Journal of Tropical Biodiversity and Biotechnology
Volume 10, Issue 02 (2025): jtbb16510
DOI: 10.22146/jtbb.16510

Research Article

Partially Purified L-asparaginase with Low Glutaminase and Urease Co-Activities of Bacteria from the Rancabuaya Coast, Indonesia

Wulan Pertiwi[1]*, Intan Nuraeni[1], Azmy Jasmine Namira[1], Maelita Ramdani Moeis[1], Muhammad Fauzi[1], Toto Subroto[2,3]

1)Department of Biotechnology, Faculty of Science and Technology, Universitas Muhammadiyah Bandung, Jl. Soekarno-Hatta No 752, Cipadung Kidul, Panyileukan, Bandung, Jawa Barat 40614, Indonesia
2)Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia
3)Research Center for Molecular Biotechnology and Bioinformatics, Universitas Padjadjaran, Bandung 40133, Indonesia
* Corresponding author, email: wulanpertiwi@umbandung.ac.id

Keywords:
16S rRNA
Enzyme
Indonesia
L-asparaginase
Marine
Sequencing
Submitted:
18 September 2024
Accepted:
03 January 2025
Published:
13 June 2025
Editors:
Miftahul Ilmi
Liya Audinah

ABSTRACT

L-asparaginase, enzyme that can hydrolyse L-asparagine into L-aspartate and ammonia is used as a therapeutic enzyme in treating Acute Lymphoblastic Leukemia (ALL). The use of this enzyme is restricted by the presence of dual substrate specificity towards asparagine and glutamine, which causes side effects. The objectives of this research were to isolate and to identify marine bacteria from Rancabuaya Coast that generate L-asparaginase with low glutaminase and urease co-activity and to produce and to measure the asparaginase activity using the Nessler reagent. Bacteria were isolated from seawater, and screened using Zobell Marine media containing either L-asparagine, glutamine or urea, with phenol red as an indicator. The bacterial isolate with the highest asparaginase activity and relatively lower glutaminase and urease activity, was identified by 16S rRNA gene sequence. This bacterial isolate, RB3, was identified as *Pseudoalteromonas tetraodonis* GFC with greater than 99 % homology. Enzyme specific activities of crude extracellular and intracellular enzymes were 72.30 U mg^{-1} and 67.18 U mg^{-1} respectively, while the highest enzymes specific activity from ammonium sulphate fractionation was found at 40-60% saturation (F3), which was 136.03 U mg^{-1}. SDS-PAGE of the enzyme solutions showed the presence of a 35 kDa band suspected to be the L-asparaginase protein.

How to cite:
Pertiwi, W. et al., 2025. Partially Purified L-asparaginase with Low Glutaminase and Urease Co-Activities of Bacteria from the Rancabuaya Coast, Indonesia. *Journal of Tropical Biodiversity and Biotechnology*, 10(2), jtbb16510. doi: 10.22146/jtbb.16510

INTRODUCTION

L-asparaginase (E.C. 3.5.1.1) is an enzyme used extensively for the treatment of Acute Lymphoblastic Leukemia (ALL) (Shrivastava et al. 2016). The function of this enzyme is to hydrolyse asparagine, forming aspartic acid and ammonia (Zuo et al. 2014). Asparagine, a non-essential amino acid, is needed for protein synthesis and growth of cancer cells. L-asparaginase would reduce the availability of asparagine to the cancer cell, eventually resulting in cancer cell death (Nagarethinam et al. 2012). Even though L-asparaginase is found in higher organisms like animals and plants (Prihanto et al. 2018), bacterial sources are preferable for L-asparaginase production due to the ease in culturing, extraction, and large scale production (Prihanto et al. 2019).

Several types of L-asparaginase formulations have been produced commercially, such as *Escherichia coli* asparaginase, PEGylated *E. coli* asparaginase, and *Erwinia chrysanthemi* asparaginase (Erwinase) (Pieters et al. 2011). While these formulations were effective in treating leukemia, they had side effects which were probably due to the presence of by-products such as glutaminase and urease (Doriya & Kumar 2016). The side effects included mild to serious immune reactions (hypersensitivities, allergies such as skin rash, fever and anaphylactic shock), central nervous system toxicity and other toxicities such as pancreatic disfunction, liver disfunction, edema, diabetes, leukopenia, and bleeding (Batool et al. 2016).

To address these challenges, it is essential to produce L-asparaginase freely from glutaminase and urease activities. Urease breaks down urea in the bloodstream, generating ammonia and carbon dioxide, which can have toxic effects (Bano & Sivaramakrishnan 1980; Doriya & Kumar 2016; Ashok et al. 2019). Glutaminase- and urease-free L-asparaginase is considered beneficial for cancer therapy (Ashok et al. 2019). Examples include *Bacillus licheniformis*, isolated from the Red Sea, Saudi Arabia, produced L-asparaginase with no glutaminase activity (Alrumman et al. 2019), and from *Pseudomonas stutzeri* ATCC 17588 (T) isolated from medicinal plants (Sulistiyani & Kusumawati 2019). These findings suggested that marine and coastal environments are promising yet underexplored source of novel enzymes (Prihanto & Wakayama 2016). Additionally, glutaminase-free L-asparaginase also had been isolated from *Streptomyces exfoliates*, *Streptomyces cyaneus*, and *Streptomyces phaeochromogenes* (Saxena et al. 2015). Enzymes from bacteria in marine environments exhibit unique properties, such as tolerance to high salt concentrations, hyperthermostability, barophilicity and high adaptation to cold temperatures (Zilda et al. 2021). Screening of thermohalophilic bacteria from Wawolesea hot springs of Southeast Sulawesi identified 14 isolates capable of producing L-asparaginase. Among these, the AAT3.2 isolate exhibited the highest activity at 86.61 IU mL^{-1}, while the CAT1.1 isolate showed the lowest activity at 38.24 IU mL^{-1}. In terms of specific activity, CAT3.2 had the highest value at 6767.98 IU mg^{-1}, whereas CAT1.1 had the lowest at 684.54 IU mg^{-1} (Muzuni et al. 2024).

Research on L-asparaginase-producing bacteria from Indonesian marine environments, such as the Rancabuaya Coast, remains limited. This area holds potential for discovering enzymes with novel characteristics. Therefore, this study focused on isolating bacteria from the seawater of Rancabuaya Coast with L-asparaginase activity that could potentially be used for ALL treatment.

MATERIALS AND METHODS

Sampling of seawater

A total of 1000 mL of seawater samples were collected from the Rancabuaya Coast area in West Java, Indonesia. Sampling took place at 08:00 AM, at a water depth of 30 cm and 5 meters from the shoreline. The samples were col-

lected along with algae and marine sediment. The seawater had a pH of 7.27 and a salinity of 3.5 %. It contained dissolved organic substances such as carbohydrates, amino acids, organic-rich particulates, and chemical elements like magnesium and bromine, which influence microbial activity (Byrne & Mackenzie 2024). The samples were placed in sterilised bottles and transported in an icebox during a six-hour journey to the Biotechnology Laboratory at Muhammadiyah University, Bandung, Indonesia.

Isolation and purification of marine bacteria

Isolation of bacteria was carried out by spreading 100 µL of seawater directly on Zobell Marine Agar (ZMA) in a petri dish using an L-shaped cell spreader and incubating at 30 °C for 16 hours. Bacterial colonies with different morphologies were then subcultured separately on ZMA medium using the four-quadrant-streak method and incubating for 16 hours at 30 °C, to obtain pure cultures. Subculturing was repeated until uniform single colonies were obtained in one petri dish. Single colonies were grown on ZMA slants and stored at 4°C or maintained and stored in 10 % glycerol at -20 °C.

Characterization of bacteria

The characteristics of the pure bacterial cultures were then determined based on the standard colony shape: 1) Colony shape: circular, irregular, and punctiform (in the form of dots); 2) Margin shape (colony edge shape): entire (smooth and flat), undulate (wavy), filamentous, rhizoid (branched like roots), and lobate (grooved edges); 3) Colony elevation: can be seen from the side in the form of flat, raised, convex, umbonate, plateau, raised, raised spreading edge, flat raised margin, and growth into; 4) The colour of the colonies formed can also be determined by colour appearance, such as dull (pale), shiny, translucent (slightly transparent), and opaque (Leboffe & Pierce 2012).

