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Isolation and identification of nitrogen-fixing bacteria in the corn rhizosphere (*Zea mays* l.) originating from Jeneponto Regency, South Sulawesi

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Abstract. This study aims to determine the type of isolates and the characteristics of nitrogen-fixing bacteria in the rhizosphere of corn (*Zea mays* L.) from Jeneponto regency, South Sulawesi. This research is an explorative study consisting of nitrogen-fixing bacteria isolation and identification using nitrogen free bromohymol blue (Nfb) medium, and an ammonium excretion test on nitrogen-fixing bacteria using spectrophotometry methods. Bacteria identification methods include Gram staining, and physiological and biochemical characterization which consist of temperature resistance at 5°C, 25°C, and 40°C, pH resistance at 4, 6, and 8 and salt tolerance at 1%, 6% and 10 %. The study has found 20 bacterial isolates that can be characterized as round, mucoid, non-mucoid, white, clear, Gram-negative and bacilli. Physiological characteristics (biochemical tests) have shown growing pellicles and discoloration in the liquid Nfb medium. The ammonium excretion test showed there are 5 isolates excreting fairly high ammonium levels, namely BKr_1, BKr_2, BKr_3, TKN_2 and $BBTg_1$, with concentrations of 42.3, 39.1, 41.5, 41.0 and 39.4 µM, respectively.

1. Introduction

Indonesia is one of the countries with megadiversity and a high degree of plant diversity so that it is useful to increase the efficiency and production of food crops. The high intensity and productivity of plants results in natural nutrient depletion of the ground. The availability of nutrients nitrogen, phosphate and potassium play a role in the level of soil productivity. The availability of the nutrients is determined by two factors, namely innate factors and dynamic factors. The innate factor is the soil parent material, which influences the soil order. Dynamic factors are factors that can change such as tillage, irrigation, fertilization, and plant composting [11].

Nitrogen is a basic requirement for all organisms because the element is needed in complex protein molecule synthesis. Moreover, it influences organism growth and reproduction. Therefore, nitrogen is an important nutrient for plants because of all the components of fertilizer, it is the one which is needed in the greatest amount, with the result that a plant cannot grow normally when there is nitrogen deficiency Excessive use of chemical fertilizers can cause a decrease in the quality of agricultural land [10]. One solution that can be applied is the use of biological fertilizer.

Bacteria that live freely on the roots and in the tissues of rice plants, such as *Pseudomonas spp.*, *Enterobacteriaceae, Bacillus, Azotobacter, Azospirillum*, and *Herbaspirillum* have been shown to be

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capable of fixing nitrogen [9]. Nitrogen-fixing bacteria in the rhizosphere of *Gramineae* plants, such as *Azotobacter paspali* and *Beijerinckia spp*, are a few of a group of aerobic bacteria that colonize the surface of roots [1]. Nitrogen-fixing bacteria can tether nitrogen from the atmosphere because this type of bacteria has a specific enzyme in the cell known as Nitrogenase. The enzyme is composed of two mutually supporting components: Fe protein and Mo-Fe protein. *Azospirillum* is one of the most characteristic genera and currently includes 15 N-fixing species and one non-fixing species. *A. brasilense* is a bacterium used in agriculture in many countries since its discovery in 1978. *A. brasilense* is found to be associated with many plants throughout the world and can be isolated from the soil as well. The main strategy used to isolate and to count bacteria from this genus is using N-free semisolid media [2].

Organic fertilizers in the form of *Azotobacter* increase yields with positive effects on height, weight, leaf index, and corn yield [12]. In field experiments, *Azotobacter* inoculation without the addition of inorganic fertilizers increased corn yields by 15-30% compared to plants that were uninoculated [3]. Positive effects on plants can also due to a better balance of nutrients and improved absorption of nitrogen and other nutrients by plants [12]. The role of *Azotobacter* in rice and maize indicates that these bacteria can be developed as biofertilizers for lowland rice, maize, or plants which belong to the same family as lowland rice and corn, such as sorghum, which has not been widely cultivated in Indonesia. This study aims to determine the type of isolates and identify the nitrogen-fixing bacteria in the rhizosphere of corn (*Zea mays* L.) from Jeneponto Regency, South Sulawesi. Bacterial isolates obtained will be used as ingredients to make biological fertilizers which are expected to help provide a source of nitrogen and micro and macro nutrients to fertilize soil for agricultural plants.

