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***ISOLATION, IDENTIFICATION, AND METAL RESISTANCE
TESTING OF SOIL FUNGI (RHIZOSPHERE) FROM BANGKA TIN
POST-MINING***

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Abstract

The ecological impacts of tin mining activities in the Bangka island is the presence of heavy metal contamination that accumulated in the environment. One of the heavy metals that was often found in post-tin mining areas is lead (Pb). Mycoremediation is a method that can be used to reduce lead pollution in post-tin mining areas. This study aimed to isolate, resistance test and identify to Pb-resistant soil fungi (rhizosphere) in the post-tin area of Bangka. The research methods included isolation, test of fungal resistance to Pb metal (Control, 150ppm, 250ppm, 350ppm, 500ppm, 1,000ppm, 5,000ppm and 10,000ppm), macroscopic and microscopic identification. The results showed that 9 isolates tested, there were 6 isolates that were resistant to Pb up to the concentration of 5,000 ppm, namely isolates I1(Rhizoctonia), I2(Penicillium sp1), I5(Papulaspora), I6(Penicillium sp2), I7(Penicillium sp3) and I9 (Sclerotium). Three isolates of soil fungus were not resistant to Pb up to a concentration of 5,000 ppm, namely isolates I4 (Sclerotium), I8(Microsporium), and I10(Cladosporium). All isolates did not grow anymore at a concentration of 10,000 ppm of Pb.

Keywords: Micoremediation; Pb metal; Resistance; Soil fungi; Tin mining

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INTRODUCTION

Tin mining activities on Bangka island have an ecological impact on the environment. The negative impacts that appear on the land around mining are the increase in critical land due to reduced forest area, the loss of germ plasm due to the loss of forest ecosystems and habitats for animals, causing the environment to be below quality standards due to decreasing soil quality (Suryati, 2017; Kurniawan & Mustikasari, 2019). Another negative impact caused by tin mining is the presence of heavy metal contamination that accumulates in the environment (Kurniawan & Mustikasari, 2019). One of the heavy metals that is often found in post-tin mining areas in Bangka Belitung is lead (Pb). Several studies have shown that the concentration of lead heavy metal contamination in post-tin mining areas is much higher than other metals such as Cr, As, Cu and Cd. The results of study by Kurniawan *et al.*, (2019) showed that the concentration of lead metal in post-mining land was 4.62-8.21 ppm.

Heavy metal lead that accumulates in the environment can cause a decrease in microbial activity, soil fertility and quality, as well as the entry of lead metal into the body of organisms. Lead accumulation that exceeds the tolerance limit has a toxic effect

on an organism, 2011). The lead enters the body of organisms through the food chain and then forms food webs and ends up in humans as the highest level of consumers. The accumulation of lead in humans can cause dangerous diseases and even death. The impact of lead on humans is that it can damage body organs, especially the nervous system, blood formation system, kidneys, heart system and reproductive system. Lead can also cause anemia, hypertension, impaired blood synthesis, hyperactivity, brain damage and liver disease (Amaral *et al.*, 2010).

One of the efforts that can be done to reduce lead pollution in post-tin mining areas is to use the microremediation method. Mycoremediation is a detoxification process for toxic materials from a polluted environment using fungi (Asiriuwa *et al.*, 2013). Microremediation can be done by multiplying the fungus (biostimulation) that is already in the polluted soil by providing the necessary growth environment and increasing the fungal population (bioaugmentation) (Ahmad, 2018). The microremediation mechanism consists of separation of cellular extracts through chelation and cell wall binding and physical intracellular separation of metals through binding of proteins or other ligands in order to

prevent damage to metal sensitive cellular targets.

Fungi isolated from an environment contaminated with heavy metals are very potential to be used as bioremediation agents, because these fungi have resistance and tolerance for heavy metals in the surrounding environment (Ahmad, 2018). Research on the isolation of fungi that have resistance to lead found in post-tin mining areas in Bangka has never been done before. This research needs to be carried out to obtain the type of fungus that is resistant to lead, so that it can be used as a candidate for microremediator in post-tin mining fields through the identification and resistance test process.

