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**6310 Words**

## CHARACTER COUNT

**34383 Characters**

## PAGE COUNT

**13 Pages**

## FILE SIZE

**491.0KB**

## SUBMISSION DATE

**May 13, 2024 9:29 AM GMT+7**

## REPORT DATE

**May 13, 2024 9:30 AM GMT+7**

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## Bioprospecting of Rhizobia as Plant Growth Promoting Rhizobacteria Potential from Root Nodules of Groundnut (*Arachis hypogaea* L.)

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Received: 25 October 2023, Revised: 31 January 2024, Accepted: 7 February 2024, Published: 1 May 2024

### Abstract

Rhizobia are bacteria that symbiosis with host plant, as shown in the root nodules formation, and provide nitrogen that can be absorbed by plants in greater quantities than rhizobacteria. Available Nitrogen, which absorbed by plants, is the essential requirement for plant growth because its role in increasing yield and quality, hence it is needed in greater quantities than other nutrients. The study aimed to determine the macroscopic and microscopic diversity of rhizobia isolates from the groundnut nodules and their potential as PGPRs, and to identify 16S rRNA isolates with the best potential as PGPRs molecularly. The methods used were isolation from root nodules, screening of PGPR potential, molecular identification based on the 16S rRNA gene, and phylogenetic analysis to determine their kinship. Based on the isolation results, 17 Gram-negative isolates were obtained white to pink or orange color on AG media with various colony characteristics in terms of shape, margin, elevation, and texture. KT 20, which was selected as rhizobia isolate with the best potential as PGPR, has ammonium concentration of 23.12 ppm, synthesizes IAA with a concentration of 10.36 ppm, and phosphates solubilization activity, although its ability to synthesize proteases is low. The results of molecular identification of 16S rRNA showed that KT 20 belongs to the *Rhizobium* genus with a similarity of 99.48 % and bootstrap value of 96 %.

**Keywords:** *Arachis hypogaea*, IAA synthesise, Nitrogen fixation, Proteases, Phosphate solubilization, *Rhizobium* sp.

### Introduction

Plant growth promoting rhizobacteria (PGPR) are rhizobacteria (soil bacteria on the root surface and surrounding) that support plant growth through their various activities, including nitrogen fixation, phosphate solubilization [1], synthesizing phytohormones such as indole-3-acetic acid (IAA) [2], as well as synthesizing lysos enzymes, such as proteases [3]. There is a huge number of bacterial species that identified as PGPR, at least 15 genera, including *Bradyrhizobia*, *Sinorhizobia*, *Rhizobia*, *Devosia*, *Azorhizobia* and *Ochrobactrum* [4]. However, their ability as PGPR were limited to the specific host plant [5] and some species find it difficult to adapt when studied *in vivo*.

Rhizobacteria that have symbiosis with legumes are called rhizobia. The symbiosis between rhizobia and host was shown by root nodules formation on legume plants [6]. Symbiotic bacteria were able to provide nitrogen that can be absorbed by plants in greater quantities than rhizobacteria. Furthermore, Meghvansi *et al.* [7] stated that indigenous rhizobia capable to associate with other soil microbes and adaptable to extreme conditions with ease.

One of the plants that is adaptable too is groundnuts (*Arachis hypogaea* L.), one of the legumes that is produced commercially [8]. This plant has been cultivated at least in 118 countries, including Indonesia

[9]. In 2020, FAOSTAT reported that the groundnut crop harvest area of 345,000 tons from a planting area of 364,000 ha and a total production of groundnuts with shell of 860,000 tons in Indonesia.

Moreover, rhizobia which infect groundnut has a different way of nodulation than rhizobia in general. These rhizobia do not develop infection threads, but enter through lateral root crack on the epidermis, then immediately go to cortical cells by a process that like an endocytosis [10]. Due to the presence of “crack entry” method, these rhizobia symbiotic with non-legume plants more easily [11].

Based on these, the research related to bioprospecting was conducted. Bioprospecting is the process of exploring materials from various organisms to obtain valuable products as the need for the material increases, including in agroindustry [12]. Bioprospecting plays a significant role in the conservation and sustainable use of biodiversity. According to that, this study aimed to determine the macroscopic and microscopic diversity of rhizobia isolates from the groundnut nodules and their potential as PGPRs, and to identify 16S rRNA isolates with the best potential as PGPRs molecularly.

