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KEMAMPUAN BAKTERI PENAMBAT NITROGEN UNTUK MENGEKSKRESIKAN AMONIUM YANG DIISOLASI DARI RIZOSFER JAGUNG

ABILITY OF NITROGEN-FIXING BACTERIA TO EXCRETE AMMONIUM ISOLATED FROM MAIZE RHIZOSPHERE

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ABSTRAK

Bakteri penambat nitrogen adalah bakteri yang memiliki peran penting dalam proses asimilasi nitrogen di dalam tanah. Bakteri ini terdiri dari bakteri penambat nitrogen yang bersimbiosis dan tidak bersimbiosis, yang banyak ditemukan di rizosfer. Penelitian ini bertujuan untuk mengisolasi, mengkarakterisasi, dan mengevaluasi kemampuan bakteri rizosfer pada tanaman jagung di tanah marginal tropis dalam mengekskresikan amonium. Isolasi bakteri penambat nitrogen dilakukan dengan menggunakan media *nitrogen free bromothymol blue* (NFB). Sampel tanah diinokulasi pada media NFB padat menggunakan metode sebar, kemudian diinkubasi pada suhu 30°C selama 6-7 hari untuk mengamati pertumbuhan bakteri penambat nitrogen, dan kemudian dikarakterisasi. Karakteristik fisiologis isolat bakteri penambat nitrogen dari rizosfer jagung yang diamati meliputi suhu, pH, dan salinitas. Untuk menentukan ketahanan isolat bakteri penambat nitrogen terhadap suhu, dilakukan uji ketahanan suhu. Uji ekskresi amonium dilakukan dengan metode spektrofotometri. Sebanyak enam isolat bakteri penambat nitrogen telah diidentifikasi secara molekuler, yaitu *Rhizobium pusense* (LC585441.1), Beijerinckia fluminensis (MG547695.1), *Microbacterium neimengense* (LC430078.1), *Microbacterium binotii* (MN428150), *Rhizobium pusense* (MK542929.1), dan *Rhizobium sp.* strain FNF3 (MH910714.1). Hasil uji ekskresi amonium menunjukkan bahwa konsentrasi tertinggi diproduksi oleh *Microbacterium binotii* sebesar 42,0 µM, diikuti oleh *Microbacterium neimengense* sebesar 41,0 µM. Sementara itu, untuk spesies *Beijerinckia fluminensis*, *Rhizobium sp.* strain FNF3, serta dua isolat *Rhizobium pusense* masing-masing menghasilkan 38,8 µM, 38,6 µM, 38,0 µM, dan 36,2 µM.

KEYWORDS:

Ammonium excretion, maize rhizosphere, nitrogen-fixing bacteria, tropical marginal soil

ABSTRACT

Nitrogen-fixing bacteria are bacteria that have an important role in the process of nitrogen assimilation in the soil. Nitrogen-fixing bacteria consist of symbiotic and non-symbiotic nitrogen-fixing bacteria found abundantly in the rhizosphere. This research seeks to isolate, characterize, and evaluate the ability of rhizosphere bacteria in maize on tropical marginal soils to excrete ammonium. Isolate nitrogen-fixing bacteria using nitrogen-free bromothymol blue (NFB) medium. Soil samples were inoculated on solid NFB medium using the spread method and then incubated at 30°C for 6-7 days to observe nitrogen-fixing bacterial growth and were then characterized. The physiological characteristics of nitrogen-fixing bacterial isolates from the maize rhizosphere that were observed include temperature, pH, and salinity. A temperature resistance test was conducted to determine the resistance of nitrogen-fixing bacterial isolates to temperature. Ammonium excretion test using the spectrophotometric method. Six nitrogen-fixing bacteria isolates had been identified molecularly were Rhizobium pusense *(LC585441.1),* Beijerinckia fluminensis *(MG547695.1),* Microbacterium neimengense *(LC430078.1),* Microbacterium binotii *(MN428150),* Rhizobium pusense (*MK542929.1) and* Rhizobium sp. *strain FNF3 (MH910714.1). The results of the ammonium excretion test showed that the highest concentration was produced by* Microbacterium binotii *at 42.0 µM,* Microbacterium neimengense *at 41.0 µM while for* Beijerinckia fluminensis *species,* Rhizobium sp *strain FNF3, and 2* Rhizobium pusense *isolates respectively 38.8 µM, 38.6 µM, 38.0 µM, and 36.2 µM.*

1. INTRODUCTION

Maize is one of Indonesia's local commodities that can be relied on to support food security. This Indonesian food commodity has an annual production volume of 12.45 million tons of dry shells. The need for imported maize is intended to meet domestic needs, among others, for animal feed, seeds, and the food industry. Self-sufficiency in maize to meet domestic needs continues to be improved. This enhancement can be accomplished through multiple methods, such as inorganic fertilization, especially inorganic nitrogen (N) (Razaq *et al*., 2017).

Most of the biomass depends on the availability of N as a building block for amino acids, nucleotides, and chlorophyll in maize (Hirel *et al*., 2011; Faisal Mahmood *et al*., 2017). Although the use of inorganic nitrogen fertilizers can enhance agricultural productivity, it may reduce soil quality, contaminate groundwater, and release greenhouse gases, leading to global warming *(Bageshwar et al*., 2017; Jumadi *et al*., 2020). Among the various alternatives to nitrogen intake into the soil, biological nitrogen fixation needs to be further explored because it can reduce dependence on the use of inorganic N. Research on nitrogen-fixing bacteria is rare, especially for marginal lands in the tropics.

