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Exploration of Nyamplung Rhizosphere Nitrogen Fixing Bacteria in Limestone Mine Reclamation Land

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Abstract *– Nitrogen fixing bacteria have the ability to utilize free nitrogen as a nitrogen source for their growth. These bacteria are able to make efficient availability of N- in the soil, so that it can be utilized by plants. The aim of this study was to obtain nitrogen fixing bacteria isolates from the rhizosphere of nyamplung plants in limestone reclamation lands. Isolation and testing of nitrogen fixation ability by nitrogen fixing bacteria was carried out using selective Ashby's Mannitol Agar media. The isolation results obtained 5 isolates on Ashby's Mannitol Agar media. The morphological characters of the colonies of the five isolates showed round and irregular shapes, flat and convex elevations with flat edges, white, cloudy white and clear. Microscopic observation showed that the isolates consisted of gram-positive and negative bacteria with bacilli cell shape. The catalase test showed 4 positive isolates and 1 negative. The conclusion of this study was that 5 nitrogen fixing bacteria isolates were obtained from the rhizosphere of the nyamplung limestone land plant. Isolates 1, 3, and 5 showed the character of the genus Clostridium, isolate 2 led to the genus Bacillus, and isolate 4 had the same character as the genus Klebsiella.*

Keywords: *Limestone, nitrogen fixing bacteria, Nyamplung rizosphere*

INTRODUCTION

Tuban Regency is known as one of the main lime producers in East Java (Majid & Sukojo, 2017). This is due to the abundant mineral resources in the form of limestone which are spread over several sub-districts, starting from the sub-districts of Palang, Semanding, Merakurak, Montong, Kerek, and Tambakboyo.

Limestone is a type of carbonate rock and contains the main mineral in the form of calcite. This limestone is used as an ingredient in the cement, quicklime, and slaked lime industries (Aziz, 2010), where the cement industry is a widespread consumer in the use of this limestone. Along with the rapid development of the cement industry, the mining of limestone is also carried out more intensively.

Limestone mining activities are generally carried out openly (open pit). Mining with this system has negative impacts including increased dust and vibration flows, decreased water quality, changes in fauna habitat, decreased soil quality, damaged landscapes, and loss of vegetation (Andriani et al., 2019; Mahardi, 2019). As a result, when the mining process has been completed and leaving post-mining land, this land becomes nutrient-poor marginal land (Aisah et al., 2022; Sriwulan et al., 2022).

Reclamation according to the regulations of Undang-undang no. 3 of 2020 is written as an activity in all stages of mining activities for the purpose of managing, restoring and improving the quality of the environment and ecosystem so that they can return to functioning as they should. This activity must be carried out holistically and as soon as possible, even though the mining process has not been completed as a whole (Zainuddin, 2021). Thus, reclamation carried out by mining companies is directed at revegetating in order to restore forest areas.

Revegetation is an effort to replant on land that has finished mining. This process is carried out by preparing technical designs, providing fields, procuring seeds, planting and caring for plants (Setyowati et al., 2017). One way to revegetate land after limestone mining can be done by using the nyamplung plant (*Calophyllum inopyllum*).

Nyamplung (*Calophyllum inopyllum*) is a type of plant that can live in dry areas. This ability is supported by the presence of adaptive microorganisms which generally colonize the area around plant roots. These adaptive micro-organisms generally have a certain ability to adapt to unfavorable environmental conditions, such as nutrient-poor limestone post-mining land. Such microorganisms need to be explored to obtain sources of isolates which in turn have potential as biological fertilizer agents, especially for application to nutrient-poor lands such as post-limestone mining lands.

Microorganisms, in this case bacterial isolates with potential as biological fertilizer agents, one of which is nitrogen fixing bacteria. This group of bacteria is one of the rhizosphere bacteria that can increase the use of available N in the soil by fixing free Nitrogen.

Nitrogen itself is an essential nutrient, which is absolutely needed by plants to grow optimally. Plants need this element in large quantities in order to support its growth. The source of nitrogen needed by plants is generally in the form of N_2 gas in the atmosphere which can be fixed due to an electric jump, from a biological fixation process by the symbiosis of certain plants with bacteria, or from industrial N fertilizer processes.

