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Optimization of L-asparaginase production from endophytic bacteria isolated from the mangrove *Rhizophora mucronata*

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Abstract. *Nafisaturrahmah A, Susilowati A, Pangastuti A. 2023. Optimization of L-asparaginase production from endophytic bacteria isolated from the mangrove* Rhizophora mucronata. *Nusantara Bioscience 15: 279-287.* L-asparaginase is an enzyme that hydrolyes L-asparaginase to L-aspartate and ammonia. L-asparaginase has the potential to treat acute lymphoblastic leukemia and other malignant cancers. So far, purified L-asparaginase from *Escherichia coli* and *Erwinea chrysanthemi* has been available and applied clinically in humans. However, this treatment has side effects such as allergy, cross-interaction, immune system stimulation, drug resistance, and nonspecific L-glutaminase activity. These side effects can be overcome by discovering new sources of L-asparaginase, which are serologically different but have similar therapeutic effects. This study aims to determine the optimal conditions of endophytic bacterial culture in producing L-asparaginase. Endophytic bacteria were screened using an M9 medium with asparagine as a substrate; the L-asparaginase-producing isolates showed pink zones around the colonies. Optimization of L-aparaginase production by endophytic bacteria is carried out by One Factor at A Time (OFAT). Optimization of enzyme production includes incubation time, temperature, pH, ammonium sulfate levels, and glucose concentration in the bacterial growth medium; determination of enzyme production by Nesslerization method. The results showed that 8 isolates could produce high L-asparaginase, 14 isolates had medium ability, 30 isolates had low ability, and 2 bacterial isolates did not produce L-asparaginase. Endophytic isolates were able to produce the highest L-Aparaginase under different optimal conditions. The optimal incubation time for endophytic isolates in this study was 60-84 hours, the optimal temperature was 37ºC, the optimal pH was 7, the nitrogen content was 0.25 mg/L, and the optimal glucose level was 3%.

Keywords: Endophytic bacteria, enzyme production, L-asparaginase, mangrove *Rhizopora mucronata*, optimization

INTRODUCTION

L-asparaginase is a catalytic enzyme in the hydrolysis of L-asparagine to L-aspartate and ammonia. L-asparagine is a nonessential amino acid used for protein synthesis and cell growth. L-asparaginase has the potential to treat acute lymphoblastic leukemia and other malignant cancers. Asparagine synthetase can work normally in normal cells, producing sufficient amounts of asparagine (Micu et al. 2020). Meanwhile, in patients with acute lymphoblastic leukemia, the amount of asparagine synthetase in cells is limited, so additional asparagine is needed from nutrients outside the cells (Konecna et al. 2004; Lomelino et al. 2017). L-asparaginase degrades L-asparagine, which leukemia cells need, so the cancer cell's need for L-asparagine is not met sufficiently. Consequently, cell growth will be inhibited, and death will occur in leukemia cells (Narta et al. 2007; Radadiya et al. 2020). L-asparaginase activity cannot interfere with normal cells since the normal cells can produce asparagine synthetase in the proper amount so that the cells' needs for asparagine are sufficiently fulfilled by the normal cells. This L-asparaginase potential will be developed as an alternative treatment for cancer cells.

The mangrove forest in Kulon Progo, Indonesia is a mangrove tourist area directly adjacent to residential areas. This area has a high risk of anthropogenic threats in the form of final waste storage, such as agricultural waste, household waste, and oil spills, affecting water quality (Saru 2013). Mangrove ecosystems are coastal ecosystems composed of many vegetation and living organisms with biological and physiological adaptations in synthesizing metabolites or enzymes specific to environmental stress (Prihanto et al. 2019). In this habitat, *Rhizophora mucronata* Lam. adapts to grow resistant to stress conditions by producing different metabolites compared to a serene environment, allowing specific types of endophytes to be found.

L-asparaginase can be produced by animals, plants, and microorganisms such as fungi, yeast, and bacteria (Talluri et al. 2014). Bacteria are a better source of L-asparaginase; it is easier to culture, and optimizing enzyme production and extraction is easier to do (Savitri et al. 2003; Cachumba et al. 2016). Bacteria need a substrate in the form of asparagine as the main source to be processed and broken down into L-aspartate and ammonia. Asparagine is abundant in plants, about 60-80% in the roots, leaves, and fruit, 40-45% in the xylem and 20-30% in the phloem. One of the plants that contain asparagine and can be used as a source of L-asparaginase-producing microorganisms is the mangrove *R. mucronata* (Nur et al. 2013). However, L-asparaginase purified from *Escherichia coli* and *Erwinea chrysanthemi* has been available and has been clinically applied to humans, but this treatment has side effects such

as allergies, cross interactions, stimulation of the immune system, drug resistance, and nonspecific L-glutaminase activity (Kotzia and Labrou 2007). These side effects can be reduced by discovering new sources of L-asparaginase, which are serologically different but have similar therapeutic effects. This requires screening samples from various sources to obtain isolates with L-asparaginase non-glutaminase, which have the same or greater therapeutic effect and different serology characteristics (Joshi and Kulkarni 2018).