Screening for bacteria producing L-asparaginase low in glutaminase and urease

Screening for bacteria producing L-asparaginase low in glutaminase and urease was carried out by inoculating purified colonies onto Zobell Marine Agar (ZMA) with added 5 g L^{-1} of amino acids of either asparagine, glutamine or urea and 0.009 % phenol red, together with *E. coli* ATCC 8739 as positive control and distilled water as negative control. The culture was then incubated at 30 °C for 16 hours. Observation of bacteria producing L-asparaginase low in glutaminase and urease was carried out visually, by observing the zone index of each bacterium. Colony diameter and diameter of red zone formed around each colony was measured using a caliper after 16 hours of incubation. The phenol red indicator would turn red due to the increase in pH caused by the appearance of ammonia as a result of the hydrolysis of asparagine by L-asparaginase (Qeshmi et al. 2018). The zone index was calculated as the ratio of the diameter of the red zone and the colony (Ashok et al. 2019).

$$\text{Zone Index} = \frac{\textit{Diameter of red zone}}{\textit{Diameter of colony}}$$

DNA isolation

Bacteria were cultured in 5 mL of Zobell Marine Broth (ZMB) medium with optimum growth conditions. A total of 1.5 mL of culture was centrifuged at 5000 x *g* for 10 minutes, the supernatant was discarded, 1.5 mL culture was added, recentrifuged at 5000 x *g* for 10 minutes, then the supernatant was discarded. DNA from the pellet was isolated using GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific) and performed in accordance to the instruction manual.

PCR amplification and electrophoresis

The template for the PCR reaction used 1 µL of isolated genomic DNA from samples, 1 µL of *E. coli* genomic DNA, and 1 µL of nuclease-free water (as a negative control). DNA was amplified with DreamTaq master mix with a pair of primers BactF1(5'-AGAGTTTTGATC(A/C)TGGCTCAG-3') and UniB1 (5'-GGTTAC(G/C)TTGTTACGACTT-3') to amplify a 1400 bp fragment of the 16S rRNA gene (Baker et al. 2001; Gaffar et al. 2014). Each tube consisted of 10 pmol BactF1 primer, 10 pmol UniB1 primer, 12.5 µL of DreamTaq master mix, 9.5 µL of nuclease-free water, and 1 µL of template DNA, resulting in a total reaction volume of 25 µL. Amplification was carried out under the following PCR conditions: initial denaturation 95 °C for 2 minutes, followed by 25 cycles consisting of denaturation 95 °C for 1 minute, annealing 50 °C for 1 minute, elongation 72 °C for 1 minute and ending with final elongation 72 °C for 10 minutes. The amplicons were analysed in agarose gel electrophoresis, using 1 % agarose gel in TAE buffer. To 5 µL of sample, 1 µL of loading dye and 1 µL of GelRed were added. Electrophoresis was carried out at a voltage of 75 mV for 45 minutes. The electrophoresis results were observed using a UV-transilluminator.

Sequencing 16S rRNA gene and phylogenetic analysis

The PCR products were sequenced at 1st BASE using the Sanger method, which relies on a DNA synthesis technique that selectively terminates the synthesis at specific nucleotide bases (Sanger et al. 1977; Lander et al. 2001; Moorcraft et al. 2015). Data were processed using BioEdit software (RRID:SCR_007361) (https://scicrunch.org/resolver/SCR_007361) and search for sequence similarity by performing BLAST (https://blast.ncbi.nlm.nih.gov) (Altschul et al. 1990). Phylogenetic tree analysis using the neighbor-joining (NJ) method with a 2-parameter Kimura model and Gamma distribution, implemented in MEGA 11 (Tamura et al. 2021) with a bootstrap value of 1000x.

Bacterial growth curve

The optimal isolate from the screening, which exhibited high asparaginase activity and low glutaminase and urease activities, was used as an inoculum to prepare a starter culture by inoculating 10 mL of Zobell Marine Broth containing 10 g L^{-1} asparagine. This culture was incubated at 30 °C with shaking at 150 rpm for 24 hours. Subsequently, 100 mL of Zobell Marine Broth with 10 g L^{-1} asparagine was inoculated with 1 mL of the starter culture and incubated on a shaker at 30 °C and 150 rpm for 50 hours. Samples (1 mL) were taken every two hours to measure optical density at 600 nm (Kumar et al. 2010a).

L-asparaginase production

The method for L-asparaginase production was based on (Kumar et al. 2010a) with modification. A preculture was made by inoculating 10 mL of Zobell Marine Broth with 10 g L^{-1} asparagine, then incubating at 30 °C, 150 rpm until the logarithmic phase. This preculture was used as a 2 % v v^{-1} inoculum to 500 mL Zobell Marine Broth with 10 g L^{-1} asparagine, then the culture was incubated at 30 °C, 150 rpm until the logarithmic phase. After incubation, the culture was centrifuged at 4000 x *g* for 15 minutes at 4 °C. The cell-free supernatant contained crude extracellular L-asparaginase. Meanwhile, the cell pellet was suspended in sonication buffer (50 mM Tris-Cl and 10 mM EDTA pH 7.5), transferred to a Falcon tube, and sonicated on ice at 20 MHz, 35 % amplitude, 20 minutes (15 seconds on and 5 seconds off). The lysate was centrifuged at 20,000 x *g* for 10 minutes (4 °C). The supernatant contained intracellular L-asparaginase.

Partial Purification of the crude extracellular L-asparaginase

Partial purification was based on the method described by (Kumar et al. 2010b) with modification. Crude extracellular L-asparaginase was fractionated with ammonium sulphate to obtain five fractions with the following percent saturation: 0-20 % (F1), 20-40 % (F2), 40-60 % (F3), 60-80 % (F4) and 80 -100 % (F5) (Scopes 1994). Ammonium sulphate was weighed according to the desired fraction to obtain the desired concentration, then was added to the supernatant solution little by little while stirring with a magnetic stirrer at 4 °C for 30 minutes. The mixture was then incubated at 4 °C overnight, centrifuged at 3000 x *g* for 30 minutes. The precipitate obtained was dissolved in 50 mM Tris-Cl pH 8.6. Cellophane membrane dialysis tubings (cutoff 12-14 kDa) were soaked in distilled water for 30 minutes at 40 °C, then rinsed with distilled water. One end of the tubes was tied with a thread. Then the tubes were filled with ammonium sulphate fractions of L-asparaginase, after which the other end of the tube was tied with a thread. The dialysis tubes were then soaked in 0.025 M Tris-Cl buffer pH 8.6 and stirred with a magnetic stirrer at 4 °C. Every two hours the buffer was changed to reduce the concentration of salt ions in the tubes. The presence of ammonium sulphate was tested with 0.1 M $BaCl_2$. Dialysis was stopped when testing with $BaCl_2$ did not result in a white precipitate of $BaSO_4$. The solution in the dialysis tube which contained the partially purified intracellular L-asparaginase of *Pseudoalteromonas tetraodonis* RB3 was transferred into a new bottle.

Determination of L-asparaginase activity

L-asparaginase assay was carried out using the Nessler method with modifications from (Kumar et al. 2010a). Nessler's reagent (K_2HgI_4) reacted with ammonia in a basic solution to form a yellow-brown colloidal dispersion. This reaction produced a yellow-brown solution which followed the Lambert-Beer law (Vogel 1951). The intensity of the colour in the sample was measured by absorbance using a UV-Vis spectrophotometer at a wavelength of 420 nm, which was chosen based on the highest absorbance. This wavelength was chosen to minimise errors, because the greater the absorbance, the measurement will tend to be constant for repeated measurements (Kresnadipayana & Lestari 2017). The assay was performed on samples *Pseudoalteromonas tetraodonis* RB3 enzymes, negative control (distilled water), and positive control. To 100 µL samples, 900 µL substrate L (10 mM asparagine in 50 mM Tris-Cl pH 8.6) was added. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped by adding 100 µL of 1.5 M trichloroacetic acid (TCA) solution. The reaction mixture was centrifuged at 10,000 rpm for 5 min at 4 °C. The ammonia released in the supernatant was determined by taking 100 µL of the supernatant and adding 100 µL of Nessler's reagent and 3.8 mL of distilled water. This mixture was vortexed and incubated at room temperature for 20 minutes. Then the absorbance of the solution was measured with a UV-Vis spectrophotometer at the optimum wavelength with a blank (asparaginase substrate in Tris-Cl + TCA buffer). The ammonium ions produced was determined based on the linear regression equation of ammonium chloride standard curve. One IU of L-asparaginase was defined as the amount of enzyme required to generate 1 µmol of ammonia per minute at the experimental conditions. The enzyme activity was calculated using the following formula:

$$\text{Enzyme Activity (IU mL}^{-1}) = \frac{(y-b)}{a} \times \frac{V\ total}{V\ analysis} \times \frac{1}{V\ enzyme} \times \frac{1}{T\ incubation}$$

where:

y : Absorbance

a : Slope

b : Intercept
V. Total : Volume of enzyme + substrat + buffer + TCA
V. Analysis : Analyzed volume
V. Enzyme : Volume of enzyme
T. Incubation : Incubation time

