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# SELECTION OF INDIGENOUS N-FIXING RHIZOBACTERIA FROM POST-TIN MINING AREAS

D Wulandari[1*], R Rosita[2], A F Maulana[3], I Mansur[4], Kasam[1]

[1] Department of Environemntal Engineering, Faculty of Civil Engineering and Planning, Universitas Islam Indonesia, Yogyakarta, IndonesiaJl Kaliurang KM 14, 5 Yogyakarta, Indonesia. 55584
[2] SEAMEO BIOTROP, Jl Raya Tajur Bogor 16134, Indonesia
[3] Department of Bioresources Technology and Veterinary, Vocational College, Universitas Gadjah Mada, Gedung SV UGM, Sekip Unit 1, Depok Sleman Yogyakarta 55281, Indonesia
[4] Faculty of Forestry, IPB University, Dramaga Bogor 16680, Indonesia

## ABSTRACT

Tin mining is one industry that contributes to Indonesia's economic development. However, because tin is always in high demand, this activity creates an environmental problem. Silica sand, the dominant soil in post-tin mining, is easily eroded by water and wind, resulting in soil nutrient deficiency including N. Because this condition makes it difficult for organisms to survive, rehabilitation is essential. Nitrogen (N) is an essential nutrient for plant growth. N-fixing rhizobacteria are well-known for fixing N from the atmosphere, whether through symbiosis or otherwise. The goal of this study was to isolate indigenous N-fixing rhizobacteria from a post-tin mining area. The soybean plant was used as the testing plant. Three types of N-fixing rhizobacteria were isolated from the nodule of Acacia mangium growing in a post-tin mining area: B1, B2, and B3. To minimize the nutrient content in the growth media, the plant was grown in sterilized sand. Inoculated and non-inoculated soybean were grown in a greenhouse for three months without fertilizer. There were seven replications. Among the treatments, B3 had the highest soil and pod N content, best growth performance, nodule formation, and soybean production. This finding suggests that B3 could be used for future rehabilitation in the post-tin mining area.

*Keywords:* post-tin mining, rehabilitation, rhizobacteria. N deficiency

## 1. INTRODUCTION

Surface mining is the only method used for tin mining practise in Bangka, Indonesia (Oktavia et. al., 2014). Though tin mining contributes on economic development in Indonesia, however, this process of mining resulted on a wide of land degradation in this area. Abandoned post tin mining remained fragile soil dominated by white quartz sand, deleterious soil fertility, water scarcity, change soil biophysiochemical characteristics, and finally create it to be unproductive land (Sukarman et. al., 2020). This condition lead to the unfavourable condition for organism to survive. Therefore, it is important to find a potential environmental friendly approach to overcome thus problem of post tin mining area.

[1*] Corresponding author's email: dewi.wulanndari@uii.ac.id
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Nitrogen is one of the most important nutrient for plant to growth. Nitrogen is playing a vital role in biochemical and physiological function, particularly growth, development, and reproduction of the plant by increasing yield and its quality (Leghari et. al., 2016). Nitrogen occupies 15 – 40 mg/g of dry matter in above ground tissue of healthy plants (White and Brown, 2010), indicating as the highest important nutrient compares to other nutrients for plant living. Nitrogen is the major component in forming chlorophyll (Bassi et. al., 2018), which means crucial for photosynthesis to occur in plants. Furthermore, nitrogen is part of component amino acids forming protein (White and Brown, 2010), which influence biochemical reactions in the plant cell. Without protein, plants will wither and die.

Nitrogen is commonly a deficiency in the degraded area regarding the lack of organic matter that is playing as the source of N. Nitrogen in the soil can be found as organic nitrogen compounds, ammonium ($NH_4^+$) ions, and nitrate ($NO_3^-$) ions, of which more than 90% are available in the form of organic nitrogen ( Li et. al., 2013). This form of organic nitrogen in the soil cannot be used directly by plants. In addition to nitrogen in the soil, it is estimated about 80% of N, in the form of N2, is in the atmosphere (Bernhard, 2010). However, this N2 also cannot be used directly and therefore is not useful for the plant. The N2 in the atmosphere and organic nitrogen in the soil need to be converted to be available for the plant to uptake. This conversion is only can be done by a specific microorganism.

N-fixing rhizobacteria, both symbiotic and symbiotic/associative bacteria, is a microorganism well known for its ability in providing available nitrogen for plants. Bradyrhizobium, Sinorhizobium, Azorhizobium, and Mesorhizobium are examples of symbiotic bacteria belonging to Rhizobium forming an association with leguminous plants (Wang et. al., 2018). Meanwhile, Azotobacter and Azospirillum are free-living nitrogen-fixing bacteria (Steenhoudt and Vanderleyden, 2000). These bacteria may fix N2 from the atmosphere and convert it into ammonium ($NH_4^+$) (Aasfar et. al., 2021). In addition to N2 fixation, this microorganism contributes to nitrogen mineralization in soil, which converts organic nitrogen into inorganic nitrogen.

