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AA Prihanto[1,2,3*], G Swardhika[1], and KA Pradarameswari[1,3]

[1]Department of Fishery Product Technology, Faculty of Fisheries and Marine Science, Brawijaya University, Jl. Veteran Malang 65145, East Java, Indonesia
[2]Halal Thoyib Research Center, Brawijaya University, Jl. Veteran Malang 65145, East Java, Indonesia
[3]BIO-SEAFOOD, Reasearch Unit (Aquatic Biotech Exploration Sustainable), Faculty of Fisheries and Marine Science, Brawijaya University, Jl. Veteran Malang 65145, East Java, Indonesia

[*]Corresponding author: asep_awa@ub.ac.id

Abstract. Mangrove ecosystem is a reservoir for natural product such as enzyme. One of the enzymes which able to be isolated from mangrove is L-asparaginase. The aim of this research was to obtain endophytic fungi which capable of producing L-asparaginase from mangrove. Sonneratia alba was obtained from Aeng Sareh Beach, Madura. Endophytic fungi were isolated from leaf, stem, and root of Sonneratia alba. The fungi were screened its capability to produce L-asparaginase using selective media namely modified Czapex Dox agar. The best producer of L-asparaginase was further identified with microscopic and macroscopic identification method. The result showed that seven strain produced L-asparaginase. Based on the analysis, Trichoderma sp. was the best producer for L-asparaginase.

Keywords: L-asparaginase, endophytic, mangrove, Trichoderma sp.

1. Introduction

Mangrove ecosystem is an abundant reservoir for many bioactives. Bioactive compounds can be obtained from estuary. Marine microbes which were isolated from estuary and coastal area exhibited bioactive substances with unique pharmaceutical characteristic [1]. Both pharmaceutical compounds and enzymes which had application on health and food can be obtained from marine area [2]. Hence, by exploring the mangrove ecosystem will probably obtain a unique enzymes.

In recent years, abundant producers of L-asparaginase from coastal and marine area was reported [3]. Many, indicated the high producer of L-asparaginase. From the other source, bacteria and fungal isolates were also reported. *Aspergillus terreus, Aspergillus niger, Cladosporium* sp., *Alternaria* sp. and *Fusarium* sp. which were isolated from algae were reported t*o* produce L-asparaginase [4] . The objective of this study is to achieve the L-asparaginase producer from endophytic fungi which was isolated from mangrove, *Sonneratia alba*.

2. Methodology

The chemical compounds for this research were bromothymol blue (BTB), D-glucose, PDA *(Potato Dextrose Agar)* $NaHPO_4$, KH_2HPO_4, $MgSO_47H_2O$, CaCl, HCl, NaOH, and modified Czapek Dox Medium. All chemical compounds were analytical grade. All chemical compounds were purchased from Sigma-Aldrich co.

2.1. Location and Collection of Plant

Plant materials were collected from Aeng Sareh Beach (7°22'00.58''S, 113°20'28.60''E), Madura Island, East Java, Indonesia. The mangrove species, *Sonneratia alba* was used for present study. Plant parts (leaf, stem, and root) were cut off with ethanol-disinfected cutter. Each part was placed separately in sterile polythene bags to avoid moisture loss. The materials were transported to laboratory within 12 h and stored at 4 °C until isolation procedures were completed.

2.2. Isolation of Endophytic Fungi

The isolation of endophytic fungi followed the method [5]. The samples were washed thoroughly with sterile distilled water. The materials were then surfaces sterilized using ethanol 75% (1 min), 0.5% sodium hydrochloride (3 min), and ethanol 75% (30 s) and rinsed thoroughly with sterile distilled water. The samples were grinded using sterile mortar. 1 g of samples was put in 9 mL NaFis and vortexed. Furthermore, 1 mL of aliquot was plated onto potato dextrose agar (PDA; 12 g Difco potato dextrose broth, 20 g agar/L, with streptomycin 100 mg/L) using spread plate technique. The plates were then incubated at room temperature until fungal growth appeared (1-2 weeks). Each fungal colony was transferred into PDA slant tubes for purification of fungal strain. The fungal isolates were identified based on their morphological.

2.3. Screening of L-asparaginase producing fungi

The screening method followed [6] while the medium for the screening of L-asparaginase was Modified Czapek Dox (MCD). The composition was 6 gr/L Na_2HPO_4, 2 gr/L, KH_2PO_4, 0.5 gr/L NaCl, 20 gr/L L-methionine, 2 gr/L glucose, 0.2gr/L $MgSO_4$, 0.005 gr/L $CaCl_2$, 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. Fungal identification

Two fungal identification have been used, which were microscopic and macroscopic observations. The macroscopic morphology observation was based on the pure culture plate of the fungi sample which was incubated at room temperature (27 °C) for three to four days. The growth rate, colour and elevation of the colony were observed. For microscopic observation, a very small amount of the fungus are stained with lactophenol cotton blue (LPCB). Visualization was done under under a microscope. Data and photos from microscope observations were matched with the fungus atlas Pictorial Atlas of Soil and Seed Fungi [7].

