Biodiversity of Endophytic Fungi in Sembilang National Park of South Sumatera

Rifka Rimbi Anggraini[1*], Uun Yanuhar[2], Yenny Risjani[2]
[1]*Master Program in Faculty of Fisheries and Marine Science, Brawijaya University, Jalan Veteran Kota Malang 65145, Indonesia*
[2]*Departement of Aquatic Resource Management, Faculty of Fisheries and Marine Science, Brawijaya University, Jalan Veteran Kota Malang 65145, Indonesia*
**corresponding author: Rifka Rimbi Anggraini*

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Abstract: Endophytic fungi originating from areas affected by tidal water are microbes that are rich in natural bioactive products and secondary metabolites. The purpose of this study was to determine endophytic fungi that live in symbiosis with mangrove plants from Bruguiera gymnorrhiza species taken from the Sembilang National Park, South Sumatra. The research method in isolating symbiont mushrooms was carried out using the Direct Planting method with PDA (Potato Dextrose Agar) media. The results in this study indicate that there are three types of endophytic fungi, namely *Aspergillus flavus*, *Penicillium* sp., and *Aspergillus niger*.

1 INTRODUCTION

The potential possessed by the diversity of natural resources, especially plants, still needs to be studied. According to Prihatiningtias (2005), sources of bioactive compounds are obtained from plants, animals, microbes and marine organisms which are continuously being explored as more and more new diseases emerge. These endophytic microbes were first discovered by Darnel *et al* on 1904 and from then on, the definition of endophytic microbes was agreed as microorganisms that live in plant tissue systems and symbiotic mutualism (Stone *et al*., 2000).

Endophytic fungi are one of the endophytic microbial organisms (Strobel, 2003). In-plant tissues that have endophytic fungi can produce compounds that have the same properties as the host plant, although the types of compounds are different. The activity of compounds produced by endophytic fungi is usually greater than that of the host compound (Strobel *et al*., 2004).

One of the plants that contain a lot of bioactive compounds produced by endophytic fungi in mangrove plants. According to several researchers in Noor *et al*. (2012) mangroves are plants that live between sea and land, in the form of shrubs and trees and at high tide, the roots of these mangroves will be flooded by water and the receding time of the roots will be seen. *Bruguiera gymnorrhiza* is a species of mangrove that grows on muddy soils, is flooded during high tide and does not like hard substrates such as sand.

So far, many researchers have succeeded in isolating endophytic fungi and secondary metabolite compounds from various types of plants. However, researchers who isolate endophytic fungi from *B. gymnorrhiza* mangroves and information on endophytic fungi in mangroves as producers of natural ingredients are still limited in Indonesia, especially in the Mangrove Ecosystem in South Sumatra. Limited information, the authors have conducted research on the isolation of endophytic fungi in mangrove *B. gymnorrhiza* plants taken from the mangrove area of the Sembilang National Park in South Sumatera.

2 MATERIALS AND METHODS

The research sample was taken using a purposive sampling method which is located in Sembilang National Park, South Sumatera (Fig. 1).