Determination of protein concentration

Protein concentrations of standard solutions, crude extracellular, partial purified, intracellular L-asparaginase of *Pseudoalteromonas tetraodonis* RB3, and positive control (*E. coli* ATCC 8739) were measured by the Lowry method. The blank consisted of 50 mM Tris-Cl buffer. Lowry B reagent (2 mL) was added to 0.25 mL samples, then the mixture was homogenised and incubated for 10 minutes. Then 0.25 mL Lowry A reagent was added and incubated at room temperature for 20 minutes. The absorbance of the solution was measured using a UV-Vis spectrophotometer at the optimum wavelength relative to the blank solution (Lowry et al. 1951; Waterborg 2009). Protein concentration was determined based on the linear regression equation of the Bovine Serum Albumin (BSA) standard curve, using the following formula:

$$\text{Protein concentration (mg mL}^{-1}) = \frac{(y-b)}{a}$$

y : Absorbance of sample
a : Slope of the linear regression equation of the BSA standard
b : Intercept of the linear regression equation of the BSA standard

Analysis of L-asparaginase enzyme by SDS-PAGE

Proteins in the enzyme solutions were separated by SDS-PAGE on 12 % acrylamide gel and stained with Coomassie Brilliant Blue stain (Sambrook et al. 1989). Samples were prepared by adding 5 µL sample buffer (60 mM Tris-Cl pH 6.8, 25 % glycerol (v v^{-1}), 2 % SDS (w v^{-1}), 0.1% (w v^{-1}) bromophenol blue) to 10 µL sample, heated in a waterbath at 95 °C for 15 minutes, centrifuged at 10,000 x *g*, 4 °C, for 2 minutes. Samples (15 µL) and protein molecular weight markers were placed inside the wells of the gel. Electrophoresis was performed at 100 volts for 100 minutes. After that, staining was carried out using a staining solution for 30 minutes, and color removal was carried out using a destaining solution for 60 minutes. Protein bands would appear and the molecular mass was calculated using GelAnalyzer 23.1.1 (Lazar Jr. et al. 2023).

RESULTS AND DISCUSSION

Isolates of bacteria from sea water at Rancabuaya Coast

From the resulting colonies, four colonies were taken that had differences in shape, margin, elevation, and colour (Table 1). These four bacterial colonies were subcultured and purified, and were named RB1, RB2, RB3 and RB4.

L-asparaginase producing bacteria low in glutaminase and urease

The bacterial isolates were screened using a semi-quantitative rapid plate test method by means of direct visualization (Figure 1). Enzyme activity could be measured by calculating the zone index (Ashok et al. 2019; Chakraborty & Shivakumar 2021). Rancabuaya Coast seawater bacteria had higher L asparaginase activities compared to the positive control. Isolate RB3 had the highest L-asparaginase activity as indicated by a zone index value of 2.5 (Table 2).

Molecular identification of RB3

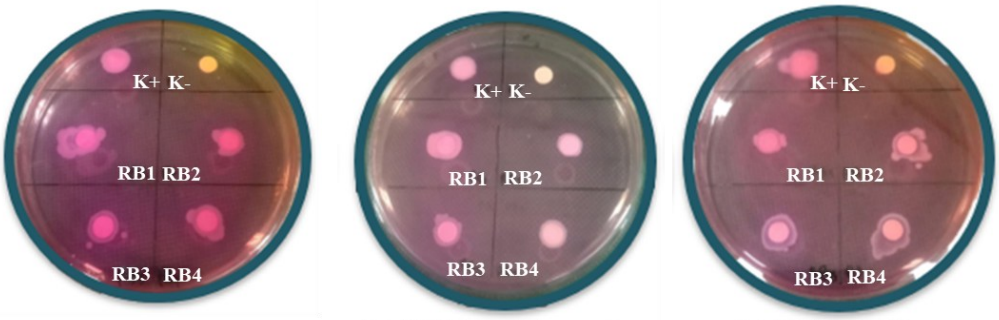
Molecular identification was carried out on RB3 which had the highest L-asparaginase activity. Amplification of the 16S rRNA gene from the genomic DNA of RB3 resulted in a 1.5 kb fragment (Figure 2).

Table 1. Colony Morphology of Rancabuaya Coast Seawater Bacterial Isolates.

No.	Bacterial Isolate	Colony shape	Colony Margin	Colony Elevation	Colony color
1.	RB1	Undulate	Entire	Convex	white
2.	RB2	Circular	Irregular	Convex	white
3.	RB3	Circular	Entire	Convex	white
4.	RB4	Undulate	Irregular	Convex	white

Table 2. Screening for bacteria producing L-asparaginase low in glutaminase and urease on ZMA medium given different substrates (L-asparagine, glutamine and urea).

Bacterial Isolates	ZMA + L-asparagin + *Phenol red*	ZMA + glutamin + *Phenol red*	ZMA + urea + *Phenol red*
K-	1.0	1.0	1.0
K+	1.7	1.3	1.7
RB1	1.7	1.7	2.0
RB2	1.7	0.8	1.7
RB3	2.5	1.3	2.2
RB4	1.7	0.8	1.7



(a) ZMA + Asparagine (b) ZMA + Glutamine (c) ZMA + Urea

Figure 1. Screening for bacteria producing L-asparaginase low in glutaminase and urease.

Sequencing and phylogenetics

Sequencing of the 16S rRNA gene of RB3 produced a DNA sequence with a length of 1491 bp. BLAST (Basic Local Alignment Search Tools) analysis of the sequence was carried out online on the NCBI website (http://www.ncbi.nlm.nih.gov) to obtain a list of sequences and information on sequence alignments of RB3 16S rRNA gene with sequences in the data bank (Table 3).

Pseudoalteromonas tetraodonis GFC strain KMM 458 (NR_114547.1) had a maximum Score of 2577, total score of 2577, query coverage of 98 %, E value equals 0.0 and homology of 99.51 %. Isolates with a 16S rRNA sequence identity of more than 97 % would represent the same species. Meanwhile, sequence identities between 93-97 % would represent the same genus, but different species. A 16S rRNA sequence similarity of 97 % has been proposed as delimiter for bacterial species (Hagström et al. 2002). To state species similarity, 16S rRNA gene sequences must have at least 97 % nucleotide base similarity (Stackebrandt & Goebel 1994).

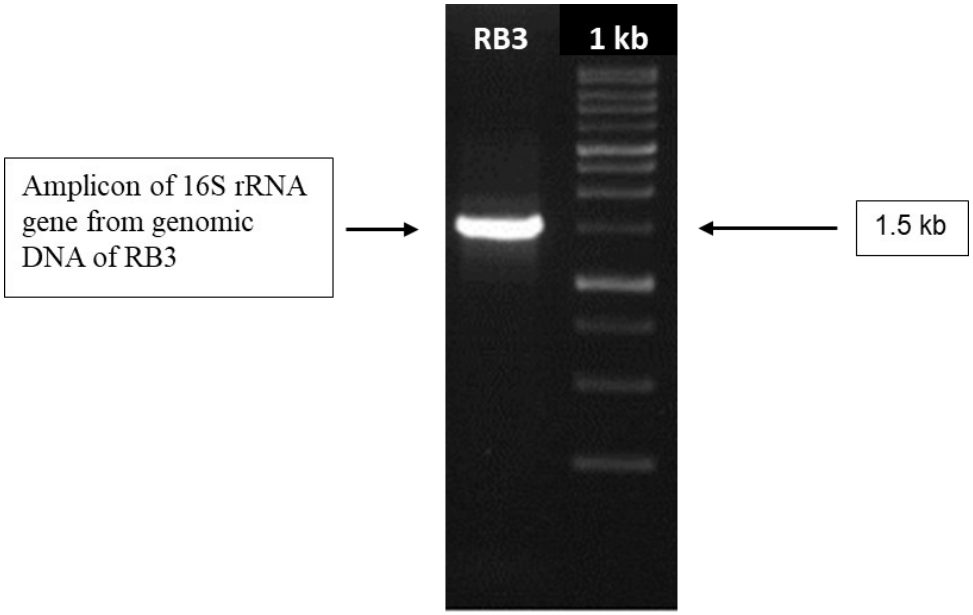


Figure 2. Electropherogram of the 16S rRNA gene amplicon from RB3.

Eight sequences were used for the construction of a phylogenetic tree for RB3 and the resulting phylogenetic tree (Figure 3) showed that the RB3 had the closest relationship to *Pseudoalteromonas tetraodonis* GFC strain KMM 458 with a boostrap value of 100. A bootstrap value between 70-100 indicated that the branches were robust and difficult to change. However, if the bootstrap value was less than 70, then the possibility of branching changes being formed would be very high (Apollos et al. 2017). Since RB3 sequence has a high identitity with all of the *Pseudoalteromonas tetraodonis* species (>99.29 %), RB3 is now named *Pseudoalteromonas tetraodonis* RB3.

