of macroalga *Sargassum polycystum*

The similarity with the nucleotide sequences derived from other bacteria can be analyzed by BLASTN program on online sites at http://www.ncbi.nlm.nih.gov/blast.

16S Ribosomal RNA gene expressed by SBJCT while the sample is expressed with QUERY. There are 778 bp Query length. The length of the 16S Ribosomal RNA gene was used for 1419 bp with a range of 518 bp to 1277 bp. The use of 518 bp to 1277 bp by the system because in that section is the closest approximation to the nucleotide sequence of the sample with 98% cover query value.

Molecular identification of the identification results is Pseudomonas putida strain E16 16 ribosomal RNA, Accession KC820813.1 (fig 2 with 221A code). The same result research with the study of El-Bessoumy et al., (2004) who identify bacteria producing the enzyme L-Asparaginase from Pseudomonas aeruginosa 50071.

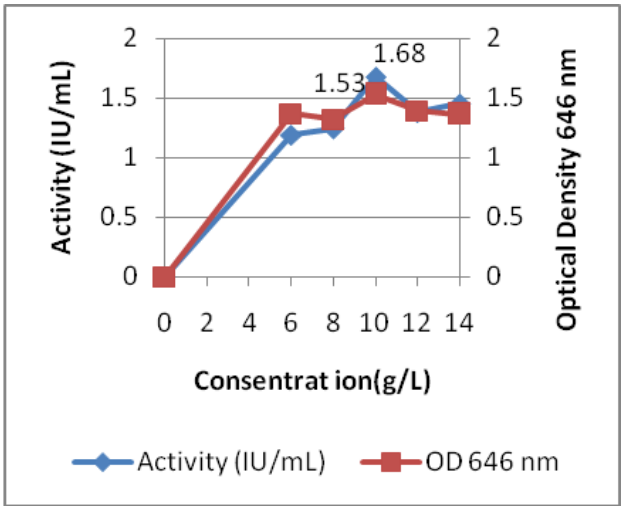
Fig 2. Agarose gel electrophoresis of isolates of bacterial symbionts macroalgae Sargassum polycystum.



1. Determination of Optimum Concentration of substrate *L-Asparagine in medium M-9*

The results of measurements of enzyme activity at a wavelength of 450 nm at several variations of concentration as seen in Figure 3 shows that the concentration of L-Asparagine 0 g/L activity generated at 0 IU/mL, and the increase in the concentration of 6 g/L with the activity of 1.19 IU/mL, a concentration of 10 g/L with the activity of 1.68 IU/mL, but decreased at a concentration of 12 g/L and 14 g/L, respectively by 1.38 IU/ml and 1.45 IU/ml, therefore concluded that the concentration of 10 g/L was the optimum concentration. Once the optimum activity is reached, the resulting activity declined despite the increase in the concentration of L-Asparagine.

Fig 3. The concentration of L-Asparagine variation on bacterial growth and enzyme activity of L-Asparagine



The decline in activity at concentrations above 10 g/L because of the media there is excess L-Asparagine which causes an unbalanced nutrient composition of the growth of bacteria, so that the enzyme L-Asparaginase production decreased. This is in accordance with the opinion Suhartono (1992) which states that 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, so that when the substrate concentration is increased there will be a decrease in enzyme activity.

3. Determination of Optimum incubation time for bacterial growth and enzyme production of L-Asparaginase

A wavelength of 450 nm was used for the determination of the optimum incubation time by measuring enzyme activity. The results showed that the optimum incubation time required to produce the enzyme L-Asparaginase activity was 48 hours with the highest activity value of 1.68 IU/mL and the highest OD values showed bacterial growth of 1.53 IU/mL with incubation time of 36 hours. Determination of enzyme activity using the Nessler method with ammonia as the standard solution.

Figure 4 shows that adaptation is not found in bacterial growth phase, this was due to have taken place during the adaptation phase bacterial inoculum was the media that occurred during the 24 hours prior to being in the media production.

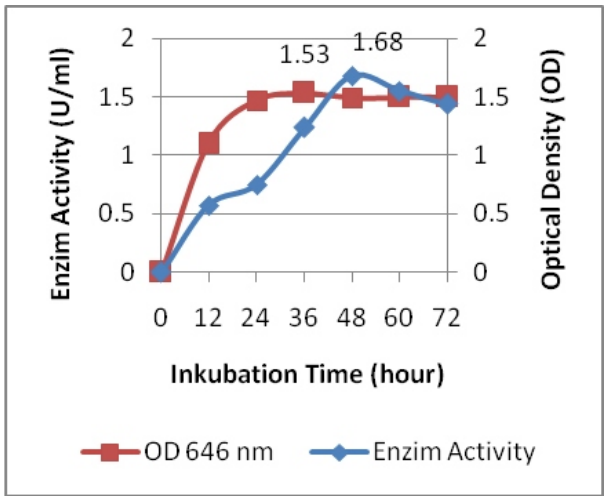


Fig 4. Effect of Incubation Time on enzyme activity and growth of bacteria on the concentration of L-Asparagine 10 g/L and the temperature 37oC.