Endophytic microbes can produce compounds or metabolites similar to bioactive compounds in host plants because of their close symbiotic relationship with the host plant (Chow et al. 2018). This was proven by Harper et al. (2003), who stated that taxol, an anticancer agent, was originally only found in the *Taxus brevifolia* tree, but many researchers found that the endophytic *Taxomyces andreanae* in this plant also contained taxol. Salini et al. (2016) research stated that the stems of the mangrove *R. mucronata*, originating from the mangrove forest on the south coast of Kerala, India, contain many endophytic bacteria that produce L-asparaginase, capable of treating cancer.

Further research and promising applications in L-asparaginase is that the L-asparaginase gene (GkASN) from the thermophilic bacterium, *Geobacillus kautophilus*, has been cloned and expressed in *E. coli* Rosetta™2 (DE3) cells using the pET-22b(+) vector. GkASN-induced asparagine deficiency effectively reduced metastatic synergy in SNU387 HCC cells on the xCELLigence system with differentiated epithelial Hep3B and poorly differentiated metastatic mesenchymal HCC SNU387 cells (Özdemir et al. 2022). Another study (Aktar et al. 2023) performing a structural comparison of ten L-ASNases with very different kinetic properties showed that residues with a catalytic role are conserved and some differences at position 59 close to the substrate may influence the kinetic parameters. L-ASNase type I from the yeast *Lachancea thermotolerans* (LtASNase) showed the highest specific activity (313.82 U/mg) and catalytic efficiency for L-Asn. Therefore, LtASNase is a promising oncological therapeutic candidate to be further investigated in pharmaceutical applications. Furthermore, research that can successfully modify L-ASNase such as reducing glutaminase activity, increasing the in vivo stability, and enhancing thermostability, will increase its commercialization in the pharmaceutical field (Wang et al. 2021) and also improve the knowledge about its production current biotechnological developments in L-ASNase production and biochemical characterization isis also being carried out (Castro et al. 2021).

This research was conducted to determine whether the roots, stems, and leaves of the mangrove *R. mucronata* in the Wana Tirta, Yogyakarta mangrove area contain endophytic microorganisms that can produce L-asparaginase.

MATERIALS AND METHODS

Study area

The Wana Tirta mangrove forest is located in Kulon Progo District; one of five districts/cities in the Special Region of Yogyakarta Province, Indonesia; located in the western part, with coordinates -7.89267, 110.01983. The climate in Kulon Progo can be characterized by two main seasons - the rainy season and the dry season. The rainy season generally falls between November and April. Most of the rain falls in a short time and is heavy, but sometimes there is prolonged rain that can last all day. January is usually the wettest month. During the rainy season, the average daily high temperature ranges from 28-30°C. The dry season occurs between May and October. Although there may be occasional rainfall at the beginning and end of this period. During these months, the average daily high temperature is around 32°C and the nighttime low is 25°C. During 2021 in Kulon Progo District the average monthly rainfall was 213.56 mm and 12.10 rainy days per month, the lowest humidity was 78.6% (August), and the highest was 85.9% (January). Pemerintah Kabupaten Kulon Progo https://kulonprogokab.go.id/v31/detil/7672/kondisi-umum. 23 Juni 2020

The mangrove *R. mucronata* was taken from the Wana Tirta ecotourism area, Kulon Progo, Yogyakarta. The sampling technique was carried out based on the cluster sampling method. Approximately 10% of the mangrove area of Wana Tirta Kulon Progo is divided into 3 regions, namely region 1 (beach), region 2 (coastal), and region 3 (land). Each area has 3 sampling points with a distance of 200 m in each area. At each sampling point, samples were taken from 3 plants. The samples used in this study were the roots, stems, and leaves. Fresh samples were taken, put in a newsprint, and taken to the laboratory. Next, samples were stored in the refrigerator (Prihanto et al. 2019). The following is a map of the sampling locations for the mangrove *R. mucronata* (Figure 1).

Isolation of endophytic bacteria

Endophytic bacteria were isolated from samples of roots, stems, and leaves of mangrove *R. mucronata*. The surface sterilization of the sample was done by washing it with running water and immersing it in 70% alcohol for 30 seconds and 2% sodium hypochlorite for 2 minutes. Samples were rinsed with 70% alcohol for 30 seconds and ddH_2O 3-4 times (Lashgarian et al. 2021). Sterile samples were cut and grown on Nutrient Agar (NA) growth media. The bacterial colonies from the plant tissue were purified as pure cultures using the streak-plate method, then observed for morphology and screened for their ability to produce L-asparaginase.

Screening of L-asparaginase-producing endophytes

Screening of L-asparaginase-producing endophytic is based on increased pH due to enzymatic release of ammonia on asparagine, glutamine, urea, or $NaNO_3$ substrates by L-asparaginase-producing bacteria. Rapid L-asparaginase activity detection is demonstrated by staining techniques using a pH indicator mixed in culture media

containing asparagine as an N source (Gulati et al. 1997). The commonly used pH indicator is phenol red, which is yellow under acidic conditions and changes color to pink under alkaline conditions due to increased pH. Endophytic bacteria producing L-asparaginase were screened qualitatively based on the method in Gulati et al. (1997). Pure cultures of endophytic isolates were inoculated using the single dot-plate method on a modified M9 medium; this M9 medium is minimal, and the only source of N is added L-asparaginase. The composition of M9 medium is D-Glucose 2 g/L, Na_2HPO_4 6g/L, KH_2PO_4 3g/L, $MgSO_4.7H_2O$ 0.02g/L, NaCl 0.5g/L, $CaCl_2$ 0.002g/L, Agar 3g/L, L-asparagin 5g/L, and phenol red 0.009%). Cultures were incubated at 37°C for 24-48 hours. The pink zone around the colony indicates that the isolate produces L-asparaginase; the pink zone diameter formed around the colony was measured. Screening is done with two repetitions; next, the 10 isolates with the highest ability were tested for optimization of L-asparaginase production.