## Materials and methods

The research started with isolating the nodules from groundnut on land with low levels of N-available and acidity level of around 6.02 - 7.02 (BPTP, 2022) in July 2022. The position of the land is in Sayung District, Demak, Central Java, Indonesia, to be precise at 6°56'16"S and 110°36'31"E with an altitude of 19 m AMSL and the air temperature of 28 °C. Selected nodules were characterized by a slightly red or pink color (leghemoglobin) in response to roots-colonized by rhizobacteria [13].

### Rhizobia isolation from nodules

First, surface sterilization was conducted to nodules according to the modified procedure of Pang *et al.* [14], then the isolates were diluted and grown in Arabinose Gluconate (AG) agar media that prepared following the steps of Parks *et al.* [15] and Soares *et al.* [16]. The isolates were incubated at 28 - 30 °C. After 3 - 10 days, isolates, which grew white to slightly pink, as well as orange with various macroscopic characteristics, were purified using the streak plate method [17]. Then, the single colonies were characterized microscopically by observing the shape of the cells and the results of Gram's staining which refers to the study of Thairu *et al.* [18].

### Qualitative screening of the potential PGPR

#### Nitrogen fixation activity

Analysis of nitrogen fixation activity conducted to select rhizobia isolates, which have these main characteristics, by refers to the study of Day and Döbereiner [19]. Isolates were grown in semi-solid nitrogen-free bromothymol blue (NFB) media for 7 days, then the blue color that appears was observed and categorized based on the intensity [20].

#### Phosphate solubilization activity

Analysis of phosphate solubilization activity conducted by refers to the study of Pikovskaya [21]. After growing isolates in AG broth media for 3 days, 5 µL of each isolate culture was drip on paper disc and it was placed on Pikovskaya's agar media for 24 - 48 h [22]. Then, the clear zone that appears was observed and calculated to determine the phosphate solubilization index (PSI) using the equation below [23]:

$$PSI = \frac{\text{Clear Zone Diameter} + \text{Colony Diameter}}{\text{Colony Diameter}}$$

The PSI values were classified (**Table 1**) according to the categories used by Halimursyadah *et al.* [23].

**Table 1** Phosphate Solubilization Index (PSI) Category.

PSI value	Efficiency
< 1.6	Low
1.6 - 2.14	Moderate
2.15 - 2.59	High
> 2.6	Very High

### **Indole-3-Acetic Acid (IAA) activity**

Analysis of IAA activity conducted by refers to the study of Sarwar and Kremer [24]. Isolates were grown in nutrient broth (NB) media added 200 ppm L-tryptophan in dark condition with a shaker at 112 rpm. After 3 days of incubation, the Salkowski's reagent was added to the isolate culture as an indicator [25]. Then, the pink to dark red that appears was observed and categorized based on the intensity.

### **Proteases activity**

Analysis of protease activity conducted by refers to the study of Mehta *et al.* [26]. After growing isolates in AG broth media for 3 days, 5  $\mu$ L of each isolate culture was drip on paper disc and it was placed on 12.5 g/L skim milk agar (SMA) media for 24 - 48 h [27]. Then, the clear zone that appears was observed and calculated to determine the proteolytic index (PI) using the equation by Lim *et al.* [28], as follows:

$$PI = \frac{\text{Clear Zone Diameter}}{\text{Colony Diameter}}$$

The PI values were classified according to the categories used by Dewiyanti *et al.* [29] (**Table 2**).

**Table 2** Proteolytic Index (PI) category.

PI value	Efficiency
< 1	Low
1 - 2	Moderate
> 2	High

### **Selection of potential rhizobia isolates as PGPR**

Three rhizobia isolates, which had the best ability as PGPR qualitatively, were analyzed further quantitatively to select the preeminent. Two quantitative analyzes were accomplished by measuring the absorbance of the supernatant at a wavelength of 610 nm for nitrogen fixation activity [20] and at a wavelength of 535 nm for IAA synthesise activity [25]. Then, the absorbance values were compared with the standard curves that has been created according to Cordova-Rodriguez *et al.* [20] and Pant and Agrawal [25] respectively.

