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To cite this article: A A Prihanto *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **230** 012101

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# *Aspergillus* sp. as a potential producer for L-Asparaginase from mangrove (*Avicennia germinans*)

A A Prihanto<sup>1,2,3</sup>, I O Caisariyo<sup>1</sup> and K A Pradarameswari

<sup>1</sup> Department of Fishery Product Technology, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Malang, Indonesia

<sup>2</sup> BIO-SEAFOOD Research Unit, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Malang, Indonesia

<sup>3</sup> Halal Thoyib Research Center, Universitas Brawijaya, Malang, Indonesia

E-mail: asepa\_awa@ub.ac.id

**Abstract.** L-asparaginase, (EC 3.5.1.1) is an important enzyme for food and non-food industries. In food industry, it is used to inhibit the formation of acrylamide compounds. In non-food industry, it can be used as anticancer agent. The objective of this study was to obtain L-asparaginase-producing fungi from mangroves, *Avicennia germinans*. The first step of this research was to isolate and screen L-asparaginase-producing fungi. The second step was to identify the species by using macroscopically and microscopically method. Nine fungal isolates were successfully isolated. Four isolates, P2, D1, B4, A4 were L-asparaginase positive. The D1 isolate had the highest activity. Based on macroscopic and microscopic analysis result, it can be concluded that the D1 is *Aspergillus* sp. This finding is the first report on the fungal endophytic fungi from mangrove (*Avicennia germinans*) which produced L-asparaginase.

## 1. Introduction

L-Asparaginase (EC 3.5.1.1) is an enzyme that can hydrolyze L-asparagine to aspartic acid and ammonia [1]. L-asparaginase can be used in the field of food processing for reducing the risk of acrylamide formation, because it reduces asparagine levels [2]. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma [3].

It has been observed that eukaryote microorganisms such yeast and fungi have a potential for asparaginase production. For example, the mitosporic fungi genera such as *Aspergillus*, *Penicillium*, and *Fusarium*, are commonly reported to produce asparaginase. Endophytic fungi are microorganisms that colonize internal plant tissue. They can live there for all or part of their life cycle without causing any apparent damage or disease [4]. These endophytes can be transmitted from one generation to the next through tissue of host seeds, vegetative propagules, or horizontally, external to host tissue, by spores [5].

Mangrove is a reservoir of terrestrial and marine microorganisms. This area is a unique habitat for many organisms. The marine ecosystem is a rich source of enzymes [6, 7]. The mangrove habitat has proven to be a rich source of new fungal species and these now form the second largest ecological subgroup of marine fungi. Mangroves are plants used in folklore medicine, and extracts from mangroves



and mangrove-dependent species have proven activity against human, animal and plant pathogens but only limited investigations have been carried out to identify the metabolites responsible for their bioactivities [8]. Some of the mangrove plants that have been studied for their endophyte associations including *Rhizophora apiculata* [9], *R. mucronata* [7] *Aegiceras corniculatum*, *Avicennia marina*, *A. officinalis*, *Bruguiera cylindrica*, *Ceriops decandra*, *Excoecaria agallocha* and *Lumnitzera racemosa* [10]. To our best knowledge, the screening on fungal endophytic from *A. germinans* was never be conducted. Therefore, further exploration of the potential of endophyte fungi from *Avicennia germinans* is needed as a new source of L-asparaginase.

## 2. Materials and Method

### 2.1. Location and collection of plant

Plant materials were collected from Bentar Probolinggo Beach, East. The mangrove for the present study is *Avicennia germinans*. Three parts of plant (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 performed.

### 2.2. Isolation of endophytic fungi

Isolation method followed [11]. The samples were washed again 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 buffer and vortexed. Furthermore, 1 mL of aliquot was plated onto potato dextrose agar (PDA; 12 g Difco potato dextrose broth, 20 g agar/L, supplemented with streptomycin 100 mg/L) using spread plate technique. The plates were then incubated at room temperature until fungal growth appeared (1-3 weeks). Each fungal colony was transferred into PDA slant tubes for pure culture of fungal strain. The pure fungal isolates were identified based on their morphological.