MATERIALS AND METHODS

The research was carried out from November 2020 to March 2021. The sampling location was carried out in Bangka post-tin mining (Figure 1). Samples were taken from the soil around the roots for fungal isolation and measurement of Pb. Isolation of fungi in soil samples was carried out at the Microbiology Laboratory, Faculty of Agriculture, Fisheries and Biology, Bangka Belitung University. Tests for Pb levels were carried out by the Chemical Laboratory, PT. Timah Tbk. Identification of the fungus was carried out at Microbiology, Faculty of Agriculture,

Fisheries and Biology, Bangka Belitung University and Fish Quarantine Station, Quality Control and Safety of Fishery Products in Pangkalpinang.

The main object of this research project is a soil sample taken from Bangka Tin Post Mining. The materials used include distilled water, 70% alcohol, Bengal Rose Agar, HCL 37%, HNO₃ 65%, PDA (Potato Dextrose Agar) media, 0.9% NaCl, Pb nitrate (Pb(NO₃)), and paper, strain. The tools used in this research include autoclave, soil drill, petri dish, incubator, caliper, Laminar Air Flow Cabinet (LAFB), microscope, and vortex.

Research Sampling

The sampling method was carried out by purposive sampling by considering the presence of plants in post-mining area. The plants used were Australian pine (*Casuarina equisetifolia*). Sampling was carried out at three stations S1, S2 and S3 with each point originating from one plant. Soil was taken from each 1 point of collection with a soil depth of ± 20 cm (Edhar *et al.*, 2017).

Fungal Isolation and Purification

Isolation of the fungus was carried out using the dual pour cup method on Rose Bengal Agar media up to a dilution of 10⁻⁵. Samples from the 10⁻³, 10⁻⁴, and 10⁻⁵ dilutions as much as 100 L were inoculated into Rose Bengal Agar media and the

inoculation results were then incubated at room temperature 26°C-28°C for 7 days. The fungal isolates that grew were then calculated for their population using the Total Plate Number (ALT) calculation formula (Wijaya, 2018). The fungal isolates that grew were selected Based on macroscopic morphological differences (colonial shape, colony color front view, colony color rear view, surface texture, presence or absence of mycelium) and then inoculated back into new media (Ed-har *et al.*, 2017).

Fungal Resistance Ability Test

The fungal isolate resistance test was carried out in two stages, namely qualitative and quantitative tests. Qualitative tests were carried out by inoculating fungal isolates on media that had added Pb with concentrations of 150, 250, 350, 500, 1,000, 5,000, and 10,000 ppm, then incubated at room temperature 26°C-28°C for 7 days. Qualitative tests were carried out to see whether the fungus grew or not on media with the addition of metal. Quantitative test to see the growth of the fungus through the diameter of the isolate colonies. The increase in the colony diameter of the isolates was measured daily using a caliper. The results of fungal growth on agar media with heavy metals were compared with control media

without the addition of heavy metals (Rose & Devy, 2018).

Identification of Fungal Isolates

Microscopic characterization of isolates (hyphal shape, hyphal branching, presence or absence of septa, presence or absence of conidia, conidia form) was carried out by direct observation of fungal isolates on a slide in a microscope with 1000x magnification (Ed-har *et al.*, 2017). The identification process was carried out by comparing the morphological characters of the isolates both macroscopically and microscopically with the identification book *Identifying Filamentous Fungi: Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species: Third Edition* (Watanabe, 2010).

Data analysis

The research data obtained were analyzed descriptively. Data types and growth ratio values are displayed in the form of tables and graphs using Microsoft Office 2007.

