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Bacillus U3 and *Pseudomonas* U4 are Able to Provide NPK and Produce Ligninase Enzymes Qualitatively

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***Abstract*: Peat land is known as land that has low productivity because it contains very few macro and micro nutrients, making it less suitable for use as land for cultivating plants. One way to improve peatland fertility is by adding lignocellulolytic and NPK solubilizing bacteria which have the potential to act as biofertilizers. This study aims to evaluate the potential of *Bacillus* U3 and *Pseudomonas* U4 as biofertilizers by testing the solubilizing NPK and enzymatic activity of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. The test was carried out qualitatively using standard methods to detect the presence and activity of NPK and the enzyme. The results showed that both bacterial isolates had significant resulte in solubilizing phosphate and enzymatic activity, indicating their potential as biofertilizers. The activities of LiP, MnP, and laccase in *Bacillus* U3 and *Pseudomonas* U4 show their ability to degrade lignin, which can increase nutrient availability in the soil. This shows the potential of both as effective biofertilizers in increasing the decomposition of organic matter and improving soil fertility, especially in peatlands that have low productivity.**

***Keywords*: biofertilizer, ligninase, NPK, peatland.**

1. Introduction

Indonesia has around 10.8% peat land with an area of 14.9 million hectares, especially in Sumatra, Kalimantan and Papua [1]. Peatlands tend to be unproductive due to the low content of macro and micro nutrients, as well as a low pH between 3.0-4.5 [2]. To increase productivity, land engineering is needed which involves adding nutrients [3].

Peat land is land that has low productivity because it contains very few macro and micro nutrients, making it less suitable for use as land for cultivating plants. So that peatlands can be planted and used as land for cultivating plants, macronutrients can be added to increase their productivity. Peatlands formed from plant remains experience slow decomposition due to acidic conditions that do not support the life of decomposing microorganisms [4]. The addition of acid-resistant microorganisms, such as bacteria, yeast, or fungi, can accelerate decomposition [5], [6].

The macro nutrient NPK has a very important role in plant growth and production, where these three elements interact with each other to support plant growth. Based on its fertility level, peatlands have a macronutrient content of N 0.7%, P 0.05-0.25%, and K 0.10-0.03%, but the amount that can be absorbed by plants is very small. The minimum amount of macro nutrients based on SNI (19-7030-2004) that plants need is N 0.40%, P 0.10% and K 0.20%, so the macro nutrients in peatlands need to be increased so that they can be used as cultivation land.

Lignin is the main component in organic materials which makes it difficult to decompose. The lignin decomposition process requires the ligninase enzyme, which consists of lignin peroxidase, manganese peroxidase, and laccase. These enzymes help break down complex lignin structures, which contributes to improving the quality and fertility of peat soil [3]. The aim of this research is to determine whether *Bacillus* U3 and *Pseudomonas* U4 are able to provide NPK elements and produce ligninase enzymes.

2. Methods

A. The Isolates Used

The isolates used are Bacillus U4 and Pseudomonas U3, collected from the Microbiology and Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, and were isolated from Bereng Bengkel Village, Central Kalimantan [7]. *Bacillus* U4 (B U4) was chosen because it can produce cellulase enzymes and degrade cellulose, while *Pseudomonas* U3 (P U3) is a gram-negative bacterium used as a comparison to the gram-positive Bacillus U4 [8].

B. Screening NPK

Nitrate screening is carried out to determine whether isolates are able to provide nitrogen in the form of nitrate. One isolate was subcultured and inoculated into JNFb medium (James Nitrogent Free Malat Bromthymol Blue) agar plates (which have been sterilized using an autoclave) using the point method. JNFb medium per liter consists of 5 g of malic acid; 0.013 g NH_2CONH; 0.1 g NaCl; 1.8 g KH_2PO_4;0,2 g $MgSO_4.7H_2O$; 0.6 g K_2HPO_4; 0,2 g $CaCl_2.2H_2O$; 2 ml bromothymol blue; 0,02 g yeast extract; 4,5 g KOH (pH=5,8); 17 g agar; 2 ml

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micronutrien (0,2 g $Na_2MoO_4.2H_2O$ 0,235 g $MnSO_4.H_2O$, 0,28 g H_3BO_3, 0.008 $CuSO_4.5H_2O$, 0.024 g $ZnSO_4.7H_2O$, dissolved in up to 200 ml of distilled water). All ingredients are put into a 1 liter measuring flask, added with distilled water up to 1000 ml, the pH is measured up to pH=7. Then sterilized by autoclave (121°C, 15 minutes). The culture was incubated for 4 days at room temperature, the formation of a blue zone around the colony showed that the isolate was able to provide nitrate [9].

