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Research Article

Characteristics of Nitrogen-Fixing Bacteria Isolated from the Rhizosphere at Kaolin Lake, Belitung, Indonesia

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ABSTRACT

Former kaolin mining has poor physical and chemical properties for plants. The low nutrient content makes difficult for plants to grow. Nitrogen (N_2) is an important macronutrient for plants, but it can only be absorbed in the form of ammonium ions (NH^{4+}) or nitrate ions (NO^{3-}). Therefore, it is necessary to transform nitrogen, which is able to change the molecular form so that it can be absorbed by plants. The strategy that can be done is using nitrogen-fixing bacteria. This study aimed to explore and analyze the diversity of nitrogen-fixing bacteria based on 16S *r*RNA and *nif*H gene from the rhizosphere of the Kaolin Lake area. The method started with bacterial isolation from rhizosphere soil samples. Selected isolates were tested for ammonium content to determine the ability of isolates to fix nitrogen. In this study, 13 isolates had the ability to fix nitrogen, two of which had a high ammonium concentration, namely RBN 5.6, and RBA 2.3. The highest ammonium concentration was produced by isolate RBN5.6 amounting to 7.55 µg/ml. Based on 16S rRNA gene identification, isolate RBN5.6 was similar to *Burkholderia cepacia* and isolate RBA2.3 was similar to *Bacillus aquimaris*. Based on the detection of the *nif*H gene, isolate RBN5.6 was identified as a protein-encoding dinitrogenase reductase gene group cluster from the *Stutzerimonas stutzeri*.

1. Introduction

Mining activities are carried out in several regions of Indonesia, including lead mining in the Bangka Belitung Province. Mining activities caused the emergence of an excavation area in the form of a basin, which over time became a puddle of water so that it was shaped like a lake. The former mine is known as the Kaolin Lake. Kaolin Lake is a former tin mining site located in Nibung Village, Koba District, Central Bangka Regency (Savira *et al.* 2023). The soil in former kaolin mining has poor physical and chemical properties for plants, with a pH of 3.46, which is classified as very acidic. The N-total content was approximately 0.22%, with low P availability of 4.05%, C-organic content of 0.106%, and low cation exchange capacity (CEC) of 6.53% (Hamid *et al.* 2017). The low it nutrient content makes difficult for plants to grow, and it causes damage to the soil structure, a decrease in the number of soil microbes, a reduction in organic matter, and high levels of heavy metals (Hilmi 2018).

Plants need macronutrients that are used for growth, development, and productivity. Plants that lack macronutrients experience decreased growth (Kumar and Mohapatra 2021). Nitrogen (N_2) is an important macronutrient for plants in large quantities, but availability is limited to plants. The availability of nitrogen in the soil is lower than that in the air (Leghari *et al.* 2016). Nitrogen in the atmosphere is abundant, containing approximately 78%, but nitrogen is still in the form of molecules, most of which are not reactive, so plants are not able to absorb it directly. Plants can only absorb nitrogen in the form of ammonium (NH^{4+}) or nitrate (NO^{3-}) ions (Martinez-Dalmau *et al.* 2021). Therefore, it is necessary to transform nitrogen into

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air, which can change its molecular form so that it can be absorbed by plants. The strategy that can be implemented is to utilize microorganisms, such as non-symbiotic nitrogen-fixing bacteria, that live freely. Non-symbiotic nitrogen-fixing bacteria have several potential applications, including the ability to fertilize soil and provide micronutrients, and are widely used as biofertilizers (Widiyawati *et al.* 2014). Widawati and Suliasih (2019) successfully isolated eight isolates identified as nitrogen-fixing bacteria originating from the former tin mine land on the island of Bangka. However, there were not many reports on nitrogen-fixing bacteria from former kaolin mining. This study aims to explore and analyze the diversity of nitrogen-fixing bacteria based on 16S *r*RNA and *nif*H genes from the rhizosphere of the Kaolin Lake area, Belitung.

2. Materials and Methods

2.1. Rhizosphere Soil Sampling

Samples were obtained from the rhizosphere soil of the Kaolin Lake area in Bangka Belitung Province, taken from five sampling locations (Figure 1). The first location point with coordinates 2°44'17.1" S 107°41'10.8" E, the second location point 2°44'18.3" S 107°41'11.9" E, the third location point 2°44'18.5" S 107°41'13.6" E, the fourth location point 2°44'15.1" S 107°40'59.8" E, and the fifth location point 2°44'09.1" S 107°40'54.5" E.

2.2. Isolation of Bacteria from Soil Samples

A sample of 1 g was placed in 9 ml of physiological NaCl for multilevel dilution of up to 10^{-6}. Dilutions of 10^{-2} to 10^{-6} were taken as much as 0.1 L to be inoculated using the spread plate method on nutrient agar (NA) medium for heterotrophic bacteria (Ekowati *et al.* 2021). Isolation of bacteria from soil samples was also used enrichment culture techniques which were then grown on selective nitrogen-free bromthymol blue (NFB) agar medium for nitrogen-fixing bacteria. The NFB medium used was composed of DL-malic acid, KOH, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, NaCl, CaCl2, $FeSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and bacto agar for solid NFB medium while for semi-solid NFB medium 0.23% bacto agar and 0.5% bromthymol blue (BTB) were added. Colonies that grew on both media were counted to determine bacterial cell density per gram (Ding *et al.*2005). The selected isolates were subjected to macroscopic and microscopic morphological characterization.

2.3. Screening of Nitrogen Fixing Bacteria

2.3.1. Ammonium Content Test

The ammonium content test was performed qualitatively by growing the isolates on a semi-solid NFB medium. Changes in the media color from green to bluish indicate positive results for nitrogen fixation (Jannah *et al.* 2022). Quantitative testing was performed by growing the isolates on an NFB liquid medium

Figure 1. Map of sampling locations. Kaolin Lake, Bangka Belitung Province, Indonesia

for 8 days. The culture was centrifuged to obtain the supernatant. A total of 2 ml of supernatant was added with 0.08 ml of 10% phenol alcohol, 0.08 ml of nitroprusside solution, and oxidizing solution in a ratio of 1:4 (0.2 ml sodium hypochlorite and 20% alkaline citrate solution), each reaction was stirred. The solution was allowed to stand for 1 h in the dark until it changed color to bluish. The color changes formed from the reaction results were measured using a spectrophotometer at a wavelength of 640 nm (Eaton *et al.* 2005).

2.3.2. Hypersensitivity Test

A hypersensitivity test was conducted on tobacco plants. Liquid culture was obtained from a cell density of ±10^8 cells/ml, then injected as much as 1 ml into the lower surface of the tobacco leaf using a 1 ml syringe. *Pseudomonas aeruginosa* was used as the positive control, whereas the negative control remove media without isolates. Symptoms of necrosis were observed up to 48 h after culture injection (Harca *et al.* 2014).