RESULTS AND DISCUSSION

Isolation of Fungi (Rhizosphere) from Tin Post Mining

The highest number of fungal populations (rhizosphere) was at Station 1 with a total of 5.7×10^4 CFU/g and the

lowest number of fungi was at Point 3 with a total of 0.9×10^4 CFU/g (Table 1). The results of the isolation of the fungus (rhizosphere) on Rose Bengal media obtained 10 isolates of fungi with different macroscopic characteristics. The fungal isolates found in Station 1 were 6 isolates (I1, I2, I5, I6, I7, I8), Station 2 found 2

isolates (I3, I4), and Station 3 found 2 isolates (I9, I10) (Figure 1). The number of fungal isolates (rhizosphere) that were successfully purified in Rose Bengal media amounted to 9 isolates, namely isolates I1, I2, I4, I5, I6, I7, I8, I9, and I10, while 1 other isolate, isolate I3 died during purification.

Table 1. Number of fungi (rhizosphere) in post-mining

Station	Total fungal population (CFU/g)
1	5.7×10^4
2	0.9×10^4
3	2.3×10^4

Figure 1. Fungal (rhizosphere) isolates from post-tin mining on Rose Bengal agar media 7th day of incubation

Fungal Resistance Ability Test (Rhizosphere)

The results of the resistance test against 9 isolates of fungi (rhizosphere) showed that 6 (six) isolates of fungi (rhizosphere) were resistant to Pb up to a concentration of 5,000 ppm, namely

isolates I1, I2, I5, I6, I7 and I9. The other three isolates of fungi (rhizosphere) were not resistant at a concentration of 5,000 ppm, namely isolates I4, I8, and I10. The results of further research showed that all isolates did not grow again at a concentration of 10,000 ppm (Table 2).

Table 2. Test of resistance of the fungus (rhizosphere) to Pb . metal

Isolation Code	Pb . metal concentration						
	150 ppm	250 ppm	350 ppm	500 ppm	1000 ppm	5,000 ppm	10,000 ppm
I1	+	+	+	+	+	+	-
I2	+	+	+	+	+	+	-
I4	+	+	+	+	+	-	-
I5	+	+	+	+	+	+	-
I6	+	+	+	+	+	+	-
I7	+	+	+	+	+	+	-
I8	+	+	+	+	+	-	-
I9	+	+	+	+	+	+	-
I10	+	+	+	+	+	-	-

Description: +(resistance)

-(not resistant)

Heavy metal resistance is one of the abilities of microorganisms to develop resistance to toxic heavy metal ions. The resistance test was carried out through 8 concentrations of lead, namely Control (0), 150, 250, 350, 500, 1,000, 5,000, and 10,000 ppm. The provision of different concentrations refers to previous research, namely Wijaya's research, (2018) which showed that from concentrations of 150, 250 and 400 ppm, the optimal concentration for the resistance test was in the range of 250 ppm, so the concentrations of 150, 250, 350, 500, 1,000, 5,000 and 10,000 ppm.

The results of the resistance test to 9 isolates of fungi (rhizosphere) showed 6 (six) isolates of fungi (rhizosphere) were resistant to Pb up to a concentration of 5,000 ppm, namely isolates I1, I2, I5, I6, I7 and I9, 3 (three) isolates of fungi (rhizosphere) not resistant at a concentration of 5,000 ppm, namely isolates I4, I8, and I10, and at a

concentration of 10,000 ppm all isolates did not grow again (Table 2). These isolates were able to be resistant to certain metal concentrations due to the ability or resistance to metals which allowed the fungus to be able to live in media with the addition of heavy metal Pb. It is supported by the statement of Anahid *et al.*, (2011), which stated that the ability of fungal isolates to be resistant in the environment with the addition of metals was due to the ability of these isolates to survive by adapting to high concentrations of toxic heavy metals. Several other isolates were not resistant due to the different ability of the isolates in the process of the resistance mechanism to these metals. Based on Iram *et al.*, (2013), differences in metal resistance are caused by the various strategies of different resistance mechanisms shown by the fungus.