Preparation of ammonium chloride standard solution

A standard solution was prepared by diluting a standard ammonium chloride solution with a concentration of 100 ppm (Karimullah et al. 2018). The standard solution is prepared by dissolving 0.01g of NH_4Cl in 100 mL of distilled water. Standard solutions were made with concentrations of 1 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, and 25 ppm.

Preparation of the standard curve

The standard curve between ammonium chloride's absorbance and ammonium chloride concentration is obtained by adding 100 mL of standard solution with 15 drops of Nessler's reagent. The solution was allowed to stand at room temperature for 10 minutes, and the absorbance was measured at a wavelength of 450 nm.

Determination of optimum incubation time

The optimum incubation time is the optimal time for L-asparaginase production. L-asparaginase production is indicated by the enzyme activity shown by breaking asparagine into ammonium sulfate. The isolate that produced L-asparaginase was grown in liquid M9 medium and incubated on a 200 rpm incubator shaker at 26-29°C for 96 hours. Every 12 hours, the samples were centrifuged at 3,000 rpm for 10 minutes at 4°C to separate the supernatant and cells. The supernatant was taken to estimate enzyme production (Abhini and Zuhara 2018).

Measurement of L-asparaginase production

Enzyme production is expressed as enzyme activity U/mL. Enzyme production was measured using the Nesslerization method (Abhini and Zuhara 2018). The reaction will be seen by adding 0.5 mL of the supernatant containing the enzyme into 1.7 mL of 0.04 M L-Aparagine and 1 mL of 0.05M Tris-HCl pH 8.6. The supernatant containing enzymes and substrates was incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.5 mL of 1.5 M Trichloroacetic Acid (TCA). 0.2 mL of Nessler's reagent was added to a tube containing 0.1 mL of the reaction mixture and 3.75 mL of ddH_2O. The tube was incubated at room temperature for 10 minutes. The absorbance of the sample was measured at a wavelength of 450 nm with a spectrophotometer. Next, 1 unit of L-asparaginase enzyme is defined as the amount of enzyme that breaks down 1 µmol of ammonia for 1 minute at 37°C.

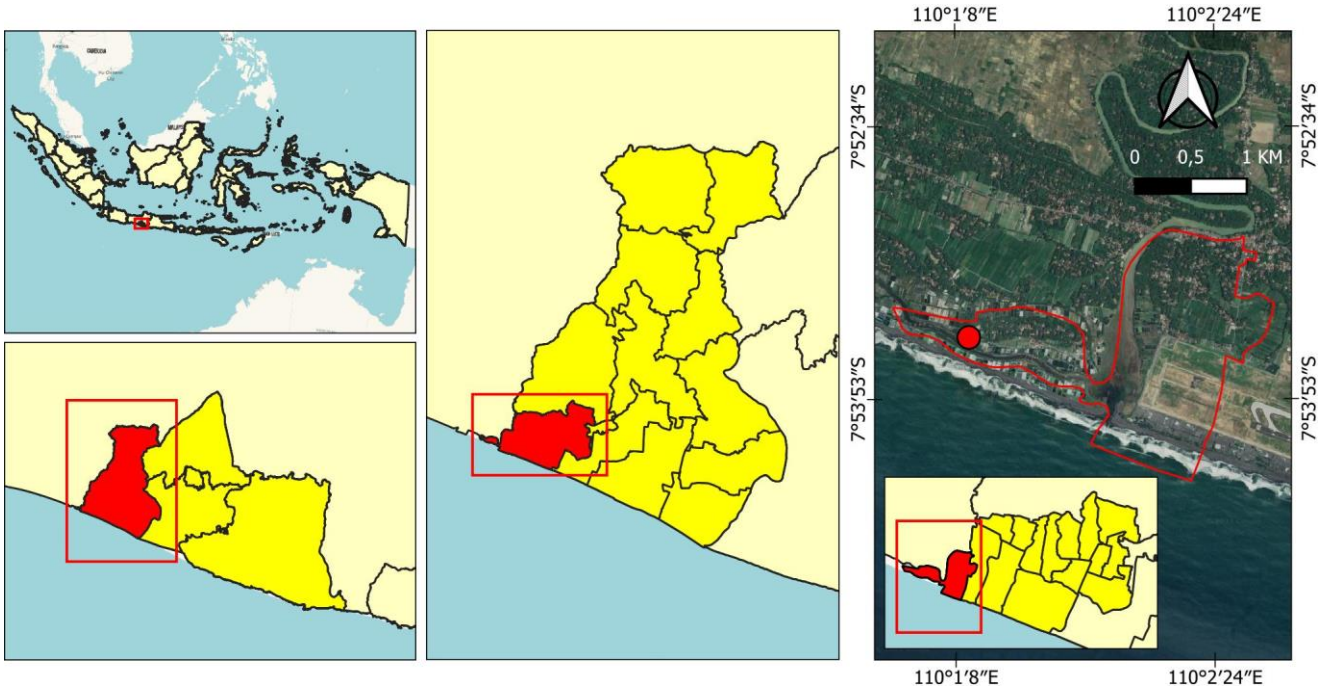

Figure 1. Locations of mangrove sampling: the Wana Tirta ecotourism area, Jangkaran, Temon, Kulon Progo, Yogyakarta, Indonesia

