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Bacillus subtilis* UBTn7, a potential producer of L - Methioninase isolated from mangrove, *Rhizophora mucronata

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Abstract. L-methioninase is an enzyme that degrades sulfur-containing amino acids to α -keto acids, ammonia, and thiols. L-methioninase could be found in plants, bacteria, and fungi. The aims of this study was to obtain L-methioninase-producing endophytic bacteria isolated from mangrove *Rhizophora mucronata*. The mangrove was collected from Jenu Beach, Tuban, East Java, Indonesia. The samples were roots, stems, and leaves of *Rhizophora mucronata*. Endophytic bacteria were pure isolated using LB agar medium. Each bacteria were screened its capability to produce L-methioninase using selective media namely modified Czapek Dox agar. The best producer of enzyme was further identified with morphological and biochemical analysis. The result showed that three bacteria produced L-methioninase. Based on the result of morphological and biochemical analysis, the best producer was *Bacillus subtilis* UBTn7.

1. Introduction

L-methioninase is an enzyme that degrades sulfur-containing amino acids to α -keto acids, ammonia, and thiols. It belongs to the family of lyases enzyme, a class of carbon-sulfur lyases [1]. This enzyme is found in many microorganisms and has a specific substrate that varies depending on the type of microorganism. In addition, this enzyme also has different physiological characteristics. For example, the type of bacteria *P. gingivalis* acts as a determinant of its pathogenicity, whereas in some groups of anaerobic bacteria, this enzyme plays a role in the metabolism of ATP [2].

The potential application of this enzyme in the health sector is as a cancer inhibiting agent, especially in acute lymphoblastic leukemia. The basis of this application is the fact that cancer cells often have unique characteristics and generally require higher nutrients. Furthermore, the restriction on nutritional components that become precursors or limit the development of these cells will be able to inhibit the growth of cancer cells.

Rhizophora mucronata is an unexploited source for endophytic microorganism. Endophytic bacteria plays an important role in the ecosystem. Some of their capabilities include nitrogen fixation, sulfate reduction, etc. Endophytic bacteria is a source for new and unique metabolites [3, 4]. The expectation in the exploration of *Rhizophora mucronata* is to gain bacteria that produces unique metabolites, such as high salt stability due to its extreme habitat.

Jenu Beach, Tuban is fully covered by mangrove plants. This natural resource has largely been free from exploitation and has a large diversity of mangrove. However, there is no exploration of this diversity to the best of our knowledge. Moreover, several studies have indicated that the coastal area is a myriad source for mining bioactives such enzymes and pharmaceutical compounds [5]. In this study, the mangrove *Rhizophora mucronata* was explored as a source of endophytic bacteria which produces L-methioninase.



We also report the preliminary exploration of L-methioninase producing endophytic bacteria isolated from the mangrove, *Rhizophora mucronata*.

2. Methodology

The equipment used in this study were glass beakers, erlenmeyer, measuring glasses, autoclaves, incubators, pH-meters, digital scales, petri dishes, micro pipettes, stirrers, refrigerator, freezer, mortar and pestle, and thermocyclers. The chemical compounds for this research were DNA extraction kit, bromothymol blue (BTB), aquades, yeast extract, pepton, NaCl, agar, D- glucose, L-methionine, Na₂HPO₄, KH₂HPO₄, MgSO₄.7H₂O, CaCl₂, NaOH, HCl. All chemical compounds were analytical grade.

2.1. Sample preparation

Rhizophora mucronata were obtained from Jenu Beach, Tuban, which is located in East Java, Indonesia. The samples used were the parts of the mangrove such as the root and leaves. The isolation of the mangrove bacteria was according to the method of Prihanto *et al.* [6] with modification.

2.2. Culture of the endophytic bacteria

All samples were washed with tap water to remove soil and dust. The samples were then surface sterilized using ethanol 75 % (1 min), 0.5 % sodiumhydrochloride (3 min), and ethanol 75 % (30 s). Lastly, they were rinsed with sterile aquadest. The samples were then grinded and diluted. One mL of aliquot was plated onto LB agar (LB; 10 g/L pepton, 5 g/L Yeast extract, 10 g/L NaCl, 15 g/L agar) and the plates were incubated for 24-48 hours with the temperature of 37°C. Prior to the collection of pure culture, the purity of each colony was checked by using the steak quadran method.