Even though N-fixing rhizobacteria is well known in providing N, however, there is a variation in N production by these microorganisms. This variation includes introduced and indigenous bacteria. Some researchers reported that indigenous bacteria have a better contribution in providing N than introduced bacteria. Therefore, related to this fact and the role of N-fixing rhizobacteria, the selection of indigenous N-fixing rhizobacteria is important to get the best microorganism in providing N for future rehabilitation in degraded the post-tin mining area.

## 2. MATERIALS AND METHODS

### 2.1. Isolation of N-Fixing Bacteria

Isolation of nodulating bacteria with N-fixing bacteria as a target was carried out from root nodules of Acacia mangium and Pueraria javanica collected from the post-tin mining area. A. mangium and P. javanica were uprooted carefully. Healthy nodules were detached carefully from the root and collected carefully for further isolation of root nodulating bacteria (N-fixing bacteria). The detached root nodules were washed in tap water to remove soil particles following sterilization by immersing it in 70% alcohol solution for 10 seconds and washing it with sterilized deionized water at least 10 times to remove the alcohol. The sterilized nodule was weighed 0.1 gram and transferred into a test tube containing 0.9 ml sterilized deionized water and crashed it by sterilized glass rod

(pestle). About 0.1 ml suspension was transferred into a sterilized petri dish containing Congo Red Yeast Manitol (CRYEMA) agar by pour plate method under aseptic conditions (Roychowdhury et al, 2015). Inoculated CRYEMA was incubated at 28 oC under dark conditions for 3 – 5 days (Somasegaran et al, 1985). The colony that appears on CRYEMA media is reinoculated into a new sterilized CRYEMA by streaking one loopful of the colony into the petri dish. To avoid contamination, the petri dish is sealed with parafilm and incubated it at 28 oC for 24 – 48 h. The N-fixing bacteria colonies will remain white, translucent, elevated and mucilaginous, after 24-72 h. The colony was picked up and transferred to Yeast Manitol Agar (YEMA) for further characterization.

### 2.2. Preservation and Subculture of Isolated N-Fixing Bacteria

There were three successes in isolated N-fixing bacteria (B1, B2, B3), and were prepared for pure culture isolation. A single colony of N-fixing bacteria that appears in YMA media was isolated and transferred to other sterilized YMA media. Transferred isolated N-fixing bacteria were incubated at 25 oC under dark conditions. Re-culture of N-fixing bacteria was carried out every month.

### 2.3. Inoculum production

All isolates of N-fixing bacteria were cultivated into nutrient broth in 250 ml Erlenmeyer flasks shaken at 125 rpm at 28 oC. The 78 hours old culture of this isolated culture was used as inoculants.

### 2.4. Inoculation of N-Fixing Bacteria

There were three isolates of N-fixing bacteria tested to get the best isolates from the post-tin mining area. Selected seeds of soybean were immersed in alcohol 70% for 3 minutes to minimize contamination on the surface of the seedling. Sterilization was continued by the first inoculation of N-fixing bacteria by immersing the sterilized seed into liquid inoculums for 20 minutes. All sterilization and inoculation process was carried out under aseptic condition. Sterilized seed without inoculation was prepared as well as control. For reliable nodulation, sand as grows media was sterilized and about 500 grams of it was used to grow soybean. Each pot received five seeds of the soybean. One week after germination, only one seedling was allowed to grow in each pot. There were seven replications for each treatment. Three weeks after germination, the seedlings received the second inoculation of N-fixing bacteria (~ 200 ml/L of the substrate) suspension containing 106 CFU (cell forming unit), which was poured onto the rhizosphere of the seedling. A sterilized pipette was used for inoculation. Seedlings were allowed to grow for two months under greenhouse conditions. Fertilizer of modified Hoagland solution with free nitrogen was applied once in every two days.

### 2.5. Seedling growth parameter

Earlier pod production of soybean was measured one month after sowing. The number of soybeans, soybean fresh weight, number of nodules, N concentration of pod, and N total of soil were analyzed at harvest time two months after sowing.

### 2.6. Statistical analysis

Statistical significance of inoculation and non-inoculation treatments was analyzed using Kaleida Graph 4.1 software (Synergy Software 2012, USA) for analysis of variance (ANOVA). Post-hoc analysis was performed using the Tukey HSD test ($P < 0.05$).