Optimization of L-asparaginase enzyme production by endophytic bacteria

Optimization of media and endophytic growth conditions for enzyme production follows Lashgarian et al. (2021). Optimization of L-aparaginase production by bacteria is carried out with One Factor at A Time (OFAT) method. Optimization factors performed include incubation time, incubation temperature (27, 37, and 50°C); pH media (6,7,8); and ammonium sulfate 0.25 mg/L, 0.5 mg/L, 1 mg/L; glucose level 0.5, 1, 3%. Bacterial isolates producing L-Asparaginase were cultured in 100 mL of liquid M9 medium. In the temperature optimization treatment, the bacterial culture was incubated in an incubator shaker with a speed of 200 rpm at 27°C, 37°C, and 50°C, respectively.

In the pH variation treatment, the growth medium was adjusted until it reached a pH of 6, 7, and 8. In the glucose variation, the growth medium was adjusted with D-glucose levels to 0.5%, 1%, and 3%, respectively. In the ammonium sulfate variation, the growth medium was added as much as 0.25 mg/L, 0.5 mg/L, and 1 mg/L. Endophytic cultures were incubated on an incubator shaker at 200 rpm at 37°C. Endophytic cultures were incubated during the optimal incubation time that had been obtained. After reaching the optimum incubation time, the culture was taken and centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was taken for measurement of enzyme activity.

The absorbance value obtained is substituted into the ammonium-chloride standard curve equation with the regression equation y = ax+b

Ammonia level:
y = ax+b (y-b)
x = ((y-b))/a

Where: x: ammonia content (µmol/L); y: absorbance; a: slope; b: intercepts

The value of L-Asparaginase production is obtained by using the equation:

Enzyme activity (Unit/mL) = (y-b)/a x (V.Total)/(V.Analysis) x 1/(V.Enzyme) x 1/(t.Incubation)

Where: V.Total = Enzyme Volume + Substrate + Buffer +TCA; V. Analysis = Total Volume analyzed; V. Enzymes = Volume of enzymes analyzed; t. Incubation time = 30 minutes

Data analysis

The data obtained in this study were the isolation and screening of endophytic bacteria from the mangrove *R. mucronata,* which produce L-asparaginase. Optimization of L-asparaginase production was done using a One-Factor-at-The-Time method by calculating the concentration of ammonia and enzyme activity. The data obtained was then analyzed descriptively by displaying the data in the form of pictures and tables.

RESULTS AND DISCUSSION

Endophytic bacteria in the mangrove *Rhizopora mucronata*

A total of 54 isolates of endophytic bacteria have been isolated from the mangrove plant *R. mucronata* from 3 sampling areas, namely the beach, the coastal, and the land; the beach area had the highest number of isolates 35 and the most isolates obtained from leaf samples as many as 20. The number of endophytic isolates from all samples is presented in Table 1.

In these beach areas, water circulation is higher when compared to other areas, so the amount of nutrients dissolved in the water is also relatively high. Plants submerged in water absorb nutrients dissolved and are used for bacterial growth. The coastal area has the fewest bacterial isolates; in this area, the plants are not always submerged in water and sometimes experience dry conditions. This area will be submerged by brackish water during high tide, whereas during low tide, this area will not be submerged. A total of 15 bacterial isolates obtained were from roots, 19 isolates were derived from stems, and 20 isolates were derived from leaves. Most of the isolates obtained in this study came from leaves, and the least isolates came from roots.

Screening of L-asparaginase-producing endophytic bacteria from the mangrove *R. mucronata*

The screening process determines whether the production of the L-Aparaginase by endophyte isolates is large, moderate, or low. Enzyme production is estimated from an increase in the pH of the medium, which forms a pink zone due to the breakdown of the substrate by the growing bacteria. M9 medium is a minimal medium, so it will not affect changes in pH when bacterial growth occurs. The M9 medium negates the influence of other carbon and nitrogen sources, which may influence L-asparaginase production (Chow and Ting 2017). Determination of the ability to produce enzymes based on the pink zone formed on the M9 medium. The larger the pink zone formed, the greater the production of L-asparaginase in the bacterial isolates (Figure 2.B, C, D). Bacterial isolates that did not produce L-Aparaginase did not form pink zones around the colonies (Figure 2.A).

Table 1. Number of endophytic bacteria isolated from mangroves *Rhizopora mucronta*

	Number of Endophytic Isolates		
	Root	**Stem**	**Leaf**
Beach	12	12	11
Coastal	2	3	2
Land	1	4	7
Total	15	19	20

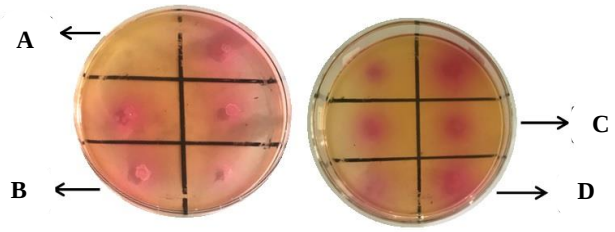

Figure 2. Pink zone showing L-asparaginase production of an endophytic isolate from *Rhizopora mucronata.* Note: a. Endophyte bacterial colony does not produce L-asparaginase (-), b. Endophyte bacterial colony that produces low L-Asparaginase (pink zone diameter <1.5 cm), c. Endophyte bacterial colony that produces moderate L-asparaginase (diameter of the pink zone 1.5-2.5 cm), d. Endophyte bacterial colony that produces large L-asparaginase (pink zone diameter >2.5 cm)