### **16S rRNA molecular identification of rhizobia isolate**

Molecular identification of 16S rRNA was accomplished in several stages starting with reviving the isolate for DNA extraction following the Genomic DNA Wizard kit (Promega, Wisconsin, USA) procedure. Then, the purity and concentration of DNA were measured with the NanoDrop-2000 Spectrophotometer (Thermo-Fisher Scientific, Massachusetts, USA) in order to get good PCR results. The PCR mixture was prepared following the 2X GoTaq PCR mix (Promega, Wisconsin, USA) protocol with universal bacterial primers 27F and 1492R (**Table 3**). Running PCR accomplished using a thermocycler following the amplified condition by Raissa *et al.* [30]. Furthermore, the PCR results were visualized on agarose gel and observed using UV through the GelDoc (UVITEC, England, UK) System. Then, the good PCR results were sent to PT Genetika Science Indonesia for sequencing.

**Table 3** Concentration of PCR mix components.

Components	Concentration
GoTaq PCR Mix	2X
Primer 27F	100 pmol/ $\mu$ L
Primer 1492R	100 pmol/ $\mu$ L
DNA Templates	20 ng
ddH <sub>2</sub> O	

### Phylogenetic analysis

The isolate sequences were re-read and contig with BioEdit version 7.0.5.3, then being compared with gene sequence data on the National Center for Biotechnology Information (NCBI) website (<https://www.ncbi.nlm.nih.gov/>) using the basic local alignment search tool-nucleotide program (BLAST-N). A phylogenetic tree was created with MEGA XI software under the Neighbor-Joining Tree construction using the bootstrap 1,000 method [31].

### Results and discussion

The soil sample is moderately acidic, with a  $\text{pH}_{\text{KCl}}$  of 6.02 and a  $\text{pH}_{\text{H}_2\text{O}}$  of 7.02, and it exhibits low N-available levels, as categorized by Azizan *et al.* [32], with only 0.12 % N-available content. These conditions supported the growth of N-fixing bacteria. According to Friel and Friesen [33], the number of nodules, where rhizobia live, is relatively increased under limited nitrogen conditions. Besides that, based on the statement of Lin *et al.* [34], acidic soil has also been known as a factor for symbiotic of N-fixing bacteria since low pH in soil limits plants from absorbing nutrients.

#### Isolation and selection of rhizobia

##### Macroscopic characterization

Isolation and selection of rhizobia from nodules on AG media resulted in 27 various macroscopic isolates with white to pink, as well as orange colors. The isolates that encode with KT 1 - KT 27 varied in terms of colony shape, margin, elevation, and texture (**Table 4**). Ondieki *et al.* [17] reported that, rhizobia isolates that they found were mostly had a circular in shape, while some others were irregular or punctiform. Meanwhile, rhizobia isolates explored by Kapembwa *et al.* [35] reported that, 88 % had an entire edge, 10 % undulate and 2 % lobate. The rhizobacterial isolates found by Shankar and Prasad [36] had flat, raised, convex, crateriform, or umbonate elevations. In addition, based on the exploration of Odori *et al.* [37], rhizobia colonies that they found appeared mucoid or glistening. Moreover, rhizobia colonies explored by Janczarek and Skorupska [38], some looked dry and wrinkled, i.e. the mutant *Rhizobium leguminosarum* bv. *trifolii*.

**Table 4** Macroscopic characteristics of isolates on AG media.

Colony shape		Margin		Elevation		Texture	
Circular	62.96 %	Entire	55.56 %	Flat	40.74 %	Moist	44.44 %
Irregular	29.63 %	Undulate	40.74 %	Raised	29.63 %	Mucoid	22.22 %
Punctiform	7.41 %	Lobate	3.7 %	Convex	14.81 %	Glistening	18.52 %
				Umbonate	7.41 %	Wrinkled	11.11 %
				Crateriform	7.41 %	Dry	3.7 %