3. Results and Discussion

3.1. Screening of L-asparaginase producer

The endophytes were isolated using mycological medium, namely potato dextrose agar (PDA). A total of seven strains were obtained from the leaves, stems and roots of *Sonneratia alba*. Endophytic fungi can be found in all plant parts. Isolation of fungi from parts of mangroves have cultured and incubated for 3-5 days (Figure 1). Based on Figure 1, some fungus have different results. All fungus would be screened to find the fungus L-asparaginase-producer. The screening process by Modified Czapek Dox (MCD) medium indicated that All isolates produce-asparaginase. The result was depicted on Figure 2.

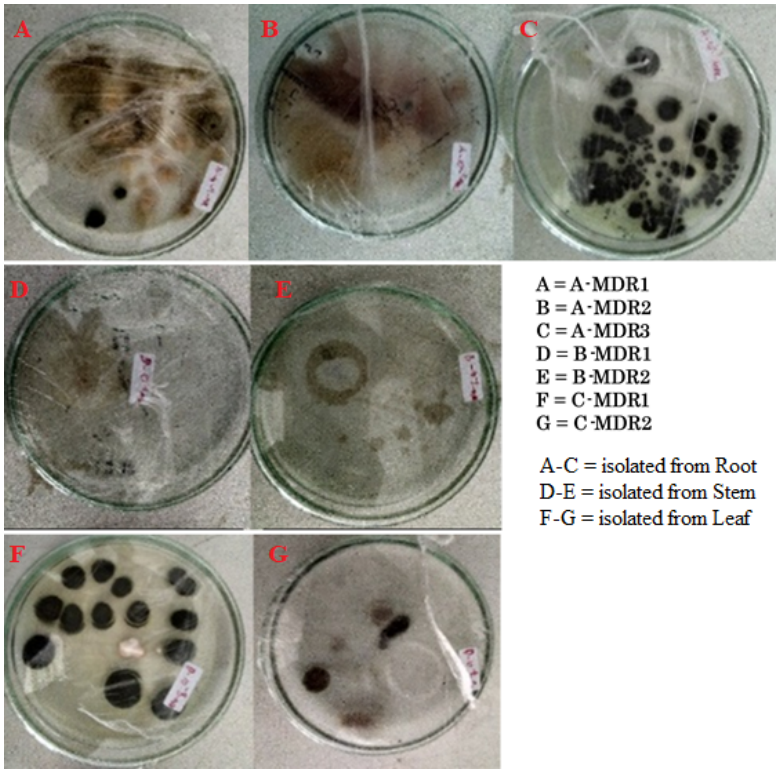

Figure 1. Endophytic fungal isolates from Sonneratia *alba*

Results of screening on the last day of incubation showed significant color changes of the endophytic fungal isolates. Screening results of enzyme activity zone values by assigning a value (+). The (+) value in table refer to the blue density level of the media in Table 1.

Table 1. Zone of enzyme activity

NO	Sample code	Zone of enzyme activity
1	A –MDR1	++
2	A – MDR2	+
3	A – MDR3	+
4	B – MDR1	++
5	B – MDR2	++
6	D – MDR1*	+++
7	D – MDR2	+

Notes : (+) Zone of low enzyme activity
(++) Zone of medium enzyme activity
(+++) Zone of less enzyme activity

Screening of L-asparaginase producers indicated that all seven positive produced L-asparaginase. The highest yield obtained mushrooms with code D-MDR1 which was isolated from stem. The highest producer was determined based on the time needed for the fungi to excrete L-asparaginase. The highest was indicated by the triple plus. The triple plus indicate that the fungi excrete L-asparaginase in the first to second days of the culture (24 hour). Hence, it produced the most concentrated color (Figure 2.). Furthermore, B - MDR1 and B-MDR2 produced less active. Judging from the result, further analysis was performed only for D-MDR1.