Pseudoalteromonas tetraodonis, classified as gram-negative bacteria, are aerobic and have the form of bacilli (rods) measuring 1.0 µm 2.4 µm. Generally, these bacteria are halophilic organisms, namely bacteria that can grow optimally in environments with high salt concentrations and can move using flagella. Halophilic bacteria can be divided into three groups based on their

Table 3. Bacterial strains with the highest 16S rRNA partial sequence homology with RB3 16S rRNA gene.

Species	Max Score	Total Score	Query Cover	E Value	Homology	Acc. Len	Accession
Pseudoalteromonas tetraodonis GFC strain KMM 458	2577	2577	98 %	0.0	99.51 %	1491	NR_11457.1
Pseudoalteromonas tetraodonis GFC strain NBRC 103034	2573	2573	98 %	0.0	99.44 %	1459	NR_114187.1
Pseudoalteromonas tetraodonis GFC strain IAM 14160	2545	2545	98 %	0.0	99.29 %	1428	NR_119142.1
Pseudoalteromonas spiralis strain Te-2-2	2510	2510	98 %	0.0	98.73 %	1468	NR_114801.1
Pseudoalteromonas carrageenovora strain NBRC 12985	2507	2507	98 %	0.0	98.59 %	1461	NR_113605.1
Pseudoalteromonas distincta strain KMM 3548	2507	2507	98 %	0.0	98.59 %	1506	NR_025654.1
Pseudoalteromonas agarivarons DSM 14585	2501	2501	98 %	0.0	98.52 %	1505	NR_025509.1
Pseudoalteromonas atlantica strain NBRC 103033	2497	2497	98 %	0.0	98.38 %	1461	NR_114186.1
Pseudoalteromonas espejiana strain NBRC 102222	2497	2497	98 %	0.0	98.45 %	1461	NR_114049.1

ability to live, namely low halophilic (slight halophiles) grow optimally in the range of 2-5 % NaCl content, moderate halophiles in the range of 5-20 % NaCl content, and extreme halophilic (extreme halophiles) range of 20-30 % NaCl content. *P. tetraodonis* grows between 4-35 °C. This bacterium was capable of producing tetrodotoxin, gelatinase and lipase enzymes (Simidu et al. 1990; Ivanova et al. 2001; Hassanshahian & Jafar 2011). Halotolerant microorganisms grow both at high concentration of salt and without salt, while halophilic microorganisms are salt-loving microorganisms (Kanekar & Kanekar 2022). The type strain *P. tetraodonis* GFC can grow in 2-10 % NaCl and cannot grow in 0 % NaCl (Reimer et al. 2022; BacDive 2024). Therefore, it is halophilic, not halotolerant. *Pseudoalteromonas* had been characterized and identified as L-asparaginase producing bacteria, such as *Pseudoalteromonas arabiensis* that produced L-asparaginase from marine sources (Lakshmi et al. 2020).

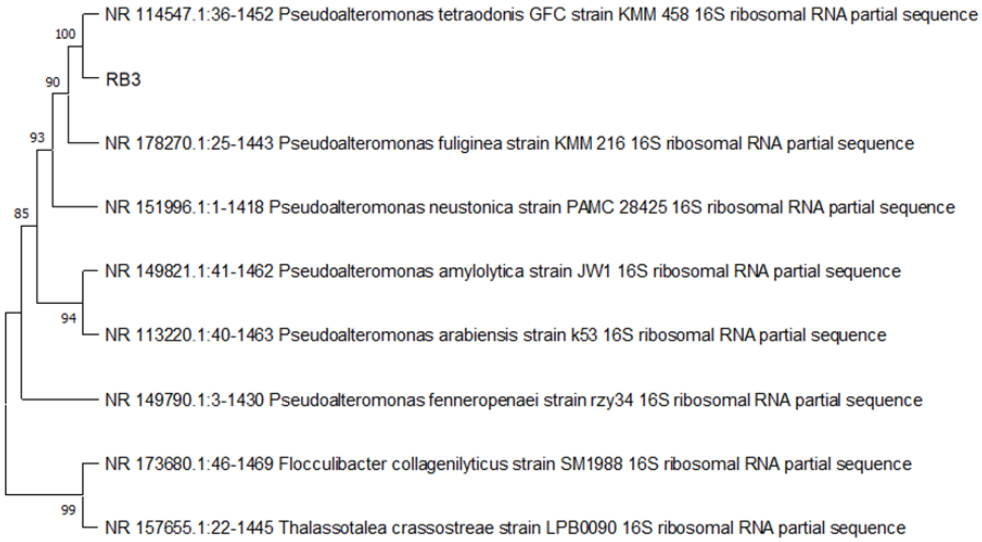


Figure 3. Phylogenetic tree based on 16S rRNA gene sequence of RB3 using the neighbour-joining method, 2-parameter Kimura model and Gamma distribution with a boostrap value of 1000 replicates.

P. tetraodonis belonged to the phylum Proteobacteria, where several species from this phylum have been tested to produce L-asparaginase. *Pseudomonas aeruginosa* was obtained from fish intestines, belonging to the phylum Proteobacteria, which produced L-asparaginase (Qeshmi et al. 2022). *Bacillus licheniformis* of the Proteobacteria phylum was isolated from the Red Sea, Saudi Arabia which was reported to produce L-asparaginase (Alrumman et al. 2019). *Pseudomonas stutzeri* ATCC 17588(T) from the phylum Proteobacteria, could produce glutaminase-free L-asparaginase, was isolated from Indonesian medicinal plants (Sulistiyani & Kusumawati 2019). Four strains of bacteria belonging to Gammaproteobacter, a subdivision of the Proteobacteria phylum, namely *Pseudomonas* sp., *Bacillus* sp., *Zobellella* sp. and *Oceanimonas* sp. which could produce L-asparaginase were obtained from the Persian Gulf (Qeshmi et al. 2014).

Growth curve

The growth of *Pseudoalteromonas tetraodonis* RB3 that was isolated from Rancabuaya Coast is characterised by an increase in density or turbidity in the ZMB medium according to (Kumar et al. 2010a). Determining the culture time used to measure enzyme activity was based on the growth curve (Kurniawati et al. 2019). Figure 4 shows the growth curve of *Pseudoalteromonas tetraodonis* RB3. The growth curve of *Pseudoalteromonas tetraodonis*

RB3 was needed to determine the right time to produce L-asparaginase, because from the growth curve, there is a bacterial logarithmic phase that actively produces primary metabolites such as enzymes (Alrumman et al. 2019). From two to eighteen hours, the exponential phase occured which was characterised by significant bacterial cell growth. Logarithmic phase of *Pseudoalteromonas tetraodonis* RB3 begins to end at hour 18. In this phase, the cells metabolism was constant and growth was quite balanced. The logarithmic phase was the best time to use the cells, because the bacteria had an optimal growth rate so they could be used as inoculum in a treatment (Wijanarka et al. 2016; Mahjani & Dwi 2020).

In addition, because in the logarithmic phase there was an increase in the number of bacterial cells, this would increase the synthesis of enzymes for metabolic processes in cells (Saropah et al. 2012). Metabolite began to form when they entered the middle of the exponential phase and decreased in production in the stationary phase (Judoamidjojo et al. 1990; Ruiz et al. 2010; Hidayatulloh et al. 2019). The samples used for testing enzyme activity were crude extract samples of enzymes produced during the logarithmic phase, where during this phase bacteria produced primary metabolites in the form of enzymes (Kurniawati et al. 2019).

From the explanation above, it showed that primary metabolite compounds were produced optimally in the logarithmic phase. Therefore, L-asparaginase activity of the *Pseudoalteromonas tetraodonis* RB3 culture was tested at 18th hour, just before the end of the logarithmic phase and before entering the stationary phase.

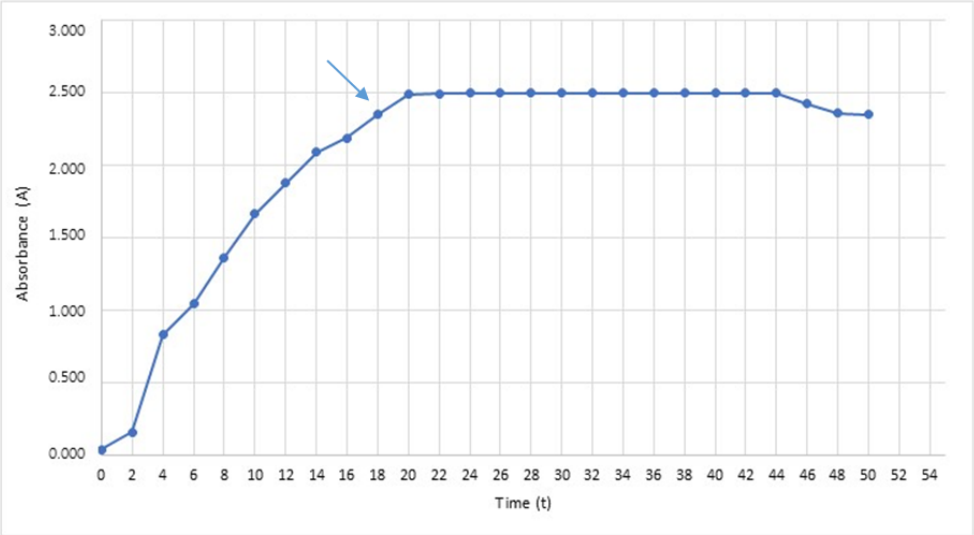


Figure 4. Growth curve of *Pseudoalteromonas tetraodonis* RB3. Arrow showed growth at the 18th hour where L-asparaginase activity was tested.