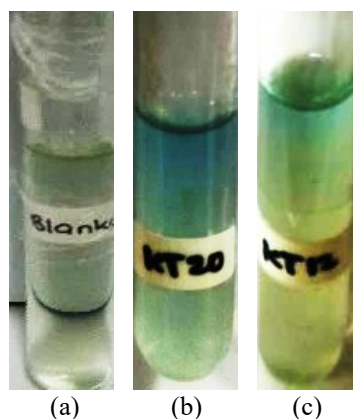
##### Microscopic characterization

Twenty-seven isolates that had been selected macroscopically were re-selected through Gram staining, thus proving that all isolates were Gram negative, except for KT 6. This was in accordance with the stated by Etesami [39] that, N-fixing bacteria in nodules are Gram-negative bacteria. Based on the Gram stain's results, 26 isolates were Gram negative which was characterized by a pink color with 69.23 % being rod-shaped. Thairu *et al.* [18] stated that, Gram-positive and Gram-negative bacteria are differentiated based on the thickness of their cell wall (peptidoglycan) and permeability of their membranes. Gram-positive have thick peptidoglycan as their cell wall, thus they are kept the crystal violet-iodine complex when decolorized. While bacteria Gram negative has a thin peptidoglycan and lipids-rich (lipopolysaccharides) that cause the cell wall is damaged and loses the crystal violet-iodine complex, then gain safranin.

#### Characterization of rhizobia main properties

Following macroscopic and microscopic selection, isolates were selected based on the main characteristic of rhizobia, i.e. its ability to fix nitrogen. According to Piromyou *et al.* [40], selection of its ability to fix nitrogen can increase the possibility of selecting isolates with higher survival properties in soil with limited N-available. In addition, Han *et al.* [41] stated that, one of the rhizobia's roles as PGPR is by fixing  $\text{N}_2$  from the atmosphere into ammonium, because plants just absorb nitrogen in inorganic forms,

such as  $\text{NH}_4^+$  and  $\text{NO}_3^-$ . Out of the 26 isolates, 17 showed positive results for fixing nitrogen, namely KT 1, KT 2, KT 3, KT 7, KT 11, KT 13, KT 14, KT 15, KT 16, KT 17, KT 18, KT 19, KT 20, KT 21, KT 22, KT 24, and KT 25, which indicate by the appearance of blue color after incubation for 7 days (**Figure 1**).



**Figure 1** Nitrogen fixation test (a) control, (b) positive (KT 20), and (c) negative (KT 12).

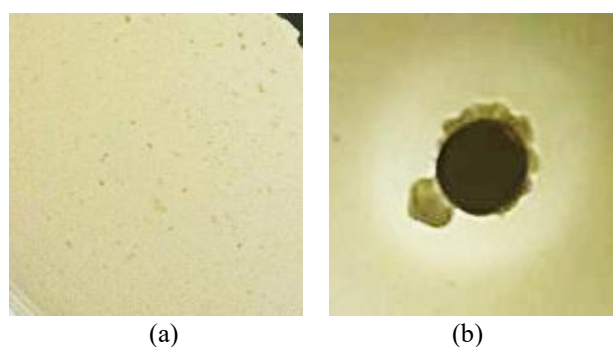
In NFB media, there is bromothymol blue reagent whose color is affected by pH. Thus affect the appearance of blue color, since the isolates fix nitrogen and then formed ammonium, which is alkaline. This statement supported by Shimada and Hasegawa [42] that, the green color could change to blue according to the level of acidity in the NFB medium, so that it is used as an indicator of nitrogen fixing bacteria.

#### Qualitative screening of the potential PGPR

Apart from fixing nitrogen, the isolates were also tested for other abilities as PGPR, such as the ability to solubilize phosphate, synthesize IAA and synthesize protease.

#### Phosphate solubilization activity

The others potential as PGPR showed that all isolates were able to solubilize phosphate. This was indicated by the formation of a clear zone around the colony on Pikovskaya's agar media after incubation for 24 h (**Figure 2**). The phosphate, which was previously insoluble, was assimilated by bacteria 'till it was soluble. According to Joe *et al.* [43], the clear zone is an indicator because these bacteria produce enzymes that dissolve tricalcium phosphate [ $\text{Ca}_3(\text{PO}_4)_2$ ] by releasing organic acids.