### 2.3. Screening of L-asparaginase producing fungi

The screening method followed [12]. The medium for the screening of L-asparaginase was Modified Czapek Dox (MCD). The composition was 6 gr/L Na<sub>2</sub>HPO<sub>4</sub>, 2 gr/L, KH<sub>2</sub>PO<sub>4</sub>, 0.5 gr/L NaCl, 20 gr/L L-methionine, 2 gr/L glucose, 0.2gr/L MgSO<sub>4</sub>, 0.005 gr/L CaCl<sub>2</sub>, 20 gr/L agar and 0.007 % BTB. pH for the medium was set at 5.5 - 6. The fungal media isolates turn to blue colour were accounted as L-asparaginase positive.

### 2.4. Fungal identification

Fungal identification in these research were using microscopic and macroscopic. The macroscopic morphology observation was based on the pure culture plate of the fungi sample which was incubated at room temperature (28 °C) for three to four days. The growth rate, colour of the colony and texture of the culture were the most remarkable features during this observation. For microscopic observation, a very small amount of the fungus are stained with lactophenol cotton blue (LPCB) for better cells and cell components visualization under a microscope. From the microscopic observation, the colour, shape, appearance and arrangement of the fungus structures were identified.

Procedure for conducting microscopic observation was preparing tools and ingredients in the form of sterile cotton in sterile petri dishes, then added distilled water to soaking all the cotton. Object glass is placed on cotton, then cut off PDA media with a rectangular shape and placed at the end of a glass object. The next stage, the fungi starter was scratched on all four sides of the PDA media, followed by incubation for 5-6 days. After that, coverslip is taken with tweezers, and put on an object glass that has been dripped with Lacto Phenol Cotton Blue (LPCB) and placed on the preparation. Next, observed with 100x and 400x magnification using a condenser. Data and photos from microscope observations were matched with the fungus atlas Pictorial Atlas of Soil and Seed Fungi.

### 3. Results and Discussion

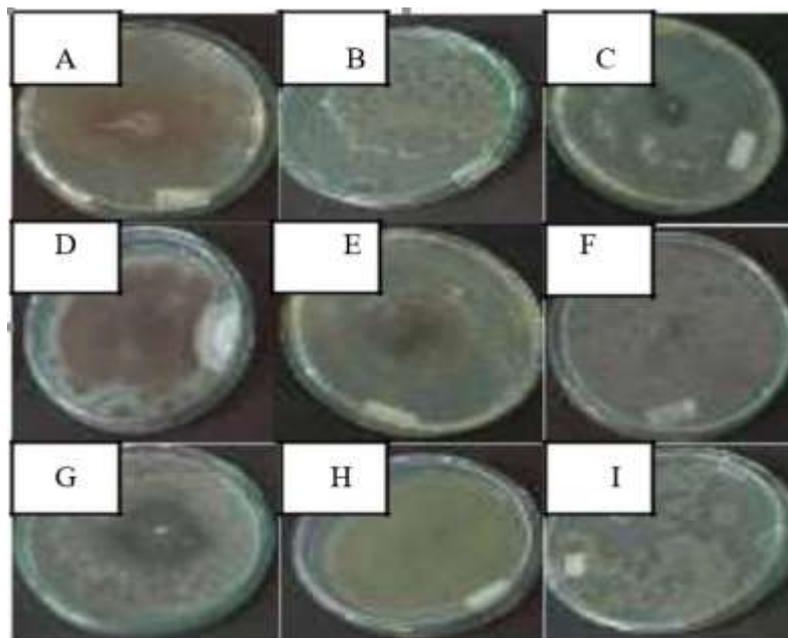
#### 3.1. Isolation of fungus

Fungal were obtained from three samples of leaf, steams, and roots (Figure 1)