Logarithmic phase occurs directly on the incubation time of 0-24 hours with a peak value of 1.53 OD bacterial growth. The highest phase for maximum enzyme activity with the value of 1.68 IU/mL occurred 24 hours after the optimum bacterial growth phase. This is because most bacterial growth but the largest enzyme secretion occurred after 24 hours after the optimum bacterial growth phase.

1. Ammonium sulfate fractionation

In this research centrifugation at 3000 rpm at 4 ° C for 30 min to separate the filtrate and precipitate liquid cell culture medium results. By centrifugation, cell debris and organelles will settle at the bottom of the tube centrifuges, whereas macromolecules including proteins whose size is much smaller than the cell debris and organelles would not settle but dissolved in buffer (supernatant). These supernatants were used as samples for the analysis of proteins and tissues 23.

Table 1. Total addition of Ammonium Sulfate for each stage of FractionationProteinVolume added total of ammonium Sulphate (gr)Fraction(mL) Crude Extract 100000 - 20%1690190.9720 - 40%1740210.5440 - 60%1810235.360 - 80%1940271.680 - 100%2020307.04

Table 2. Enzyme Activity and Protein Concentration for Each stage Ammonium Sulfate FractinationProtein Protein ConcentrationEnzimFraction(mg/mL)activity (IU/ml)Crude Extract1.2466.510 - 20%0.3591.3120 - 40%0.0560.7340 - 60%0.1060.7460 - 80%1.2742.0380 - 100%0.1350.65

The highest fraction obtained at 60-80% fraction with the highest protein content of 1.274 mg / ml and the enzyme activity of

2.03 IU/mL (Table 2). In table 2 shows that the highest activity of the crude extract artifacts on the fraction with a value of 6.51 IU/mL, and this is because in the crude extract there are many other types of proteins that have activity, but after being given ammonium sulfate fraction which begins with 0-20% to 80-100%. 60-80% fraction has the highest activity of 2.03 IU/mL.

This is because the enzyme L-Asparaginase is an enzyme with a protein consisting of more hydrophilic amino acids L-Asparagine which is a major component of the enzyme L-Asparaginase is a hydrophilic amino acid.

This is in accordance with Chaplin (2004) stated that proteins containing hydrophobic amino acids will precipitate at lower salt concentrations than the protein containing hydrophilic amino acids.

5. Sephadex G-100 gel filtration

The results showed that the F9 and F14 have the highest activity of L-Asparaginase compared to other fractions (Fig 5). This is the basis for the selection of F9 and F14 to proceed to the next stage of purification on CM-Sephadex C50 ion exchange chromatography. F9 and F14 mixture is a mixture of proteins which bind strongly to the matrix.

Sephadex G-100 gel filtration indicated that in this study used sephdex swelling of 100 times, the matrix on Sephadex G100 gel is porous grain carbohydrates and insoluble (matrix) where the pores are smaller molecules will be caught in the matrix, while the molecular great going out first. Sephadex G100 gel filtration consists of two phases namely the mobile phase is a liquid buffer B and a static phase porous grain carbohydrates and insoluble (matrix).

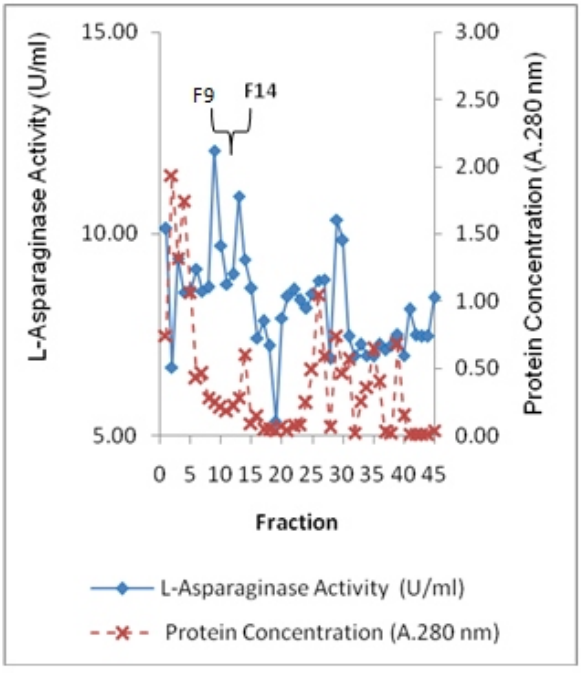


Fig 5. Graphics of L-Asparaginase enzyme activity and protein Concentration Each fraction of Gel Filtration Chromatography Sephadex G-100 Purification.

