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# Isolation and Characterization of *Azotobacter* from Neems Rhizosphere

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**Abstract.** Nitrogen-fixing bacteria can be isolated from rhizosphere plants, and one of the N<sub>2</sub>-fixing bacteria is *Azotobacter*. One of the plants that can grow well in dry and barren soil conditions is Neem (*Azadirachta indica* A. Juss)(Mimba). The purpose of this study was to determine any *Azotobacter* isolated from the rhizosphere of the neem plant. The type of research is an experimental study, tracing the diversity of *Azotobacter* in the rhizosphere of the neem plant. The study was conducted by taking soil samples from the rhizosphere of neem plants on the Bali Island. Morphological characterization based on colony observation in terms of shape, color, margin, and texture. The isolated cells were determined based on Gram staining and determination of the presence of spores. Isolates were characterized by various biochemical methods namely nitrate, reductase, citrate utilization, methyl red, VP test, catalase (method of slip-cover), and oxidase tests. The soil sample was taken from rhizospheres of 5 plants. The results showed that the N<sub>2</sub>-fixing bacteria isolated in the five soil samples were *Azotobacter paspali* and *Azotobacter vinelandii* (Azomonas) groups. Actinomycetes were found when isolating using specific media *Azotobacter* on plant 2.

**Keywords:** *Azotobacter*, neem, *Azadirachta indica* A. Juss, plant Rhizosphere

## 1. Introduction

Nitrogen-fixing bacteria are found and can be isolated from rhizosphere plants [1]. The presence of nitrogen-fixing bacteria in the rhizosphere of plants that can live in dry, thin and information about nutrient of soils is still rarely studied. One of the plants that can grow well in dry and barren soil conditions is neem (*Azadirachta indica* A. Juss). The mimba plant belonging to the shrub plant which was first discovered in the Hindustani area, in Madhya Pradesh, India. Mimba comes or is spread to Indonesia, estimated since 1500 SM. This plant grows in the regions of West Java, East Java, and Madura at an altitude of up to 300 m above sea level. This plant grows in the tropics, in the lowlands, grows in periodic arid places that are often found on the roadside as shady trees or in forests bright [2].

The isolation of microbes from the neem rhizosphere of *Azadirachta indica* which grows in several regions in West Bengal, India shows that the Rhizosphere mimba is suitable for the growth of common microbes in the land dwellers. This allows the growth of certain bacteria that have the ability to prevent unfavorable microenvironment. This information will serve as a database for future exploration of the bioprospective potential of mimba rhizosphere bacteria [3]. The population of



rhizosphere bacteria from the mimba plant has not been studied in detail. Similarly, the presence of N<sub>2</sub>-fixing bacteria in the mimba rhizosphere has not been widely reported. Research related to Rhizosphere microbes of neem plants has a very large possibility to provide an important database for the exploration and evaluation of bioprospective potential in the future. Therefore, this research will isolate N<sub>2</sub>-fixing bacteria from the Rhizosphere of neem plants to determine their physiological diversity.

Most nitrogen-fixing heterotroph aerobic bacteria are found in the histosphere area (inside the root tissue) compared to the rhizosphere area (around the root), rhizoplane (attached to the root surface) and at the base of the plant stem. Furthermore, the activity in the phyllosphere area is completely ignored. The highest number of bacteria in the histosphere, the base of the shoot and the lowest number is in the rhizosphere [4]. ARA test results from several plants showed plants from the families Cyperaceae, Rosaceae, Oxalidaceae, Onagraceae, and Asteraceae could not reduce acetylene and these plants showed N<sub>2</sub>-fixing activity in the histosphere and rhizoplane [5].

The life of N<sub>2</sub>-fixing bacteria in the soil depends on the host plant because most of the energy source is provided by the plant through an exudation process. Some carbohydrates and amino acids which are excreted by plant roots will be used by N<sub>2</sub>-fixing bacteria. Expenditures of such substances are considered to vary widely according to species and strains. Therefore the composition of bacteria in the rhizosphere vary according to plant genotypes [6]. Meanwhile, The use of exudates by N<sub>2</sub>-fixing microbes in the rhizosphere affect microbial growth. Inoculated plants excrete more exudates than non-inoculated plants. The highest number of exudates was in the area closest to the root, and this number will decrease if the distance far from the root [7].