2. Methods

2.1 Nitrogen-Fixing Bacteria Isolation

Soil samples were taken from corn plantation land (*Zea mays* L) in 3 locations: Bangkala District, Tamalatea District and West Bangkala District in Jeneponto Regency, South Sulawesi. Samples taken were soil in direct proximity of plant roots (Rhizosphere). The method of extraction was by taking the plant roots along with the soil at a size of 20 cm (6-6.5 inches). Around 1 kg of the rhizosphere was taken by using a shovel or soil spoon and then placed in a sterile plastic sample container. Samples were taken to the FMIPA UNM Biology Laboratory. Soil samples from 3 points (left, center and right) were air dried and sifted in the amount of 50 grams, and then pounded and mixed with 5 grams of mannitol so that it became an enriched soil. Then 14-15 ml of distilled water was added and stirred until it was well-mixed. The mixture was incubated for 5-7 days at 30°C. During the incubation process, observation was made for mucous formation (bubbles) which indicated the growth of nitrogen-fixing bacteria. Ten grams of the enriched soil was then serially diluted to produce concentrations of 10^{-1}, 10^{-2} and 10^{-3}. In a 90 mL glass bottle of concentration 10^{-1}, 5 mL was taken and transferred to another bottle containing 45 ml distilled water to produce a concentration of 10^{-2}. And, from the 10^{-2} bottle, 5 mL was taken and tranferred to another bottle containing 45 ml distilled water to produce a concentration of 10^{-3}.

Inoculation was made on Nfb medium consisting of 5.0g; DL-Malic Acid, 0.5g; KH_2PO_4, 0.2g $MgSO_4.7H_2O$, 0.1g; NaCl, 0.02g; $CaCl_2.2H_2O$. 4ml, micronutrient solution (0.4g; $CuSO_4.5H_2O$; 0.12g $ZnSO_4$, 1.40g; H_3BO_3, 1.0g; $Na_2MoO_4.2H_2O$, 1.0g; $MnSO_4.2H_2O$ 1.175g) by adding the volume to 1000 ml distilled water and heating in a water bath. 2 ml (5 g L^{-1}) bromothymol blue, in 0.2 N KOH dissolved in 100 ml. 2 ml (1.64% g/ L^{-1} FeEDTA) dissolved in 100 ml distilled water. 4 ml of Vitamin Solution of 20 mg pyridoxine HCl was dissolved in 100 ml distilled water. 1 ml (4.5 g) of KOH was dissolved in 1000 ml distilled water. Next, all of the ingredients were mixed according to their measured amounts, the recommended pH is 6.8, by adding NaOH. Subsequently, 5-7 g of bacto agar was added for semisolid Nfb, and 16 g bacto agar for solid Nfb. The volume of semisolid Nfb was 1000 ml and solid Nfb was also 1000 ml [2].

2.2 Morphological and physiological identification of nitrogen-fixing bacteria

The obtained nitrogen-fixing bacterial isolates were then morphologically identified using the Gram staining method. As for physiology, temperature (5°C, 25°C, 40°C), pH (4,6,8) and salt tolerance tests (1%, 6% and 10%) were carried out according to standard procedures.

2.3 Ammonium Excretion Test

The ammonium test was conducted out after 21 ml of liquid Nfb medium was shaken for 7 days at room temperature. Isolates were taken and centrifuged at 10,000 rpm for 10 minutes. 3 ml of isolate supernatant was taken and 3 ml of each standard solution was taken, and 1 drop of 2% EDTA was added along with 1 drop of sodium potassium tartrate, and 0.13 ml of Nessler reagent. It was then homogenized and incubated for 30 minutes at 25°C. The absorbance was then read at wave length of 435 nm [6].

3. Results and Discussions

3.1 Isolation and purification of nitrogen-fixing bacteria

Isolation from 9 soil samples of the rhizosphere of maize (*Zea mays* L.) in Jeneponto Regency resulted in 20 pure bacterial isolates. The data from nitrogen-fixing bacterial isolates is shown in Table 1.