Phosphate solubilizing screening using medium pikovskaya sterilized solid. Pikovskaya medium has a cloudy white color due to the content of tricalcium phosphate ($Ca_3[PO_4]_2$) as insoluble phosphate in the medium [10]. The composition per liter consists of 5 g $Ca_3(PO_4)_2$; 10 g glukosa; 0,2 g NaCl; 0,2 g KCL; 0,1 g $MgSO_4.7H_2O$; 0,5 g $(NH_4)_2SO_4$; 0,5 g yeast extract; 0,002 g $MnSO_4.H_2O$; 15 g agar; 0.025 g BPB (Bromo-phenol-blue); and 0.002 g $FeSO_4.7H_2O$ is dissolved in distilled water up to 1000 ml measured to pH 7, then sterilized by autoclaving (121°C, 15 minutes). One dose of isolate from the subculture medium was inoculated into the medium pikovskaya agar using the dot method, incubated at room temperature for 4 days, the clear zone formed around the colony is an indication that the isolate is able to dissolve complex phosphate in the medium Pikovskaya [11].

Potassium solubilizing screening using medium Alexandrov. Medium Alexandrov. The solid composition per liter consists of: 5 g glucose; 0.5 g $MgSO_4.7H_2O$; 0,006 g $FeCl_3$; 0,1 g $CaCO_3$; 2 g $Ca_3(PO_4)_2$, 3 g mica powder, 20 g agar, adjusted to pH 6.5. All ingredients were dissolved in distilled water up to 1000 ml, then sterilized by autoclaving (121°C, 15 minutes). One dose of isolate from the subculture medium was inoculated into the medium Aleksandrov using the point method, incubated for 4 days at room temperature, isolates that have a clear zone around the colony indicate that the bacteria can dissolve the potassium in the media Alexandrov [12].

C. Lignin peroxidase (LiP) Enzyme Activity Test

The test was carried out qualitatively on solid media with the addition of a substrate that can be degraded by lignin peroxidase (LiP). Screening for bacteria capable of secreting LiP was carried out using Luria-Bertani agar medium with the addition of a synthetic dye whose chemical structure resembles lignin, namely methylene blue. The composition of Luria-Bertani agar medium consists of 5 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract and 15 g/L agar. Next, 25 mg/L of methylene blue dye was added to the medium. The medium was sterilized using an autoclave at a temperature of 121^0 C and a pressure of 1.5 atm. Inoculate the isolate on the medium using the point method. Incubated for 120 hours at 37^{0}C and observed the formation of a clear zone around the colony which indicates decolorization due to lignin peroxidase activity.

D. Manganase Peroxidase Enzyme Activity Test

Qualitative screening of bacteria that secrete MnP was carried out by growing isolates on agar plates containing 1% glucose, 0.1% peptone, 0.05% MgSO4.7H2O, 0.1% K2HPO4, 0.1 mM MnSO4, agar 1, 5% and supplemented with phenol red 0.01%. It was incubated for 48 hours at 370 C and the color change of the medium was observed from dark orange to light yellow which indicated the presence of manganese peroxidase activity [13].

E. Laccase Enzyme Activity Test

Qualitative screening of laccase-secreting bacteria was carried out using agar plates containing 1% dextrose, 0.5% peptone, 0.3% beef extract, 0.5% NaCl, 1mM $CuSO_4$, 1.5% agar and supplemented with 0.5% guaiacol. 02%. Incubated for 48 hours at 37^{0}C and observed a brownish red color change around the colonies which indicated substrate oxidation by laccase-secreting bacteria [13].

3. Results and Discussion

A. Nitrogen Screening

Pseudomonas U3 and *Bacillus* U4, after the nitrogen supply test was carried out, did not show a blue zone around the isolate, indicating that the two isolates could not provide nitrogen (Figure 1).