2.4. Molecular Identification of Potential Isolates

2.4.1 Bacterial Genome DNA Extraction

Genomic DNA was extracted from bacterial cells using the Zymo Research Quick-DNATM Fungal/ Bacterial Miniprep Kit. The DNA extraction results were measured for concentration and purity using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4.2. Identification of Nitrogen Fixing Bacteria Based on 16S rRNA Gene and nifH Gene

Amplification of the 16S rRNA gene was performed using a polymerase chain reaction (PCR) machine with primer sets 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Harca *et al.* 2014). Amplification of the nifH gene was performed using the primer set PolF-GC (5'-CGCCCGCCGCCGCCCGCGCCCGGGC-3) and PolR (5′-ATSGCCATCATYTCRCCGGA-3′) (Gaby dan Buckley 2012). PCR was performed under the following conditions: pre-denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for the 16S *r*RNA gene, 54°C for the *nif*H gene for 45 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min (Peng *et al.* 2018). The PCR products were visualized on 1% agarose gel. The amplified DNA was sent to the sequencing service for PT. Genetika Science.

2.4.3. Bioinformatics Analysis

Nucleotide base sequences that were successfully sequenced were contiguous using SeqTrace software version 0.9.0, after which they were aligned with GenBank data using the BLAST-N and BLAST-X programs. Phylogenetic tree construction was carried out using the MEGA 11 program with the Neighbor-Joining method and the Tamura Nei model with a bootstrap value of 1000x (Tamura *et al.* 2021). Target gene nucleotide sequences were reduced to amino acids using the ExPasy website. The isolate with the highest ammonium concentration had a 3D structure constructed using the I-Tasser (Zhou *et al.* 2022). The 3D structural model of the protein was visualized and superposition analyzed using the USCF ChimeraX program (Pettersen *et al.* 2021).

3. Results

3.1. The Abundances and Morphological Characteristics of Bacterial Isolates

Culturable bacterial populations of the five rhizosphere soil samples from the Kaolin Lake area in Belitung showed different abundances between heterotrophic bacteria and nitrogen fixing bacteria. The counting results showed that the total population of nitrogen-fixing bacteria was higher, 10^4-10^5 CFU/g, than that of heterotrophic bacteria, 10^3-10^4 CFU/g (Table 1). The difference in the total number of bacteria was influenced by the isolation technique.

Selected isolates from the NA and NFB media were characterized macroscopically and microscopically (Tables 2 and 3). Observation of colony morphology was carried out on 2 days for isolates with NA media origin and incubation on day 5 for isolates with NFB media origin. Observations were made by observing the characteristics of the bacterial colonies, including shape, margin, elevation, and colony color.

Table 1. Total number of bacteria in rhizosphere soil of the area around Kaolin Lake, Belitung, Bangka Belitung Province, Indonesia

Rhizosphere soil sample	Total number of heterotrophic bacteria (CFU/gr)	Total number of nitrogen fixing bacteria (CFU/gr)
Rhizosphere 1	1.5×10^4	1.0×10^5
Rhizosphere 2	2.0×10^3	3.0×10^4
Rhizosphere 3	2.1×10^3	1.4×10^4
Rhizosphere 4	5.3×10^4	1.6×10^5
Rhizosphere 5	6.6×10^3	5.1×10^4

Table 2. Characterization of macroscopic and microscopic, morphology of bacterial isolates on NA media from rhizosphere soil of the area around Kaolin Lake, Belitung, Bangka Belitung Province, Indonesia

Isolate code	Macroscopic				Microscopic	
	Color	Shapes	Elevation	Margins	Cell shape	Gram
RBA 1.1	White	Irregular	Raised	Undulate	Bacillus	+
RBA 1.2	White	Circular	Convex	Entire	Bacillus	+
RBA 1.3	White	Circular	Convex	Curled	Bacillus	+
RBA 1.4	White	Circular	Pulvinate	Undulate	Bacillus	+
RBA 1.5	White	Circular	Convex	Entire	Bacillus	+
RBA 2.1	Yellow	Circular	Convex	Entire	Coccus	+
RBA 2.2	Yellow	Circular	Convex	Entire	Bacillus	+
RBA 2.3	Orange	Circular	Convex	Entire	Bacillus	-
RBA 2.4	Cream	Circular	Convex	Entire	Bacillus	-
RBA 2.5	White	Irregular	Raised	Undulate	Bacillus	-
RBA 3.1	Yellow	Circular	Convex	Entire	Bacillus Bacillus Bacillus	+
RBA 4.1	White	Circular	Convex	Entire	Bacillus	+
RBA 4.2	Yellow	Circular	Convex	Entire	Bacillus	-
RBA 4.3	Yellow	Circular	Convex	Entire	Coccus	-
RBA 4.4	Yellow	Circular	Convex	Entire	Bacillus	+
RBA 4.5	White	Circular	Umbonate	Entire	Bacillus	+
RBA 5.1	White	Circular	Convex	Entire	Bacillus	+
RBA 5.2	White	Circular	Convex	Entire	Bacillus	+
RBA 5.3	Yellow	Circular	Convex	Entire	Coccus	+
RBA 5.4	Yellow	Circular	Convex	Entire	Coccus	+

Table 3. Characterization of macroscopic and microscopic morphology of bacterial isolates on NFB media from rhizosphere soil of the area around Kaolin Lake, Belitung, Bangka Belitung Province, Indonesia

Isolate code	Macroscopic				Microscopic	
	Color	Shapes	Elevation	Margins	Cell shape	Gram
RBN 1.1	White	Circular	Raised	Curled	Bacillus	+
RBN 1.2	White	Circular	Convex	Entire	Bacillus	+
RBN 2.1	White	Filamentous	Umbonate	Filamentous	Filament	+
RBN 2.2	White	Circular	Convex	Entire	Bacillus	+
RBN 2.3	White	Circular	Convex	Entire	Bacillus	+
RBN 2.4	White	Circular	Convex	Entire	Bacillus	-
RBN 2.5	White	Circular	Convex	Entire	Bacillus	+
RBN 2.6	White	Circular	Convex	Entire	Bacillus	+
RBN 3.1	Brown	Circular	Raised	Filamentous	Filament	+
RBN 3.2	White	Circular	Irregular	Entire	Coccus	-
RBN 3.3	White	Circular	Convex	Entire	Bacillus	+
RBN 3.4	White	Circular	Convex	Entire	Bacillus	+
RBN 3.5	White	Circular	Convex	Entire	Bacillus	+
RBN 3.6	White	Circular	Convex	Entire	Bacillus	+
RBN 3.7	White	Circular	Convex	Entire	Bacillus	+
RBN 3.8	White	Circular	Convex	Entire	Bacillus	+
RBN 3.9	White	Circular	Convex	Entire	Bacillus	+
RBN 4.1	White	Circular	Convex	Entire	Bacillus	+
RBN 4.2	White	Circular	Convex	Entire	Bacillus	+
RBN 4.3	White	Circular	Convex	Entire	Bacillus	+
RBN 4.4	White	Circular	Convex	Entire	Coccus	+
RBN 4.5	White	Circular	Convex	Entire	Bacillus	+
RBN 4.6	White	Circular	Convex	Entire	Bacillus	-
RBN 4.7	White	Circular	Convex	Entire	Bacillus	+
RBN 4.8	White	Circular	Convex	Entire	Bacillus	+
RBN 4.9	White	Circular	Convex	Entire	Bacillus	+
RBN 4.10	White	Circular	Convex	Entire	Bacillus	+
RBN 4.11	White	Circular	Convex	Entire	Coccus	-
RBN 5.1	White	Circular	Convex	Entire	Bacillus	-
RBN 5.2	White	Circular	Convex	Entire	Bacillus	+
RBN 5.3	White	Circular	Convex	Entire	Bacillus	+
RBN 5.4	White	Circular	Convex	Entire	Bacillus	-
RBN 5.5	White	Circular	Convex	Entire	Bacillus	+
RBN 5.6	White	Circular	Convex	Entire	Bacillus	-
RBN 5.7	White	Circular	Convex	Entire	Bacillus	-