**Figure 1.** Isolation of *Avicennia germinans* (A) Root, (B) steams, (C) leaves.

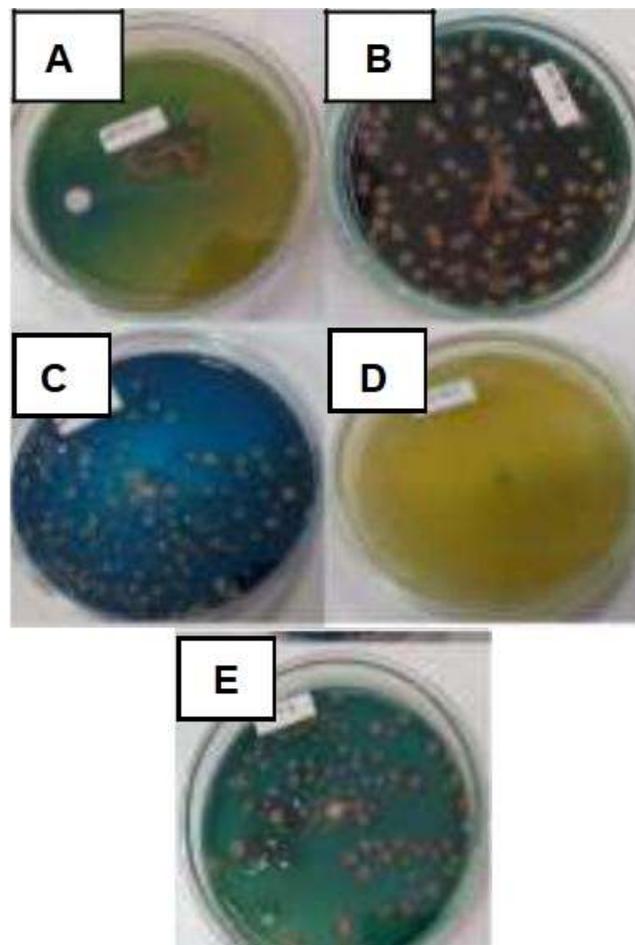
Nine fungal isolates were successfully isolated and purified. The purified fungal is shown in Figure 2.



**Figure 2.** Fungal pure culture isolated from mangrove. (A) A2, (B) A3, (C) A4, (D)D1, (E) D2,(F) D4, (G) B1, (H) B3, (I) B4.

A2 had yellowish brown spores and transparent hyphae, yellowish brown. A3 is greenish spores and spreads. A4 is centered black spores in the middle, hyphae are transparent. D1 is brown, black beige spores tend to gather in the middle. D2 had brown spores and transparent hyphae. D4 had brown spores spread and there was a clear transparent hypha. B1 has brownish black spores and thick transparent hyphae. B3 has green and centered spores. B4 has greenish spores.

Czapek Dox Modified Agar (CDMA) was used as a screening media to determine the activity of L-Asparaginase. The change of color on the media will indicate the L-Asparaginase activity.



**Figure 3.** Endophytic fungi isolates from *Aecennia germinans* incubation for 5 days in CDMA. A=D2, B=D1, C=B4, D=B1, E=A4.

The media which turn to black blue indicated L-Asparaginase positive. This color changes due to BTB (bromothymol blue) and it is because of the change of the pH medium. The color of screening media turned to yellow in acidic pH conditions. Contrastingly, it turned to blue when the pH medium is base. This base condition is because of production of ammonia on the L-Asparaginase reaction toward hydrolysis of Asparagine to Ammonia and Aspartate. Hence, when the media remained yellow, the L-asparaginase did not presence or notably low.

From the results, four positive isolates were detected for producing L-asparaginase. The highest yield was D1 (sample isolated from Leaf) (+++) with a more concentrated color. Furthermore B4, A4 (++) and D2 (+) were not strong enough for producing L-asparaginase. B1 did not produce L-asparaginase. It can be concluded that D1 had the highest L-asparaginase compared to the others isolates. Research conducted by Noverita et al. [13] shown similar result. The fungi isolated from leaves produced higher enzyme activity than those isolated from rhizomes or plant roots. This was presumably because of in the part of the plant, a supportive source of nutrients found maximally in the leaves. The quantitative data of blue zone could be found in Table 1.