Plants cannot use N in free form, but N can be used by plants in ionic form. In a reduced state, plants will absorb N in the form of ammonium (NH_4^+) while in an oxidized state, N is absorbed in the form of nitrate (NO_3^-) (Santoso & Rahmawati, 2019). This causes plants to need nitrogen-fixing bacteria so that ammonia can be absorbed and converted into nitrate ions, and then released into the environment (Widowati et al., 2012).

Much of research related to the exploration of nitrogen-fixing bacteria has been carried out, including exploring N-fixing bacteria from the rhizosphere of the mangrove plants of the Peniti Mempawah River (Santoso & Rahmawati, 2019) and Kuala Singkawang (Saputri et al., 2021). Analysis of the potential of N-fixing bacteria in peat soil was also carried out by Agustine et al. (2014) and Istina et al. (2020), Whereas on limestone land, phosphate solubilizing and nitrogen fixing bacteria have been characterized by Fitriyanti (2017). However, the research sample came from a limestone mining area in the Cirebon area, belonging to PT Indocement, Palimanan to be exact. Meanwhile, exploration of nitrogen-fixing bacteria from the rhizosphere of nyamplung plants on post-limestone mining land in Tuban Regency has never been carried out. The description shows that there is an urgency to explore phosphate solubilizing bacteria from the rhizosphere of the nyamplung plant growing on limestone post-mining land in order to obtain nitrogen-fixing bacteria isolates that are adaptive to the limestone post-mining land. Thus the isolates from the results of this study can be used for the development of further biofertilizers.

MATERIALS AND METHODS

This research was conducted in December 2022 to March 2023 at the Biology Laboratory of Universitas PGRI Ronggolawe, Tuban. This research is a descriptive exploratory study conducted to explore nitrogen fixing bacteria from the rhizosphere of nyamplung plants on limestone mine reclamation land.

1. Materials

The tools used in this study were bunsen, aluminum foil, tripod and gauze, petri dish, camera, cover glass, object glass, beaker glass, Erlenmeyer, ose needles, microscope, dropper pipette, zip lock plastict, spatula, test tube rack, test tube, analytical balance, tissue, plastic warp, colony counter, and concave glass. Meanwhile, the materials used in this study were nyamplung plant rhizosphere soil samples from limestone mine reclamation land owned by PT. Semen Indonesia (Persero) Tbk Tuban Factory, Ashby's Mannitol Agar selective media, NaCl 0,85%, gram dye reagent, H_2O_2, and distilled water.

2. Procedures

a. Soil Sampling

Soil samples were taken from the rhizosphere of the nyamplung plant at the completion of the limestone mine reclamation of PT. Semen Indonesia Persero (Tbk) Tuban Factory. Soil sampling was carried out by taking soil at a depth of 15-20 cm from the soil surface around the roots of the nyamplung plant as much as 100 grams at each point and then homogenizing. Samples were taken from 5 points on the reclamation limestone mining site after the mining was completed in 2020. The composite soil samples were then put into a plastic zip lock, labeled, and stored in a cooler box to be taken to the laboratory.

b. Isolation of nitrogen fixing bacteria

Bacteria were isolated with Ashby's Mannitol Agar selective media containing 3 g Agar; mannitol 1.5 g; Dipotassium Hydrogen phosphate 0.04 g; Magnesium sulfate 0.04 g; Sodium chloride 0.04 g; Potassium Sulpathe 0.01 g; Calcium carbonate 0.5 g (Sujatha et al., 2015), a mixture of each ingredient composition is dissolved in 200 ml of distilled water and then heated with a Bunsen burner for 15 minutes or until boiling. The choice of Ashby Mannitol Agar media in this study is because this medium does not contain nitrogen (Pambudi et al., 2017).

The isolation process was carried out by weighing 25 grams of soil sample and adding 225 ml of sterile NaCl 0,85% to obtain a 10^{-1} dilution. Then serially diluted by taking 1 ml of suspension from 10^{-1} and putting it in a test tube that has been filled with 9 ml of sterile NaCl 0,85% solution (10^{-2}). This procedure was carried out until a dilution level of 10^{-7} was obtained. Each level of dilution was inoculated using the pour plate method, where 1 ml of the suspension was put in a sterile petri dish and added to Ashby's Mannitol Agar, homogenized and waited for it to solidify. Then incubated upside down at room temperature for 3x24 hours to 7x24 hours. Every 1x24 hours an observation is carried out in order to see the growth of bacteria in the media.

c. Characterization of nitrogen fixing bacteria

Nitrogen-fixing bacterial colonies obtained were then characterized based on colony morphological characters, microscopic characters (cell shape), gram type, and catalase test.