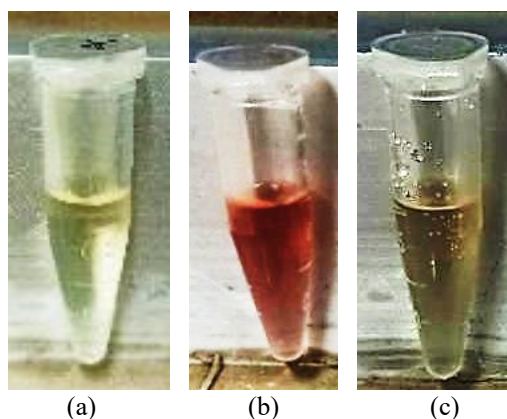


**Figure 2** Phosphate solubilization test (a) control and (b) positive (KT 20).

Based on the analysis results, KT 2, KT 3, KT 7, KT 11, KT 13, KT 14, KT 17, KT 18, KT 20, KT 21, and KT 24 have a good ability with PSI value is more than 2.16. Kumar *et al.* [44] stated that, one of the PGPR's roles is to provide the phosphate needed on plant growth by assimilating insoluble phosphate to soluble and absorbed by plants, because most of the P is absorbed in anion forms, i.e.  $\text{HPO}_4^{2-}$  and  $\text{H}_2\text{PO}_4^-$ .

### Indole-3-Acetic Acid (IAA) activity

Those 17 isolates were also synthesized IAA, except for KT 16. This was indicated by pink color that appears on NB+L-tryptophan medium with Salkowski's reagent after incubation for 3 days (**Figure 3**).

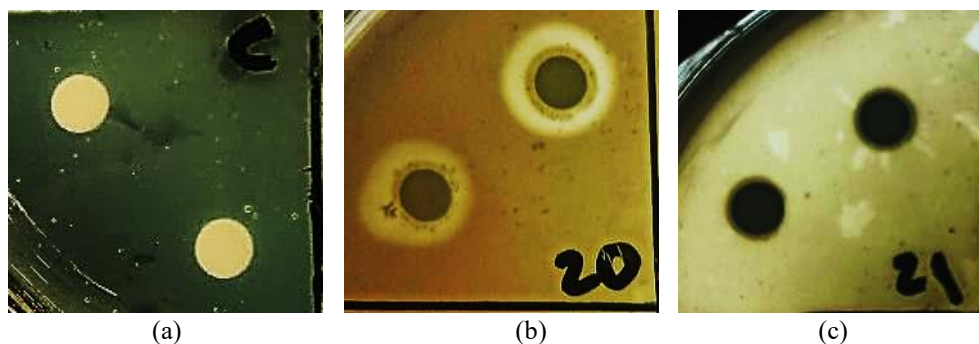


**Figure 3** IAA synthesis test (a) control, (b) positive (KT 20), and (c) negative (KT 12).

This result is supported by Gang *et al.* [45], the pink color appears as a positive reaction from the formation of indole compounds as a result of tryptophan metabolism, the synthesized IAA forms a complex with  $\text{Fe}^{3+}$  from Salkowski reduction. According to Chandra *et al.* [46], the ability to synthesize IAA is also necessary for plants, because IAA increases the root length by enhance the number of branches, lateral roots, and root hairs, thus helping the absorption of nutrients from the surroundings.

### Protease activity

Furthermore, 12 out of 17 rhizobia isolates showed the ability to synthesize proteases. This is indicated by the formation of a clear zone around the colony on SMA media because the bacteria produce enzymes that breakdown proteins in SMA media and forms a clear zone (**Figure 4**). According to Ramadhan *et al.* [47], the clear zone that formed around the colony indicated that the bacteria growing on SMA media had broken down proteins in skim milk into amino acids and peptides by producing protease enzymes.



**Figure 4** Protease synthesis test (a) control, (b) positive (KT 20), and (c) negative (KT 21).

Based on the analysis results, at most KT 22 has a good ability with PI value is more than 1.00. Bhat *et al.* [48] stated, the protease synthesized by PGPR is also known as plant-growth promoter, as they degraded pathogenic cell walls, which composed of proteins, thereby inhibiting these plant pathogens.