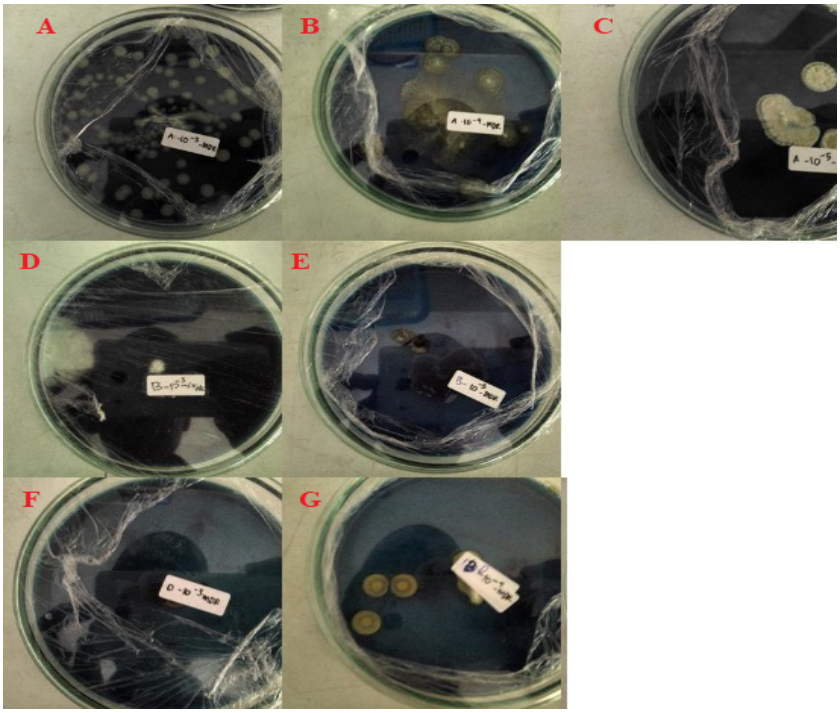

Figure 2. Endophytic fungi isolates from *Sonneratia alba* incubation for five days

3.2. Macroscopic and Microscopic Morphology Observation

Morphological observations on fungi have been performed from D-MDR1 endophytic fungi include color, surface, spores and hyphae. Color of the isolates is slightly greenish color, large and grouped from several colonies. It indicated the similar colony character of the fungus *Trichoderma sp*. Macroscopic of fungi isolated is presented on Figure 3.

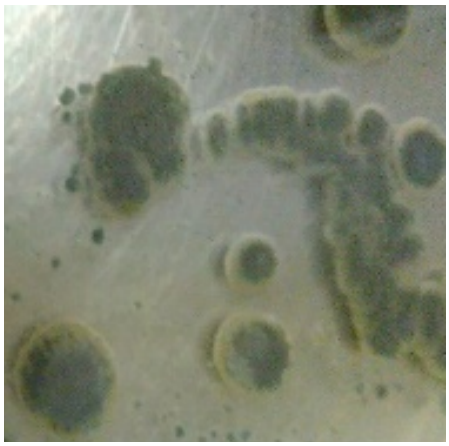

Figure 3. Macroscopic characteristic of D-MDR1 isolate

Based on macroscopic observation, the color of the mushroom is greenish green with the mycelium. It conidiophore branched. The existence of oval shape and single phialide also convinces the assumption that the fungus is *Trichoderma sp*. Based on the results of microscopic fungal observation, other traits obtained from the interal have conidia, conidiphore and fialid and several other parts. Figure 5 shows the results of microscopic fungal identification

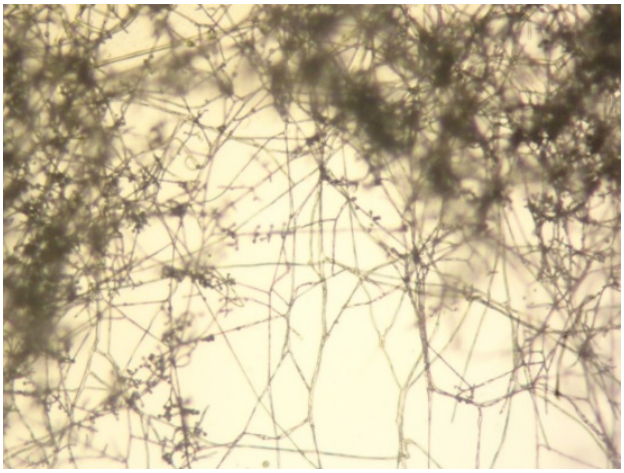

Figure 4. Microscopic characteristic of D-MDR1 isolate

Conidiophores of microscopic results are clear / bright, branched perpendicular to many, single fidium or also in groups. Conidia is bright / clear, one cell, ovoid, grows from inside / from the tip of a small group; young are recognized by their growth velocity and parts such as bearing a green membrane, generally saprophyte in soil or wood, there are several species that are parasitic to other fungi.

Figure 5. Comparison of phialides fungal (A) and fungal Atlas (B)

Figure 5 showed the similar characteristic between observed fungi and *Trichoderma* sp The similarity was single and long spore and conidiophores. In the phialide [8]. It carries mold spores. Usually, phialide mushrooms has a size of 8.5-11 x 2.4-2.7µm. The size of conidia has 2.4-2.7 x 2.1-2.5µm [7]. In general, colonies grown on this PDA have a rather greenish yellowish color, and are shaped like crystals In this study, *Trichoderma* sp. exhibited potential bioactivity producing L-asparaginase. Based on macroscopic and microscopic observation, the D-MDR1 were most likely *Trichoderma* sp.

4. Conclusion

Seven isolates have been successfully isolated from mangrove, *Sonneratia alba*. All fungi able to produce L-asparaginase. Based on macroscopic and microscopic analysis, the best producer for L-asparaginase is presumably as *Trichoderma* sp.

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