Bacteria that live around the roots, which can increase plant growth, are known as the Plant Growth Promotion Rhizosphere (PGPR). Several studies have shown that PGPR bacteria can fix nitrogen, produce auxins, gibberellins, siderophores, aminocyclopropane carboxylate (ACC) deaminase, dissolve phosphate, and provide protection to plants against pathogens (Arruda *et al*., 2013; Wang *et al*., 2016; Khalaf & Raizada, 2016; Herrera *et al*., 2016). Biological nitrogen fixation can be performed by several groups of bacteria and archaea. Bacteria and archaea that can fix nitrogen are classified as diazotroph organisms. Diazotrophs are microbial communities that supply fixed nitrogen to plants (Kumar *et al*., 2017). Diazotrophs reduce dinitrogen to ammonium using the nitrogenase enzyme system. The nitrogen-fixing ability of diazotrophs may vary based on growth medium, O_2 concentration, and method of experimentation. These factors may affect diazotroph abundance, affecting plant nutrient requirements in different seasons (Orr *et al*., 2011). Diazotrophs can live freely in soil, water, or symbiosis with legume plants. Nitrogen fixation contributes about 30% to 50% of total nitrogen in agricultural land. Several diazotrophs, including several genera *Rhanella, Pantoea, Rhizobium, Pseudomonas, Herbaspirillum, Enterobacter, Brevundimonas, Burkholderia, Bacillus, Brevibacillus, Staphylococcus, Azospirillum and Paenibacillus* are found to be in association with maize plants (Steenhoudt & Vanderleyden, 2000; Montañez *et al*., 2012; Nascimento *et al*., 2021).

A unique nitrogen-fixing regulatory mechanism is found in several species of nitrogen-fixing bacteria in which the ammonium formed is excreted out of the cell by a simple diffusion mechanism. This mechanism will cause the nitrogen-fixing activity to take place continuously, and ammonium that is excreted from cells by bacteria can be directly utilized by plants without having to wait for cells to die and decompose. Nitrogen-fixing bacteria with high ammonium excretion ability are expected to be an effective inoculum of biological fertilizers supporting plant growth. In *wild-type strain* nitrogen-fixing bacteria, ammonium excretion is caused by the low activity of the enzymes involved in ammonium assimilation, such as the glutamine synthetase (GS) enzyme. This causes ammonia to accumulate in cells without inactivating the nitrogenase enzyme. Narula & Kleiner (1992) reported that a decrease in ammonium excretion in the bacterium *Azospirillum brasilense* occurred along with the increased activity of the GS and glutamate dehydrogenase (GDH) enzymes. Research on the diversity of nitrogen-fixing bacteria capable of releasing ammonium in the rhizosphere of maize on tropical marginal soils has not been widely carried out. The aim of this study was to isolate, characterize, and evaluate nitrogen-fixing bacteria from the rhizosphere of maize (*Zea mays* L.) grown in tropical marginal soil, with a focus on assessing their ability to excrete ammonium.

2. MATERIALS AND METHODS

2.1 Soil Sampling

The soil sample for this research was gathered from maize (*Zea mays* L) plantations situated in Jeneponto Regency, South Sulawesi Province, Indonesia, at three distinct locations. The first area, is Bangkala District (coordinate point; -5,577454,119.562), has dark and slightly sandy soil characteristics (alluvial) with a pH of 4.5. The second area, Tamalatea District (coordinate point; -5.599779,119.665), has very dark and not sandy characteristics (inceptisol) with a pH of 5. The third area, West Bangkala District (coordinate point; -5,570544,119.550), soil sample typic inceptisol tending to be dark in color with a pH of 6. Geographically, Jeneponto is a low rainfall area and high air temperature because Jeneponto has a coastline parallel to the direction of the wind, both the West monsoon and the East monsoon, so water vapor does not pass through the Jeneponto mainland.

Maize planted in Jeneponto is a commercial variety, Bisi-2; it is F1 of a single cross between FS 4 and FS 9. FS 4 and FS 9 are tropical inbreds developed by Charoen Seed Co., Ltd. Thailand, and Dekalb Plant Genetics, USA.