The roots and around of the area (rhizosphere) are new ecosystems that are the place of the life of various microorganisms including bacteria, fungi, algae, etc. The plant rhizosphere generally inhabited by N<sub>2</sub>-fixing bacteria like *Azospirillum* sp. and *Klebsiella pneumonia* which will enhance the plant growth [8]. The roots of the host of plants release certain growth compounds that afford a unique microbial population in the rhizosphere. The rhizosphere-inhabiting microbes provide antimicrobial properties to harmful microorganisms or pathogens effect which that infects the root system in particular. Rhizomicroorganism has antimicrobial properties. Bacteria from the rhizosphere area of medicinal plants are also reported to produce antibiotics [9].

*Azadirachta indica* is very interesting to be developed because it contains abundant medicinal compounds. However, the rhizosphere bacteria from this plant have not been explored in deeply. The study of the population of rhizosphere bacteria from this plant has plentiful possibility to provide an important database related to the bacterial population of the new ecosystem for potential bioprospective exploration and evaluation in the future.

## 2. Research Method

### 2.1 Collection of sample

The soil was taken from the rhizosphere of five plants. The plant 1, 2, 3 and 4 were taken at the low land around University Hindu Indonesia (UNHi), Denpasar, Bali. The soil sample from plant 5 was taken around Serangan Beach, Bali, near mangrove forest. Collection of the soil was done at Mei-June 2018.

### 2.2 Determination of *Azotobacter*

The *Azotobacter* was isolated from the soil. Isolated the bacteria from the rhizosphere soil by making a series of dilution of soil from 10<sup>-1</sup> to 10<sup>-7</sup> on *Azotobacter* media and incubation for 48 h at 30°C. Bacterial culture was repeated for three times to obtaining the purity of the cultured isolate of bacteria [10]. Identification of the isolates was made by a morphological and biochemically test.

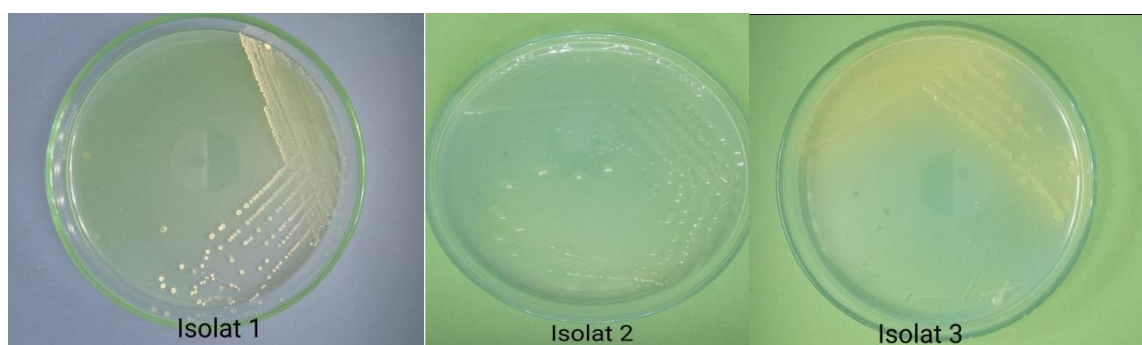
### 2.3 Characterization

Isolates were characterized by using cell Gram stain and colony (shape, color, margin, nature of colony and texture) test. Determination of biochemical properties was conducted by using different biochemical tests such as nitrate reductase, citrate utilization, methyl red, VP, catalase (cover-slip method), and oxidation test.

## 3. Results and Discussion

### 3.1 Isolation of NFB

The picture shows there are three isolates were isolated and identified from five plants rhizosphere. Among them, strains 2 and 3 show properties of *Azotobacter* sp. and strain 1 as an *Actinomycetes* sp. Isolation did on LG medium (selective media for *Azotobacter* and other N<sub>2</sub>-fixing microbial), and pure culture maintain on NA media (Figure1).



**Figure 1.** Bacterial strains isolated from neems Rhizosphere: *Actinomycetes* sp. (Isolate 1); *Azotobacter vinelandii* (Isolate 2); *Azotobacter paspali* (Isolate 3)

### 3.2 Morphological & biochemical characterization

Isolates were characterized by using cell test (Gram stain, determination of the presence of spores), and the colony (shape, color, margin, nature of colony, texture,) was observed (Table 1). Various biochemical tests were performed on isolates for characterization according to Burgy's Manual books. Some isolates showed positive results for the biochemical test (Table. 2 and Figure 2)[11].

