Screening of L-asparaginase-producing endophytic bacteria from mangrove *Rhizophora mucronata*

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Abstract. L-asparaginase is an enzyme that converts L-asparagine to L-aspartate and ammonia. L-asparaginase is recommended as medical treatment of Acute Lymphoblastic Leukemia (ALL) and other malignant cancers. Bacteria are the most effective source of L-asparaginase as they can easily be cultured so that the enzyme can be extracted and purified. The commercial L-asparaginase is now available from bacterial but has many side effects for the patients. Therefore, the alternative source of this enzyme is highly necessary to be explored for a more effective and safer future production of L-asparaginase. For this reason, this study was carried out to investigate the endophytic bacteria producing L-asparaginase from mangrove *Rhizophora mucronata*. The samples of the mangrove plants, i.e., roots, stems, and leaves, were surface sterilized with alcohol and sodium hypochlorite. Endophytic bacteria were screened for L-asparaginase production using the rapid plate assay on Minimal Salt Medium with L-Asparagine as a substrate. Asparaginase-producing endophytes were detected by a pink zone formation on the agar, indicating the hydrolysis of asparagine into aspartic acid and ammonia, which changed the phenol red dye indicator from yellow (acidic conditions) to pink (alkaline conditions). The results revealed that out of the mangroves taken from the seaside, i.e., between sea and land, there were six bacterial colonies from the roots, 12 bacterial colonies from the stems, and 20 bacterial colonies from the leaves, each showing positive L-asparaginase. The isolated bacteria had varied colony morphology and different ability to produce the L-asparaginase enzyme.

Keywords: anticancer, endophyte bacteria, L-Asparaginase, mangrove, *Rhizophora mucronata*.

1. Introduction

L-asparaginase is an enzyme that catalyzes the hydrolysis reaction of L-asparagine into L-aspartate and ammonia [1]. L-asparaginase has potential in treating Acute Lymphoblastic Leukemia (ALL) and other malignant cancers [2]. In this case, L-asparaginase decomposes L-asparagine needed by leukemia cells so that the cell’s need for L-asparagine is not met. It results in inhibited cell growth and death of the leukemia cells [3]. This potential has been developed by researchers as an alternative treatment for cancer cells. L-asparaginase enzyme purified from *E. coli* and *Erwineachrysanthemi* is available and has clinical application in humans, but this treatment has side effects, such as allergies, cross-interactions, stimulation of the immune system, drug resistance, and non-specific L-glutaminase activity [4]. These side effects can be managed by the discovery of a new source of L-asparaginase, which is serologically different but has a similar therapeutic effect, thus requiring screening of samples from various sources [5].
The mangrove ecosystem is a coastal ecosystem composed of many vegetation and living things, with specific biological and physiological adaptations to environmental stresses [6]. The existence of these stresses causes the *Rhizophora mucronata* mangrove plant to make special adaptations through the production of specific metabolites or enzymes, which can be used in the pharmaceutical industry [7]. For this reason, this study was conducted to determine whether the leaves, stems, and roots of the *R. mucronata* mangrove plant in the WanaTirta mangrove area, Yogyakarta, contained endophytic microorganisms that can produce the L-asparaginase enzyme. In addition, *R. mucronata* is expected to produce suitable L-asparaginase-producing bacteria and can later be used for medical needs.

2. Materials and Methods

2.1 Sampling sites

Mangrove *R. mucronata* was taken from the ecotourism area of WanaTirta, KulonProgo, Yogyakarta. The samples used in this study were the roots, stems, and leaves of mangrove plants. Fresh samples were taken and put in newsprint. Samples were then stored in a refrigerator [7]. The following is an image of a map of the sampling locations for *R. mucronata* mangroves (Figure 1).

![Figure 1. Mangrove Sampling Location](Source: Google Maps, 2020)

The sampling technique was carried out based on the cluster sampling method. 10% of WanaTirta's mangrove area is divided into three areas: area 1 (coast side), area 2 (the part between the coast and land), and area 3 (landside). Each region has three sampling points with a distance of 200m in each area. In one area, samples were taken from three mangrove *R. mucronata* plants (Figure 2).

![Figure 2. Sample Point Design](Source: Google Maps, 2020)

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2.2 Screening of L-asparaginase-producing endophytic bacteria

Endophytic bacteria were isolated from samples of roots, stems, and leaves of *R. mucronata* mangrove. The sample surface was sterilized by washing the sample with running water. Samples were immersed in 70% alcohol for 30 seconds and 2% sodium hypochlorite for two minutes. The samples were then rinsed with 70% alcohol for 30 seconds and sterile ddH2O 3-4 times [8].

The sterile samples were cut to a length of 5 cm utilizing a sterile scalpel and inoculated on m9 media (D-Glucose 2 gr/l, Na2HPO4 6gr/l, KH2PO4 3gr/l, MgSO4. 7H2O 0.02gr/l, NaCl 0.5gr/l, CaCl2 0.002gr/l, Agar 3gr/l, L-Asparagine 5gr/l), using the patch method. The pH indicator used was 0.009% phenol red. The isolates were then incubated at 37°C for 24-48 hours. The pink zone around the colony indicated that the isolate contained L-Asparaginase. In addition, the screening was carried out with two repetitions [9]. Then, the isolate that grew around the sample was isolated.