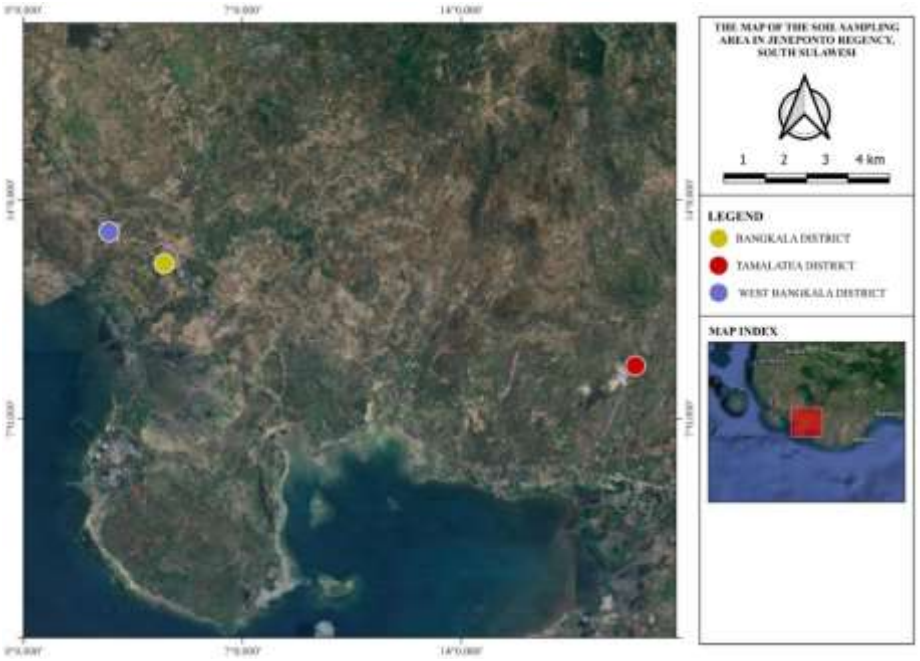

Figure 1. Soil sampling location

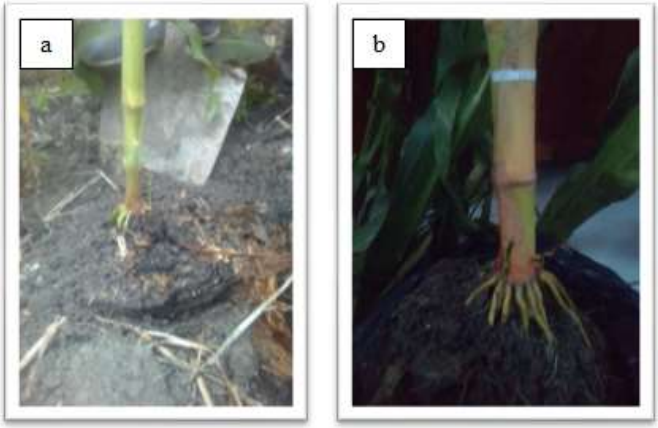

Figure 2. Soil sampling in the field. The soil in the root area is removed; (b) The plant is lifted from the soil until the roots are detached.

2.2 Bacterial Growth Medium and Isolation

The medium used isolate nitrogen-fixing bacteria (NFB) included both semi-solid and solid nitrogen-fixing bacteria, composed of 0.5 g; K_2HPO4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2.2H_2O$. 0,02 micronutrient solution, containing ($CuSO_4.5H_2O$, 0.04; $ZnSO_4.7H_2O$, 0.12; H_3BO_3,1,40; $Na_2MoO_4.2H_2O$, 1.0; $MnSO_4.H_2O$, 1.175. Add distilled water to bring the total volume to 1,000 mL, then include 2 mL of bromothymol blue (5 g/L in 0.2 N KOH), 2 mL of FeEDTA solution (16.4 g/L), and 4 mL of a vitamin solution containing 10 mg of biotin and 20 mg of pyridoxal-HCl (Baldani *et al*., 2014).

The soil samples were air-dried and sieved to obtain 50 g of material, which was then ground and combined with 5 g of mannitol to produce enriched soil. Then 14-15 ml of distilled water was added and stirred until well-mixed. Soil samples were inoculated on solid NFB medium using the spread method and then incubated at 30℃ for 6-7 days to observe nitrogen-fixing bacterial growth. Pure isolates were obtained by repeatedly streaking colonies on solid NFB medium. The isolates were then characterized employing the method reported by Holt *et al*., (1994).

Physiological features of nitrogen-fixing bacterial isolates originating from the maize rhizosphere that were observed include temperature, pH, and salinity. To determine the resistance of nitrogen-fixing bacterial isolates to temperature, a temperature resistance test was conducted. The test was performed at temperatures of 5℃, 25℃, and 40℃. The temperatures were selected to determine the temperature tolerance range of the nitrogen-fixing bacterial isolates, thereby assessing their adaptability and physiological stability under different environmental conditions. Furthermore, a test was conducted on the nitrogen-fixing bacterial isolates at pH 4, 6, and 8, and they were incubated at room temperature. Next, a salinity test was conducted on the NFB isolates in semi-solid media with NaCl concentrations of 1%, 6%, and 10% at pH 6.8.

(a) (b) (c)

Figure 3. (a) The production of Nitrogen-Free Bacteria (NFB) media; (b) Isolation of Nitrogen-Fixing Bacteria on NFB media (broth); (c) Colonies of Nitrogen-Fixing Bacteria on NFB media.

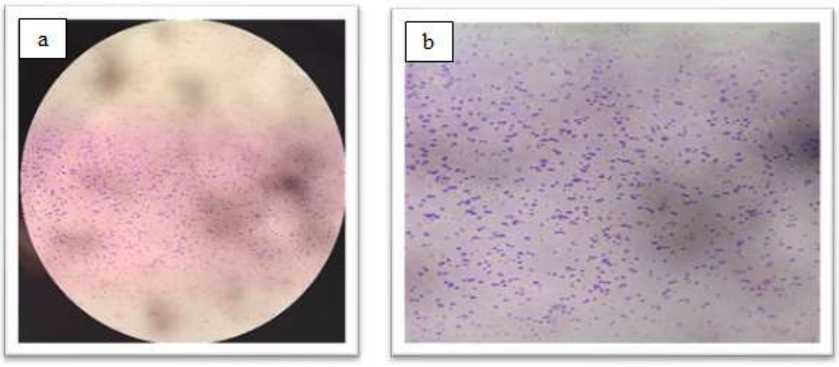

Figure 4. Gram test of Nitrogen-Fixing Bacteria magnified 10 × 100

2.3 Bacteria Identification

The selected bacterial isolates were then identified by morphology, physiology, and molecular methods. In the identification stage, observations and tests are referred to in Bergey's Manual of Determinative Bacteriology. The physiological characteristics test is based on the activity test for the presence or absence of a pellicle growing on the surface of the media and the color change that occurs on the media; if there is a white pellicle, it indicates the activity of nitrogen-fixing bacteria. The test is based on the resistance of bacterial isolates to temperature, salinity, and acidity. Molecular identification began with DNA extraction following the method of Lahiri and Schnabel (1993), followed by *PCR16S rRNA*. The primers used were universal, namely *8F (5'- AGAGTTTGATCATGGCTCAG-3')* and *15R (5'- AAGGAGGTGATCCAACCGCA-3').* The PCR results were then analyzed for sequencing, followed by a blast at NCBI to determine the type of nitrogen-fixing bacteria isolated. 16S rRNA nucelutide sequence from all isolates was analyzed and aligned using the GenBank database through the BLASTN Program (Altschul *et al*., 1990). The sequence alignment data were used to construct a phylogenetic tree and to assess sequence similarity employing the neighbor-joining approach proposed by Saitou & Nei (1987), as implemented in MEGA software version 10.0.