Table 1. The characteristics of the nitrogen-fixing bacterial isolates

Isolate code	Characteristics of Colonies						
	Form	Elevation	Surface	Margin	Color	Mucus	Whole Colony
BKr_1	Round	Raised	Smooth	Smooth	White	Dry	Round
BKr_2	Irregular	Raised	Smooth shiny	Smooth	White	Dry	Irregular
BKr_3	Round	Raised	Smooth shiny	Smooth	White	Dry	Round
BKr_4	Round	Raised	Smooth shiny	Smooth	Milky white	Dry	Round
BTg_1	Irregular	Raised	Smooth shiny	Undulated	White	Dry	Irregular
BTg_2	Irregular	Raised	Smooth	Smooth	White	Dry	Irregular
BKn_1	Round	Raised	Smooth shiny	Smooth	Milky white	Dry	Round
TTg_1	Round	Raised	Smooth shiny	Smooth	Brownish white	Dry	Round
TTg_2	Round	Convex	Smooth	Smooth	White	Dry	Round
TTg_3	Irregular	Raised	Smooth shiny	Smooth	White	Dry	Irregular
TTg_4	Round	Raised	Smooth	Smooth	Brownish white	Dry	Round
TKr_1	Irregular	Convex	Smooth	Undulated	White	Slimy	Irregular
TKn_1	Round	Raised	Smooth	Smooth	White	Dry	Dots
TKn_2	Round	Raised	Smooth shiny	Smooth	Clear	Slimy	Round
BB Kr_1	Irregular	Convex	Smooth shiny	Smooth	White	Dry	Irregular
BB Kr_2	Round	Raised	Smooth shiny	Smooth	White	Dry	Round
BB Kn_1	Round	Convex	Smooth shiny	Smooth	Clear	Slimy	Round
BB Tg_1	Round	Raised	Smooth shiny	Undulated	White	Dry	Round
BB Tg_2	Round	Convex	Smooth shiny	Smooth	Clear	Slimy	Round
BB Tg_3	Irregular	Raised	Smooth shiny	Smooth	Clear	Slimy	Irregular

Isolate Information: B = Bangkala District, T = Tamalatea District, BB = West Bangkala District, Kr = Left Rhizosphere, Tg = Middle Rhizosphere, Kn = Right Rhizosphere.

Most isolates have flat edges, except for isolates BKr_4, TTg_3, and $BBTg_1$. The overall colors are white except for isolates BKr_4, BKn_1, TTg_1, , TTg_4, TKn_2, $BBKn_1$, $BBTg_2$, and $BBTg_3$ with varied colors including milky white, brownish white, and clear. Most isolates were not slimy except for TKr_1, TKn_2, $BBKn_1$, $BBTg_2$ and $BBTg_3$ isolates. The overall colony characteristic is round except for isolates BKr_2, BTg_1, BTg_2, TTg_3, TKr_1, TKn_1, $BBKr_1$ and $BBTg_3$ having irregular and dot colonies.

The study result with regard to nitrogen-fixing bacteria such as *Azotobacter* sp. and *Azospirillum* sp. are in line with an earlier study [7] which reported that non-symbiotic nitrogen-fixing bacterial isolates had a round and irregular shape, mostly flatly elevated with a smooth shiny surface, smooth margin, with smooth shiny surface, and smooth and undulating margin with colors transparent, transparent white and opaque white.

3.2 Physiological Characteristics and Gram Staining

The physiological characteristics and gram staining of 20 isolates can be seen in Table 2.