2.3 Endophytic bacteria isolation

L-Asparaginase positive isolates were isolated using the streak-plate method. The isolates were incubated at 37°C for 24-48 hours [9]. The grown isolates were purified by the 4-quadrant streak-plate
method four times. Three isolates from all pure isolates were then observed for their morphology and tested for enzyme activity.

2.4 Enzyme Activity Measurement
In this study, measurement of enzyme activity estimation was carried out qualitatively and quantitatively. The qualitative test was conducted based on Gulati (1997) [10]. The isolates were inoculated on an m9 medium, modified by adding a substrate (Table 2) by the spot method. The pH indicator used was 0.009% phenol red. The isolates were incubated at 37°C for 24-48 hours. The size of the zone and the density of the pink zone were then observed.

Quantitatively, the enzyme activity estimation was performed based on the Nesslerization method. L-asparaginase positive isolates were grown on liquid M9 medium and incubated on a shaker incubator at 200 rpm at 37°C for 120 hours. Samples were centrifuged at 3000 rpm for ten minutes at 4°C to separate the filtrate and cells. Then, the supernatant was taken for estimation of enzyme activity measurement.

The reaction would be seen by adding 0.5 ml of the supernatant containing the enzyme into 1.7 ml of 0.04 M L-asparagine and 1 ml of 0.05 M Tris-HCl pH 8.6. The isolates were incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.5 ml of 1.5 M Trichloroacetic Acid (TCA). Then, 0.2 ml of Nessler's reagent was added to the tube, which contained 0.1 ml of the reaction mixture and 3.75 ml of ddH2O. Tubes were incubated at room temperature for ten minutes. The samples were then checked for a spectrophotometer with an absorbance of 450 nm. The blanks employed were supernatant and TCA. In this regard, one unit of the enzyme L-asparaginase is defined as the amount of enzyme that breaks down 1μmol ammonia for one minute at 37°C [9].

3. Results and Discussion
3.1 Screening of L-asparaginase enzyme-producing bacteria
Endophytic bacteria were isolated from the roots, stems, and leaves of the mangrove plant R. mucronata and could grow well on minimal M9 media that had been modified with the addition of L-Asparagine as a substrate. M9 media was used in this study because the media contained minimal nutrients that would not affect the pH conditions of the media when bacterial growth occurred. Thus, changes in the pH of the media would occur due to the conversion of L-Asparagine by bacteria and not due to other factors.

The results obtained in the screening process were colonies growing around the sample (roots, stems, leaves) of plants. Colonies that grew around the sample were the result of bacterial growth in plant tissue. According to Nelson (2012), endophytic microbes are microorganisms found in tissues and are not pathogenic for plants [11]. Figures a, b, and c are L-Asparaginase positive endophytic bacterial colonies, while Figure 3d is a bacterial colony that could not produce the L-Asparaginase enzyme. In addition, the size of the zone and the density of the pink color in each colony varied, depending on the ability of the bacteria to produce the L-Asparaginase enzyme. The denser the pink zone and the wider the pink zone, the greater the ability of bacteria to produce the L-Asparaginase enzyme.

Bacterial colonies capable of producing L-Asparaginase enzymes will break down the substrate (L-Asparagine) into L-Aspartate and ammonia. The release of ammonia due to enzyme activity will result in increased pH. An increase in pH will cause the medium to become pink [10]. From the three areas studied (coast side, between ocean and land, and land side), there were six positive L-Asparaginase colonies in root samples, 12 colonies in stem samples, and 20 colonies in leaf samples (Table 1).

Leaf samples had the highest number compared to root and stem samples. Meanwhile, the root sample had the least number of colonies compared to the leaf and stem samples. The result obtained in each isolation is different. Prihanto’s [7] research succeed isolating 6 isolates of L-Asparaginase positive bacteria from Excoecaria agallocha mangrove and most of them were found in stems [7]. The number of isolates were obtained in this study was more than the result of the isolates of Prihanto’s study [7]. But it less than the result of the isolation of L-Asparaginase bacteria from other samples. There were 45 isolates of L-Asparaginase bacteria from Glycyrrhiza glabra roots in Karkhane’s research [8].
Figure 3. Colonies growing around the roots, stems, and leaves of the *R. mucronata* mangrove plant; (a) Positive bacterial colonies around the roots. (b) Positive bacterial colonies around the leaves. (c) Positive bacterial colonies around the stem. (d) Negative bacterial colonies around the stem.

Table 1. Number of Endophytic Bacterial Colonies on *R. mucronata*

| Sample | Number of Colonies |
|--------|--------------------|
| Root   | 6                  |
| Stem   | 12                 |
| Leaf   | 20                 |

3.2 Bacterial isolate purification

Three isolate from all isolated with the largest pink zone were purified. Purification was carried out using the 4-quadrant streak-plate method. It is supported by Ibrahim’s (2015) statement that the process to obtain a single isolate of a bacterium can be done by zigzag scratching the inoculant on a quadrant dish. The purification process was carried out four times.