2.4 Ammonium Excretion Test

A primary 1000 ppm nitrogen (N) standard solution was prepared by weighing 4.7193 g of $(NH_4)_2SO_4$ (dried at 100℃ for 4 hours), placing it in a 100 ml volumetric flask, dissolving it with ion-free water to the marked line, and then shaking. Nessler's reagent was made by weighing out 18.26 g KI, 22.72 g HgI_2, and 40.00 g NaOH. The ammonium test was conducted following the method of Molins-Legua et al. (2006). Different volumes of ammonium standard stock solutions or 0.05 mL of S2 were combined with 10 µL of 0.177 M sodium–potassium tartrate and distilled water to obtain a final volume of 2.4 mL. Subsequently, 0.1 mL of Nessler's reagent was added to the solution, and the reaction was deemed to commence immediately upon the addition of the final drop of the reagent. Absorbance was then determined at a wavelength of 425 nm using water as the blank.

3. RESULT AND DISCUSSION

Bacteria that grew on selective media were then identified physiologically, including their resistance to temperature (Figure 5), acidity (Figure 6), salinity (Figure 7), and gram staining—the results of identifying the physiology of bacterial isolates growing in selective BNF media (Table 1).

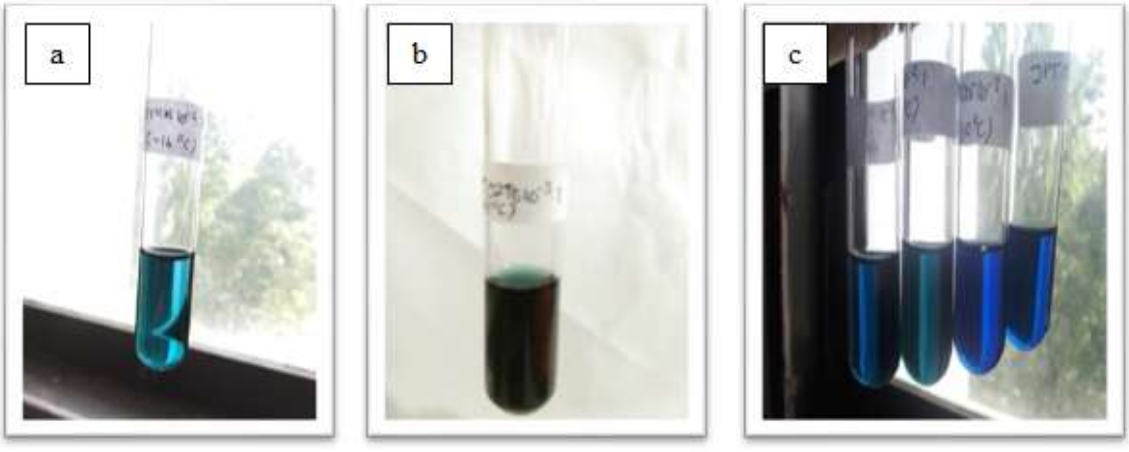

Figure 5. The results of testing Nitrogen-Fixing Bacterial isolates for temperature resistance at (a) 5ºC, (b) 25ºC, and (c) 40ºC showed variations in media color.

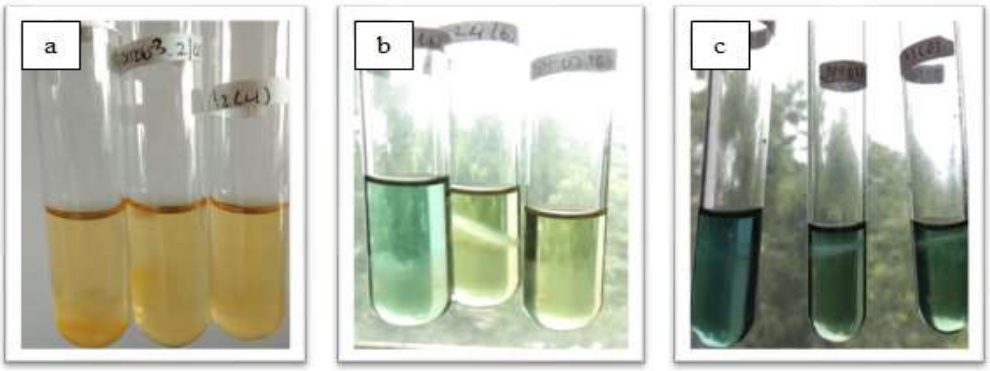

Figure 6. The results of testing Nitrogen-Fixing Bacterial isolates at pH levels of (a) 4, (b) 6, and (c) 8 showed variations in media color.