2.3. Screening of L-methioninase producing bacteria

The screening method followed Gullati *et al.* [7] while the medium for the screening of L-methioninase bacteria was Modified Czapek Dox (MCD). The composition was 6 gr/L Na₂HPO₄, 2 gr/L, KH₂PO₄, 0.5 gr/L NaCl, 20 gr/L L-methionine, 2 gr/L glucose, 0.2 gr/L MgSO₄, 0.005 gr/L CaCl₂, 20 gr/L agar and 0.007 % BTB. pH for the medium was set at 5.5 - 6. All tested bacteria were plated onto MCD and incubated at 37°C for 24-48 hours.

2.4. Bacterial identification

Bacterial identification was carried out using the Microbact kit analysis. This test is highly dependent on the results of the preceding oxidase test; if the result of the oxidation is positive then the test is done using the microbact 24E, and if it is negative the microbact 12E will be used. The microbact 24E or 12E was performed based on the manufacture protocol. In addition, the bacteria was also identified its gram type with the standard gram analysis procedure.

3. Result and discussion

3.1. Isolation of endophytic bacteria

L-methioninase enzyme-derived bacteria were isolated from the *Rhizophora mucronata* mangrove plant from the Tuban Regency. It was diluted until it reached the appropriate number for the isolation of pure colony. The culturable endophytic bacteria from each part of the mangrove is shown in Table 1. The colonies were derived from the roots, stems and leaves of *Rhizophora mucronata* and it appeared that each part of the sample had culturable bacteria.

Table 1. Culturable endophytic bacteria isolated from *R. mucronata*.

Sampel	Code	Cultured colony
Root	R1	1
Leaves	L1	1
Stem bark	S1, S2	2

Four colonies were obtained from the mangrove. Each isolate was then subjected to the screening process to test its capability to produce L-methioninase. The result of the L-methioninase screening showed that only one of the bacterial isolates produced L-methioninase (figure 1.). This is marked by the formation of a blue color surrounding the tested colony on the screening media. The formation of the blue color is due to the production of ammonia that will subsequently react with BTB which indicates the pH change in the media.

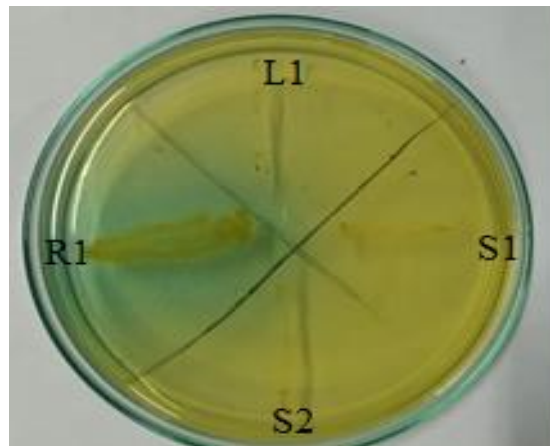


Figure 1. Positive L-methioninase producing bacteria. (L_1 = strain 1 from leaf, S_1 = strain 1 from stem, S_2 = strain 2 from stem, L_2 = strain 2 from leaf).

A larger the blue zone surrounding the isolates shows a greater activity of bacteria in producing L-methioninase. According to Suganya et al (8), L-methioninase generates ammonia from its catalysis. Selective media which was added with BTB (Bromothymol Blue) had a yellow color and turned to blue due to the change in the pH. The best isolate is the code samples of R1 which is the result of isolates from the root of the mangrove. It was further identified by using the Microbact system.

3.2. Bacterial Identification

Gram staining revealed that the isolates bacteria is a gram positive bacteria (figure 2). Further analysis using microbact system was conducted using microbact 24E.