3.2. Nitrogen Fixing Activity of the Selected Isolates

Based on the result of the qualitative test of nitrogen fixation, thirteen isolates capable of nitrogen fixation indicated by a color change in the semi-solid NFB medium from green to bluish (Figure 2) (Table 4).

A quantitative ammonium content test was conducted on 13 isolates. The ammonium concentration was determined based on the absorbance value measured using a spectrophotometer. Based on the measurement results, several isolates were obtained at various concentrations (Figure 3), and the rate of ammonium formation produced by cells per day was determined (Table 5).

3.3. Hypersensitivity Reaction to Tobacco Leaves

Hypersensitivity tests were conducted on tobacco leaves as hosts to determine the pathogenicity. None of the isolates showed any symptoms of necrosis in the tobacco leaves (Figure 4).

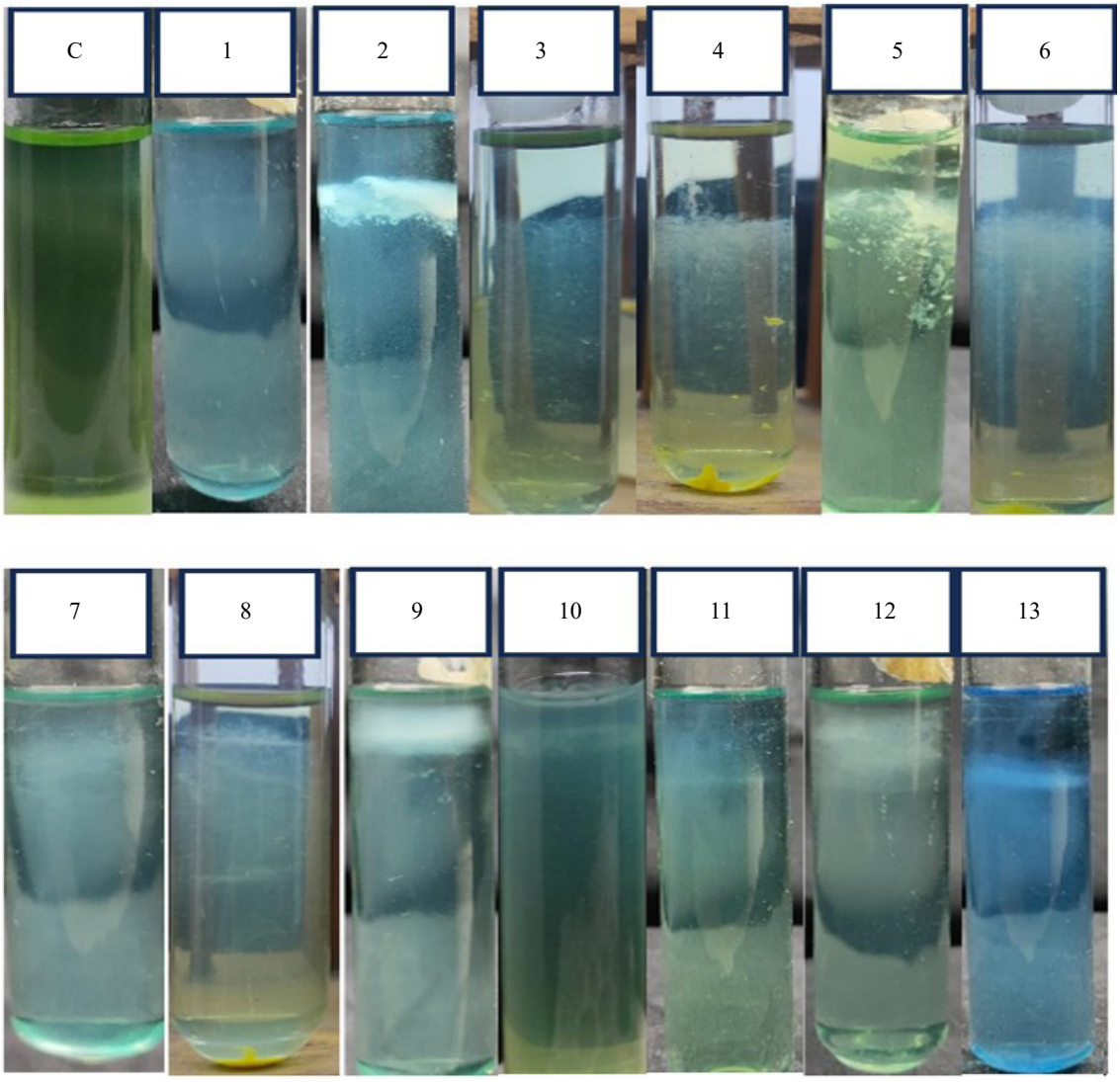

Figure 2. The result of qualitative analysis of nitrogen fixation bacteria on a semi-solid NFB medium. Description: (C) negative control, (1) RBA 1.5, (2) RBA 2.3, (3) RBA 5.13 (4) RBN 3.6, (5) RBN 3.7, (6) RBN 3.9, (7) RBN 4.1, (8) RBN 4.6, (9) RBN 4.5, (10) RBN 4.7, (11) RBN 4.11, (12) RBN 5.3, (13) RBN 5.6

Table 4. Qualitative analysis of nitrogenase activity in semi-solid NFB medium

Isolate	Nitrogenase activity	Growth on NFB
RBA 1.5	++	Aerobic
RBA 2.3	++	Aerobic
RBA 5.3	+	Aerobic
RBN 3.6	+	Microaerophilic
RBN 3.7	+	Aerobic
RBN 3.9	+	Aerobic
RBN 4.1	+	Aerobic
RBN 4.6	+	Aerobic
RBN 4.5	+	Aerobic
RBN 4.7	+	Aerobic
RBN 4.11	+	Microaerophilic
RBN 5.3	+	Aerobic
RBN 5.6	+++	Aerobic

Nitrogenase activity: + low, ++ medium, +++ high

Table 5. Nitrogen fixation activity based on ammonium accumulation of five selected rhizospheric isolates from the rhizosphere soil of the area around Kaolin Lake, Belitung, Bangka Belitung Province, Indonesia