The ability to survive at concentrations of toxic heavy metals in the fungus (rhizosphere) is better known as

the resistance of the fungus. The resistance mechanism serves to overcome the toxicity of heavy metals so as not to interfere with the biological function of microorganisms (Anahid *et al.*, 2011). The ability of fungal resistance occurs through two mechanisms, namely the extracellular mechanism and the intracellular mechanism (Ahmad, 2018). The extracellular mechanism functions to prevent the cell from entering metal, while the intracellular mechanism functions to reduce the metal load in the cytosol (Anahid *et al.*, 2011).

The extracellular mechanism occurs through biosorption and chelation formation, while the intracellular mechanism occurs through efflux pumps, production of metallothionein and phytochelatin proteins, and vacuole compartmentalization (Lotlikar, 2019). Biosorption is a process of passive absorption of metals by microorganism cells, resulting from the formation of metal-complex organics with microorganism cell walls, capsules or extracellular polymers that are synthesized and excreted by microorganisms (Ahmad, 2018). The formation of chelate is the formation of a double coordination bond between organic and metal molecules which functions for

the natural detoxification process of heavy metals (Anahid *et al.* 2011).

The efflux pump is a metal detoxification process where if the surface of living cells is saturated with metal species, the metal will penetrate into the interior of the cell through ion channels (Yang *et al.*, 2016). Metallothionein protein is a metal-binding cytoplasmic protein that can modulate the concentration and intracellular binding of both essential metals such as Cu and Zn and non-essential metals such as Cd. Phytochelatin protein is a cytoplasmic protein that functions as an intracellular chelator in fungal cells. Vacuoles in fungi have an important role in molecular degradation, storage of metabolites, regulation of cytosolic concentrations of metal ions and detoxification of potentially toxic metal ions (Siddiquee *et al.*, 2015).

The growth of the fungus was indicated by the increase in isolate diameter per day. The fungal isolates that had the fastest growth were marked by the largest isolate diameter values at the 7th day of incubation. The fastest growth of fungal isolates (rhizosphere) was at a concentration of 5,000 ppm on the 7th incubation day, indicated by isolate I9, which was 65.57 mm, while the slowest growth was found by isolate I6, which was 11.25 mm. The other five isolates, namely

I1, I2, I4, I8 and I10 did not grow at a concentration of 5,000 ppm. All fungal isolates (rhizosphere) at a concentration of 10,000 ppm did not grow (Figure 2).

Figure 2. Growth graph of fungal isolates (rhizosphere) incubation days 1, 3, 5, and 7

The fungal isolates that had the fastest growth were marked by the largest isolate diameter values at the 7th day of incubation. The results showed that the isolate that had the fastest growth at a concentration of 5,000 ppm incubation on the 7th day was indicated by isolate I9, which was 65.57 mm (Figure 2). The isolate, which belongs to the genus *Sclerotium*, is known to have the ability to be resistant to Pb metal (Sana *et al.*, 2015). In addition, the genus *Sclerotium* also has a special structure in the form of sclerotia which allows it to live in the soil for how many years and in an unfavorable environment, one of which is polluted by heavy metals (Arunasri *et al.*, 2011)

Some isolates had an increasing growth along with the increase in heavy metal concentrations and began to decrease from a concentration of 1,000 ppm to 10,000 ppm, such as isolates I8 and I9 (Figure 2). It was due to the contact with Pb metal, the fungus underwent morphological ultrastructural changes in the form of thicker mycelium with a larger diameter due to cell wall thickening. Cell wall thickening occurs due to changes in cell wall composition, namely an increase in hydroxyl proteins (Ekawati, 2019). Hydroxyl protein plays a role in the process of cell wall biosynthesis which affects the growth of fungal cells. The greater the

concentration of hydroxyl protein in the cell wall, the greater the cell wall biosynthesis process, causing the growth of fungal cells to increase (Yanti *et al.*, 2016).