**Table 1.** Zone of L-asparaginase activity.

No	Code( <i>Avicennia germinans</i> )	Zone of activity enzyme
1	root (A4)	++
2	steam (B4)	++
3	steam (B1)	-
4	leaf (D1)	+++
5	leaf (D2)	+

Notes:

-: not grow

+: low enzyme activity

++: moderate enzyme activity

+++: high enzyme activity

In the identification of fungi, namely macroscopic and microscopic observation was conducted two identifications. Macroscopic were performed to observe the fungus directly in the form of spores, hyphae, mycelium, colonies colors. Meanwhile, the microscopic analysis observed conidia and conidiophore fungi.

### 3.2. Macroscopic observations

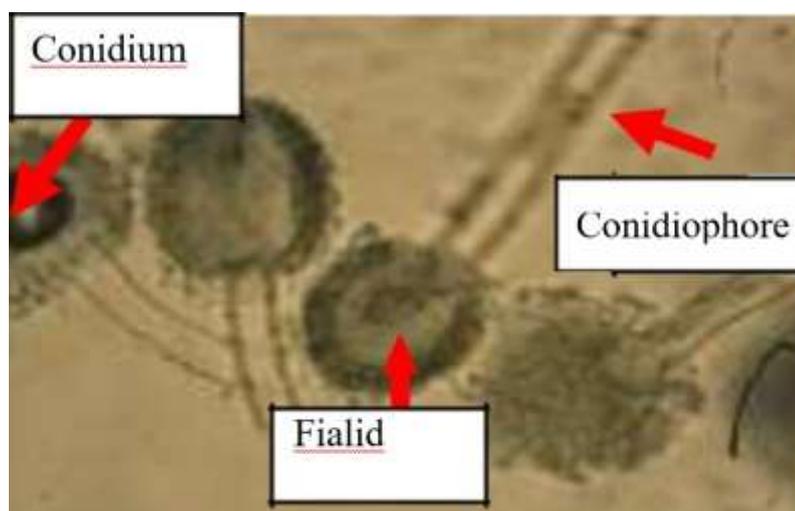
Morphological observations on fungi were conducted on color surface, spores, and hyphae. Macroscopic observation results of fungal endophytes *Avicennia germinans*. Isolates had characteristics of blackish beige mycelium and spores, large and grouped colonies. It showed that the fungal isolate was *Aspergillus sp.* The macroscopic of presumable fungi, *Aspergillus sp.* is depicted on Figure 4.



**Figure 4.** Macroscopic of fungi isolated from *Avicennia germinans*'s leaves.

### 3.3. Microscopic observations

Microscopic observations (Figure 5) include conidiophores, foot cells and heads that have conidium, consisting of fialids and sometimes metula and conidium, phialide formed directly on uniseriat or metula biseriat bubbles, columner or radial conidium heads. Thus, microscopic observations can be concluded by referring to the characteristics - type of fungus *Aspergillus sp.* *Aspergillus* is a fungus included in the Deuteromycetes class. Characteristics of the Deuteromycetes class according to Susilowati and Listyawati [14] are found in Table 2.



**Figure 5.** Identification of *Aspergillus sp.*

**Table 2.** Characteristic of Deutromycetes class.

Characteristics	Deutromycetes
Mycelium	Septa
Conidial	Asexual spores
Sexual spores	-
Natural habitats	Soil, plants, animal

**Table 3.** Identification of fungus.

Strain Code	Characteristic
DPro1	Conidiophore has a simple perpendicular shape, the end part is round, has a phialid connecting bud around the entire surface, one conidia is rounded, the color is blackish [20] So it can be concluded as <i>Aspergillus sp.</i>

The results of fungal testing can be concluded that the sample with code of D1 has been identified as fungus, *Aspergillus sp.* The results of this observation are supported by the results of macroscopic and microscopic. The results obtained lead to the characteristics of the fungus *Aspergillus sp.* which has the characteristics of misellia, spores, hyphae, color, colonies.