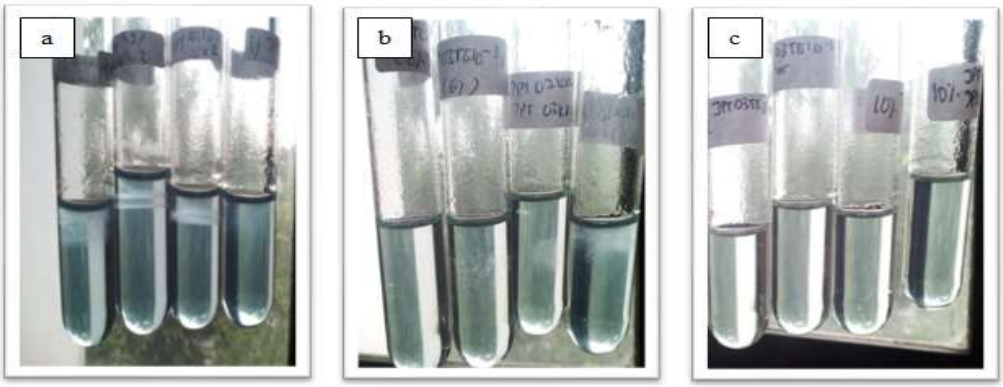

Figure 7. The results of testing Nitrogen-Fixing Bacterial isolates with NaCl salinity concentrations of (a) 1%, (b) 6%, and (c) 10% showed variations in media color.

Table 1. Physiological features of Nitrogen-Fixing Bacterial isolates inhabiting the maize rhizosphere of tropical marginal soils in Indonesia

No	Isolate Code	Gram Stain	Pellicle Temperature (°C)			Pellicle Acidity (pH)			Pellicle Salinity (%)		
			5	25	40	4	6	8	1	6	10
1	BKr_1	-	+	++	++	+	+	+	+	++	-
2	TTg_1	+	+	+	-	-	+	+	++	+	-
3	TKr_1	-	+	+	++	+	+	+	+	+	-
4	TKn_2	-	+	+	++	-	+	+	+	++	-
5	$BBKr_1$	-	+	+	+	+	+	+	++	++	+
6	$BBTg_3$	-	-	++	+	+	+	+	+	+	-

Based on the data from Table 1, the gram-staining results indicate that the average isolate is gram-negative except for TTg1, which is gram-positive. The temperature of 5°C indicates that, on average, the isolates produced pellicles on the surface of the media except for BBTG3 isolates. The temperature resistance test results of 25°C showed that the average isolate produced pellicles on the surface of the media. The temperature resistance test results of 40°C mean that isolates had pellicles except for TTg1 isolates, which did not produce pellicles. Testing at pH 4 showed that, on average, the isolates produced pellicles except for TTg1 and TKn2 isolates. At pH 6 and 8, it shows that, in general, all isolates had a surface pellicle on the growth medium. The results of observations on salinity resistance at 1% and 6% NaCl concentrations were that all isolates produced pellicles on the surface of the media. At a concentration of 10%, it shows that, in general, the isolates did not produce pellicles on the surface of the media except for BBKr1 isolates. The identification results of the 6 isolates are shown in Table 2.

Table 2. Molecular Identification and Sequencing Results of 6 Selected Nitrogen-Fixing Bacterial isolates from soil samples from the rhizosphere of maize plants (*Zea mays* L.) in tropical marginal soils in Indonesia

No	Isolates code	Species	Identity	Accesion Number
1	TTG_1	*Rhizobium pusense*	99,16%	LC585441.1
2	TKR_1	*Beijerinckia fluminensis*	98,03%	MG547695.1
3	TKN_2	*Microbacterium neimengense*	98,57%	LC430078.1
4	BKR_1	*Microbacterium binotii*	99,65%	MN428150.1
5	$BBKR_1$	*Rhizobium pusense*	96,33%	MK542929.1
6	$BBTG_3$	*Rhizobium sp.* strain FNF3	98,63%	MH910714.1