### Selection of potential rhizobia isolates as PGPR

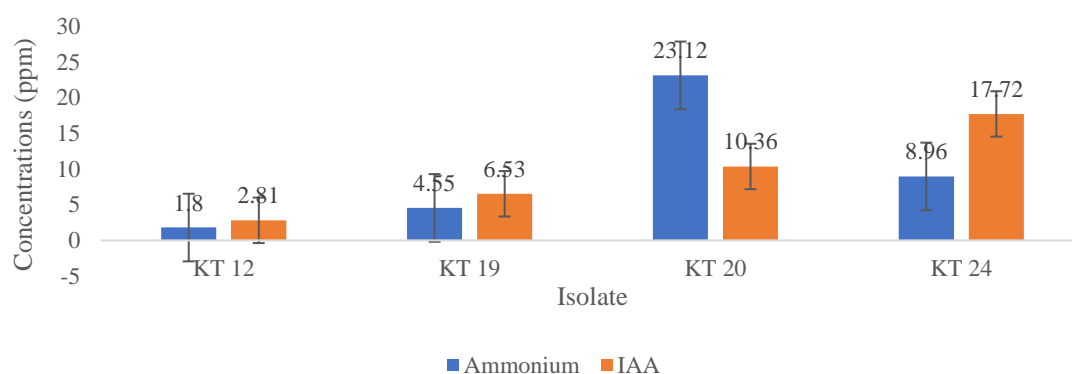
Based on the **Table 5**, KT 20 and KT 24 were the best PGPR isolates qualitatively. Therefore, the absorbance values of KT 20 and KT 24, also KT 12 as a negative isolate and KT 19 as a positive isolate were compared with the standard curves of ammonium and IAA, respectively, for further analysis.

**Table 5** Qualitative test results of rhizobia isolates ability as PGPR.

Isolate	Nitrogen Fixation	Phosphate Solubilization	IAA Synthesis	Protease Synthesis	Gram
6 KT 1	++	+	+++	-	Negative
KT 2	+	++	++	-	Negative
KT 3	++	++	++	+	Negative
KT 7	+++	++	++	+	Negative
KT 11	+	+++	++	+	Negative
1 KT 13	+	++	++	+	Negative
KT 14	+	++	+	+	Negative
KT 15	+	+	++	+	Negative
KT 16	+	+	-	-	Negative
KT 17	+	++	++	+	Negative
KT 18	+	+++	+	-	Negative
KT 19	+	+	+	+	Negative
KT 20	++++	++	+++	+	Negative
KT 21	+	++	++	-	Negative
KT 22	+	+	+	++	Negative
KT 24	+++	+++	+++	+	Negative
8 KT 25	+	+	+	+	Negative

Notes: “+” = Low; “++” = Moderate; “+++” = High; “++++” = Very High.

According to the concentrations of  $\text{NH}_4^+$  and IAA, KT 20 has much better potential in fixing nitrogen (Figure 5). This statement is supported by Shimada and Hasegawa [42] that the color change between yellow, green to blue in media with bromothymol blue follows changes in the pH of the media, so that it is along with the concentration of the ammonium produced. Shoukry *et al.* [49] also reported that the concentration of IAA is along with the level of red color as a reaction between IAA and the Salkowski reagent. According to Simon *et al.* [50], rhizobia are soil microorganisms that can infect the roots of the *Leguminosae* family and fix nitrogen, thus it can be used by plants.