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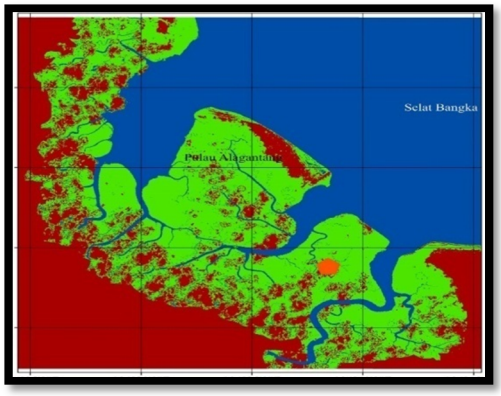
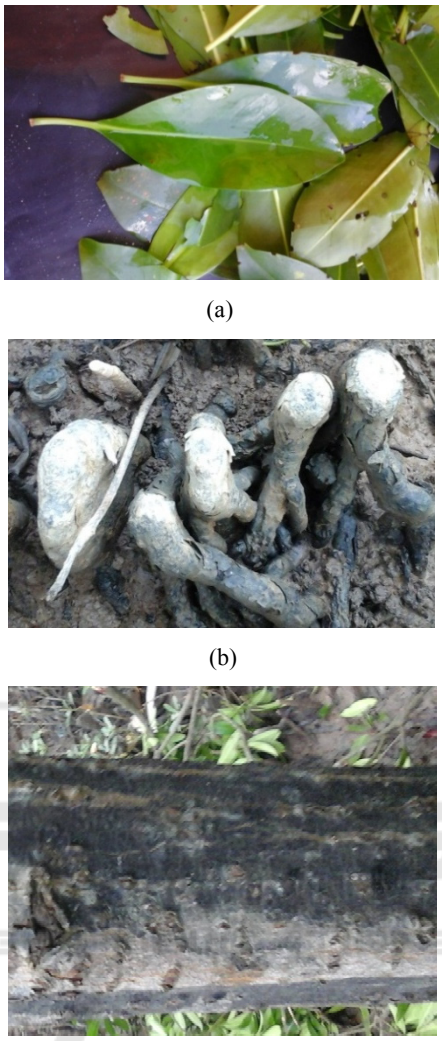


Figure 1. Research Location

The tools used in this research are aluminum foil, autoclave, identification book, bunsen, petri dish, cover glass, cutter, erlenmeyer, freezer, measuring cup, hot plate, incubator, inoculating loop, cotton, filter paper, laminar air flaw, masks, microscopes, analytical balances, glass objects, tweezers, and plastic wrap. The materials used in this study were apart, sterile seawater, distilled water, 70% alcohol, dextrose, 75% ethanol, seawater, chloramphenicol, lactofenol blue cotton, potato dextrose broth (PDB), potato dextrose agar (PDA), *Bruguiera gymnorrhiza* and spiritus.

2.1 Collection and Preparation of *Bruguiera Gymnorrhiza* Samples

B. gymnorrhiza mangrove samples were randomly selected from one of the tree representatives in the mangrove zoning of the Sembilang National Park area, South Sumatra. Mangrove samples taken are the roots, stems and leaves as much as ± 500 gram each part (Fig. 2). The sample taken is put into a sterile plastic sample so that the sample is not contaminated, then put in a cool box. In handling in the laboratory, samples that have been taken are washed using sterile seawater 3 times to remove impurities. Furthermore, soaked using 70% alcohol for 1-2 minutes to kill the epiphytic fungus that sticks to the surface. After that, the samples were rinsed again using sterile seawater (Kjer *et al*., 2010).



(a)

(b)

(c)

Figure 2: (a) Leave, (b) Root and (c) Stems of *Bruguiera gymnorrhiza* (Personal Documentation)

2.2 The Manufacture of Media Growth Endophytic Fungi

In this research, there are two media used, namely Potato Dextrose Broth (PDB) as liquid media and Potato Dextrose Agar (PDA) as solid media. The way of making these media is as follows:

2.2.1 Potato Dextrose Broth (PDB)

12 gram PDB dissolved with 500 ml of seawater in an erlenmeyer tube. The erlenmeyer tube is closed using a cotton swab that is coated with aluminium foil. Seawater and media are homogeneous using a hot plate. The media is waited until completely homogeneous. Then the media is sterilized by

autoclave for 15 minutes with a temperature of 121°C and a pressure of 1 atm (Ariyono *et al*., 2014).

2.2.2 Potato Dextrose Agar (PDA)

PDA media as much as 19.5 gram in 500 ml of seawater was dissolved in an erlenmeyer tube. The erlenmeyer tube is closed using a cotton swab that is coated with aluminium foil. Seawater and media are homogeneous using a hot plate. The media is waited until completely homogeneous. Then the media is sterilized by autoclave for 15 minutes with a temperature of 121°C and a pressure of 1 atm. Then chill the media a few moments then put Chloramfenicol as much as 0.1 gram. Modifications from Ariyono *et al*. (2014).