Table 2. Physiological characteristics of microscopically evaluated Gram-stained nitrogen-fixing bacteria

Isolate Code	Gram staining		Physiological Characteristics								
			Temperature (Pellicle)			pH (Pellicle)			Salinity (Pellicle)		
	Form	Gram	5°□	25°C	40°C	4	6	8	1%	6%	10%
BKr $_1$	Basil	-	+	++	++	+	+	+	+	++	-
BKr $_2$	Coccus	-	-	++	+	+	+	+	++	++	-
BKr $_3$	Basil	-	+	+	+	+	+	+	+	++	-
BKr $_4$	Basil	-	-	+	-	-	+	+	+	++	-
BTg $_1$	Basil	-	-	+	+	+	+	+	++	++	-
BTg $_2$	Basil	-	-	+	+	+	+	+	++	++	-
BKn $_1$	Basil	-	-	+	+	-	+	+	++	-	-
TTg $_1$	Basil	+	+	+	-	-	+	+	++	+	-
TTg $_2$	Basil	-	-	+	+	-	+	+	+	-	-
TTg $_3$	Basil	-	-	+	+	+	+	+	+	+	-
TTg $_4$	Basil	-	-	++	-	+	+	+	++	+	-
TKr $_1$	Coccus	-	+	+	++	+	+	+	+	+	-
TKn$_1$	Coccus	-	-	+	-	+	+	+	+	++	-
TKn $_2$	Basil	-	+	+	++	-	+	+	+	++	-
BBKr $_1$	Coccus	-	+	+	+	+	+	+	++	++	+
BBKr $_2$	Basil	-	+	++	++	+	+	+	++	+	-
BBKn $_1$	Basil	-	-	+	-	+	+	+	+	++	-
BBTg $_1$	Basil	-	-	+	-	+	+	+	++	+	-
BBTg $_2$	Basil	-	-	++	++	+	+	+	++	+	-
BBTg $_3$	Coccus	-	-	++	+	+	+	+	+	+	-

Isolate Description:

a. B = Bangkala District, T = Tamalatea District, BB = West Bangkala District, Kr = Left Rhizosfer, Tg = Middle Rhizosphere, Kn = Right Rhizosphere.
b. + = there is a pellicle, ++ = many pellicles, - = no pellicles

Table 2 shows, based on Gram staining, that nitrogen-fixing bacteria isolates are usually bacilli except for isolates BKr2, TKr1, TKn1, BBKr1 and BBTg3 which showed the form of coccus. Almost all are Gram negative except for BTg1 isolate, a Gram positive. The physiological characteristics show that in most cases, the bacteria survived at 25°C and 40°C, while at 5°C only 7 isolates survived: BKr1, BKr3, TTg1, TKr1, TKn2, BBKr1, BBKr2. Based on the pH tolerance test, most isolates survived at a pH of 4.6 and 8 except for 4 isolates: BKr4, BKn1, TTg1, TTg2. The salt tolerance test showed that most survived at 1% and 6% salinity, while at 10% only 1 isolate survived: BBKr$_1$. The result showed that when stained with Gram, most of the bacteria were colored red, indicating Gram-negative bacteria. Most Gram-negative bacteria have the form of bacillus and coccus and are red-stained. The presence of

a pellicle on the surface of the media is a positive indicator that this type of nitrogen-fixing bacteria can adapt to its environment. Most nitrogen-fixing bacteria only live in the range of 25°C to 35°C.

Azotobacter sp. survived at 25°C on the semisolid Nfb media, for which the media color ranges from green to blue. There were also pellicles present on the surface of the media. The pellicle was white and thin indicating that there is oxygen diffusion on the surface of the media. The nature of these bacteria is obligate aerobe. Whereas the type of *Azotobacter* which can survive at 40°C is *Azotobacter indigenous* which is notable for its capability to survive in a hot environment. A study by Caceres [5] stated that the change in color of the Nfb medium occured because bromthymol blue turns green to blue at higher pH, a result of nitrogenase activity due to Nfb medium's ability to provide nutrients needed by non-symbiotic nitrogen-fixing bacteria. The formation of pellicles characterizes the growth of non-symbiotic diazotrophic bacteria as an indicator that the bacteria is able to reduce the source of N from the media as an activity of nitrogenase [7].