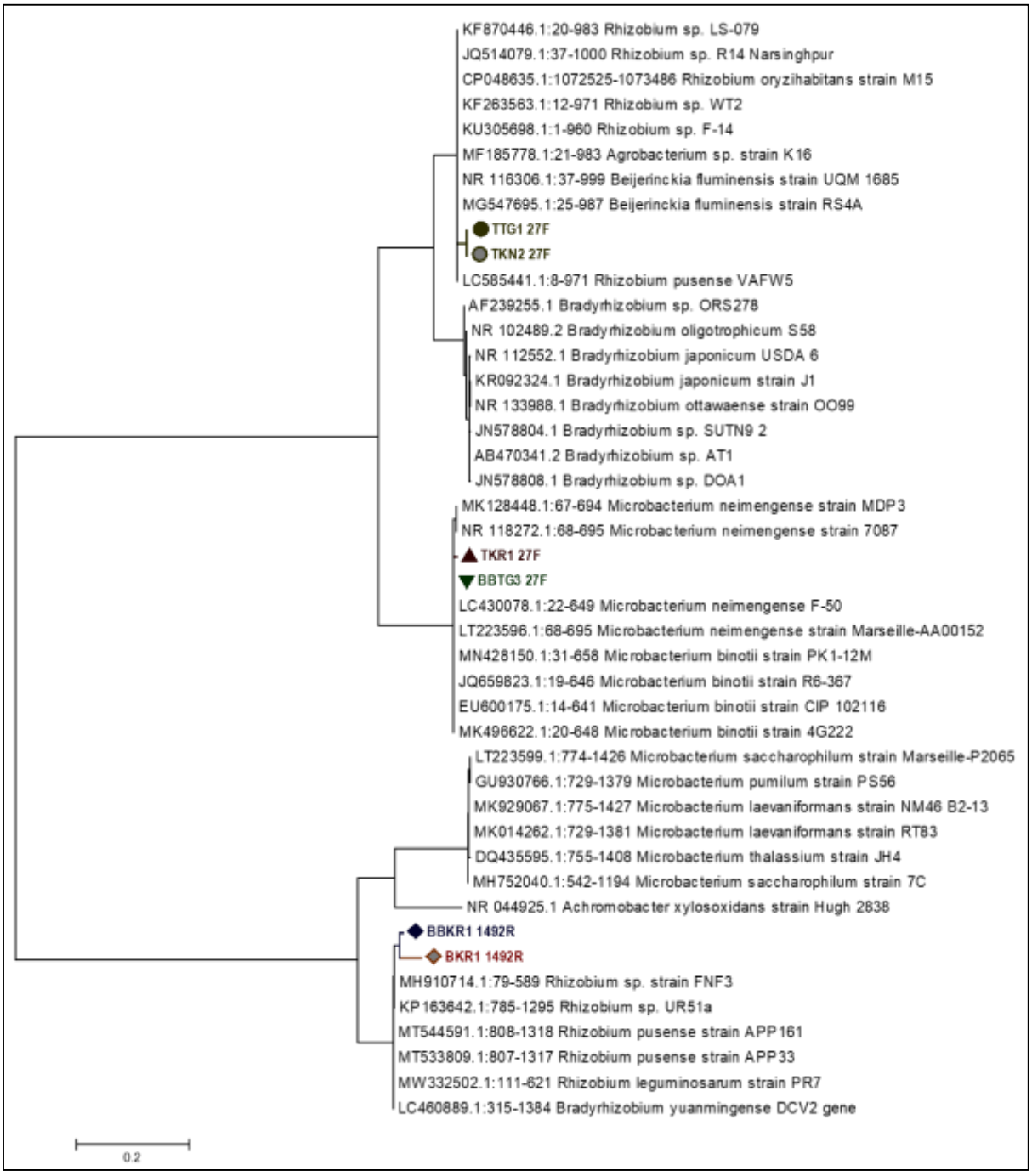

Figure 8. Phylogenetic tree analysis of 6 Nitrogen-Fixing Bacterial isolates from soil samples derived from soil samples from the rhizosphere of maize (*Zea mays* L.)

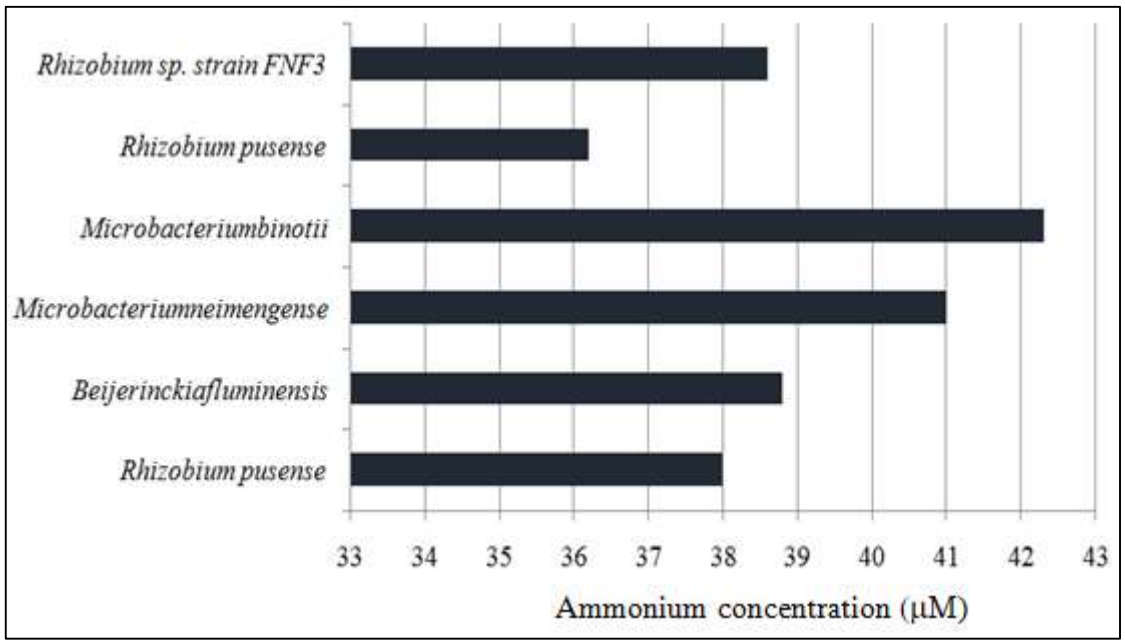

Figure 9. Ammonium Excretion (µM) from 6 Isolates of Nitrogen-Fixing Bacteria

According to molecular identification results of 6 nitrogen-fixing bacteria isolates, information was obtained that the 6 bacteria were nitrogen-fixing bacteria. The six nitrogen-fixing bacteria are Rhizobium pusense, Beijerinckia fluminensis, *Microbacterium neimengense*, *Microbacterium binotii*, *Rhizobium pusense*, and *Rhizobium sp.* strain FNF3. Furthermore, phylogenetic analysis was carried out to determine the relationship between one bacteria to another. The phylogenetic analysis of the nitrogen-fixing bacterial isolates is as follows (Figure 8).

Based on the phylogenetic analysis results of 6 selected nitrogen-fixing bacteria isolates, information was obtained that these bacteria were nitrogen-fixing. The bacterial isolates were TTG1 isolates with 99.16% similarity to *Rhizobium pusense* (LC585441.1), TKR1 isolates with 98.03% similarity to *Beijerinckia fluminensis* (MG547695.1), TKN2 isolates with 98.57% similarity to *Microbacterium neimengense* (LC430078.1), BKR1 isolates with 99.65% similarity to *Microbacterium binotii* (MN428150.), BBKR1 isolates with 96.33% similarity to *Rhizobium pusense* (MK542929.1) and BBTG3 isolates with 98.63% similarity to *Rhizobium sp.* strain FNF3 (MH910714.1). After knowing the type, the next step was to test the ammonium excretion from the 6 isolates of the nitrogen-fixing bacteria. The results of ammonium excretion from the 6 isolates are shown in the graph (Figure 9).