Isolate code	Ammonium accumulation (µg/ml)	Standard deviation	Ammonium accumulation rate µg NH4/cell/day)
RBA 1.5	0.16	0.00	9.25E-05
RBA 2.3	7.28	0.06	0.46
RBN 4.1	1.16	0.03	0.04
RBN 4.6	2.79	0.04	0.02
RBN 5.6	7.55	0.04	0.38

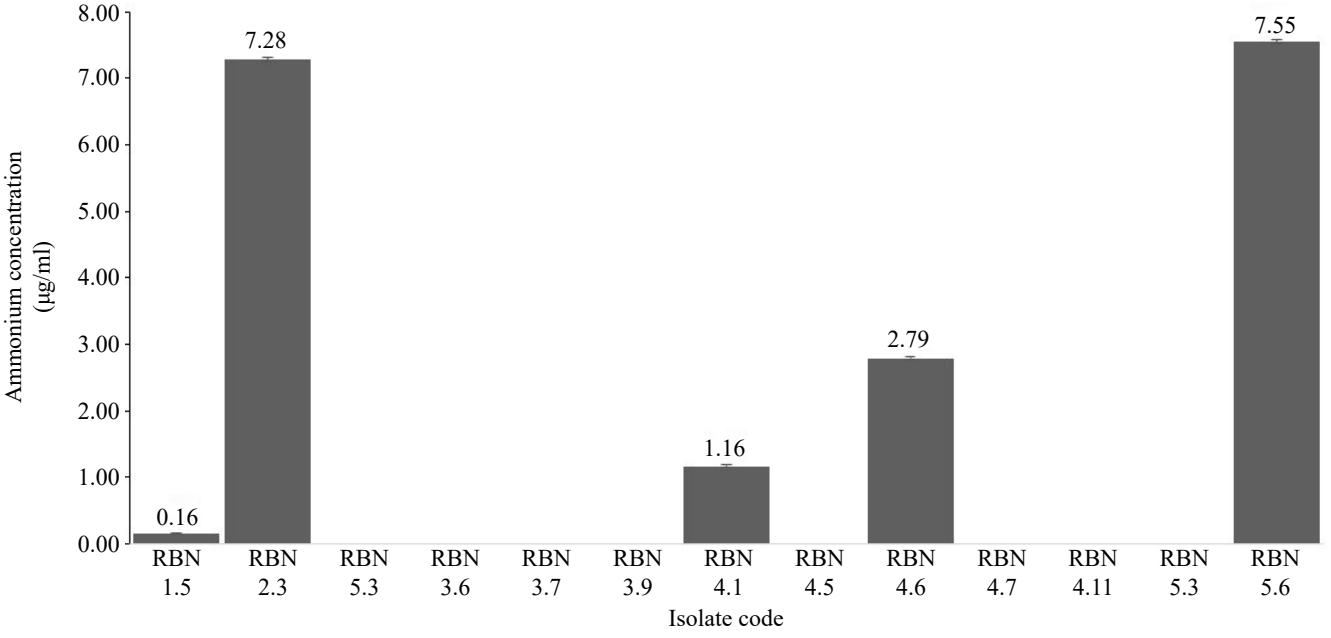

Figure 3. Ammonium content produced by bacterial isolates from rhizosphere soil

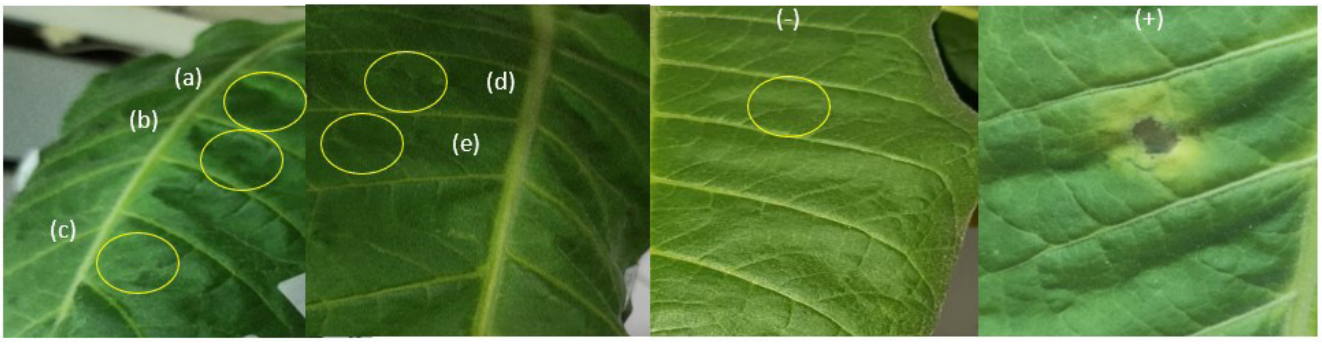

Figure 4. Hypersensitivity test result on tobacco leaf. (a) RBN 5.6, (b) RBN 4.6, (c) RBA 5.1, (d) RBN 4.1, and (e) RBA 2.3, negative control (-) blank medium without isolates, positive control (+) *Pseudomonas aeruginosa*

3.4. Identification of Potential Isolates with 16S rRNA Gene Analysis

Identification of the 16S rRNA gene was carried out on potential isolates RBN 5.6 and RBA 2.3 based on the ability of the isolates to produce high concentrations of ammonium. Visualization of 16S rRNA gene amplification formed a DNA band of about ~1300 bp. The 16S rRNA gene sequence was analyzed using GenBank data in the BLAST-N program (Table 6).

A phylogenetic analysis based on 16S *r*RNA gene sequence was conducted to determine the relationships between the species. The analysis showed that isolate RBN 5.6 was similar to *Burkholderia cepacia*, while isolate RBA 2.3 was similar to *Bacillus aquimaris* (Figure 5).

3.5. Identification of Potential Isolates by *nif*H Gene

The nifH gene was detected in the potential isolate RBN 5.6. BLAST-X results showed that the partial sequence of the nifH gene from isolates RBN 5.6 was identified as a protein-encoding dinitrogenase reductase (Table 7). Based on phenetic tree analysis, amino acid dinitrogenase reductase isolate RBN 5.6 is related to dinitrogenase reductase protein from *Stutzerimonas stutzeri* strain AWA45398.1 (Figure 6).

The partial amino acid sequence of nifH from isolate RBN 5.6 aligned with the reference sequence (Figure 7), resulted in 17 conserved amino acid residues. These amino acid residues function as catalytic sites, which are dominated by Alanine (A), Glutamate (E), Glycine (G), Asparagine (D), Leucine (L), and Valine (V).