2.3 Growth of Endophytic Fungi Isolates

Samples (roots, leaves and stems) were cut to size ± 1x1 cm. The sample was put into the GDP medium with a ratio of 1: 9 (g/v) where 10 gram of sample was added 90 mL of PDB media. Samples are stirred using a shaker for 4-7 days until the colour of the water turns brownish turbid at a speed of 150 rpm at room temperature (Kjer *et al*., 2010).

Samples on PDB media are put into test tubes with the principle of multilevel dilution. The last three dilutions (10^{-4},10^{-5} and 10^{-6}) were taken for planting by the pouring method on 1 mL petri dishes. PDA media that have been made are taken, poured into a petri dish while homogenized until the media becomes solid and incubated for 7 days at 25°C (Benson, 2002).

2.4 Purification of Endophytic Fungi in Potato Dextrose Agar (PDA) Media

Fungal colonies that have grown on PDA media were previously regrown on sterile PDA media based on morphological differences from each growing colony to obtain endophytic fungal colonies according to their respective morphology. Fungal colonies on PDA media were taken using a sterile round inoculating loop then etched on aseptic sterile PDA media in laminar airflow. If one mushroom colony is still mixed with other colonies, then it is refined repeatedly until a pure mushroom colony is obtained (Ariyono *et al*., 2014).

2.5 Characterization of Endophytic Fungi

Characterization was carried out on each fungal colony macroscopically and microscopically. The results of observations are used as ingredients for identification of endophytic fungi. Gandjar (1999) mentions macroscopic observations include the colour and surface of the colony, radial lines from the centre of the colony towards the edge of the colony, and concentric circles in concentric or non-concentric Petri dishes and colony growth (cm/day). Microscopic observations include hyphae bulkhead, hyphae growth, hyphae colour, presence or absence of conidia and conidia form. Microscopic observations were made on the last day observations (5-7 days) using a microscope.

This microscope observation was carried out using the slide culture method. A sterile petri dish is provided, a buffer ring is placed inside and 5 mL of distilled water is added to maintain moisture. The top of the ring is placed with glass preparations/object glass and sterile PDA media pieces on it. Fungi culture is taken and applied to the entire surface and closed using a glass cover. Fungi cultures were incubated for 5-7 days at 25°C. Cultures that have grown on the cover glass are placed at the top of the glass preparation which is dripped with lactofenol blue cotton to increase the transparent effect on the fungi to be more easily observed under a microscope at magnifications of 10X and 40X (BKIPM, 2014).

2.6 Identification of Endophytic Fungi

Observations obtained from the characterization of fungi will be used observation results obtained from the characterization will be used for the identification stage based on the guide book identification Introduction to Food-Borne Fungi (Samson *et al*., 1995), Introduction of General Tropical Molds (Gandjar *et al*., 1999) and Identifying Filamentous Fungi (St-Germain and Summerbell, 1996).

3 RESULT

3.1 Endophytic Fungi Pure Colony

In the growth of fungi in PDA media for 7 days at a temperature of 25ºC found seven types of pure isolates in mangrove *B. gymorrhiza*. Of the six types, there are three types of isolates that differ in shape, colour and texture. There are several differences in visually purified endophytic fungi. The macroscopic

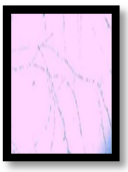
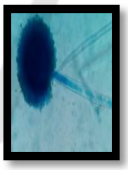
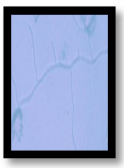
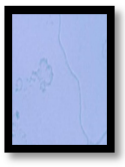
observation of endophytic fungi can be seen in Table 1.