Based on the graphs above, it can be seen that the highest ammonium excretion is in the nitrogen-fixing bacteria *Microbacterium binotii* species with a concentration of ammonium of 42.0 μM, then *Microbacterium neimengense* with 41.0 μM while for *Beijerinckia fluminensis species, Rhizobium sp* strain FNF 3, and 2 *Rhizobium pusense* isolates the concentrations are 38.8 μM, 38.6 μM, 38.0 μM, and 36.2 μM respectively.

The results of the temperature resistance test from the temperature tests of 5°C, 25°C, and 40°C showed that, on average, the isolates produced pellicles on the surface of semi-solid NFB media. A transition in the NFB medium color from green to blue occurred. The appearance of pellicle layers on the medium surface indicates that this nitrogen-fixing bacterium possesses the ability to adapt effectively to its surrounding environment. Testing at a temperature of 25°C can confirm that 6 nitrogen-fixing bacterial isolates can grow well. Nitrogen-fixing bacteria that grow at room temperature are evidenced by the presence of pellicles in a semi-solid NFB medium that already contains isolates. The resulting media color is turquoise blue. The pellicle in the NFB medium is white

and floats on the surface of the media, shaped like a ring. The growth test results of the N-fixing bacteria showed better growth at 30-35℃ or room temperature. These results indicated that the isolates obtained are mesophilic. Of all the species tested, it was seen that the colonies could increase from day 3 at an incubation temperature of 25-35℃.

The observation of the effect of pH on the activity of nitrogen-fixing bacteria shows that the pellicle color is brownish-yellow, which is influenced by the acidity of the media. The resulting color is *Broom yellow*, which is a brownish yellow. The pH range for growth that produces additional nitrogen is 4.5-8.5, while the pH optimal for growth and nitrogen fixation is 7-7.5. Isolates that could not grow and had no pellicle at an acidic pH were due to their inability to adapt to an acidic environment where micronutrients from the media were lacking. The growth of the isolates was indicated by the presence of pellicles floating on the surface of the media. As nitrogen builds up in the medium, bacteria form a pellicle at the surface so that the pellicle will move to the surface of the media. Rhizosphere bacteria move in the media (motile) because they have an aerotactic capability to find a balance of oxygen diffusion. When the oxygen diffusion rate is equal to the rate of respiration of bacteria, these are favorable conditions for nitrogenase activity.

The test results on the salinity test with a concentration of 1% and 6% showed that the isolates produced many pellicles on the surface of the semi-solid NFB media. After an incubation period of 7 days, the color changed from green to turquoise blue. Overall, these observations found many pellicles on the surface of the media, indicating that this type of bacteria could adapt to salinity levels with concentrations of 1% and 6%. The results of the salinity test with a concentration of 10% showed that only 1 isolate could live with high salt content. The capacity of the isolates to develop pellicles across a range of environmental conditions indicates a high level of adaptability to stress. Pellicle production was observed at 5°C in all isolates except BBTG3 and reached its maximum at 25°C and 40°C, although TTg1 exhibited diminished growth at the highest temperature. Low pH conditions (pH 4) suppressed the growth of TTg1 and TKn2, whereas all isolates demonstrated favorable growth under neutral to alkaline conditions (pH 6–8). Salinity assays revealed that the isolates could tolerate NaCl concentrations up to 6%, with BBKr1 displaying notably high tolerance at 10% NaCl. These physiological characteristics are presumably associated with the presence of stress-related genes, including groES/groEL and dnaK involved in heat-shock response, as well as otsA/otsB responsible for trehalose synthesis, which collectively contribute to improved resistance against thermal and osmotic stress (Gao *et al*., 2013). Therefore, the $BBKr_1$ isolate is found in the Jeneponto area of West Bangkala and close to the coast so that it can adapt to high levels of salinity.

The results of the test of ammonium excretion from the 6 isolates were ammonium concentrations ranging from 30-50 μM. This indicates that the nitrogen-fixing capacity of bacteria is reflected in their ability to convert atmospheric nitrogen into ammonia, which is subsequently incorporated into amino acids utilized by plants for growth. Higher levels of ammonium production correspond to a greater efficiency of microbial nitrogen fixation and ammonia synthesis. Nitrogen-fixing bacteria are capable of assimilating free nitrogen (N_2) from the atmosphere and transforming it into ammonia (NH_3), which is then converted into amino acids that support plant growth and development. Some of the microbes that live in the rhizosphere are known as fixers of N_2 gas in the atmosphere. The limited amount of nitrogen in the soil is a limiting factor for plant productivity.

The direct role of rhizosphere bacteria in assisting plant growth is fixing free nitrogen in the environment and converting it to ammonium. Ammonium ($NH4^+$) is one of the primary forms of nitrogen that plants can absorb. The availability of ammonium sources for plants greatly influences plant growth (Liu and von Wirén 2017). Ammonium is a mineral source for plants that functions to build and maintain cells. Ammonium is known to increase tolerance to salinity stress in Sorghum bicolor plants by increasing photosynthetic ability and reducing the effect of Na^+ toxicity on plants.

Ammonium is also known to increase water absorption in rice seeds (*Oryza sativa* L.) under water stress, thereby increasing the ability of plants to cope with drought (Gao *et al*., 2013).