Table 6. Similarity analysis of the 16S rRNA gene sequences of nitrogen-fixing bacterial isolates using the BLAST-N program

Isolate	Description	Query length	Identity (%)	Accession
RBN 5.6	*Burkholderia cepacia*	1231/1299	99.92	MN508424.1
RBA 2.3	*Bacillus aquimaris*	1259/1283	96.62	MK841571.1

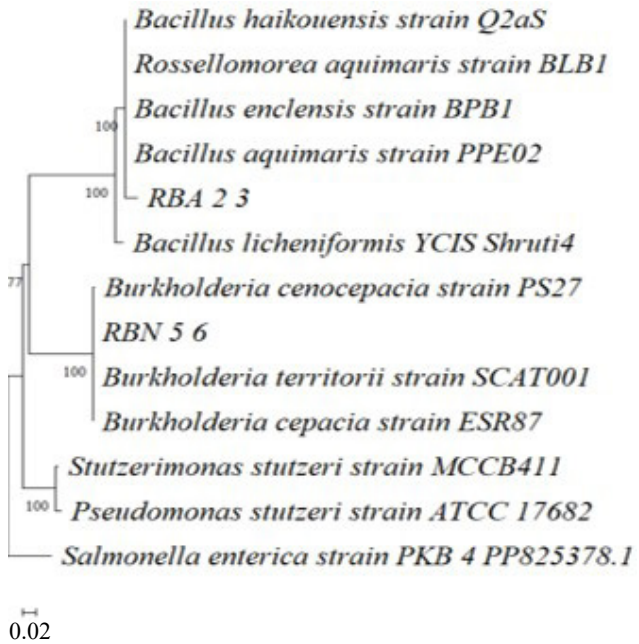

Figure 5. Construction of a phylogenetic tree of 16S *r*RNA gene sequences. The phylogenetic tree was constructed using the Neighbor-Joining method, the Tamura-Nei model with a bootstrap value of 1000x

3.6. 3D Structure Analysis of Amino Acid Dinitrogenase Reductase

The 3D structure model of the partial amino acid sequence of dinitrogenase reductase sequence isolate RBN 5.6 (Figure 8) is similar to the reference protein model of Fe-protein nitrogenase from *Azotobacter vinelandii*. The protein model was created using I-Tasser with protein quality values including C-score 0.99 (range of values -5 to 2), TM-score 0.85±0.08 (range of values between 0-1), and RMSD 2.0±1.6 Å (< 2, Å). Based on the results of the superposition analysis, there was a structural overlap between the partial sequence model of dinitrogenase reductase isolate RBN 5.6, and the Fe-protein nitrogenase reference protein model from *A. vinelandii*. In addition, several amino acid residues are known to play a role in ligand-binding and active sites.

4. Discussion

The total population of bacteria in the rhizosphere soil of the former kaolin mine was quite low. Previous research has reported that the former tin mine land obtained a number of bacterial populations ranging from 10^5-10^7 CFU/g (Novalia *et al.* 2022). The total population of rhizosphere soil bacteria generally ranges from 10^8-10^9 CFU/g (Glick 2012). Ex-mining land has poor physical and chemical properties and low nutrient content. The high and low numbers of bacterial populations in the soil can be caused by the availability of nutrients in the soil

Table 7. Similarity analysis of the gene encoding dinitrogenase reductase (*nif*H) isolates RBN 5.6 with BLAST-X analysis

Isolate	Description	Query length	Identity (%)	Accession
RBN 5.6	Dinitrogenase reductase *(Stutzerimonas stutzeri)*	292/354	100	AYK03524.1