The purification process resulted in colonies that did not spread, and separate colonies were obtained. Three types of isolates were identified by morphology. The morphological identification results obtained were that isolate 1 had an irregular shape, elevation raised, undulate margins, wrinkles on the colony surface (curled), bone-white in color, and was opaque to light (Figure 4a, d). Isolate 2 had a bone-white colony morphology, circular form, elevation raised, undulate margins, opaque colonies, and a solid white core (Figure 4b, e). Isolate 3 was grayish-white with circular colonies, elevation raised, undulate margins, and transparent (translucent) colonies. In the center of the colony of isolate 3, there was a gray core (Figure 4c, f).

In Prihanto’s research [7], most of the L-Asparaginase enzyme-producing bacterial isolates from the Rhizophora mangrove plant had a rounded colony morphology, with undulated margins, white colonies, and a convex elevation (raised)[7]. The morphological colony characteristics are in accordance with the isolate 2 morphology obtained from the bacterial isolation process carried out.
3.3 Enzyme activity measurement

The bacterial isolates obtained were measured for enzyme activity using the Nesslerization method. According to Shakambari [12], the method of determining enzyme activity can be done by determining the amount of ammonia released during the reaction due to the degradation of L-Asparagine [12]. Nessler's reagent serves as an indicator of color change due to the release of ammonia during enzymatic reactions.

The measurement results of the L-Asparaginase enzyme showed that isolates 1, 2, 3 had different abilities to produce L-Asparaginase enzymes. Based on the quantitative test, bacterial isolate 1 had the lowest activity of 29.1 IU/mL compared to bacterial isolates 2 and 3. It was evidenced by the low absorbance value. The highest activity of L-Asparaginase enzyme production was found in isolate 3, with a total enzyme value of 32.5 IU/mL (Table 2).

| Isolate | Absorbance value | Ammonia Amount (µmol/L) | Enzyme Amount (IU/mL) |
|---------|------------------|-------------------------|-----------------------|
| Isolate 1 | 1.022            | 95.4                    | 29.1                  |
| Isolate 2 | 1.125            | 105.8                   | 32.8                  |
| Isolate 3 | 1.134            | 106.7                   | 32.5                  |

The enzymatic value is vital to evaluate the efficiency of the enzyme. In addition, the amount of enzyme is directly proportional to the absorbance value and the amount of ammonia. The greater the L-Asparaginase enzyme activity, the greater the enzyme activity degrading L-Asparagine into L-Aspartate and ammonia. The greater the ammonia formed, the more concentrated the solution, resulting in a higher absorbance value.

Figure 4. Purification of 4 quadrant bacteria. (a) Colony 1 front view. (b) Colony 2 front view. (c) Colony 3 front view. (d) Colony 1. (e) Colony 2. (f) Colony 3.
4. Conclusion
Isolation of endophytic bacteria producing L-asparaginase enzyme in R. mucronata mangrove plants obtained six positive colonies of L-asparaginase in root samples, 12 colonies in stem samples, and 20 colonies in leaf samples. Three isolate with the largest pink zone were purified. Each isolate had a different ability of L-Asparaginase enzyme production activity. The highest activity was owned by isolate 1, while the lowest activity was owned by isolate 2.

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References
[1] Shi R, Liu Y, Mu Q, Jiang Z, and Yang S 2017 Intrenat. J. of Biological Macromolecules.96 93-99
[2] Shrivastava AAA, Khan M, KhursidMA, Kalam SK, Jain, dan Singhal PK 2016 Critical Reviews in Oncology/Hematology. 100 1-10
[3] Narta UK, Kanwar SS, dan Azmi W 2007 Critical Review in Oncology/Hematology. 61 208-221
[4] Kotzia GA, Labrou NE 2007 J. Biotechnol.127 657-669
[5] Joshi R.D and Kulkarni NS 2018 DAV Int. J. of Science. 7(1) 1-8
[6] Saru A 2013 MengungkapPotensiEmas Hijau di Wilayah Pesisir (Makasar: Masagena Press)
[7] Prihanto AA, Firdaus M, dan Nurdiani R 2011 J. of Food and Engineering 1 386-389
[8] KarkhaneM, Lashgarian HE, Mirzaei SZ, Marzban A 2020 Biointerface Research in Appl. Chem. 11(2) 9113-9125
[9] AbhiniKN, Zuhara KF 2018 J. of Chem. and Pharm. Sci.. 11(1) 73-76
[10] Gulati R, Saxena RK, Gupta R 1997 Lett. In appl. microbiology. 24(1) 23-26
[11] Nelson., K. E. Nelson., B. J. 2012. Genetic Diversity of Microbial Endophytes and Their Biotechnical Applications. In: Strobels G (ed) Genomics applications for the developing world, advances in microbial ecology. (New York: Springer Sciences).
[12] Shakambari., G. Ashokkumar., B, and Varalakshmi., P 2019 J of Biocatalyst and Agricultural Biotech. 17 213-224