The diameter of the pink zone is formed based on an increase in the media's pH due to the substrate's breakdown by growing bacteria. The bacteria convert L-asparagine to L-aspartate and ammonia in the media, resulting in a change in pH as an indication of the bacteria producing L-asparaginase. The ammonia that is formed cause the pH of the media to become more alkaline, bringing about a color change in the media from yellow to pink (Mahajan et al. 2014). Endophytic bacteria in solid media break down L-asparagine into L-aspartate and ammonia in the media, indicating that bacteria can produce L-asparaginase. The ammonia that is formed cause the pH content of the media to become more alkaline; it will change the media's color from yellow to pink (Mahajan et al. 2014). A total of 10 bacterial isolates with high and moderate enzyme activity were selected for further optimization of enzyme production.

Based on screening on the M9 medium, endophytic bacteria produced L-asparaginase; of all endophytic bacterial isolates, 52 endophytic isolates produced L-asparaginase, and 2 endophytic isolates did not produce L-asparaginase. Endophytic isolates have different L-asparaginase production abilities. A total of 8 isolates produced high L-asparaginase, 14 isolates produced moderate L-asparaginase, 30 isolates produced low L-asparaginase, and 2 bacterial isolates could not produce L-asparaginase (Table 2).

The grouping of low, medium, and high enzyme production is based on Abhini and Zuhara (2018). Endophytic isolates were categorized as having low enzyme production if the diameter of the pink zone formed around the colony was less than 1.5 cm and classified as moderate enzyme production if they had a pink zone diameter of 1.5 cm to 2.5 cm. Endophytic isolates were categorized as having large L-asparaginase enzyme production if the diameter of the pink zone formed around the colony was more than 2.5 cm.

Optimization of L-asparaginase enzyme production

A total of 10 endophytic isolates with high and medium L-asparaginase production were optimized for enzyme production. Optimization of enzyme production was carried out by the Nesslerization method. The production of the L-aparaginase enzyme is very important for evaluating the efficiency of enzyme production so that it can be increased several times by manipulating environmental conditions and growth factors (Pola et al. 2018). Bacterial L-asparaginase activity is distinguished based on different levels of stability, and production levels are influenced by concentrations of C and N sources, temperature, aeration rate, pH, incubation time, and inoculum (Ali et al. 2016).

Table 2. Qualitative production of L-asparaginase endophytic bacterial isolate from *Rhizopora mucronata* on M9 medium

Endophytic Isolate	Enzyme Production* (cm)	Endophytic Isolate	Enzyme Production* (cm)	Endophytic Isolate	Enzyme Production* (cm)
1	3.6	21	1.3	41	1.1
2	1.7	22	1.3	42	0.9
3	2.7	23	1.9	43	0.6
4	2	24	1.7	44	0.4
5	3	25	1.3	45	1.6
6	1.8	26	0	46	1.5
7	1.3	27	1.4	47	0.6
8	1	28	1	48	0.9
9	1.5	29	0.8	49	1.6
10	3.2	30	0.9	50	0
11	1.2	31	0.3	51	0.6
12	2.6	32	0.5	52	1.7
13	1.5	33	0.4	53	0.2
14	1	34	0.3	54	0.4
15	2.4	35	0.3		
16	3.3	36	0.4		
17	3.2	37	0.3		
18	3.7	38	0.2		
19	1.9	39	1.5		
20	1	40	0.3		

Note: *Diameter of the pink zone around the colony

Optimal incubation time

Each bacterial isolate grown on culture media has its own incubation time to produce L-asparaginase. Each isolate will experience an increase in enzyme activity along with the longer the incubation period. The increase in enzyme activity will continue until it reaches the optimum point, and then the enzyme activity decreases. The optimal incubation time for the bacterial isolates in this study was 60-84 hours. The relationship curve for L-asparaginase production and incubation time for each endophytic bacterial isolate can be followed as shown in Figure 3.

The relationship curve of L-asparaginase production and incubation time for each bacterial isolate has almost the same pattern (Figure 3). In the first 12 hours, the amount of L-asparaginase produced by endophytic bacterial isolates was low. The bacterial isolates still adapt to the culture media, so L-asparaginase production is low. Bacterial isolates experienced increased growth and enzyme production as the incubation continued until it reached the optimal point. The optimal point is the optimal incubation time of an endophytic bacteria in producing the highest enzyme, and each bacterial isolate is different. The optimal incubation time for the bacterial isolates in this study was 60-84 hours. A total of 3 bacterial isolates produced L-Aparaginase optimally at the 60th hour of incubation, namely isolate 3, isolate 45, and isolate 49. Moreover, 4 bacterial isolates produced enzymes optimally at the 72th incubation time, namely isolate 1, isolate 5, isolate 39, and isolate 46. A total of 3 isolates of endophytic bacteria obtained optimally produced L-Aparaginase at the 84th hour incubation period, namely isolate 12, isolate 18, and isolate 23. Once at the optimal point, the enzyme activity will experience a slight decrease but is still relatively stable. Optimal incubation time, ammonia levels as a result of substrate breakdown by the presence of L-asparaginase, and enzyme production expressed as measured enzyme activity are presented in Table 3.