**Figure 5** Graph of ammonium and IAA concentrations produce by isolates.

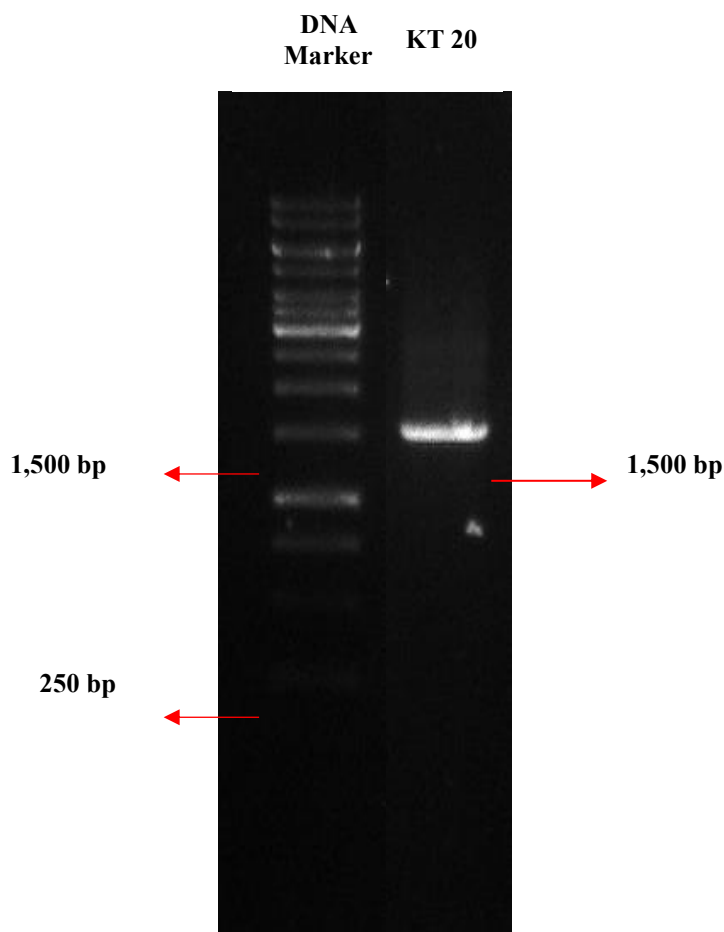


KT 20 was chosen as the superior rhizobia isolate for molecular identification of 16S rRNA, although KT 24 had a better ability to synthesize IAA and increase soluble phosphate, because the legume plant needs of N-available was very high. Kabir *et al.* [51] and Xu *et al.* [52] stated that, N-available is the most important condition for plant growth and needed in greater quantities than other nutrients. Even though the *Leguminosae* family, such as the groundnut is able to fix as much as 40-80 kg/ha N in a year, these plants still require about 86 - 92 % N (125 - 178 kg/ha N) fixed by microbes.

#### 16S rRNA molecular identification of KT 20 isolate

The DNA extraction results showed that KT 20 has a quite good concentration of 67.0 ng/ $\mu$ L and a purity of 1.55. According to Lucena-Aguilar *et al.* [53], DNA samples with A260/A280 values between 1.8 - 2.0 are accepted for their purity in general. Even so, the ratio that might indicate the presence of protein and phenol contaminants, namely if the ratio is far below 1.6. Moreover, the high ratio should be considered that it might be caused by the presence of RNA. Multinu *et al.* [54] reported that, the workable DNA concentration is more than 200 ng/ $\mu$ L, when the minimum is around 40 ng/ $\mu$ L. However, the concentration of the DNA template must exceed the minimum to get a good PCR result with ease.

The visualization results of KT 20 amplicon showed a 1,500 bp sized DNA band (**Figure 6**). Abellan-Schneyder *et al.* [55] stated that, the length of the 16S rRNA gene structured is about 1,500 bp interspersed with 9 variable regions in the highly conserved 16S sequence. According to Baker *et al.* [56], primers can also be bound to conserved areas so that it is possible to obtain more different bacterial taxa when sequencing.