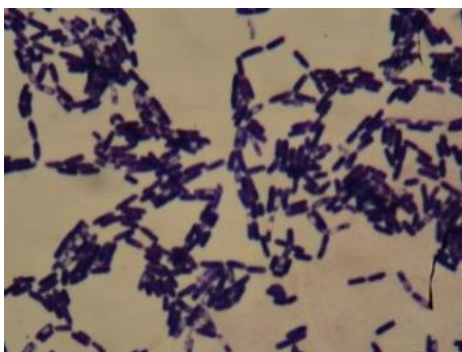


Figure 2. Gram staining of isolate R1.

The microbact system test is a test used to identify microorganisms which is highly dependent on the results of the preceding oxidase test. If the result of the oxidation is positive then the test is done using microbact 24E; while if the result is negative the microbact 12E will be used. This system is a standard micro-substrate system designed to simulate conventional biochemical substrates used to identify negative gram bacilli. The first stage of this test is placing the pure isolate in a centrifuge at 3000 rpm for 15 minutes. From the results of the centrifuge the supernatant and pellet was obtained but only part of the pellet was used. The resulting pellets were then given 5 ml of NaFis and then fed on Microbact 24E or 12E depending on the oxidation yield of 0.1 ml and incubated for 18-24 hours at 37°C. Evaluation of the results can be seen in the microbact wells, whether the results are positive or negative can be seen by comparing it with the color chart and the results are written on the Patient Record form. The octal numbers are derived from the sum of positive reactions alone from each group. The bacteria species can be identified from this total number by entering data into computer software [9].

The Microbact analysis revealed that the isolates R1 is *Bacillus subtilis* (table 2). Since this is a new strain from the coastal of Jenu, we have further named this bacillus as *Bacillus subtilis* UBTn7

Table 2. The result of microbact test.

No.	Biochmeical test	Research	Bergey's	Reference
1	Spore	+	+	+
2	Oxidase	+	+	+
3	Motility	-	+	+
4	Nitrate	+	+	-
5	Lysin	-	Not tested	-
6	Ornithin	-	Not tested	-
7	H ₂ S	-	Not tested	-
8	Glucosa	+	+	+
9	Mannitol	+	+	+
10	Xylose	+	+	+
11	ONPG	+	Not tested	+
12	Indole	-	Not tested	-
13	Urease	-	Not tested	-
14	V-P	+	Not tested	+
15	citric	-	+	-
16	TDA	-	Not tested	-
17	Gelatin	-	+	-
18	Malonat	-	Not tested	-
19	Inositol	-	Not tested	-
20	Rhamnose	-	Not tested	-

21	Sucrose	-	+	-
22	Lactose	-	-	-
23	Arabinose	+	+	+
24	Adonitol	-	Not tested	-
25	Raffinose	-	Not tested	-
26	Salicin	-	Not tested	-
27	Catalase	+	+	+
28	Arginin	-	Not tested	-
29	Coagulase	-	Not tested	-
30	Beta Hemolysis	+	Not tested	+
32	Starch hydrolysis	+	+	+
33	Casein hydrolysis	+	+	+

The bacteria is a motilbacteria with bacilform with a colony diameter of 3.11 mm, has a oxidase reaction and is catalase positive. The isolate were creamy and globular colonies with uneven edges to the colony, flat, and having a wet consistency. *Bacillus subtilis* is a gram-positive bacteria, with the form of the stems forming a chain; there are several species that are aerobic obligate and also anaerobic facultative; and have endospores as a structure to be able to survive harsh environmental conditions [10]. *Bacillus subtilis* is a non-pathogenic and non-toxic bacteria that is gram-positive and has characteristics that is much used in the production of food-grade enzymes. This bacteria is a saprophytic microorganism that can be found everywhere, usually in water, land, air, and residue of a decaying plant.

4. Conclusion

L-methioninase-producing endofit bacteria is able to be isolated from the *Rhizopora mucronata* mangrove derived from Jenu Beach, Tuban regency, East Java. The best isolate in producing l-methioninase is R1 which is known as *Bacillus subtilis* UBTn7.

5. References

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