Characterization based on colony morphology is based on colony shape, elevation, surface, margins, and colony color. The isolated colonies were then purified on the surface of the selective Ashby Mannitol Agar medium using a streak plate. The purification process was carried out 4 times with the aim of getting a single colony.

After obtaining a single colony from the purification process, the microscopic character of the bacteria was observed using a Gram stain reaction. The staining process begins by taking 1 ose of bacterial suspension and spreading it on a glass object then fixing it on a busen lamp. Drop as much as 1 drop of Crystal Violet solution (gram A). Left for 1 minute then rinsed using running water and dried. Then the preparation was flooded

with Lugol's Iodine solution (gram B) for 1 minute, rinsed again and dried. Furthermore, the 96% alcohol solution (gram C) was dripped and left for 30 seconds, rinsed again and dried. Then 1 drop of Safranin solution (gram D) is dripped and left for 45 seconds. Wash it with running water and let it dry (Fajrin et al., 2017). Stained preparations were observed under a microscope with a magnification of 400x. Isolates stained purple or blue are gram-positive bacteria while those that appear stained pink are gram-negative.

The catalase test was carried out using 3% hydrogen peroxide (H_2O_2) and dripped on a clean object glass. The culture is smeared on the glass object using an ose needle. The suspension is mixed slowly using a loop, positive results are shown by the formation of air bubbles (Pulungan & Tumangger, 2018).

d. Nitrogen fixation ability test

Testing the ability of nitrogen fixation by bacteria in this study was carried out qualitatively by observing colonies growing on nitrogen-free Ashby's Mannitol Agar media.

RESULT AND DISCUSSION

1. Isolation and characterization of nitrogen fixing bacteria

In the isolation process in this study, 5 different isolates were obtained based on the results of the characterization carried out. Colony morphological characteristics of the isolates obtained are shown in Table 1.

Table 1 shows the rhizosphere bacterial isolates of nyamplung plant from limestone mining reclamation land obtained by the pouring cup method, in which 5 isolates with different bacterial colony morphology were obtained. The differences in the morphological characters of the colonies indicated that the five isolates obtained were different bacteria (Fulyani et al., 2022). Nitrogen-fixing bacterial isolates found in this study were non-symbiotic nitrogen-fixing bacteria. The number of nitrogen-fixing bacterial isolates found was influenced by the type of plant roots and plant root exudates where they were found (Istina et al., 2020; Lengkong et al., 2022). In addition to plant factors, environmental conditions can also affect the type of bacterial isolates found. This is in line with the opinion of Prihastuti (2011) which states that the presence of bacteria in the rhizosphere area of plants is closely related to the health of plant roots and environmental factors such as temperature, humidity, aeration, soil type and texture, and organic matter content in the soil. In post-lime mining land, it is known that the pH tends to be alkaline and the temperature is relatively high (Alfariza et al., 2021). This high pH and temperature will affect the composition of the bacteria found. The tolerable pH range for non-symbiotic nitrogen-fixing bacteria itself ranges from 5.0-8.5. However, there are certain types that can also survive at a pH below 5.0 (Agustine et al., 2014).

Table 1. Morphological characteristics of rhizosphere nitrogen-fixing bacteria colonies of nyamplung plants on post-limestone mining land

Isolate	Shape	Edge	Elevation	Colour
1	Round	Flat	Convex	Cloudy white
2	Round	Flat	Convex	Clear
3	Irregular	Flat	Flat	Cloudy white
4	Irregular	Flat	Flat	White
5	Dot	Flat	Convex	Cloudy white

While the isolation of bacteria in the rhizosphere of the nyamplung plant has never been done. However, Widawati & Suliasih (2019) stated that plant roots will secrete nutrients that are beneficial for bacterial growth and these nutrients are generally specific from one plant to another. Root exudate is a mixture of complex sugar compounds, amino acids, organic acids, and others which are closely related to the metabolism of each plant (Pinton et al., 2007). This root exudate will affect the presence of microbes through the provision of nutrients, especially nitrogen and phosphorus (Sapalina et al., 2022). Therefore the population and number of bacterial isolates from one plant rhizosphere can be different from other plants. Another Research by Saputri et al. (2021) found 5 isolates of nitrogen-fixing bacteria from the mangrove rhizosphere in Kuala Singkawang. While Santoso & Rahmawati (2019) only found 4 isolates of nitrogen-fixing bacteria from the mangrove forest soil of the Peniti River, Mempawah Regency. Likewise, Lengkong et al. (2022) who also found 4 isolates of nitrogen-fixing bacteria from the rhizosphere of bamboo plants.