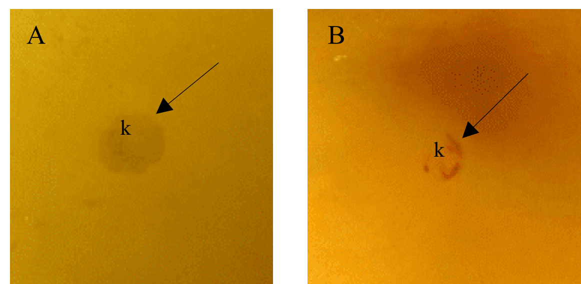

Fig. 1. Screening Results for Nitrogen Providers Arrows Show that There Are No Blue Zones Around the Colony (k: colony, A: Pseudomonas U3; B: Bacillus U4)

Bacteria will provide nitrogen if there is a blue zone around the colony. Nitrogen-providing bacteria have the ability to increase the efficiency of use of N-available in the soil [14]. The blue zone around the colony occurs due to an increase in the pH of the medium which indicates an alkaline reaction by the isolate in the medium [15]. Based on research, no blue zone was found which indicates that Pseudomonas U3 and Bacillus U4 cannot provide nitrogen.

B. Phospat Screening

Pseudomonas U3 and Bacillus U4 after carrying out the phosphate supply test showed a clear zone which indicated that both isolates could dissolve phosphate (Figure 2).

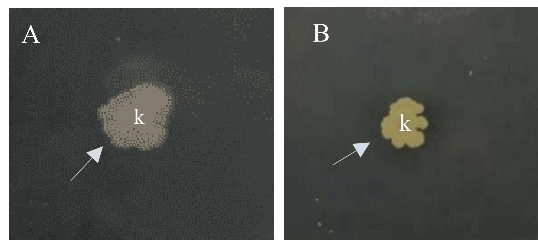

Fig. 2. Screening Results for Phosphate Providers Arrows show that Around the Colony There is a Clear Zone (k: colony, A: Pseudomonas U3; B: Bacillus U4)

The formation of the clear zone is caused by the activity of the phosphatase enzyme in Pseudomonas U3 and Bacillus U4, apart from that, both isolates also produce organic acids which can release phosphoric acid in the media resulting in a change in the color of the media from white to clear.

According to Zulaika and Rositawati (2015) media pikovskaya cloudy white because it contains calcium phosphate ($Ca_3[PO_4]_2$) as a source of phosphate. The clear zone is formed due to the release of bound phosphate ($Ca_3[PO_4]_2$) contained in the media pikovskaya. Bacteria that dissolve phosphate produce organic acids with low molecular weight such as oxalate, succinate, tartaric, citrate which can bind Ca ions from ($Ca_3[PO_4]_2$) and released H_2PO_4 thus forming a clear colored area and causing the phosphate to bind into an available form [11]. The phosphatase enzyme that can be produced by phosphate solubilizing bacteria is an enzyme that plays an important role in providing dissolved phosphate in the soil as organic phosphate [16].

It can be concluded that *Pseudomonas* U3 and *Bacillus* U4 is an isolate that can dissolve phosphate so it has the potential to be used as biofertilizer as a phosphate provider.

C. Kalium Screening

Pseudomonas U3 d Bacillus U4 after carrying out the potassium provider test showed that there was no clear zone around the isolate which indicated that the two isolates could not dissolve potassium (Figure 3).

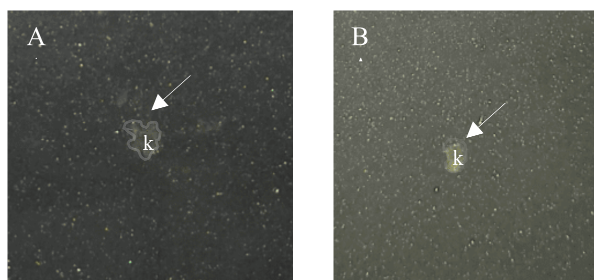

Fig. 3. Screening Results for Potassium Providers Arrows Show that Around the Colony There is No Clear Zone (k: colony, A: Pseudomonas U3; B: Bacillus U4)