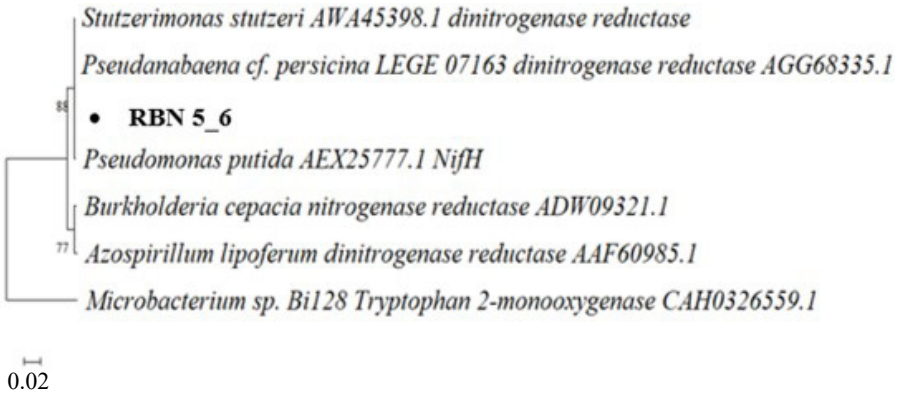

Figure 6. Phenetic tree amino acid sequence of nifH partial dinitrogenase reductase isolate RBN 5.6. The phenetic tree was constructed using the Neighbor-Joining method, with a bootstrap value of 1000x

```
Burkholderia    ------------IGKSTTSQNTLAALVELDQKILIVGCDPKADSTRLILHAKAQDTILS   47
Azotobacter     -AMRQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMIVGCDPKADSTRLILHSKAQNTIME   59
Pseudomonas     MAMRQCAIYGKGGIGKSTTTQNLVAALAELGKKVMIVGCDPKVDSTRLILHSKVQNTIME   60
RBN_5_6         -------------------------------------------------LHSKAQNTIME   11
Pseudanabaena   ------------------------------------CDPKADSTRLILHSKAQNTIME   22
                                                                 **:*.*:**:.

Burkholderia    LAAEAGSVEDLELEDVMKIGYKDIRCVESGGPEPGVGCAGRGVITSINFLEENGAYD-GV   106
Azotobacter     MAAEAGTVEDLELEDVLKAGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDDL   119
Pseudomonas     MAAEAGTVEDLELEDVLKTSYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDDL   120
RBN_5_6         MAAEAGTVEDLELEDVLKTGYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDDL   71
Pseudanabaena   MAAEAGTVEDLELEDVLKTGYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDDL   82
                :*****:*********:* .* .::*********************:******:***: .:

Burkholderia    DYVSYDVLGDVVCGG---------------------------------------------   121
Azotobacter     DFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNISKGIVKYANSGSVRL   179
Pseudomonas     DFVFYDVLGDVVCGGFAMPIRENKAQEIYVVCSGEMME----------------------   158
RBN_5_6         DFVFYDVLGDVVCGGFAMPIRENKA-----------------------------------   96
Pseudanabaena   DFVFYDVLGDVVCGGFAMPIRENKAQEIYVVCSGEMMAL---------------------   121
                *:* ***********
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Figure 7. Deduced amino acids of nifH partial dinitrogenase reductase isolate RBN 5.6 aligned with reference amino acids. Yellow highlights: conserved amino acids

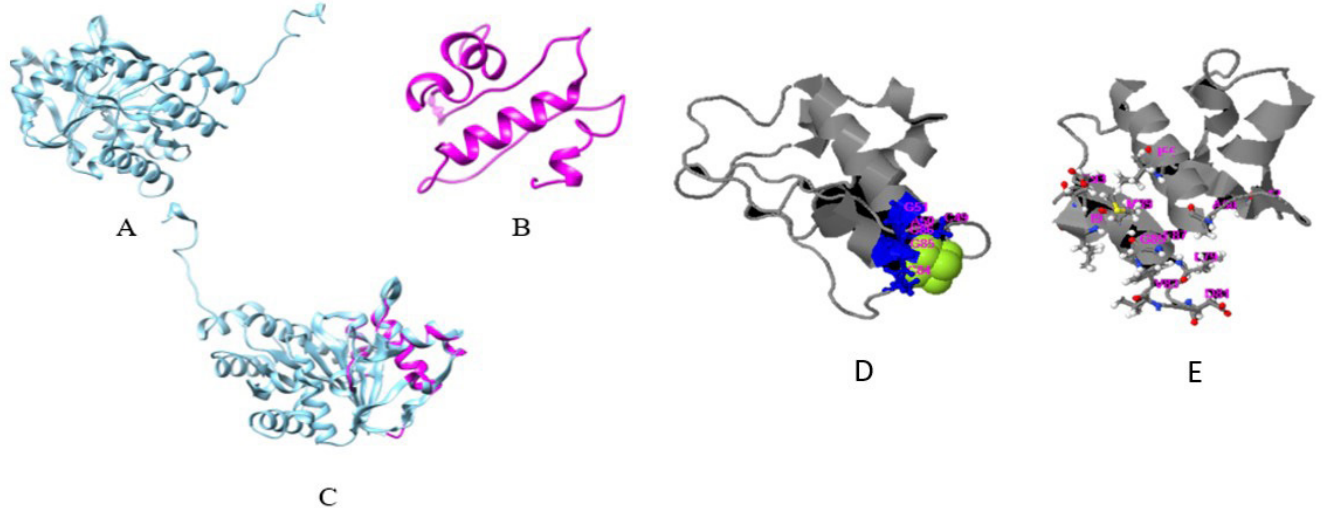

Figure 8. 3D protein structure analysis of dinitrogenase reductase isolate RBN 5.6 using I-Tasser. (A) protein structure of *A. vinelandi* PDB model (6N4J), (B) protein structure of amino acid dinitrogenase reductase isolate RBN 5.6, (C) Superposition analysis of the 3D model of dinitrogenase reductase partial protein RBN 5.6 against the Fe-protein nitrogenase reference protein from *A. vinelandii*, (D) ligand-binding site, (E) active site

(Abna *et al.* 2020). Based on macroscopic morphological observation of the 55 isolates, it can be seen that the isolates have varied colony shapes, dominated by circular shapes (round) and convex elevations with entire (smooth) edges. It has a variety colony colors, namely white, yellow, beige, orange, and brown. Microscopically, the cell shape was dominated by rod-shaped Gram-positive bacteria.

Qualitative testing was performed on all isolates to determine the color changes in the media, indicating their ability to accumulate ammonium. Color changes were caused by the nature of the bromthymol blue color indicator in the media. Bromthymol blue changes the color of the media when there is an increase in pH, which is known to accumulate ammonium from nitrogen-fixing bacteria in the media (Baldani *et al.* 2014). The results of bacterial metabolism, in the form of ammonia compounds (NH_3), are released into the environment and then reacted with water (H_2O) to form ammonium (NH^{4+}) and hydroxide ions (OH^-). These hydroxide ions cause the pH of the environment to become more alkaline (Haruta *et al.* 2004).

Qualitative analysis of nitrogen-fixing bacteria on semi-solid NFB media indicated different abilities based on the color changes formed on the media. The isolates RBA 5.3, RBN 3.6, RBN 3.7, RBN 3.9, RBN 4.1, RBN 4.6, RBN 4.5, RBN 4.7, RBN 4.11, and RBN 5.3 had nitrogenase activity classified as low, whereas isolates RBA 1.5 and RBA 2.3 had nitrogenase activity classified as medium. Meanwhile, isolate RBN 5.6 showed high nitrogenase activity. Oxygen demand affects the growth pattern of bacteria, which can be observed when bacteria are grown in liquid or semi-solid media. Aerobic bacterial colonies are scattered on the surface of the media because they require more oxygen for growth (Keenleyside 2019). Conversely, anaerobic bacterial colonies grow at the bottom of the medium because they do not require oxygen. This study shows that isolates that have the ability to fix nitrogen are isolates RBA 1.5, RBA 2.3, and RBN 5.6, which are aerobic with colonies tending to grow on the surface of the tube. In general, bacteria that grow close to the surface of the medium have better access to oxygen than bacterial colonies that grow at the bottom of the medium. However, in the process of oxygen greatly affects the activity of nitrogenase. Oxygen as a barrier that can affect the performance of nitrogenase enzymes (Ohayama *et al.* 2014).

A high oxygen concentration causes the nitrogenase enzyme to become inactive, thus reducing the ability to fix nitrogen. Therefore, non-symbiotic nitrogen-fixing bacterial protect themselves through several different mechanisms. Previous research by Wang *et al.* (2017) explained that bacteria can form biofilms as a strategy used by bacteria to survive under aerobic conditions, so they can carry out nitrogen fixing naturally. Pseudomonas stutzeri A1501 has been reported to be able to fix nitrogen under aerobic conditions by forming biofilms (Wang *et al.* 2017). Azotobacter protect nitrogenase against oxygen by forming a mucus capsule (Danapriatna 2010; Castillo *et al.* 2020). Meanwhile, the Cyanobacteria group forms a heterosis to protect the nitrogenase enzyme. Heterosis is a part of the cells with thick wall that protects the nitrogenase complex enzyme. Bacterial that do not have heterosis usually protect themselves by temporally separating oxygen from the nitrogen fixation process and O_2 production, where the nitrogen fixation process is carried out under dark conditions to avoid the production of O_2 (Sapalina *et al.* 2022).

Nitrogen-fixing bacteria have the ability to fix free nitrogen and convert it into ammonia (NH_3) compounds using the nitrogenase enzymes (Zulfarina *et al.* 2017). Ammonia fixation converts ammonia into ammonium ions (NH^{4+}) when it reacts with water (Grzyb *et al.* 2021). This is supported by previous studies, Hartono and Jumadi (2014) mentioned that nitrogen-fixing bacteria tether N_2 and convert it into ammonia, after which it is excreted outside the cell form of ammonium ions. These ammonium ions can then be utilized by plants for growth (Hartono 2014). The ammonium concentration produced in this study was fairly high compared with the results of previous studies. Dewi dan Trimulyono (2024) successfully isolated nitrogen-fixing bacteria from rhizosphere soil with ammonium concentrations ranging from 0.57-0.91 µg/ml. Ummah *et al.* (2019) successfully isolated nitrogen-fixing bacteria that obtained ammonium concentrations of 1.84 µg/ml to 2.24 µg/ml. Each type of microbe has a different ability to produce ammonium, which can be caused by variations in the type of bacteria, the amount of oxygen contained in the growth medium, and variations in carbon sources, which can affect the ability of bacterial cells to produce ammonium (Hartono and Jumaidi 2014).