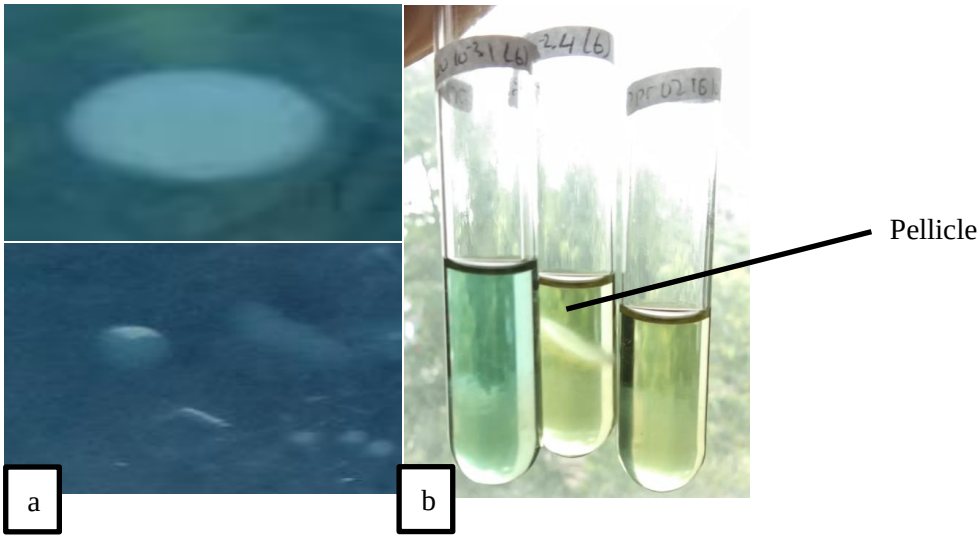

Figure 1. Nitrogen-fixing bacterial isolates BKn_1 and Kn_2 (a) and pellicle formation in the semisolid Nfb medium (b)

3.3 **Ammonium Excretion Ability Test**

The result of the ammonium excretion ability tests from the rhizosphere of corn plants containing nitrogen-fixing bateria is shown in Table 3.

Ammonium excretion tests from 3 regions: Bangkala, Tamalanrea and West Bangkala, showed that only 5 isolates had high ammonium concentrations. This showed that the ability of bacteria to fix nitrogen can be seen from the ability of bacteria to convert free nitrogen into ammonia which will be converted into amino acids that plants will use to grow. From five isolates that are high in ammonia, the ones that dominate the high levels of ammonium are isolates from Bangkala region, as we know that the higher the ammonium produced, the higher the ability of microbes to tether nitrogen and convert it into ammonia. With regard to the five isolates with high ammonium levels it cannot be determined whether the isolates can be categorized as *Azospirillum* sp. or *Azotobacter* sp. except by conducting molecular or genetic testing, so that these results can only be compared with previous studies.

Table 3 Ammonium concentrations for 20 nitrogen-fixing bacterial isolates from the rhizosphere of corn plants from Jeneponto Regency

Isolate Code	Ammonium concentration (μM)
BKr_1	42.3
BKr_2	39.1
BKr_3	41.5
BKr_4	35.0
BTg_1	36.7
BTg2	35.1
BKn1	38.3
TTg1	38.0
TTg2	38.6
TTg3	37.8
TTg4	37.5
TKr1	38.8
TKn1	37.0
TKn2	41.0
BBKr1	36.2
BBKr2	35.7
BBKn1	38.3
$BBTg_1$	39.4
$BBTg_2$	36.5
$BBTg_3$	38.6

Previous studies are in line with this research and have successfully tested the ability of ammonium excretion in some wild type nitrogen fixing bacteria such as OP strains of *Azotobacter vinelandii* with concentrations of 260,251 μM [8]. Research conducted by [8] reported that the isolate of *Azotobacter vinelandii* can excrete ammonium at a concentration of around 200 μM. Nitrogen-fixing bacteria have the ability to tether free nitrogen (N_2) from the air and convert it to ammonia (NH_3) which then becomes amino acids that can be used by plants to grow and develop.

4. Conclusion

Isolation and identification of nitrogen-fixing bacteria in the rhizosphere of corn plants from Jeneponto Regency obtained 20 isolates of nitrogen-fixing bacteria, which morphologically and physiologically showed a variety of bacterial cells with diverse forms. In temperature, pH and salinity tolerance tests, the presence of nitrogen-fixing bacteria was proved by the presence of pellicles and changes in the color of Nfb media containing isolates. Ammonium excretion tests showed that 5 isolates produced it with concentrations ranging from 42.3 μM to 35.0 μM.

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