Rhizobium represents a group of bacteria capable of fixing nitrogen and inhabiting the rhizosphere. Rhizobium usually lives in symbiosis with legume plants, but some live freely in the environment (Wang *et al*., 2019). Several strains of Rhizobium can live in extreme conditions such as low temperatures (4ºC) and high temperatures (44ºC). The GMF14 strain isolated from soybean plants increases plant growth and maintains nitrogen-fixing ability in low-temperature conditions. *Rhizobium pusense* is a type of Rhizobium bacteria that can grow at temperatures of 16 - 41°C and pH conditions of 5.0 - 11.0. This non-nodulating Rhizobium species does not cause nodulation for symbiosis with plants because it does not have nodA and nifH genes (Panday *et al*., 2011). *Rhizobium pusense* is also known to increase cowpea plants' growth and production (*Vigna unguiculata* L.), making it very effective as a biofertilizer.

Another study states that *Rhizobium pusense* isolated from the rhizosphere of curcuma has an excellent nitrogen-fixing activity to increase growth and yield in curcuma plants (Chandran, 2019). Besides having a role in nitrogen-fixing and increasing plant growth, Rhizobium bacteria can also reduce or inhibit diseases that appear in plants. *Rhizobium* acts as a biocontrol agent against fungal plant pathogens. Its antagonistic effects are attributed to the production of diverse antibiotic substances, hydrogen cyanide, and mycolytic enzymes. In addition, rhizobium bacteria have also been reported to induce systemic resistance and increase the expression of defense genes in plants that protect them from pathogens (Das *et al*., 2017).

Based on the analysis of 16s rRNA in bacterial cultures of the rhizosphere of maize, there are about 4% *Microbacterium* (Qaisrani *et al*., 2019). *Microbacterium* is taxonomically affiliated with *Actinobacteria* which can be found in non-legume plants such as corn, rice, and wheat, and is also found in legumes, namely beans. *Microbacterium* is also a microbial biocontrol agent inhibiting plant pathogens (Yadav *et al*., 2018). *Microbacterium neimengense* is a species of microbacterium found in the rhizosphere of maize. It is gram-positive, non-motile, and aerobic. It grows optimally in conditions of temperature 37ºC and pH 7, tolerant of NaCl up to 3% but very sensitive to lysozyme. *Microbacterium neimengense* is phylogenetically very close to *Microbacterium binotii* (Gao *et al*., 2013). *Microbacterium binotii* is also found in Korean rice plants. The production of auxin by *Microbacterium binotii* in Korean rice is not too high. Still, these bacteria also have antifungal activity to inhibit pathogenic fungi in plants (Ji *et al*., 2014).

Beijerinckia is a genus of nitrogen-fixing bacteria with a stem cell shape and a rounded tip. This genus is both motile (has flagella) and non-motile (has no flagella) and is aerobic, so its nitrogen-fixing activity can occur aerobically or microaerobically. *Beijerinckia* grows optimally at 20-30°C and a pH range of 3.0-9.5. In the stationary phase of its growth, *Beijerinckia* can excrete IAA. *Beijerinckia* is also known as nitrogen-fixing heterotrophic bacteria, which can use various multicarbon compounds as carbon sources. One of the *Beijerinckia* species was isolated in Java (1950) with the species name *Beijerinckia indica*, while *Beijerinckia fluminensis*, a species discovered in Brazil (1958), has a similar sequence to *Rhizobium radiobacter* (Laskar *et al*., 2010). Baldani & Baldani (2005) reported that *Beijerinckia fluminensis* was found to be abundant in the rhizoplane area of sugarcane and positively affected plant growth on the roots, stems, and leaves with nitrogenase activity.

Molecular analyses revealed that all six isolates were affiliated with established nitrogen-fixing bacterial genera, namely Rhizobium pusense, Beijerinckia fluminensis, *Microbacterium neimengense*, *Microbacterium binotii*, and *Rhizobium* sp. strain FNF3, exhibiting sequence similarity values between 96.33% and 99.65%. These taxa are recognized for possessing nif gene clusters, including *nifH*, *nifD*, and *nifK*, which code for the nitrogenase enzyme complex involved in the fixation of atmospheric nitrogen (N_2). In addition, regulatory elements such as *nifA* and *fixL/fixJ* (particularly in *Rhizobium*) are thought to optimize nitrogenase function under microaerobic conditions commonly

found in the rhizosphere. Ammonium release is also likely regulated by genes such as *amtB* and *glnA/glnB*, which play key roles in controlling ammonium transport, assimilation, and excretion (Bizjak *et al*., 2023). Among the tested isolates, *Microbacterium binotii* (BKR1) showed the greatest level of ammonium release (42.0 µM), followed by *Microbacterium neimengense* (41.0 µM). This pattern indicates a higher efficiency of nitrogen fixation and potentially lower ammonium assimilation relative to *Beijerinckia fluminensis* (38.8 µM), *Rhizobium* sp. strain FNF3 (38.6 µM), and *Rhizobium pusense* isolates (38.0 and 36.2 µM). The comparatively superior performance of the *Microbacterium* isolates highlights their promise as biofertilizer candidates, especially for soils experiencing fluctuating pH and salinity conditions, as their genetic characteristics may support enhanced nitrogenase activity and improved tolerance to environmental stress.

4. CONCLUSION

Based on the research results, it is concluded that the ammonium excretion test showed that the highest concentration was produced by *Microbacterium binotii* at 42.0 µM, *Microbacterium neimengense* at 41.0 µM while for *Beijerinckia fluminensis* species, *Rhizobium sp* strain FNF3, and 2 *Rhizobium pusense* isolates respectively 38.8 µM, 38.6 µM, 38.0 µM, and 36.2 µM.

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