Table 1. Macroscopic Characteristics of Endophytic Fungi

Organ	Shape	Colour	Texture	Figure
Stem	Round	Brownish	Smooth Powder	
	Round	Black	As if cotton	
Root	Round	Green	Velvety	
	Round	White	Smooth Powder	
Leaf	Round	White	Smooth Powder	
	Round	Black	Velvety	

Fungi that have been made macroscopic observations with visual observations, then the microscopic observation stage is carried out to make it easier to identify the results that have been obtained. Microscopic characterization is a continuation of the stage of identifying fungi. This observation was carried out by observing hyphae, spores, and conidia formed under a microscope lens with a magnification of 400X. Macroscopic observations can be seen in Table 2.

Table 2. Microscopic Characteristics of Endophytic Fungi

Organ	Figure		Explanation
	Sporae	Hyphae	
Stem			The hyphae form has a divider and is branched on hyaline Conidia are round and black
			
Root			The hyphae form has a divider and is branched on hyaline Conidia are round and black
			
Leaf			The hyphae form has a divider and is branched on hyaline Conidia are round and black
			

4 DISCUSSION

In general, the fungi has been found to grow clearly. Pure colonies were obtained based on differences in the visual appearance of each isolate. The fungi obtained at the stems has a brownish to black colour,

smooth texture like cotton. The diameter size of fungi colonies taken from stems tends to be smaller because the growth rate is relatively slow. The fungi obtained at the root looks even more different from other types of fungi because of the difference in colour. This fungi has a green colour with a larger colony diameter than fungi obtained from the leaves. The speed of growth of this fungi is also relatively faster than the fungi from the isolation of the leaves.

The fungi obtained from the leaves has a black spore colour. The size of the diameter of this fungi colony is the biggest compared to the fungi isolated from the stem and roots. The speed of growth of this fungi is much faster compared to other fungi. Therefore, the type of fungi obtained from these leaves grows to meet the entire surface of the petri dish.

The purification stage of the leaf part is more abundant with endophytic fungi compared to roots and stems. This is consistent with what was stated by Noverita *et al*. (2009); Sinaga *et al*. (2009) where more endophytic fungi isolates were obtained from the leaves. This phenomenon is suspected because the nutrients present in the leaves are more supportive of the growth of endophytic fungi. Endophytic fungi isolated from one host plant contain different types of isolates, even from one living tissue obtained from a plant can be isolated more than 1 type of endophytic fungi. This is an adaptation mechanism of endophytic fungi to the microecology and specific physiological conditions of each host plant.

Macroscopically, the fungi of *Aspergillus niger* is found in the leaves. This fungi is black and has white hyphae. Based on Summerbell (1996) that the *Aspergillus niger* fungi grow rapidly until its diameter fills the entire surface of the PDA media. The *Aspergillus niger* fungi itself is black and fills the entire surface of the media. This is also supported by microscopic results in which the *Aspergillus niger* fungi has the characteristics of large enough black spores covering all spore bubbles where the conia spreads tightly and has clear conidiospores. These characteristics are also found in microscopic images of the fungi of *Aspergillus niger* based on references from the identification book Summerbell (1996).

The endophytic fungi of *Aspergillus niger* is a fungus that is isolated from every organ of the *B. gymnorrhiza* mangrove plant. The characteristics of *Aspergillus niger* are having a large black conidia head and a round shape. The hyphae were insulated and branched mycelium. This fungi also have vesicles (bubbles) at the ends of the conidiophores and becomes a place of conidia to grow. The conidia shaped chain and black. This fungi grows well at room temperature (Wuryanti, 2008).

The fungi of *Aspergillus niger* is a type that produces quite a lot of compounds and enzymes. According to Handajani and Purwoko (2008), the fungi of *Aspergillus niger* can produce ochratoxin compounds and can produce lipase enzymes. Lipase enzyme is an enzyme that plays an important role in the world of modern biotechnology because it has high activity in hydrolysis and synthesis and chemical reactions.