Based on the hypersensitivity test, it shows that all tested isolates, namely RBN 4.1, RBN 4.6, RBN 5.6, RBA 1.5, and RBA 2.3, showed a negative response, indicating that none of the isolates were pathogenic to plants. The hypersensitivity reaction is a plant response characterized by rapid cell death at the point of pathogen injection (Balint-Kurti 2019). Hypersensitivity reactions can inhibit pathogens by damaging cells and localizing and can cause symptoms such as discoloration of leaves, drying, and necrosis (Mardhiana *et al.* 2017).

The results of 16S rRNA gene sequencing of two potential isolates compared with data from GenBank show that isolate RBN 5.6 is closely related to *Burkholderia cepacia*, and isolate RBA 2.3 is closely related to *Bacillus aquimaris*. Isolate RBN 5.6 was detected to have the *nif*H gene with an amplified gene length in the same range as the nifH gene sequence from previous studies. The nucleotide sequence of the partial *nif*H gene of isolate RBN 5.6 was successfully sequenced, and nucleotides measuring approximately ~400 bp were obtained. Isolate RBN 5.6 has homology with *B. cepacia*. The Burkholderia group is currently the center of research because it has various abilities, one of which can fix nitrogen (Perez-Pantoja *et al.* 2012). The *Burkholderia* genus can fix free nitrogen and is associate with legume plant roots. The ability to fix nitrogen has also been reported in several species, namely *B. Vietnamiensis, B. kururienis, B. tropicalis*, and *B. brasiliensis*. In addition, *B. cepacia* species has been reported to have the potential to be used as biofertilizers that can stimulate growth and increase the yield of corn plants (Tang *et al.* 2020).

Nitrogen is one of the components of various biomolecules that is important for supporting the growth and development of organisms, especially plants. Nitrogen fixation includes biological processes that can naturally convert free N_2 in the air into ammonia (NH_3), involving the nitrogenase complex enzyme (Kroneck *et al.* 2014; Ma'ruf *et al.* 2023). Nitrogen fixation is carried out by nitrogenase enzymes with several subunits encoded by several nif genes, namely *nif*D, *nif*K, and *nif*H genes. One of these three nif genes is *nif*H, which encodes the most sequential nitrogenase reductase subunit and is often used by researchers to study the phylogenetics, diversity, and abundance of nitrogen-fixing microorganisms. The nitrogenase enzyme belongs to a group of complex enzymes consisting of two subunits; dinitrogenase and dinitrogenase reductase. Dinitrogenase is component I, which is a Mo-Fe protein component that contains molybdenum, iron, and inorganic sulfur. Component II, ditinrogenase reductase, is a smaller protein component called the Fe protein (Rana *et al.* 2023).

Based on the prediction of the ligand-binding site using the protein structure from the Protein Data Bank (PDB) as a template, it is known that several residues located in the ligand-binding site play a role in electron transfer. Some residues that are in the ligand binding site are C49 (Cystein), A50 (Alanine), G51 (Glycine), C84 (Cystein), G85 (Glycine), and G86 (Glycine). While the residues that play a role in the active site on this protein are G42 (Glycine), A50 (Alanine), I55 (Isoleucine), L79 (Leucine), D81 (Aspartic acid), V83 (Valine), G85 (Glycine), F87 (Phenylalanine), M89 (Methionine), I91 (Isoleucine), and E93 (Glutamic acid) (Zhang *et al.* 2023).

In conclusion, in this study, 13 isolates had the ability to fix nitrogen, two of which had a high ammonium concentration, namely RBN 5.6, and RBA 2.3. The two potential isolates based on the 16S rRNA gene are known to have similarities to *Burkholderia cepacia* and *Bacillus aquimaris*, respectively. Isolate RBN 5.6 was detected to have a nifH gene with an amino acid sequence that belongs to the dinitrogenase component of the *Stutzerimonas stutzeri* group cluster. Further research needs to be carried out based on the 16S *r*RNA gene for three other potential isolates and detection of the *nif*H gene. Implementation tests can be conducted on selected isolates to plants and can be applied on a field scale.

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References

Abna, I.M., Mahayasih, P.G.M.W., Amir, M., 2020. Isolation and characterization of soil bacteria in Kampung Melayu Village, East Jakarta. *Arch Pharm.* 2, 102-111. https://doi.org/10.47007/ap.v2i2.3393

Baldani, J.I., Reis, V.M., Videira, S.S., Boddey, L.H., Baldani, V.L.D., 2014. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. *Plant Soil* 384, 413-431. https://doi.10.1007/s11104-014-2186-6

Balint-Kurti, P., 2019. The plant hypersensitive response: concepts, control and consequences. *Mol Plant Pathol.* 20, 1163-1178. DOI:10.1111/mpp.12821

Castillo, T., Garcia, A., Padilla-Cordova, C., Diaz-Barrera, A., Pena, C. 2020. Respiration in *Azotobacter vinelandii* and its relationship with the synthesis of biopolymers. *Electronic Journal of Biotechnology*. 48, 3645.

Danapriatna, N. 2010. Biochemistry of nitrogen fixing by nonsymbiotic bacteria. *J Agri Region Develop*. 1, 1-10.

Dewi PR, Trimulyono G. 2024. Isolation and characterization of nitrogen-fixing bacteria from the rhizosphere of pineapple plants on the slopes of Mount Kelud Kediri. *LenteraBio.* 13, 73-85. https://doi.10.25134/quagga.v12i2.2794

Ding, Y., Wang, J., Liu, Y., Chen, S. 2005. Isolation and identification of nitrogen fixing bacilli from plant rhizospheres in Beijing region. *J Appl Microbiol*. 99, 1271-1281.

Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenberg, A.E., 2005. *Standard Methods for the Examination of Water and Wastewater*, twenty-first ed. American Public Health Association (APHA) Press, Washington, DC.

Ekowati, C.N., Mirani, M., Handayani, K., Agustrina. 2021. Detection of nitrogenase producing bacteria from the soil of Liwa Botanical Garden. *J. Ilm Biol Eksperiment Biodiv*. 8:53-58.

Gaby, J.C., Buckley, D.H., 2012. A comprehensive evaluation of PCR primers to amplify the *nif*H gene of nitrogenase. *PloS one*. 7, 1-12. https://doi.10.1371/jurnal.pone.0042149