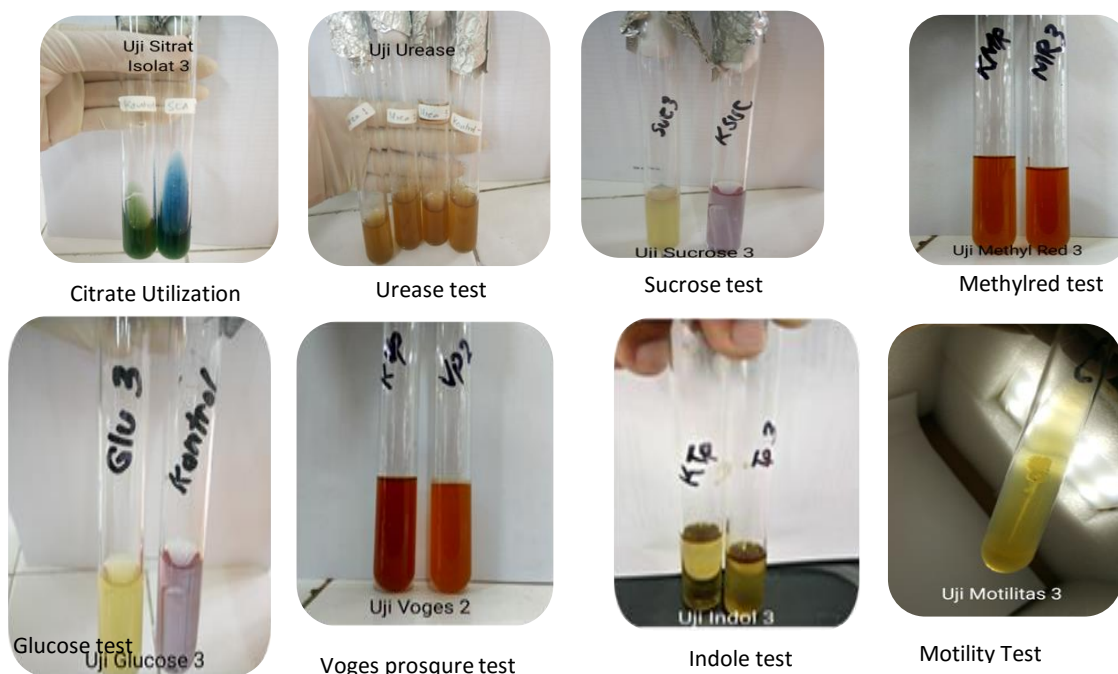
**Table 1.** Morphological of bacterial isolates

Isolates	Colony shape	Cell shape	Colony color	Trans-parancy	Nature of colony	Margin of colony	Surface of colony
Isolate 1 <sup>a</sup>	circular	rod	white	unopaque	unglistening	entire	unsmooth
Isolate 2 <sup>b</sup>	circular	rod	white	opaque	glistening	entire	smooth
Isolate 3 <sup>c</sup>	circular	rod	yellow	opaque	glistening	entire	smooth

Note: <sup>a</sup>) From Plant 2; <sup>b</sup>) From Plant 1, 2, 3, 4, 5; <sup>c</sup>) From Plant 1, 2, 3, 4

**Table 2.** Biochemical characterization of bacterial isolates

Test name	Isolate 1	Isolate 2	Isolate 3
Gram staining	Gram positive	Gram Negative	Gram Negative
Motility	+	+	+
Citrate utilization test	-	-	+
Methylred test	+	+	+
Voges proskure test	+	+	+
Catalase test	-	+	+
Indole test	-	+	+
Ureas test	-	-	-
Glucose test	-	+	+
Lactose test	-	-	-
Sucrose test	-	-	+

**Figure 2.** Several tests on biochemical characterization of bacterial isolates

### 3.3 Discussion

Various strains were found during the isolation and characterization of bacteria from the root of plants and the soil of the rhizosphere. These strains probably represent many species, and between them, we can find new species with biotechnological and ecological agricultural purpose. The significance of our research is the examination for the first time *Azotobacter* which inhabits neem roots [11]. The

bacteria isolated from soil rhizosphere by using dilution series from  $10^{-7}$  to  $10^{-9}$  on selective media for Azotobacter (LG medium) [10]. Isolates were characterized by morphological & biochemically according to Bergey's Manual method, to shown properties of Azotobacter spp [11]. From the results, it was found that the Azotobacter is the species of Nitrogen-fixing strains which can substitute the chemical fertilizer in dry and barren soil conditions, be used to reduce the alkalinity of soil by neutralization phenomenon through organic acid exudation and can survive in the soil system to retain the Nitrogen-fixing potential for a long time.

#### 4. Conclusion

Based on the result, that successful isolated Nitrogen-fixing strains bacteria from neem plants by using dilution series from  $10^{-7}$  to  $10^{-9}$  on selective media and founded that samples were *Azotobacter paspali* and *Azotobacter vinelandii* (Azomonas) groups on LG medium. *Actinomycetes* were found when isolating using specific media.

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