Aspergillus niger fungi which belong to the group of phosphate solvent fungi. Phosphate solvent fungi are able to be used as biofertilizer which is the result of biotechnology engineering in the field of soil science. Aspergillus niger has the ability to dissolve phosphate compounds that are difficult to dissolve into a form available to plants by producing organic acids so that availability becomes faster (Artha *et al*. 2013).

In addition to secondary metabolites produced by *Aspergillus niger*, this fungi is also able to produce cellulase enzymes (Sa'adah *et al*. 2010), proteases (Ramdhani *et al*. 2015), and chitinase (Purkan *et al*. 2016). Protease enzymes produced by *A. niger* fungi are able to be grouped in alkaline proteases which are one of the groups of hydrolytic enzymes that is able to catalyze the hydrolysis process or the proteins damage into their constituent amino acids (Ramdhani *et al*. 2015). The forms of commercial products in the application of alkaline proteases in the industrial sector include the detergent industry, the food industry, the pharmaceutical industry, milk, skin, and meat processing (Ramdhani *et al*. 2015).

Described in the book Gandjar *et al.* (1999) Aspergillus flavus is a fungi commonly found in nuts (especially peanuts), spices, oilseeds, cereals, and sometimes in dried fruit. The fungi of *Aspergillus flavus* is a fungi that is green and shaped like soft hair because it has fairly long hyphae. This fungi has a fairly large size because it almost fills the entire surface of the PDA media. Microscopic observations show that these fungi have quite large spores, with conidial heads scattered throughout the bubble surface and has rough conidiospores walls (Summerbell, 1996).

A. flavus fungi is also a fungus that can produce aflatoxin compounds. The main aflatoxin compounds are produced by the fungi of *A. flavus*, which is one of the causes of cancer in humans (Handajani and Setyaningsih, 2006). In addition to aflatoxin compounds, Setiarto (2011) also suggested that the fungi of *A. flavus* can produce ochratoxin and zearalenone compounds. In extreme conditions, this

type of fungi can infect grains directly which can later cause aflatoxin accumulation. This can cause health problems in animals and even humans due to contamination of feed ingredients by aflatoxins. *A. flavus* fungi can also be used as antibacterial metabolites. *A. flavus* can inhibit the growth of *Echercia coli* which is a bacterium that causes diarrhoea.

Based on the results of Hidayati's research (2010) the selection of six endophytic fungi isolates produced antibacterial metabolites using the Kirby-Bauer test method where the results were all isolates could form inhibitory zones against the test bacteria. *A. flavus* fungi can inhibit the growth of *E. coli* bacteria by 9.33 mm. Research Raharjo *et al*. (2007) said that this fungus is able to dissolve phosphate which cannot be dissolved so that plants can be used in growth.

Gandjar *et al*. (1999) describe the fungi of *Penicillium* sp. has a surface with a velvety texture although sometimes like cotton. The colours in the colonies are sometimes yellow to brownish, greyish-green to yellowish-green and greyish green. Conidiophores in fungi arise from the substrate and generally has many branches and smooth-walled. Habitat from this fungi is very common in various food products, as well as food items that are low in the water. *Penicillium* sp. used in industry to produce antibiotics (Crystovel, 2017).

These fungi are known as fungi that produce antibiotic metabolites. Amaria *et al.* (2013) said that *Penicillium*, *Trichoderma* and *Aspergillus* are fungi that can release antibiotic-like substances that can inhibit the growth of pathogens so that these fungi are antagonistic fungi that can be used as biopesticide and biofertilizer fungi. Subowo (2015) explains that the fungi of *Penicillium* sp. able to decompose cellulose and lignin compounds into simple carbon compounds needed by soil microbes as an energy source so that this fungi is very good for soil fertility.

5 CONCLUSION

The results of isolation and identification of endophytic fungi from Bruguiera gymnorrhiza mangroves taken from the Sembilang National Park, South Sumatera are known that there are two types of fungi from the Aspergillus genus namely *Aspergillus niger* and *Aspergillus flavus*, and one of the Penicillium genera namely *Penicillium* sp.

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