It was indicated that the *Aspergillus* had similar characteristic with morphology of *Aspergillus sp.*, Sect. *Wentii* (conidiophores are pale brown, erect, simple, smooth surface, gray spores (composed of conidia coils carried on phialides uniseriate developed in pale brown globel vesicles)). Further investigation was needed to analysis the exact species of the *Aspergillus sp.* From this research we concluded that the *Aspergillus sp* isolated from *A. germinans* was indeed a potential producer of L-asparaginase.

#### 4. Conclusion

The endophytic fungal from Mangrove *Avicennia germinans* can be used as a source of L-asparaginase. Four isolates produced L-Asparaginase. Based on macroscopic and microscopic analysis, the highest producer for L-asparaginase among these four isolates was *Aspergillus sp.* Further investigation is needed to confirm the potential of this fungal as a new source of L-asparaginase.

## References

- [1] Cedar H, Schwarts J H 1967 Localization of two L-asparaginases in anaerobically grown *Escherichia coli* *J. Biol. Chem.* **243** 3753–3755.
- [2] Trilokchandran B, Agrawal P, Krishna V 2016 Screening studies of microbial species For L-asparaginase production *J. Environ. Res. Dev.* **10** 4 712-716.
- [3] Verma N, Kumar K, Kaur G, Anand S 2007 L-asparaginase: a promising chemotherapeutic agent *Crit. Rev. Biotechnol* **27** 1 45-62.
- [4] Petrini O, Sieber T N, Tooi L, Vivet O 1992 Ecology, metabolite production and substrate utilization in endophytic fungi *Nat. Toxin* **1** 185-196.
- [5] Carrol G C 1988 Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont *Ecol.* **69** 2-9.
- [6] Prihanto A A, Wakayama M 2016 Marine microorganism: An underexplored source of L asparaginase *Adv. Food Nutr. Res.* **79** 1-25.
- [7] Prihanto A A, Nursyam H, Yufidasari H S, Lutfiana L 2018 Mangrove ecosystem as a source of L-methioninase-producing bacteria *Malay. Appl. Bio.* **47** 3 141-145.
- [8] Bandaranayake W M 2002 Bioactivities, bioactive compounds and chemical constituents of mangrove plants *Wet. Ecol. Manag.* **10** 6 421–452.
- [9] Prihanto A A 2018 *Bacillus subtilis* UBTn7, a potential producer of L-Methioninase isolated from mangrove, *Rhizophora mucronata* *IOP Conf. Series: Earth Environ. Sci.* **137** 1 012077 1-5.
- [10] Kumaresan V, Suryanarayanan T S 2001 Fungal Endophytes: the Tropical Dimension. In: JK Misra and BW Hom (Eds.) *Trichomycetes and other Fungal Groups* pp. 197-207 Science Publisher Enfield UK.
- [11] Prihanto AA, M Firdaus, and Nurdiani R 2011 Endophytic fungi isolated from mangrove (*Rhizophora mucronata*) and its antibacterial activity on *Staphylococcus aureus* and *Escherichia coli* *J. Food Sci. Eng.* **1** 386-389.
- [12] Gulati R, Saxena R K, Gupta R 1997 A rapid plate assay for screening l-asparaginase producing micro-organism *Lett. Appl. Microbiol.* **24** 1 23-26.
- [13] Noverita., Fitria, D, Sinaga E 2009 Isolasi dan uji aktivitas antibakteri jamur endofit dari daun dan rimpang *Zingiber ottensii* Val (Isolation and test for the antibacterial activity of endophytic fungi from leaves and rhizomes of *Zingiber ottensii* Val). *Jurnal Farmasi Indonesia* **4** 4 171-176. [In Indonesian]
- [14] Susilowati A, Listyawati S 2001 Source of contaminant microorganisms in in vitro culture at Sub lab. Biology, Central Laboratory of Mathematics and Sciences, Sebelas Maret University *Biodiversitas* **2** 1 110-114. [In Indonesian]
- [15] Watanabe T 2002 Pictorial Atlas of Soil and Seed Fungi. Morphologies of Cultured Fungi and Key to Species 2<sup>nd</sup> Ed. CRC Press LLC Florida USA.