The levels of ammonia formed from the breakdown of asparagine by L-asparaginase endophytic isolates at the optimal incubation time varied (Table 3). Ammonia levels obtained from endophytic bacterial isolates ranged from 188.1 µmol/L to 333.9 µmol/L. Bacterial isolate 39 had the lowest L-asparaginase activity compared to other bacterial isolates, 188.1 µmol/L ammonia, and 57.3 IU/mL enzyme activity. Isolate 18 had the highest L-asparaginase activity at 333.9 µmol/L ammonia and 101.7 IU/mL enzyme activity. The research of Joshi and Kulkarni (2018) stated the L-aparaginase activity of the bacteria was relatively the same as the enzyme activity values from this study; namely, *B. pseudomycoides* of 70.4 IU/mL, *B. licheniformis* of 80 IU/mL, and *P. denitriformis* of 77.56 IU/mL enzyme activity has a linear relationship with the level of ammonia. The greater the level of ammonia formed, the greater the enzyme activity value.

Optimum temperature

Enzyme biosynthesis is influenced by environmental factors, one of which is temperature. Each endophytic bacterial isolate has a different relationship curve between incubation temperature and L-asparaginase production. Apart from isolates 39 and 45, endophytic bacterial isolates had a low ability to produce enzymes at 27°C and continued to increase until they reached the optimum temperature, namely 37°C. At 50ºC, enzyme production will decrease (Figure 4).

Generally, the optimal temperature of enzyme production of most endophytic isolates is 37°C. However, isolate 39 had optimal temperature of production of L-asparaginase at 27°C, and the enzyme production continued to decrease at higher temperatures at 37°C and 50°C. This result was inversely proportional to the results of isolate 45. Isolate 45 had low enzyme production activity at 27ºC and increased to 50ºC. Isolate 45 can produce L-asparaginase optimally at 50ºC. Optimal incubation temperature, ammonia levels as a result of substrate breakdown by the presence of L-asparaginase, and enzyme production expressed as measured enzyme activity are presented in Table 4.

Table 3. Optimal incubation time for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Optimum Incubation Time (hours)	Absorbance	Ammonia Level (µmol/L)	Enzyme Activity (U/mL)
Isolate 1	72	2.234	301.2	91.7
Isolate 3	60	2.176	293.4	89.3
Isolate 5	72	2.393	322.8	98.3
Isolate 12	84	2.132	287.4	87.5
Isolate 18	84	2.476	333.9	101.7
Isolate 23	84	2.506	202.9	61.8
Isolate 39	72	1.397	188.1	57.3
Isolate 45	60	1.45	195.3	59.5
Isolate 46	72	1.503	202.5	61.7
Isolate 49	60	1.416	190.7	59.1

Table 4. Optimal incubation temperature for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Optimum Incubation Temperature (ºC)	Ammonia Level (µmol/L)	Enzyme Activity (U/mL)
Isolate 1	37	300.9	91.6
Isolate 3	37	293.1	89.2
Isolate 5	37	322.2	98.2
Isolate 12	37	287	87.4
Isolate 18	37	333.6	101.6
Isolate 23	37	337.7	102.8
Isolate 39	27	243.4	74.1
Isolate 45	50	201	61.2
Isolate 46	37	202.1	62.6
Isolate 49	37	190.4	58

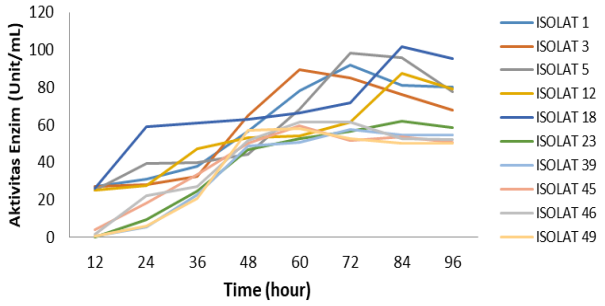

Figure 3. The relationship curve between incubation time and enzyme production by endophytic isolates

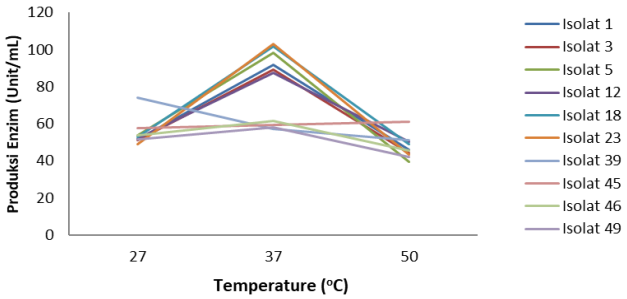

Figure 4. Relationship curve between incubation temperature and enzyme production of endophytic isolates

Isolate 23 had the highest production of L-asparaginase when compared to other endophytic isolates, namely 337.7µmol ammonia content and an enzyme activity value of 102.8 U/mL at 37ºC. Isolate 49 had the lowest ability to produce L-asparaginase, which was 190.4 µmol of ammonia content and 58U /mL of enzyme activity at 37ºC. Endophytic bacterial isolates could produce L-asparaginase optimally at 37°C, except for isolates 39 and 45 (Table 4). This is following Mohtashami et al. (2019), which stated that a temperature of 37ºC is the most suitable for L-asparaginase production using *B. licheniformis* and will experience a decrease in enzyme activity at 42ºC by 5.19% and at 47ºC by 32.19%. Isolate 39 was produced L-aparaginase optimally at 27ºC with an ammonia level of 243.4 µmol/L and an enzyme activity of 74.1 U/mL. Isolate 45 produced L-aparaginase optimally at 50ºC with an ammonia level of 201µmol/L and an enzyme activity of 61.2 U/mL. This follows Joshi and Kulkarni (2018), which showed that *E. carotovora* showed optimum activity at 50ºC.