Glick, B.R. 2012. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica.* 2012, 1-15. https://doi.10.6064/2012/963401

Grzyb, A., Wlna-Maruka, A., Niewiadomska, A. 2021. The significance of microbial transformation of nitrogen compounds in the light of integrated crop management. *Agron.* 11, 1415. https://doi.org/10.3390/agronomy11071415

Hamid, I., Priatna, J., Hermawan, A., 2017. Characteristics of some soil physical and chemical properties on former tin mine land. *J. Penelit Sains.* 19, 23-31. https://doi.10.36706/jps.v19i1.8

Harca, N.N., Mubarik, N.R., Wahyudi, A.T., 2014. Isolation and identification of nitrogen fixing and indole acetic acid producing bacteria from oil plantation in Jambi, Indonesia. *J. Int Environ Appl Sci.* 9, 147-154. https//doi.org/10.5575/biodjati.v4i1.4481

Hartono, 2014. Ammonium secretion in nitrogen-fixing bacteria and its effect on plant growth. *J. Bionature.* 15, 35-40. https://doi.org/10.35334/jpen.v5il.2262

Hartono, O., Jumadi., 2014. Selection and characterization of ammonium-excreting non-symbiotic nitrogen-fixing bacteria in maize (*Zea mays* L.) and rice (*Oryza sativa* L.) cropping soils from Baru district, South Sulawesi, Indonesia. *Sainsmat.* 3, 143-153.

Haruta, S., Kato, S., Nakamura, K., Nishizawa, T., 2004. Effect of ammonium ion on the pH dependence of hydrogen oxidation activity and the structure of the microbial community in a microbial fuel cell. *J. Biocsi Bioeng.* 98, 22-27.

Hilmi, M., 2018. Growth response, physiology and anatomy of hazelnut (*Reutealis trisperma*) to gold mine tailings [Thesis]. Bogor, Indonesia: IPB University.

Jannah, N.A., Mustafa, I., Jatmiko, Y.D., 2022. Isolation and identification of nitrogen-fixing rhizobacteria associated with cocoa plantation (*Theobroma cacao* L) as biofertilizer agent. *J. Exp Life Sci.* 12, 75-80. https://doi.10.21776/ub.jels.2022.012.03.01

Keenleyside, W. 2019. *Microbiology*. University of Guelph, Canadian Edition.

Kroneck., Peter., M.H., Torres., Martha, E., Sosa., 2014. *The Metal-driven Biogeochemistry of Gaseous Compounds in the Environment*. Springer, Mexico.

Kumar, S., Mohapatra, T., 2021. Interaction between macro and micronutrients in plants. *Front Plant Sci.* 12, 1-9. https://doi.org/10.3389/fpls.2021.665583

Leghari, S.J., Wahocho, N.A., Laghari, GM., 2016. Role of nitrogen for plant growth and development: a review sustainable agriculture view project agronomical study view project. *Adv Environ Biol.* 10, 209-218.

Mardhiana., Adiwena, M., Pradana, A.P., 2017. Characterization of bacteria from Nepenthes mirabilis roots for biological control for *Fusarium oxysporum. BUL LITTRO.* 31(1), 48-58. https://doi.10.21082/bullittro.v31n1.2020.48-58

Martínez-Dalmau, J., Berbel, J., Ordóñez-Fernández, R., 2021. Nitrogen fertilization. A review of the risks associated with the inefficiency of its use and policy responses. *Sustainability.* 13, 5625. https://doi.org/10.3390/su13105625.

Ma'ruf A., Syahminar., Zulia, C., 2023. A comprehensive process of nitrogen fixation in plants. *J. Agrium.* 20, 299-305. https://doi.org/10.29103/agrium.v20i4.13994

Novalia., Nurtjahya, E., Santi, R., Sari, E., 2022. Characterization of *Azotobacter* and *Azospirillum* bacteria from the rhizosphere of pepper plants on former tin mine land. *J. Bios Logos.* 12, 46-54. https://doi.org/10.35799/jbl.v12i1

Ohyama, T., Momose, A., Ohtake, N., Sueyoshi, K., Sato, T., Nakanishi, Y.A., Asis, J.R.C., Ruamsungri, S., Ando, S. 2014. Nitrogen fixation in sugarcane. (Eds.), *Advances in Biology and Ecology of Nitrogen Fixation.* Tokyo University of Agriculture, Japan, pp. 47-70.

Peng, W., Li, X., Wang, C., Cao, H., Cui, Z., 2018. Metagenome complexity and template length are the main causes of bias in PCR-based bacteria community analysis. *J. Basic Microbiol.* 58, 987-997. https://doi.10.1002/jobm.201800265

Pérez-Pantoja, D., Donoso, R., Agulló, L., Córdova, M., Seeger, M., Pieper, D.H., González, B., 2012. Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. *Environ Microbiol*. 14, 1091-117. https://doi.org/10.1111/j.1462-2920.2011.02613.x

Pettersen, E.F., Goddard, T.D., Huang, C.C., Meng, E.C., Couch, G.S., Croll, TI., Morris, J.H., Ferrin, T.E., 2021. UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Prot Sci*. 30, 70-82. https://doi.10.1002/pro.3943

Rana, K.L., Kour, D., Kaur, T., Negi, R., Devi, R., Yadav, N., Rai, P.K., Singh, S., Rai, A.K., Yadav, A., Sayyed, R.Z., Yadav, A.N., 2023. Endophytic nitrogen-fixing bacteria: untapped treasurer for agricultural sustainability. *J. Appl Biol*. 11(2):75-93. https://doi.10.7324/JABB.2023.110207

Sapalina, F., Ginting, E.N., Hidayat, F. 2022. Bakteri penambat nitrogen sebagai agen biofertilizer. *Warta PPKS.* 27, 41-50.

Savira., Nurtjahya, E., Santi, R., 2023. Stomata rumput-rumputan di lahan bekas tambang timah di Bangka. *J. Ilmu Dasar*. 24, 1-8. https://doi.10.19184/jid.v24i1.19435

Tang, A., Haruna, A.O., Majid, N.M.A., Jalloh, M.B., 2020. Effects of selected functional bacteria on maize growth and nutrient use efficiency. *Microorganisms.* 8, 854. https://doi.10.3390/microorganisms8060854

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol Biol Evol.* 38. 3022-3027. https://doi.10.1093/molbev/msab120

Ummah, R., Asri, M.H., Yakub, P., 2019. Potential of endophytic bacterial isolates of shallot plant roots (*Allium ascalonicum*) as nitrogen fixers. *LenteraBio*. 8, 143-149.

Wang, D., Xu, A., Elmerich, C., Ma, L.Z. 2017. Biofilm formation enables free-living nitrogen fixing rhizobacteria to fox nitrogen under aerobic conditions. *The ISME Journal.* 1, 1602-1613. https://doi.10.1038/ismej

Widawati, S., Suliasih., 2019. Role of indigenous nitrogen-fixing bacteria in promoting plant growth on post tin mining soil. *Makara J Sci.* 23, 28-38. https://doi.10.7454/mss.v23il.10801

Widiyawati, I., Sugiyanta., Junaedi, A., Widyastuti, R., 2014. The role of nitrogen-fixing bacteria to reduce the dose of inorganic nitrogen fertilizer in wetland rice. *J. Agron Indonesia*. 42, 96-102. https://doi.org/10.24831/jai.v42i2.8424

Zhang, C., Zhang, X., Ferddolino, P.L., Zhang, Y., 2023. BioLip2: an updated structure database for biologically relevant ligand-protein interactions. *Nucleic Acids Res.* 52, 404-412. https://doi.10.1093/nar/gkad30

Zhou, X., Zheng, W., Li, Y., Pearce, R., Zhang, C., Bell, E.W., Zhang, G., Zhang, Y., 2022. I TASSER MTD: a deep-learning-based platform for multi-domain protein structure and function prediction. *Nat Protoc.* 17, 2326-2353. https://doi.10.1038/s41596-022-00728-0

Zulfarina, Z., Rusmana, I., Mubarik, N.R., Santosa, D.A., 2017. The abundance of nitrogen-fixing, nitrifying, denitrifying, and ammonifying bacteria in the soil of tropical rainforests and oil palm plantations in Jambi. *Makara J. Sci.* 21, 187-194. https://doi.org/10.7454/mss.v21i4.8841