6. CM-Sephadex C50 ion-exchange chromatography

Selection Fraction F28 with the consideration that the F28 has a protein content and activity of L-Asparaginase highest total of 45 fractions. Additionally ranging from F1 to F27 and activity levels were relatively up and down but tends to decrease, and the protein concentration of L-Asparaginase activity increased dramatically in fractions F28 (Fig. 6).

Ion exchange chromatography is CM-Sephadex C50 ion-exchange chromatography using CM (carboxymethyl) as Cation Exchanger (catcher cation) where CM is itself charged anions.

Cations are captured in the form of positively charged amino acids which in this case is L-Asparagine thus negatively charged amino acids will come out ahead of the column. To remove the L-Asparagine, added salt with which the multilevel gradient fractions 1-15 in this study added Tris HCl, added HCl fractions 16-30 and fractions 31-45 and NaCl was added HCl, NaCl and CaCl2.

Increased salt gradient rise is intended that the negatively charged CM will react with the positively charged Na+ and ultimately the bond between the CM with L-Asparagine will be released that make the amino acid L-Asparagine will come out of the column.

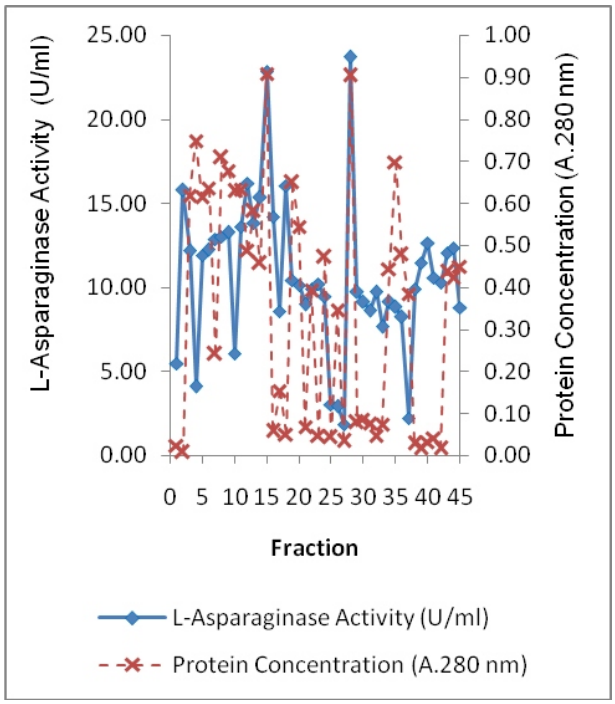


Fig 6. Graphics L-Asparaginase Enzyme Activity and Protein Concentration Each fraction CM-Sephadex C50 ion-exchange chromatography.

CM-Sephadex C50 ion-exchange chromatography consists of two phases namely the mobile phase is a liquid buffer B and a static phase of the receptor that binds to the matrix.

The procedure is in line with research studies Aly (2013) which purify the enzyme L-Asparaginase by gel filtration chromatography purification followed by ion exchange chromatography.

1. Pattern Purity Level L-Asparaginase from Pseudomonas putida strain E16 16S Ribosomal RNA at each stage of purification

Measurement of the enzyme L-Asparaginase activity in each stage of purification is meant to see how much ammonia is formed in each of the hydrolysis reaction of L-Asparagine into aspartic acid and ammonia. This study shows that a growing number of purification stages undergone by the enzyme L-Asparaginase, the higher the value of total activity, specific activity and purity. This can be seen on the stages of CM-Sephadex C50 with the highest total activity of 23 653 IU and the specific activity of 1084.33 U/mg and a purification rate of 197.112 compared to the crude extract.

Table 3. Pattern Purity Level L-Asparaginase from *Pseudomonas putida* strain E16 16S Ribosomal RNA at each stage of purification.

Step	Collected volume (ml)	Total Protein (mg)	Total activity (IU)	Spesific Activity (U/mg)	Purification (fold)
Crude Extract	2000	1.246	6.854	5.50	1.000
(NH4)2SO2 precipitation	10	0.829	8.019	9.68	1.759
Gel Filtrarion on sephadex G100 column	9	0.157	9.950	63.29	11.505
CM-sephadex C50 column	3	0.022	23.653	1084.33	197.112

Conclusion

based on the research that has been done can be concluded as follows :

1. L-Asparaginase optimum fermentation time of 48 hours and the optimum substrate concentration of 10 g / L to increase the growth and the number of colonies of bacterial symbionts macroalgae Polycystum sargassum E16 16S Ribosomal RNA.

2. Pure L-Asparaginase enzyme (fraction 28) from the bacterium Pseudomonas putida strain E16 16S Ribosomal RNA obtained through stages: 60-80% ammonium sulfate precipitation, dialysis, gel filtration chromatography sefadeks G-100 and CM-Sephadex C50 ion-exchange chromatography has a purity of 197 112 times crude enzyme extract.

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