According to Etesami et al., (2017), a clear zone forms around the colony on the media Aleksandrov indicates the activity of bacteria in dissolving potassium. The clear zone indicates the solubility of potassium in the media and the secretion of organic acids produced by potassium-solubilizing bacteria [17]. Organic acids produced by potassium solubilizing bacteria such as oxalic acid, tartaric acid, gluconic acid, 2-ketogluconic acid, citric acid, malic acid, succinic acid, lactic acid, propionic acid, glycolic acid, malonic acid, and fumaric acid are effective in releasing K from minerals containing K. Organic acids produced by potassium solubilizing bacteria can also release K ions from K minerals by chelating (forming a complex) Si ions^{4+}, Al^{3+}, Fe^{2+}, and Ca^{2+} which is associated with mineral K^+ [10].

D. Ligininase Activity

Pseudomonas U3 and Bacillus U4 were able to produce lignin proxidase (LiP) and manganese peroxidase (MnP) enzymes, indicated by the formation of LiP and MnP degradation zones around the colony, and were unable to produce laccase enzymes, indicated by the color of the isolate not changing (Figure 4).

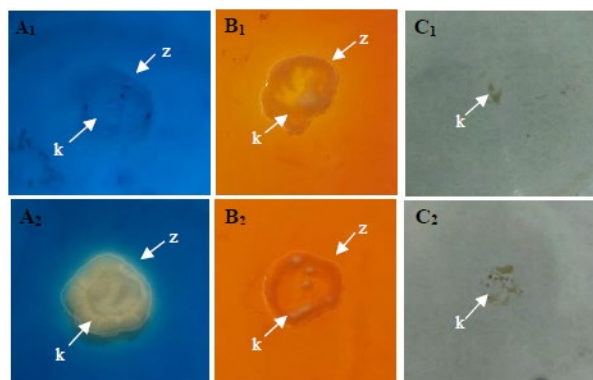

Fig. 4. Ligninase test results on Pseudomonas U3 and Bacillus U4 Qualitatively. (A. LiP activity; B. MnP activity; C. Laccase activity; 1. Pseudomonas U3; 2. Bacillus U4; k: colonies; zd: zona degradasi)

Methylene blue decolorization is used as an indicator of LiP enzyme activity. The existence of a degradation zone in the media is caused by the degradation of methylene blue contained in the media.

According to Solikhah and Zulaika (2018), the degradation zone around the colony occurs due to the degradation of methylene blue dye by lignin peroxidase (LiP) [7]. LiP contains heme which catalyzes the oxidative degradation of hydrogen per oxide of lignin [17].

MnP oxidizes Mn^{2+} ions to Mn^{3+} which is stabilized using organic acids which act as redox reaction mediators that are able to diffuse and degrade phenolic compounds such as phenol red. The phenol red color change occurs due to the oxidation of glucose by the sugar oxidase enzyme, resulting in the production of H_2O_2 and acidification of the media required for melanoidin degradation [13].

Pseudomonas U3 and Bacillus U4 did not show any brownish red color changes around the colonies, indicating that the two isolates did not have laccase activity. Bacteria that have laccase activity are indicated by a brownish change in the color of the media around isolates that have been supplemented with guaiacol.

According to Solikhah and Zulaika (2018), a change in the color of the media around the colony to a brownish red color indicates the presence of laccase activity. Laccase is a copper-containing polyphenol oxidase. The laccase enzyme catalyzes the four-electron reduction of oxygen to water and is usually accompanied by the oxidation of phenolic substrates to phenoxyl radicals. In the oxidation reaction catalyzed by laccase, guaiacol, which is the substrate, will be oxidized and lose one electron, forming a free radical in the form of o-quinone. Guaiacol then undergoes a non-enzymatic reaction in the form of polymerization of the compound in pairs with other radical substrates, resulting in a colored compound being formed [7].

Pseudomonas U3 and *Bacillus* U4 can produce lignin peroxidase and manganese peroxidase enzymes which can degrade some of the lignin structure, but cannot produce laccase.

4. Conclusion

Bacillus U3 and *Pseudomonas* U4 isolates have potential as biofertilizers for peatlands by solubilizing phosphate and through the production of ligninase enzymes, especially lignin peroxidase (LiP) and manganese peroxidase (MnP).

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