pH media optimal

Enzyme production is very sensitive to pH media fluctuations, and enzyme production will be inhibited at a certain pH range. Endophytic isolates have different optimum pH media in L-asparaginase production. A total of 5 endophytic isolates produced the highest L-aparaginase in media with a pH of 7, namely isolate 1, isolate 5, isolate 12, isolate 18, isolate 23, and isolate 45. Isolate 3 and isolate 39 produced optimal L-asparaginase in media with low pH at 6. Isolate 46 has optimal L-asparaginase production at a pH of 8 (Figure 5).

Isolate 39 had the highest enzyme production, with 382.4 µmol/L ammonia content and 116.5 U/mL enzyme activity at pH 6. Isolate 49 had the lowest enzyme production, with ammonia levels of 250.3µmol/L and 76. 2 U/mL of enzyme activity at pH 6 (Table 5).

Enzyme production can increase or decrease depending on pH changes because it affects bacteria growth. pH media plays an important role in changes in bacterial physiology and enzyme production (Purnawan et al. 2016). The pH of the media affects the absorption of nutrients and physiological activity, so it can affect the growth of biomass and the formation of its products (Mohtashami et al. 2019). The optimum concentration of ammonium sulfate in the media.

Certain nitrogen sources are preferred in increasing the production of L-asparaginase, both organic and inorganic forms. Each endophytic isolate has a relationship curve for L-asparaginase production with different optimal ammonium sulfate concentrations in the media. A total of 5 endophytic isolates produced the highest L-aparaginase in the medium with the lowest ammonium sulfate concentration (0.25 mg/L), namely isolate 3, isolate 12, isolate 18, isolate 39, and isolate 45. Isolate 1 and isolate 23 produced L-asparaginase optimal at the highest ammonium sulfate concentration, i.e., 1 mg/L. Isolate 5 and isolate 46 produced the highest L-aparaginase at a concentration of 0.5 mg/L (Figure 6).

Table 5. Optimal pH media for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Optimum pH Media	Ammonia Level (µmol/L)	Enzyme Activity (U/mL)
Isolate 1	7	300.9	91.6
Isolate 3	6	367.9	112
Isolate 5	7	322.4	98.2
Isolate 12	7	287.1	87.4
Isolate 18	7	333.6	101.6
Isolate 23	7	337.6	102.8
Isolate 39	6	382.4	116.5
Isolate 45	7	274	83.4
Isolate 46	8	251.8	76.7
Isolate 49	6	250.3	76.2

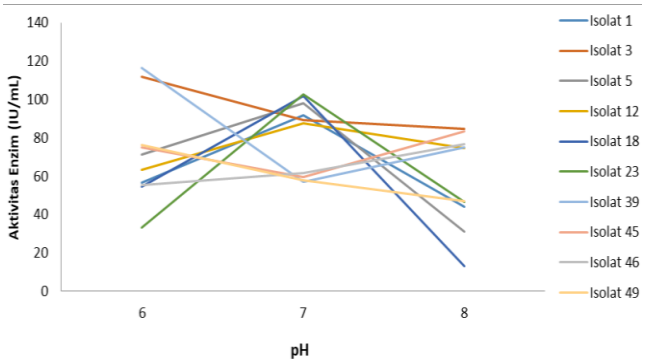

Figure 5. Relationship curve between pH media and enzyme production of endophytic isolates

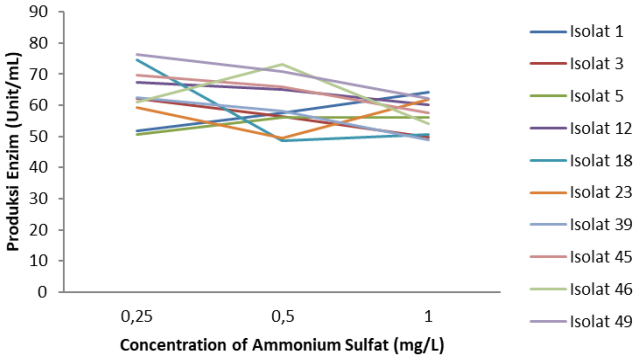

Figure 6. Relationship curve between concentration of ammonium sulfate in the media and enzyme production of endophytic isolates

Isolate 49 had the highest L-asparaginase enzyme production, with an ammonia level of 250.7 µmol/L and an enzyme activity of 76.3 Units/mL at an ammonium sulfate concentration of 0.25 mg/L. Isolate 5 had the lowest enzyme production when compared to other isolates. In comparison, isolate 5 produced an 184.4 µmol/mL ammonia level and an enzyme activity of 56.2 Units/mL at an ammonium sulfate concentration of 0.5 mg/L (Table 6). Ammonium is the best nitrogen source for L-asparaginase production. This follows Alrumman (2019), which states that ammonium sulfate is the optimal nitrogen source for *B. licheniformis*, which can increase L-aparaginase production by 35.56%.