Other isolates had slow growth as the metal concentration increased, and did not even grow at high concentrations such as 5,000ppm and 10,000ppm. The growth of the fungus decreased from a concentration of 500 ppm (Figure 2). The decrease in fungal growth at concentrations ranging from 500 ppm was due to the concentration of metal being too high and so toxic to fungal cells. It is supported by Ahmad's statement (2018), that the decreased growth and stunted growth were caused by the concentration of Pb metal which was too high so that it was toxic to the fungus. Study by Amatussalam *et al.*, (2014) also mentioned that the metal absorption process in fungi is optimal up to a concentration of 500 ppm, and is stable or decreases from a concentration of 1000 ppm.

Study by Aishwarya *et al.*, (2017) showed that an increased concentration of heavy metals caused a decrease in fungal growth due to heavy metal toxicity. Metal toxicity occurs through the displacement of the essential metal from its original binding site or through ligand interactions. Heavy metal toxicity in fungi can damage cell membranes, change enzyme structures,

disrupt cellular functions, and damage DNA structures (Olaniran *et al.*, 2013). Changes in the morphology and metabolism of fungi caused by changes in the structure of nucleic acids can cause functional and cell membrane disorders, as well as inhibit enzyme activity and oxidative phosphorylation (Fashola *et al.*, 2016). It is what causes the growth of the fungus to decrease and be hampered and even die. Based on Wijaya, (2018) metal levels that are too high can reduce or inhibit fungal growth.

Fungal isolates (rhizosphere) were said to be resistant if they were able to grow in media with the addition of Pb metal, and not resistant if they did not grow in media with the addition of Pb metal. The fungus (rhizosphere) that was resistant to Pb up to a concentration of 5,000 ppm consisted of 6 isolates from the genus *Rhizoctonia*, *Penicillium*, *Sclerotium* and *Papulaspora*. The other three isolates from the Genus *Sclerotium*, *Microsporum* and *Cladosporium* were only able to withstand a concentration of 1,000 ppm. Resistant fungi are useful in remediating environments contaminated with heavy metals because these fungi are able to survive in polluted environments and several types of fungi can reduce levels and

toxicity of heavy metals in the environment (Ahmad, 2018).

Several studies have shown that a number of fungal isolates are resistant to heavy metals because they have a special ability to bind, immobilize or reduce heavy metal toxicity in a polluted environment. The results showed that the *Penicillium* genus was able to withstand up to 5000ppm Pb metal concentrations. It is supported by a statement that explains that the genus *Penicillium* is resistant to Pb up to a concentration of 3000 g/L and is able to reduce Pb(II) up to 98.25 (Iram *et al.*, 2013; Tian *et al.*, 2018). The ability to absorb and reduce Pb(II) by the genus *Penicillium* is due to the mechanism of biosorption and bioaccumulation by fungi. Biosorption is one of the mechanisms that play an important role in the tolerance ability of fungi. Biosorption occurs passively and is an independent metabolic reaction between heavy metals and fungal cell walls. This cell wall serves as the first barrier against incoming heavy metals and reduces metal concentrations (Maini *et al.*, 2019).

Research on the genus *Sclerotium* showed that the same two genera had the ability to resist Pb at different concentrations. The genus *Sclerotium* isolate I9 was able to withstand Pb metal

up to a concentration of 5,000 ppm, while the other isolate, isolate I4 was only able to withstand Pb metal up to a concentration of 1,000 ppm. It was due to the possibility that the two isolates were from the same genus but different species. The difference in metal resistance is caused by the existence of various strategies of different resistance mechanisms shown by different fungal species (Iram *et al.*, 2013). Study by Sana *et al.*, (2015) showed that *Sclerotium rolfsii* was able to tolerate Cr(III) up to a concentration of 100 ppm.