While the isolation and characterization of nitrogen-fixing bacteria from limestone fields was carried out by (Fitriyanti, 2017) and (Mubarik & Hastuti, 2015). Fitriyanti (2017) used land from post-limestone mining land owned by PT. Indocement, Palimanan Cirebon as a sample and obtained isolates of phosphate solubilizing bacteria which also have the ability to fix nitrogen originating from the genera *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, and *Acinetobacter*. Meanwhile, research by (Mubarik & Hastuti, 2015) which used 13 post-limestone mining soil samples, found 8 isolates of nitrogen-fixing bacteria, which were divided into 2 symbiotic isolates and 6 non-symbiotic isolates.

The five isolates obtained from the isolation results were then purified and observed microscopically. Purification in this study was carried out up to 4 times to obtain a single colony. Single colonies obtained from purification results were then stained with differential staining using gram staining. Furthermore, each isolate was subjected to a catalase test, positive catalase test results were indicated by the formation of air bubbles. The results of microscopic observations and catalase test of nitrogen-fixing bacterial isolates can be seen in Table 2 and Figure 1.

Table 2. Results of microscopic observations and catalase test of nitrogen fixing bacterial isolates

No.	Isolate Code	Cell Shape	Gram Type	Catalase
1	Isolate 1	Rod	Positive	+
2	Isolate 2	Rod	Positive	+
3	Isolate 3	Rod	Positive	-
4	Isolate 4	Rod	Negative	+
5	Isolate 5	Rod	Positive	+

Table 2 shows that of the 5 nitrogen-fixing bacterial isolates obtained had bacillus-shaped cells. Meanwhile, based on the type of gram, the 4 of the isolates were gram-positive bacteria (Isolate 1, 2, 3, and 5) and isolate 4 was gram-negative.

Isolates 1, 2, 3, and 5 in this study appeared purple in color (Figure 1), where the color comes from the color of the crystal violet solution which is maintained by gram-positive bacteria due to the presence of a thick

peptidoglycan layer on their cell walls (Pratita, 2012).

Table 2 also shown that all isolates showed positive catalase results, except isolate 3. A positive catalase test was indicated by the formation of oxygen bubbles, where the presence of oxygen bubbles indicated that the bacteria produced the catalase enzyme which was used to break down H_2O_2 (Hydrogen Peroxide) into water and O_2 (Oxygen) (Pelealu et al., 2018). Bacteria that can decompose H_2O_2 compounds are indicated as bacteria that live in an aerobic environment. This is because H_2O_2 compounds are produced in aerobic metabolism (Panjaitan et al., 2020). Thus, based on the results of the catalase test in this study, isolates 1, 2, 4, and 4 are nitrogen-fixing bacteria that live in an aerobic environment. While isolate 3 is a nitrogen-fixing bacteria that lives in an anaerobic environment. Tarigan et al. (2013) also found that in general, nitrogen fixing bacteria from the plant rhizosphere were catalase positive.

Based on the characterization carried out, isolates 1, 3 and 5 lead to the characteristics of the genus *Clostridium*. *Clostridium* is a group of non-symbiotic nitrogen-fixing bacteria characterized by round to irregular colonies, flat edges, flat to convex elevations, white to cloudy white on solid media, bacilli-shaped cells and includes gram-positive results, positive or negative in the catalase test (Kaburuan et al., 2014). This group of bacteria are non-symbiotic nitrogen-fixing bacteria that are commonly found in soil. This is related to the nitrogen fixation ability of this group of bacteria which does not demand soil conditions with excessive aeration or certain organic matter content. Thus, in post-limestone mining reclamation land conditions, simple land management can still support the growth of these bacteria.