The optimum concentration of glucose in the media

Glucose as a carbon source increases bacterial cell growth and synthesizes primary metabolites such as enzymes. The concentration of carbon sources positively affects the production of the enzyme l-asparaginase and high yields can be obtained from media rich in carbon sources (Chow and Ting 2017). Each endophytic isolate had almost the same relationship curve between glucose concentration in the media and L-asparaginase production. All endophytic isolates produced the highest L-asparaginase at a glucose concentration of 3% (Figure 7). Isolate 5 had the highest enzyme production compared to other isolates, namely 343.8 µmol/L ammonia content and 104.7 U/mL enzyme activity. Isolate 45 had the lowest enzyme activity at 141.3 µmol/L ammonia content and 43 U/mL enzyme activity (Table 7).

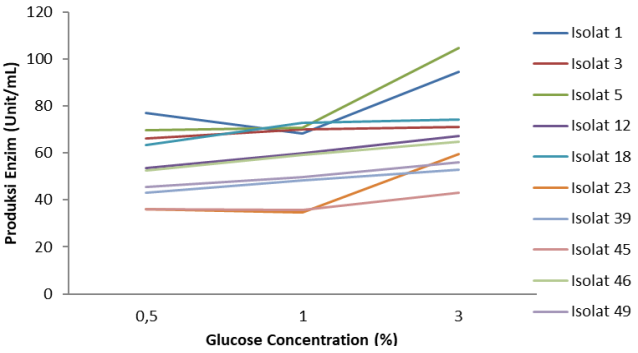

Figure 7. Relationship curve between concentration of glucose in the media and enzyme production of endophytic isolates

Table 6. The optimum concentration of ammonium sulfate in the media for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Concentration of Ammonium Sulfate (mg/L)	Ammonia Level (µmol/L)	Enzyme Activity (U/mL)
Isolate 1	1	211	64.2
Isolate 3	0.25	204.3	62.2
Isolate 5	0.5	184.4	56.2
Isolate 12	0.25	221.5	67.5
Isolate 18	0.25	245	74.6
Isolate 23	1	203.7	62
Isolate 39	0.25	205.4	62.5
Isolate 45	0.25	229.3	69.8
Isolate 46	0.5	240.3	73.2
Isolate 49	0.25	250.7	76.3

Table 7. The optimum concentration of glucose in the media for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Concentration of Glucose (%)	Ammonia Level (µmol/L)	Enzyme Activity (U/mL)
Isolate 1	3	310.4	94.5
Isolate 3	3	233.5	71.1
Isolate 5	3	343.8	104.7
Isolate 12	3	221.1	67.3
Isolate 18	3	243.7	74.2
Isolate 23	3	195.8	59.6
Isolate 39	3	173.4	52.8
Isolate 45	3	141.3	43
Isolate 46	3	213	64.9
Isolate 49	3	184.3	56.1

According to Chow and Ting (2017), incorporating glucose as a carbon source and L-asparagine as a nitrogen source affects the induction of L-asparaginase production because glucose can increase L-asparaginase production in various bacterial isolates. Alrumman et al. (2019) researched the best carbon source for *B. licheniformis* in producing L-asparaginase; the carbon source of glucose had a significant effect on enzyme activity with an increase of 11.2%, and the lowest yield was using a carbon source of starch and glycerol which resulted in a decrease in enzyme activity of 40.36% and 53.1%. Several studies on optimizing L-asparaginase activity showed several optimum conditions for each isolate used. The optimization of L-asparaginase from endophytic *Fusarium proliferatum* (isolate CCH) revealed that glucose concentration, nitrogen source, L-asparagine concentration and temperature influenced the L-asparaginase production. As such, the recommended conditions were 0.20% of glucose, 0.99% of L-asparagine and 5.34 days incubation at 30.50 °C. The L-asparaginase production of CCH increased from 16.75 ± 0.76 IU/mL to 22.42 ± 0.20 IU/mL after optimization (Yap et al. 2021). Effects of eight parameters including temperature, pH, incubation time, inoculum size, agitation speed, the concentration of starch, L-asparagine, and yeast extract were studied on L-asparaginase production by the Arctic isolate *S. koyangensis* SK4. Maximum enzyme activity of 136 IU/ml was obtained at 20°C on the seventh day of incubation in the asparagine dextrose broth maintained at pH 7.5, agitation speed 125 rpm, and L-asparagine concentration of 7.5 g/L. The statistical optimization method described in this study proved effective for increasing the L-asparaginase production by Arctic actinomycetes (Saleena et al. 2023).

In this study there were 54 isolates of endophytic bacteria were found in mangroves *R. mucronata*. Bacterial isolates produce different L-asparaginases. A total of 8 endophytic isolates could produce high L-asparaginase; 14 isolates were moderate, 30 isolates were low, and 2 bacterial isolates did not produce L-asparaginase. Endophytic isolates were able to produce the highest L-aparaginase under different optimal conditions. The optimal incubation time for endophytic isolates in this study was 60-84 hours, the optimal temperature was 37ºC, the optimal pH media was 7, the nitrogen content was 0.25 mg/L, and the optimal glucose level was 3%. L-asparaginase from endophytic isolates from mangrove *R. mucronata* shows high production and enzyme activity; in the future, it can be produced according to optimal conditions and used to treat leukemia cancer.

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