The stationary phase of *Pseudoalteromonas tetraodonis* RB3 occurred from the 20th hour to the 44th hour. Several factors could cause the number of living cells to remain constant and form a straight or constant line in the stationary phase of the growth curve. In this phase, there was a state of balance between the rate of cell growth (cell division) and the rate of cell death, which meant that the number of living cells was the same as the number of dead cells (Mardalena 2016). The availability of growth nutrients in the medium had been depleted and the population had limited space to grow, called the 'biological space'. Another factor could be the accumulation of metabolic end products which may be toxic to the growth of the bacteria themselves (Umniyati et al. 2009; Kusumaningati et al. 2013). From the 46th hour to 50th

hour, the death phase occured, which was characterised by a decrease in the number of bacteria due to a depletion of nutrients in the medium (Risna et al. 2022). In addition, the products of microbial metabolism themselves may be toxic, so they could cause cell death (Suharyono et al. 2012).

Production of crude extract of L-asparaginase

Production of L-asparaginase from *Pseudoalteromonas tetraodonis* RB3 was carried at 18 hours of culture with an OD of 2.3, namely near the end of the logarithmic phase (Figure 4). Isolation of the *E. coli* enzyme as a positive control was also carried out near the end of the logarithmic phase of the culture, namely at 16 hours. This had the largest population, so it was hoped that maximum enzyme activity would be obtained (Gunawan 2017; Ratnadewi et al. 2017).

Table 4 showed that the crude extracellular and intracellular L-asparaginase activities were different between positive control and *Pseudoalteromonas tetraodonis* RB3. The crude extracellular and intracellular L-asparaginase activities from *Pseudoalteromonas tetraodonis* RB3 were 28.34 U mL^{-1} and 25.46 U mL^{-1}, while crude extracellular and intracellular L-asparaginase activity of positive control were 25.92 U mL^{-1} and 24.20 U mL^{-1} respectively. These results showed that crude extracellular and intracellular L-asparaginase from *Pseudoalteromonas tetraodonis* RB3 had a higher activity value compared to positive control.

Table 4. Crude extracellular and intracellular L-asparaginase activity.

Source	Sample	Enzyme Activity (U mL^{-1})	Volume (mL)	Total Activity (Units)
Pseudoalteromonas tetraodonis RB3	Crude Extracellular	28.34	500	1.417×10^4
	Intracellular	25.46	6	1.528×10^2
Positive control	Crude Extracellular	25.92	500	1.296×10^4
	Intracellular	24.20	6	1.452×10^2

Several gram-negative bacteria that produced extracellular L-asparaginase were from the *Pseudomonas* genus, including *Pseudomonas ovalis* IAM 1153 which had activity of 16 U mL^{-1}, *Pseudomonas fluorescens* had activity of 20 U mL^{-1}, and *Pseudomonas dacunhae* IAM 1668 which had an L-asparaginase activity of 33 U mL^{-1} (Arima et al. 1972).

In Figure 5, the L-asparaginase activities of *Pseudoalteromonas tetraodonis* RB3 amonium sulphate fraction was different in each fraction. The activities increased from saturation level of 0-20 % (F1), 20-40 % (F2), to up to 40-60 % (F3), then decreased again from saturation level 60-80 % (F4) to 80-100 % (F5). The highest L-asparaginase enzyme activity was at a saturation level of 40-60 % (F3), namely 27.75 U mL^{-1}.

In contrast in positive control, the L-asparaginase activity of ammonium sulphate fractions increased from saturation level of 0-20 % (F1) to 60-80 % (F4), then decreased at 80-100 % saturation (F5). So, the highest L-asparaginase activity was highest at saturation level of 60-80 % (F4), namely 9.514 U mL^{-1}. The L-asparaginase activity in the F3 fraction of *Pseudoalteromonas tetraodonis* RB3 was greater than the F4 fraction of *E. coli*.

The protein content of each enzyme solution was measured using the Lowry method. The absorbance was measured 655 nm, which was chosen based on the highest absorbance. Figure 6 showed that the specific activity of the L-asparaginase in each sample was different. Fractions with large activi-

ties did not necessarily have large specific activities, nor do enzyme fractions with small protein concentrations necessarily had large specific activities. Because specific activities were determined by comparison of activity to protein levels (Arpintasari et al. 2008).

Crude extracellular and intracellular enzymes obtained from centrifugation still contained many impurities. The crude extracellular enzyme of *Pseudoalteromonas tetraodonis* RB3 had a specific activity of 72.30 U mg^{-1}, while the intracellular enzyme had a specific activity of 67.18 U mg^{-1}. Likewise, in the positive control, namely the *E. coli* crude extracellular enzyme had a specific activity of 97.80 U mg^{-1}, which was greater than the intracellular enzyme specific activity of 90.97 U mg^{-1}. *E. coli* still had higher specific activities for the L-asparaginase compared to *Pseudoalteromonas tetraodonis* RB3.

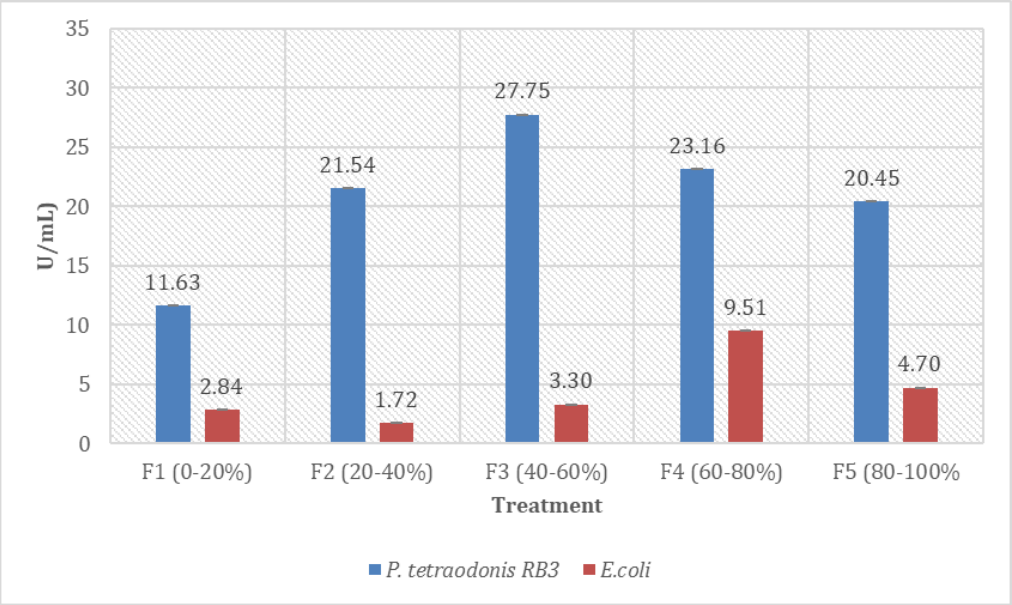


Figure 5. L-asparaginase activity in the ammonium sulphate fractions.