Meanwhile, the characters possessed by isolate 2 are close to those of the genus *Bacillus*. This group of bacteria is characterized by bacilli-shaped cells, gram-positive types, catalase-positive, with round to irregular colonies, flat edges, and convex elevations and pale white to clear colors (Riskawati, 2016). *Bacillus* is a group of non-symbiotic nitrogen-fixing bacteria that can be found in the rhizosphere of plants and soil (Suwatno, 2012). This bacterium can also live in various soil conditions, such as nutrient-poor soil, including post-limestone mining reclamation land in this study.

Isolate 4 shows the character belonging to the genus *Klebsiella*. This genus has colonial characters with irregular shapes, flat edges, flat elevation, milky white in color, bacilli-shaped cells with gram-negative and positive types on the catalase test. The *Klebsiella* genus is known as one of the soil bacteria that can produce IAA (Sari, 2014).

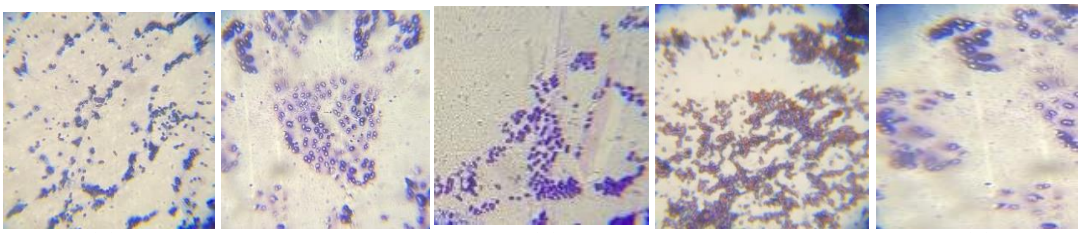

Figure 1. The results of microscopic observations of nitrogen fixing bacteria with a magnification of 40x10, (a) Isolate 1, (b) Isolate 2, (c) Isolate 3, (d) Isolate 4, (e) Isolate 5.

2. Nitrogen fixation ability test

Testing the ability of nitrogen fixation in this study was carried out qualitatively by growing isolates on selective nitrogen-free media (Lengkong et al., 2022). The nitrogen-free selective medium used in this study was Ashby's Mannitol Agar medium. Thus, the isolates that are able to grow on the media are isolates that have the ability to fix free nitrogen. This is because there is no nitrogen content in Ashby's Mannitol Agar media, therefore only nitrogen-fixing bacteria can grow on this media (Hartono & Jumadi, 2014). Thus, 5 isolates of nitrogen fixing bacteria were obtained which were able to grow on Ashby's Mannitol Agar media. These isolates have the potential to be further developed as biofertilizer agents to help provide N for plants.

The ability of nitrogen-fixing bacteria to carry out nitrogen fixation is due to the presence of the nitrogenase enzyme they are capable of producing. The enzyme is an enzyme complex with 2 components metal dinitrogenase MoFe (molybdenum-iron protein) and dinitrogenase reductase (iron protein). Dinitrogenase MoFe is a catalytic component of the nitrogenase enzyme (Mahmud et al., 2020). The role of the nitrogenase enzyme in the N_2 fixation process is to catalyze the breakdown of N_2 which requires 16 ATP molecules (Sapalina et al., 2022).

Nitrogenase enzymes are basically very sensitive to the presence of oxygen. Therefore, in the group of aerobic nitrogen-fixing bacteria, such as isolates 1, 2, 4, and 5 in this study, they have their own mechanisms to protect the nitrogenase enzymes they produce. Oxygen produced from the breakdown of H_2O_2 will be neutralized by leghemoglobin (Boyd & Peters, 2013). Under anaerobic conditions (in this study on isolate 3), the nitrogenase enzyme will work well. In some nitrogen fixing bacteria such as *Azotobacter*, the oxygen concentration is limited to low levels by increasing the respiration rate. If the oxygen concentration is high, the nitrogenase enzyme is not activated. In addition, these bacteria form an alginate capsule on their cell surface which is believed to protect nitrogenase from oxygen (Castillo et al., 2020).

CONCLUSION

The results showed that 5 isolates of nitrogen-fixing bacteria were obtained from the rhizosphere of nyamplung plants on post-limestone mining reclamation land. Isolates 1, 3, and 5 show the characters belonging to the genus *Clostridium*. Meanwhile, isolate 2 showed the characters belonging to the genus *Bacillus* and isolate 4 had the same character as the genus *Klebsiella*.

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