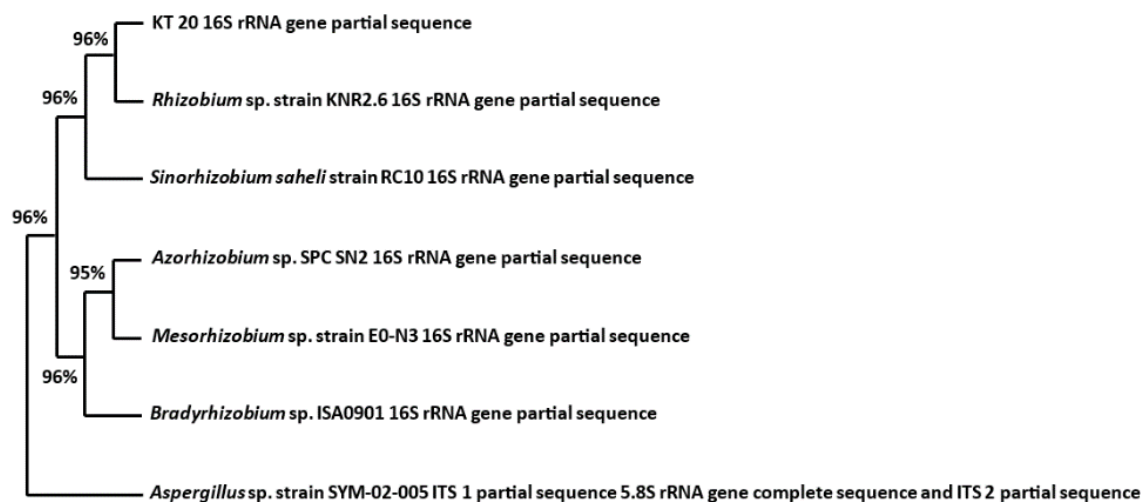


**Figure 6** Visualization of KT 20 16S rRNA amplification results.

### Phylogenetic Analysis of KT 20 Isolate

Based on the BLAST-N results, KT 20, which consists of 388 bp DNA, most closely related to *Rhizobium* sp. Strain KNR2.6 and 8 other species of *Rhizobium* genus with an identity of 99.48 %, query cover of 100 %, and E-value of 0.0. According to Samal *et al.* [57], the percent identity based on the number of identical characters from each sequence, so it describes how similar the query sequence and the target sequence, while the query cover describes the number of query sequences that are replaced by the target sequence, and the expectation value (E-value) is the significance of a given pairwise alignment. According to Karolenko *et al.* [58], identity results more than 97 % in the 16S rRNA gene sequence indicate the genus level, while > 99 % indicate the genus to species level, referring to research by Reller *et al.* [59].

Furthermore, to construct a phylogenetic tree, selection of other ingroup species, at least 4 species, and 1 outgroup species be required. The selected ingroup species were *Sinorhizobium saheli* strain RC10, *Azorhizobium* sp. SPC SN2, *Bradyrhizobium* sp. ISA0901 and *Mesorhizobium* sp. E0-N3. Meanwhile, the selected outgroup species was *Aspergillus* sp. strain SYM-02-005.



**Figure 7** Phylogenetic tree of KT 20.

The bootstrap value between KT 20 and *Rhizobium* sp. strain KNR2.6 was 96 % (**Figure 7**) indicates the accuracy level of the phylogeny. This statement is supported by Hillis and Bull [60], a bootstrap value of more than 95 % with a limit above 70 % showed a credibility.



**Figure 8** Microscopic characteristic of KT 20 (Magnification: 1,000X).

Since the BLAST-N of KT 20 showed that the query cover, percent identity and E-value has the same score for 9 species belonging to the *Rhizobium* genus, then KT 20 is also identified as a species belonging to the *Rhizobium* genus. This isolate has a circular colony-shape, undulate edge, raised elevation, and shiny light pink color. This result is supported by Silva *et al.* [61], *Rhizobium* sp. is well known as a rod-shaped Gram-negative bacterium that has a circular in shape with undulate or entire edge and raised or flat elevation (Figure 8). In addition, the results of a study by Dinkwar *et al.* [62] showed that the color of some *Rhizobium* sp. are shiny whitish pink. In addition, this bacterium also has the ability in phosphate solubilization, IAA synthesise, and protease synthesise as some of the characteristics required by PGPR. These results are supported by Sridevi and Mallaiiah [63] who reported that the phosphate solubilization ability of *Rhizobium* sp. strain 26 was the smallest compared to 40 rhizobia isolated from root and stem nodules of *Cassia absus*, *Vigna trilobata*, and 3 strains of *Sesbania sesban*. Kumar and Ram [64] also obtained *Rhizobium* sp. able to solubilize phosphate, namely *Rhizobium* sp. strains 103-JX576499 and *Rhizobium* sp. strain MRR106-KC428655. While, the ability of IAA synthesise is supported by Shokri and Emtiazi [65] and Ghosh and Basu [66] who obtained *Rhizobium* sp. from the root nodules of *Phaseolus mungo* var. PU30 synthesise IAA. In addition, although not many studies have reported the ability of *Rhizobium* sp. in synthesizing proteases, Purwaningsih *et al.* [67] reported that 8 out of 10 isolates of *Rhizobium* sp. isolated from *Arachis hypogaea* able to synthesise proteases.

## Conclusions

There are 17 rhizobia isolates from the root nodules of groundnut with various macroscopic characteristics. The potential PGPR that selected is KT 20, which has a circular colony-shape, undulate edge, raised elevation with a glistening light pink. KT 20 was chosen because it has the best nitrogen fixation ability and able to solubilize phosphate, synthesise IAA, and synthesise protease. This isolate belongs to the *Rhizobium* genus with a similarity of 99.48 % and bootstrap value of 96 %. For further studies, it is necessary to conduct *in vivo* research and identify rhizobia-specific genes molecularly to ensure that the isolates are rhizobia species which nodulation groundnut root nodules.

## Acknowledgment

AB would like to thank Diponegoro University, Indonesia for the RPI Grant No. 753-24/UN7.D2/PP/IX/2022 and WCU UNDIP Grant for Staff Exchange 2024. DW would like to thank Diponegoro University for the Postdoc Grant No. 216/UN7.A/HK/XI/2023. In memoriam Dr. Rejeki Siti Ferniah

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