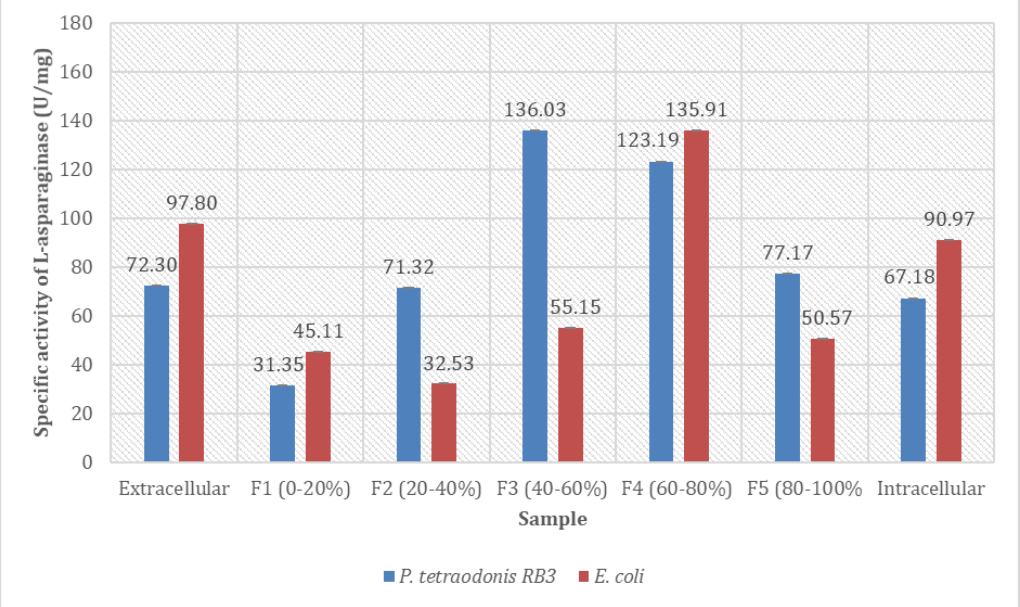


Figure 6. Specific activity of L-asparaginase.

The specific activities of L-asparaginase from each *Pseudoalteromonas tetraodonis* RB3 fractions were different and increased from ammonium sulphate saturation levels of 0-20 % (F1), 20-40 % (F2), to 40-60 % (F3) then decreased again on saturation levels of 60-80 % (F4) to 80-100 % (F5). The spe-

cific activity value of the *Pseudoalteromonas tetraodonis* RB3 L-asparaginase enzyme was highest at a saturation level of 40-60 % (F3), namely 136.03 U mg^{-1}. There was a fold purification of 1.9-fold in fraction F3. However, the specific activity between F3 and F4 was not much different, the specific activity of F4 being 123.19 U mg^{-1}. This meant that to decrease loss of enzyme, fractionation could be performed at 40-80 % saturation in the enzyme purification process and a higher fold purification could be expected to be achieved.

In contrast, the specific activities of the L-asparaginase *E. coli* ammonium sulphate fractions decreased from saturation level of 0-20 % (F1) to 20-40 % (F2), then increased from 40-60 % (F3) to 60-80 % (F4) then decreased again at saturation level 80-100 % (F5). So, the highest specific activity of *E. coli* L-asparaginase was at a saturation level of 60-80 % (F4), namely 135.91 U mg^{-1}. The fold purification in F4 was 1.4-fold. When comparing the highest L-asparaginase specific activity of the fractions between *Pseudoalteromonas tetraodonis* RB3 and *E. coli*, the *Pseudoalteromonas tetraodonis* RB3 had a higher L-asparaginase specific activity compared to *E. coli*. The L-asparaginase of *Pseudoalteromonas tetraodonis* RB3 samples was mostly found in F3 and the L-asparaginase of E. coli samples was mostly found in F4.

The difference in the level of ammonium sulphate saturation required to achieve the highest L-asparaginase enzyme activity in *Pseudoalteromonas tetraodonis* RB3 samples (in F3) and *E. coli* (in F4) could be caused by several factors such as species differences between *Pseudoalteromonas tetraodonis* RB3 with *E. coli*. Each species has unique genetic characteristics, including for certain enzymes such as L-asparaginase. These differences could affect the expression, structure, and stability of the enzyme, as well as its interactions with the ammonium sulphate ions. When the amino acid composition of the same enzyme from different species was different, it could affect the interaction with ammonium sulphate ions, the stability of the enzyme in ammonium sulphate solutions, as well as affecting the isoelectric point. The isoelectric point of a protein depended on the composition of amino acids in the polypeptide chain. Each amino acid has a different isoelectric point due to the differences in their functional groups, such as hydrophobic and ionic groups. The interaction between these groups and the ammonium sulphate ion influences the ability of the enzyme to precipitate at different levels of saturation (Scopes 1994).

Hydrophobic interactions between protein molecules in a high ionic atmosphere will cause protein precipitation, which is called salting out (Duong-Ly & Gabelli 2014). Proteins with high hydrophobicity will precipitate first, while proteins with polar residues will dissolve even at high salt concentrations. The isoelectric point is the pH of the solution at which the net charge of the protein becomes zero and at this isoelectric point the solubility of the protein decreases and the protein will eventually precipitate and clump. Thus, the difference in the level of saturation of ammonium sulphate to precipitate the L-asparaginase enzyme between *Pseudoalteromonas tetraodonis* RB3 and *E. coli* could be due to the factors above which were influenced by the enzyme properties of each species. Table 5 showed the enzyme activity and specific enzyme activity of crude extracellular, F3 (40-60 %), and intracellular enzyme from *Pseudoalteromonas tetraodonis* RB3 and positive control respectively.

SDS PAGE

In Figure 7, it could be seen that the SDS-PAGE results of *E. coli* fraction 4 (F4) there was a 35 kDa and 45 kDa band, and in the intracellular enzymes of *E. coli* the thickest band was at 35 kDa and in the 60-70 kDa range. Based on the literature, the molecular mass of *E. coli* L-asparaginase was 35 kDa (Dantas et al. 2019). F3 contained bands with sizes around 35 kDa, 45 kDa and 90 kDa. Meanwhile, in the intracellular enzyme of *Pseudoalteromonas*

tetraodonis RB3 (sample) protein bands appeared from 15 kDa to > 250 kDa. There were still many protein bands that appeared because the intracellular samples did not undergo any purification process. However, the thickest bands were at 35 kDa and 50 kDa. Most type II L-asparaginases from mesophilic sources were tetramers of identical subunits with molecular masses in the range 140-160 kDa or 35-40 kDa per subunit, and marine bacterial L-asparaginases were mostly in the range of 25-41.1 kDa (Qeshmi et al. 2018). The 35 kDa was suspected to be the L-asparaginase protein.

Table 5. Enzyme activity and specific enzyme activity of *Pseudoalteromonas tetraodonis* RB3.

Source	Sample	Enzyme Activity (U mL^{-1})	Specific Enzyme Activity (U mg^{-1})
P. tetraodonis RB3	Crude Extracellular	28.34	72.30
	F3 (40-60 %)	27.75	136.03
	Intracellular	25.46	67.18
Positive control	Crude Extracellular	25.92	97.80
	F4 (60-80 %)	9.51	135.91
	Intracellular	24.20	90.97

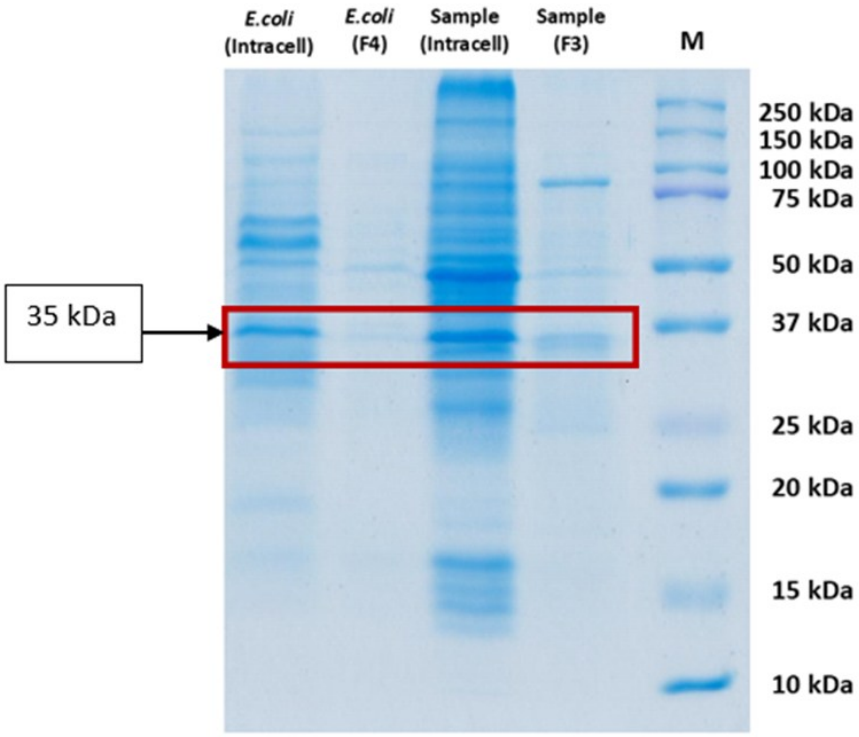


Figure 7. Electropherogram of intracellular and fractionation result of *Pseudoalteromonas tetraodonis* RB3 and *E. coli* as positive control.