The genus *Rhizoctonia* and *Papulaspora* are also capable of being resistant to Pb up to a concentration of 5,000 ppm. Research by Kredics *et al.*, (2001) showed that the genus *Rhizoctonia* was resistant to Pb . metal. Two other genera such as the genus *Cladosporium* and *Microsporum* were only able to withstand Pb metal up to a concentration of 1000 ppm. Study by Rossiana *et al.*,

(2015) showed that *Cladosporium* can reduce lead levels by 17.94% in 60 days of incubation. Research by Calvillo-Medina *et al.*, (2020) showed that the genus *Cladosporium* was psychrotolerant, pigmented and resistant to different concentrations of Pb(II).

Identification of Fungal Isolates

Based on the results of macroscopic and microscopic identification, the identity of the fungus (rhizosphere) was obtained, namely I1 is the genus *Rhizoctonia*. Isolates I2, I6, and I7 were of the genus *Penicillium*. Isolates I4 and I9 were of the genus *Sclerotium*. Isolate I5 was the genus *Papulaspora*. I8 isolate was the genus *Microsporum*. I10 isolate was the genus *Cladosporium*. The results of observations of microscopic and macroscopic characteristics are shown in Table 3. Characteristics of fungal isolates (rhizosphere) at a magnification of 10,000x are shown in Figure 3.

Figure 3. Microscopic characterization of fungal isolates (rhizosphere) at 1000x magnification of 7th day incubation, a) hyphae; b) septa; c) fialid; d) metula; e) conidia; f) papules.

The identification of fungal isolates was based on the macroscopic and microscopic characteristics of the isolates and compared with the book *Identification of Pictorial Atlas and Seed Fungi*. The results of the macroscopic characteristics of the isolates showed that 4 isolates belonged to fungi that had spores, and 5 other isolates belonged to fungi that did not have spores or were sterile fungi. Based on the identification of 9 isolates of fungi that were resistant to Pb, 6 genera of fungi were obtained, namely *Sclerotium*, *Papulaspora*,

Rhizoctonia, and *Penicillium*, *Microsporum* and *Cladosporium*.

The results of the characterization of fungal isolates showed that isolates I4 and I9 had similarities with the genus *Sclerotium*. The characteristics of the genus *Sclerotium* are that conidia are not formed, the hyphae are white, branched, with septate side branches and are limited very close to the main hyphae, sometimes with clamping connections in some isolates (Watanabe, 2010). Isolate I5 was similar to the genus *Papulaspora*. The characteristics of the *Papulaspora* genus are conidia not

formed, papules in the form of globus or subglobus, papules are located in terminal or intercalary areas, hyphae are branched with septa (Watanabe, 2010). Isolate I1 was similar to the genus *Rhizoctonia* in that conidia were not formed, hyphae branched to form a right angle, bounded by septa between the main hyphae and side branches (Watanabe, 2010).

Isolates I2, I6, I7 were similar to the genus *Penicillium*, namely the formation of conidia, hyaline conidiophores, erect, containing monoverticillate peniles consisting of several metulae, phialides and catenulated conidia at the ends, phialosporus conidia, hyaline, round in shape (Watanabe, 2010). Isolate I8 was similar to the genus *Microsporum* in that conidia were not formed, hyphae were white or yellow with a glabrous to downy texture, branched with bamboo-like septa. Several types of *Microsporum* were also known as fungi that are pathogenic, especially on the skin (Indrawati & Fakhrudin, 2016). Isolate I10 was similar to the genus *Cladosporium*, namely dark brown to brownish black colonies with powdery hyphae texture, branched and septate hyphae, with round to elliptical conidia and chain-shaped conidia collections.

CONCLUSION

This research has succeeded in isolating 6 genera of fungi (rhizosphere) which have been identified. Genus *Rhizoctonia*, *Penicillium*, and *Papuplaspora* were found to have resistance to Pb up to a concentration of 5,000 ppm, while Genus *Microsporum* and *Cladosporium* were not resistant. The genus *Sclerotium* has the ability to be resistant and not resistant to Pb up to a concentration of 5,000 ppm which raised the suspicion that the isolates came from different species. Meanwhile, all isolates were found unable to grow at the high concentration tested, namely 10,000 ppm.

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