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## 3. RESULTS AND DISCUSSION

Three isolates of Nitrogen-fixing bacteria were successfully isolated: B1 isolated from the nodule root of Pueraria javanica, B2 isolated from the nodule root of Acacia mangium, and B3 isolated from the nodule root of Acacia mangium, which is growing in the post-tin mining area. Inoculation of those three isolates resulted in a different response to the growth of soybean seedlings (Table 1). Earlier soybean formation was started only one month after planting with the highest number of soybean per seedling inoculated with B3. This result was continued for two months after planting at harvest time. Isolate B2 tended to give a higher number of soybean than B1 and control for one month after planting. The non-inoculated soybean resulted in the lowest yield of pod among treatment. This result is consistent with soybean fresh weight that shows isolate B3 as superior in producing the highest yield of soybean among treatments.

The biological interaction between N-fixing bacteria and the hostplant influenced the number of pods per seedling and the early formation of soybean in inoculated seedlings. When compared to control seedlings, inoculated seedlings produced significantly more nodules. Those interactions, which occur in a symbiotic relationship between plants and N-fixing bacteria, allow the host plant to obtain N2 directly from the atmosphere and meet its nutrient requirements (Wagner, 2011). It suggests that N-fixing bacteria are effective at providing nitrogen for soybean growth (Gelfand and Robertson, 2015). In agreement with our result, the addition of N-fixing bacteria increases the number of pods (Sankar et.al., 2015; Janagard et.., 2017).

Table 1. Effect of inoculating soybean with indigenous N-fixing bacteria on the number of pods, pod fresh weight, number of nodules per seedling, pod N concentration, and soil N concentration.

| Treatment | Number of pods per seedling | | Pod fresh weight (g) | Number of nodules per seedling | N concentration (%) | |
|---|---|---|---|---|---|---|
| | 1st month | 2nd month | | | Pod | Soil |
| Control | 0.28 a | 1.71 a | 0.53 ab | 3.28 b | 2.98 a | 0.01 a |
| B1 | 0.86 a | 2.43 a | 0.50 a | 5.60 a | 3.47 a | 0.01 a |
| B2 | 1.43 a | 2.14 a | 0.65 ab | 6.00 a | 3.74 a | 0.01 a |
| B3 | 1.71 b | 2.85 b | 0.71 b | 7.14 a | 4.10 a | 0.02 a |

B1: Bacteria 1 isolated from nodule of *Pueraria javanica*; B2: Bacteria 2 isolated from nodule of *Acacia mangium*; B3: Bacteria 3 isolated from *Acacia mangium*. A different letter in the same column indicates a significant difference between treatments according to the Tukey HSD test (P<005, n=7).

All inoculation of N-fixing bacteria resulted in higher root nodule formation than control (Table 1). Soybean nodulation occurs as a result of a mutualistic relationship between soybean plants and the bacterium. Inoculation of B3 tended to have higher root nodules followed by B2 and B1. The N-fixing bacteria immediately colonize the host plant`s root and form nodules as the house of bacteria once the bacteria were inoculated. However, each N-fixing bacteria has a different ability in colonizing and forming root nodules. Host plant influence this colonization by modulating the composition in producing root exudates (Hayley et. al., 2021). Plants secrete photosynthetically fixed carbon into the rhizosphere, creating chemical gradients that attract motile bacteria from the soil to the root surface (Mendes, et. al., 2013). Bacteria recognize root hair compounds and produce 'nod factors' (lipochitooligosaccharides) in response (Jones et. al., 2007).

All inoculation of N-fixing bacteria tended to have higher N concentration than control. Our result corresponds to Setiawati et. al (2022), which reported high N uptake of rice seedling growth inoculated with N-fixing bacteria. The N fixing bacteria was reported induced N2 fixation which contributes on N uptake by tomato (Masood et.al., 2020). This bacterial relationship is extremely valuable because, in the pod fill stage, soybeans can fix three pounds of nitrogen per acre per day (Abendroth et. al., 2006).
Inoculation of B3 tended to have the highest soil N concentration among treatments, increased by about 100% of N (Table 1). While there is no difference in soil N concentration between Bi, B2, and control. However, those values were very low for nitrogen concentration in the soil. The N-fixing bacteria support N fixation by converting the N2 in the atmosphere into nitrate/ ammonium (Saxena et. al., 2019) and transferring it directly to the host plant for plant growth. Therefore, there is no addition of nitrogen support by N-fixing bacteria to the soil. Soil N will be added only by incorporating the biomass of the host plant into the soil.

## 4. CONCLUSION

This research conclude that among indigenous N-fixing bacteria, isolate B3 resulted on better growth, improve yield, and increase nutrition of soybean. Isolate B3 improved the N concentration by fixing nitrogen from atmosphere, and therefore potential to be applied for future rehabilitation in degraded area such as post-tin mining area regarding to its nutrient deficiency particularly N.

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