CONCLUSION

Bacteria producing L-asparaginase with low glutaminase and urease activity had been isolated from seawater on the Rancabuaya Coast Indonesia. There were four different colonies named RB1, RB2, RB3 and RB4. RB3 colonies had the highest L-asparaginase activity with a zone index of 2.5 on ZMA+L-asparagine, zone index of 1.3 on ZMA+glutamine, and zone index of 2.2 on ZMA+urea. Based on the 16S rRNA gene sequence, RB3 was identified to have the closest phylogenetic relationship to *Pseudoalteromonas tetraodonis* GFC strain KMM 458, with a 16S rRNA gene homology of 99.51 % and

boostrap of 100. Enzyme specific activity of crude extracellular and intracellular enzyme were 72.30 U mg^{-1} and 67.18 U mg^{-1} respectively, while the highest enzyme specific activity from ammonium sulphate fractionation was found at 40-60% saturation (F3), which was 136.03 U mg^{-1}. SDS-PAGE of the enzyme solutions showed the presence of a 35 kDa band suspected to be the L -asparaginase protein.

AUTHORS CONTRIBUTION

W.P. designed and supervised the research and wrote the manuscript, I.N. collected and analysed the data and wrote the manuscript, A.J.N. collected and analysed the data, M.R.M. supervised the research and wrote the manuscript, M.F. collecting sample and data, T.S. supervised the research.

ACKNOWLEDGMENTS

We would like to thank to LPPM and Faculty of Science and Technology, Universitas Muhammadiyah Bandung for the facilities support of this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the research or the research funding.

REFERENCES

Alrumman, S.A. et al., 2019. Production and anticancer activity of an L-asparaginase from Bacillus licheniformis isolated from the Red Sea, Saudi Arabia. *Scientific Reports*, 9(1), 3756. doi: 10.1038/s41598-019-40512-x

Altschul, S.F. et al., 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), pp.403-410. doi: 10.1016/S0022-2836(05)80360-2

Apollos, W.P. et al., 2017. Molecular Characterization and Phylogenetic Analysis of Some Agaricomycetes (Mushroom) Fungi from Kogi State, Central Nigeria. *International Journal of Sciences: Basic and Applied Research (IJSBAR)*, 35(2), pp.276-292.

Arima, K. et al., 1972. Production of extracellular L-asparaginases by microorganisms. *Agricultural and Biological Chemistry*, 36(3), pp.356-361. doi: 10.1080/00021369.1972.10860270

Arpintasari, A. et al., 2008. Isolasi dan Uji Potensi L-Asparaginase dari Rimpang Kunyit Putih (Curcuma mangga Vall) terhadap Leukimia Tipe K562. *Jurnal Kimia Sains dan Aplikasi*, 11(3), pp.57-62.

Ashok, A. et al., 2019. Microbes Producing L-Asparaginase free of Glutaminase and Urease isolated from Extreme Locations of Antarctic Soil and Moss. *Scientific Reports*, 9(1), 1423. doi: 10.1038/s41598-018-38094-1

BacDive, 2024. *Pseudoalteromonas tetraodonis GFC is an obligate aerobe, psychrophilic, Gram-negative animal pathogen that was isolated from skin slime of pufferfish.* Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. doi: 10.1093/nar/gkab961

Baker, G.C. et al., 2001. Bacterial community analysis of Indonesian hot springs. *FEMS Microbiology Letters*, 200(1), pp.103-109. doi: 10.1111/j.1574-6968.2001.tb10700.x

Bano, M. & Sivaramakrishnan, V.M., 1980. Preparation and properties of L-asparaginase from green chillies (Capsicum annum L.). *Journal of Biosciences*, 2(4), pp.291-297.

Batool, T. et al., 2016. A Comprehensive review on Lasparaginase and its applications. *Applied Biochemistry and Biotechnology*, 178(5), pp.900-923. doi: 10.1007/s12010-015-1917-3

Byrne, R.H. & Mackenzie, F.T., 2024, 'Seawater', in *Britannica*, viewed from https://www.britannica.com/science/seawater

Chakraborty, M. & Shivakumar, S., 2021. Bioprospecting of the agaricomycete Ganoderma australe GPC191 as novel source for l-asparaginase production. *Scientific Reports*, 11, 6192. doi: 10.1038/s41598-021-84949-5

Dantas, R.C. et al., 2019. Expression of a recombinant bacterial l-asparaginase in human cells. *BMC Research Notes*, 12(1), pp.4-9. doi: 10.1186/s13104-019-4836-5

Doriya, K. & Kumar, D.S., 2016. Isolation and screening of L-asparaginase free of glutaminase and urease from fungal sp. *3 Biotech*, 6(2), 239. doi: 10.1007/s13205-016-0544-1

Duong-Ly, K.C. & Gabelli, S.B., 2014. Chapter Seven - Salting out of Proteins Using Ammonium Sulphate Precipitation. *Methods in Enzymology*, 541 (2014), pp.85-94. doi: 10.1016/B978-0-12-420119-4.00007-0

Gaffar, S., Maksum, I.P. & Julaeha, E., 2014. Identifikasi populasi bakteri dalam spons pencuci piring dengan metode Pcr-Rflp Identification of bacteria population in dishwashing sponge using pcr-rflp method. *Chimica et Natura Acta*, 2(2), pp.120-125. doi: 10.24198/cna.v2.n2.9154

Gunawan, H.V., 2017. *Aktivitas antibakteri ekstrak daun dan bunga jotang (spilanthes paniculata) terhadap bakteri Escherichia coli dan Lactobacillus acidophillus*. Universitas Atma Jaya Yogyakarta.

Hagström, Å. et al., 2002. Use of 16S ribosomal DNA for delineation of marine bacterioplankton species. *Applied and Environmental Microbiology*, 68 (7), pp.3628-3633. doi: 10.1128/AEM.68.7.3628-3633.2002

Hassanshahian, M. & Jafar, M., 2011. Isolation and Characterization of Halobacterium salinarum from Saline Lakes in Iran. *Jundishapur Journal of Microbiology*, 4(12), pp.59-65.

Hidayatulloh, A. et al., 2019. Potensi senyawa metabolit yang dihasilkan Lactobacillus plantarum atcc 8014 sebagai bahan biopreservasi dan anti bakteri pada bahan pangan asal hewan. *Jurnal Ilmu dan Teknologi Peternakan*, 7(2), pp.1-6. doi: 10.20956/jitp.v7i2.6811

Ivanova, E.P. et al., 2001. Retrieval of the species Alteromonas tetraodonis Simidu et al. 1990 as Pseudoalteromonas tetraodonis comb. Nov. and emendation of description. *International journal of systematic and evolutionary microbiology*, 51(3), pp.1071-1078. doi: 10.1099/00207713-51-3-1071

Judoamidjojo, M., Darwis, A.A. & Sa'id, E.G., 1990. *Teknologi Fermentasi*. Rajawali Pers.

Kanekar, P.P. & Kanekar, S.P., 2022. Halophilic and Halotolerant Microorganisms. In *Diversity and Biotechnology of Extremophilic Microorganisms from India*. Microorganisms for Sustainability. Singapore: Springer, pp.13-69. doi: 10.1007/978-981-19-1573-4_2

Kresnadipayana, D. & Lestari, D., 2017. Penentuan Kadar Boraks pada Kurma (Phoenix dactylifera) dengan metode Spektrofotometri UV-vis. *Jurnal Wiyata*, 4(1), pp.23-30.

Kumar, S., Dasu, V.V. & Pakshirajan, K., 2010a. Localization and production of novel L-asparaginase from Pectobacterium carotovorum MTCC 1428. *Process Biochemistry*, 45(2), pp.223-229. doi: 10.1016/j.procbio.2009.09.011

Kumar, S., Dasu, V.V. & Pakshirajan, K., 2010b. Purification and characterization of glutaminase-free L-asparaginase from Pectobacterium carotovorum MTCC 1428. *Bioresource Technology*, 102(2011), pp.2077-2082. doi: 10.1016/j.biortech.2010.07.114

Kurniawati, L., Kusdiyantini, E. & Wijanarka, W., 2019. Pengaruh variasi suhu dan waktu inkubasi terhadap aktivitas enzim selulase dari bakteri Serratia marcescens. *Jurnal Akademika Biologi*, 8(1), pp.1-9.

Kusumaningati, M.A., Nurhatika, S. & Muhibuddin, A., 2013. Pengaruh konsentrasi inokulum bakteri Zymomonas mobilis dan lama fermentasi pada produksi etanol dari sampah sayur dan buah Pasar Wonokromo Surabaya. *Jurnal Sains dan Seni ITS*, 2(2), pp.218-223. doi: 10.12962/j23373520.v2i2.4298

Lakshmi, A.V., Sheeja, L. & Devi Mari, S., 2020. Screening and identification of asparginase and glutaminase producing halophilic bacteria from natural saline habitats. *International Journal on Recent Advancement in Biotechnology & Nanotechnology*, 3(1), pp.34-53.

Lander, E.S. et al., 2001. Initial sequencing and analysis of the human genome. *Nature*, 409(6822), pp.860-921. doi: 10.1038/35057062

Lazar Jr., I., Lazar-Horvath, E. & Lazar Sr., I., 2023, '*GelAnalyzer 23.1.1* (23.1.1)', in *GelAnalyzer.com*, viewed from www.gelanalyzer.com

Leboffe, M.J. & Pierce, B.E., 2012. *Brief Microbiology Laboratory Theory & Application (2nd Edition)*, Englewood: Morton Publishing.

Lowry, O.H. et al., 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193(1), pp.265-275. doi: 10.1016/S0021-9258(19)52451-6

Mahjani & Dwi, H.P., 2020. Growth Curve of Endophyte Bacteria Andalas Plant (Morus mocroura Miq,) B.J.T A-6 Isolate. *Serambi*, 5(1), pp.29-32. doi: 10.24036/5692RF00

Mardalena, 2016. Fase Pertumbuhan Isolat Bakteri Asam Laktat (BAL) Tempoyak Asal Jambi yang Disimpan Pada Suhu Kamar. *Jurnal Sain Peternakan Indonesia*, 11(1), pp.58-66. doi: 10.31186/jspi.id.11.1.58-66

Moorcraft, S.Y., Gonzalez, D. & Walker, B.A., 2015. Understanding next generation sequencing in oncology: A guide for oncologists. *Critical Reviews in Oncology/Hematology*, 96(3), pp.463-474. doi: 10.1016/j.critrevonc.2015.06.007

Muzuni, M. et al., 2024. Skrining Bakteri Termohalofilik Penghasil L-asparaginase dari Sumber Air Panas Wawolesea Sulawesi Tenggara dan Uji Aktivitas Enzimnya. *ALCHEMY Jurnal Penelitian Kimia*, 20(1), pp.12-21. doi: 10.20961/alchemy.20.1.73523.12-21

Nagarethinam, S. et al., 2012. Microbial L-Asparaginase and its future prospects. *Asian Journal of Medical Research*, 1(4), pp.159-160.

Pieters, R. et al., 2011. L-asparaginase treatmeant in acute lymphoblastic leukemia: a focus on Erwina asparaginase. *Cancer*, 117(2), pp.238-249. doi: 10.1002/cncr.25489

Prihanto, A.A. & Wakayama, M., 2016. Chapter One - Marine Microorganism: An Underexplored Source of l-Asparaginase. *Advances in Food and Nutrition Research*, 79, pp.1-25. doi: 10.1016/bs.afnr.2016.07.005

Prihanto, A.A. et al., 2018. Identifikasi Bakteri Endofit Mangrove Api-Api Putih (Avicennia marina) Penghasil Enzim L-asparaginase. *Jurnal Ilmiah Perikanan Dan Kelautan*, 10(2), pp.84-90. doi: 10.20473/jipk.v10i2.10467

Prihanto, A.A., Ardiansyah, R.F. & Pradarameswari, K.A., 2019. Identifikasi molekuler bakteri endofit penghasil L-asparaginase yang diisolasi dari mangrove Buta-buta (Excoecaria agallocha). *Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan*, 14(1), pp.29–34. doi: 10.15578/jpbkp.v14i1.577

Qeshmi, F.I. et al., 2014. Persian Gulf is a bioresource of potent L-asparaginase producing bacteria: Isolation & molecular differentiating. *International Journal of Environmental Research*, 8(3), pp.813-818. doi: 10.22059/ijer.2014.774

Qeshmi, F.I. et al., 2018. Marine microbial L-asparaginase: biochemistry, molecular approaches and applications in tumor therapy and in food industry. *Microbiological Research*, 208, 99-112. doi: 10.1016/j.micres.2018.01.011

Qeshmi, F.I. et al., 2022. Production of a Novel Marine Pseudomonas aeruginosa Recombinant L-Asparaginase: Insight on the Structure and Biochemical Characterization. *Marine Biotechnology*, 24, pp.599-613. doi: 10.1007/s10126-022-10129-9

Ratnadewi, A.A.I. et al., 2017. Exploration of broth chicken gut as growth media of escherichia coli bl21 pet-endo for endo-β-1, 4-d-xylanase production. *Alchemy Jurnal Penelitian Kimia*, 13(2), pp.191-204.

Reimer, L.C. et al., 2022. BacDive in 2022: The knowledge base for standardized bacterial and archaeal data. *Nucleic Acids Research*, 50(D1), pp.D741–D746. doi: 10.1093/nar/gkab961

Risna, Y.K. et al., 2022. Kurva Pertumbuhan Isolat Bakteri Asam Laktat dari Saluran Pencernaan Itik Lokal Asal Aceh. *Jurnal Peternakan Indonesia*, 24(1), pp.1-7. doi: 10.25077/jpi.24.1.1-7.2022

Ruiz, B. et al., 2010. Production of microbial secondary metabolites: regulation by the carbon source. *Critical Reviews in Microbiology*, 36(2), pp.146-167. doi: 10.3109/10408410903489576

Sambrook, J., Fritsch, E.F. & Maniatis, T., 1989. *Molecular cloning: a laboratory manual* (2nd ed.). United States: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S. & Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 74(12), pp.5463-5467. doi: 10.1073/pnas.74.12.5463

Saropah, D.A., Jannah, A. & Maunatin, A., 2012. Kinetika reaksi enzimatis ekstrak kasar enzim selulase bakteri selulolitik hasil isolasi dari bekatul. *ALCHEMY: Journal of Chemistry*, 2(1), pp.34-45. doi: 10.18860/al.v0i0.2297

Saxena, A., Upadhyay, R. & Kango, N., 2015. Isolation and identification of actinomycetes for production of novel extracellular glutaminase free L-asparaginase. *Indian Journal of Experimental Biology*, 53(12), pp.786-793.

Scopes, R.K., 1994. *Protein Purification: Principles and Practice* (3rd edition), New York: Springer New York.

Shrivastava, A. et al., 2016. Recent developments in L-asparaginase discovery and its potential as anticancer agent. *Critical Reviews in Oncology/Hematology*, 100, pp.1-10. doi: 10.1016/j.critrevonc.2015.01.002

Simidu, U. et al., 1990. Taxonomy of four marine bacterial strains that produce tetrodotoxin. *International Journal of Systematic Bacteriology*, 40(4), pp.331-336. doi: 10.1099/00207713-40-4-331

Stackebrandt, E. & Goebel, B.M., 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*, 44(4), pp.846-849. doi: 10.1099/00207713-44-4-846

Suharyono, S., Rizal, S. & Kurniadi, M., 2012. Pertumbuhan L. casei pada berbagai lama fermentasi minuman sinbiotik dari ekstrak cincau hijau (Premna oblongifolia merr). *Jurnal Teknologi Hasil Pertanian*, 5(2), pp.117-128. doi: 10.20961/jthp.v0i0.13564

Sulistiyani, T.R. & Kusumawati, D.I., 2019. Keragaman bakteri endofit penghasil Lasparaginase bebas L-glutaminase. *Jurnal Kefarmasian Indonesia*, 9(1), pp.28-39. doi: 10.22435/jki.v9i1.1302

Tamura, K., Stecher, G. & Kumar, S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7), pp.3022-3027. doi: 10.1093/molbev/msab120

Umniyati, S., Astuti., B.O. & Drajat, P., 2009. Pengaruh Garam Empedu Terhadap Pertumbuhan dan Produksi Asam Laktat Streptococcus sp Dari CYME Usus Halus Ayam Broiler Strain Lohman. *Prosiding Seminar Nasional Penelitian, Pendidikan Dan Penerapan MIPA, Universitas Negeri Yogyakarta.*

Vogel, A.I., 1951. *A Text-book of Quantitative Inorganic Analysis* (2nd edition), Longmans, Green and Co.

Waterborg, J.H., 2009. The Lowry Method for Protein Quantitation. In *The Protein Protocols Handbook. Springer Protocols Handbooks.* Totowa, NJ: Humana Press, pp.7-10. doi: 10.1007/978-1-59745-198-7_2

Wijanarka, W., Kusdiyantini, E. & Parman, S., 2016. Screening Cellulolytic Bacteria from the Digestive Tract Snail (Achatina fulica) and Test the Ability of Cellulase Activity. *Biosaintifika: Journal of Biology & Biology Education*, 8(3), pp.386-392. doi: 10.15294/biosaintifika.v8i3.7263

Zilda, D.S. et al., 2021. Penapisan dan identifikasi bakteri penghasil agarase dari sampel sedimen laut Bara Caddi, Sulawesi Selatan. *Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan*, 16(1), pp.11-21. doi: 10.15578/jpbkp.v16i1.699

Zuo, S. et al., 2014. Recent research progress on microbial L-asparaginases. *Applied Microbiology and Biotechnology*, 99(3), pp.1069-1079. doi: 10.1007/s00253-014-6271-9