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Isolation and molecular identification of halotolerant diazotrophic bacteria from The Northern Coastal of Pemalang, Central Java, Indonesia

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Abstract. *Purwanto, Eka Oktaviani, Ni Wayan Anik Leana. Isolation and molecular identification of halotolerant diazotrophic bacteria from The Northern Coastal of Pemalang, Central Java, Indonesia*. Plant Growth Promoting Bacteria can fix nitrogen, a very important macronutrient for plant growth. However, the application of urea as a source of this macronutrient has a negative impact on the environment. The development of biofertilizers using N-fixing bacteria is an environmentally friendly technology. Therefore, this research aimed to isolate and analyze the diversity of N2-fixing bacteria from saline rice fields. Soil samples were taken from the rhizosphere of rice plants in Nyamplung Sari Village, Petarukan District, Pemalang Regency. Meanwhile, 9 (nine) isolates of nitrogen-fixing bacteria have been isolated, which can fix N2. The isolates can bind to N_2. but only few are capable of producing IAA. The nitrogen-fixing ability of diazotrophic bacteria ranged from 17.85 ppm to 29.05 ppm. Isolate Jn3 has the highest ability to fix nitrogen, reaching 29.05 ppm, while Jn3, J, J12, J5, Kn1, and A3 can produce IAA with values of 2.00, 2.69, 2.22, 1.76, 3.47, and 1.79 ppm, respectively. Based on the 16S rRNA analysis and phylogeny construction, the isolated bacteria were identified on 3 (three) clusters. The first cluster was identified as *Pseudomonas stutzeri* and *Acinetobacter junii* (Kn1 & Jn3), while the second was identified as *Bacillus cereus, Bacillus tropicus, Bacillus altitudinis,* and *Bacillus* subtilis (Jn, Jn1, A3, and K3 isolates). The third cluster was identified as *Bacillus pumilus, Acinetobacter baumanii*, and *Acinetobacter schindleri* (J12, J5, and J). Our study reported the findings of *Acinetobacter baumannii* and *Acinetobacter schindleri* species that can fix nitrogen.

Keywords: Daizotroph, bacteria, halotolerance, saline, N_2.

Abbreviations IAA: Indole Acetic Acid; ppm : part per millions; rRNA: ribosomal Ribo Nucleic Acid; mM: mili Molar; P : phosphate; PGPR: Plant Growth Promotion Rhizobacteria; NB: Nutrient Broth; Nfb: Nitrogen Fixing Bacteria; TPC: Total Plate Count; DNA: Deoxyribose Nucleic Acid; ng: nanogram; μL: microliter; mL: milliliter; PCR: Polymerase Chain Reaction; 16S rRNA: 16 Subunit Ribosomal Ribo Nucleic Acid; MEGA-11: Molecular Evolutionary Genetic Analysis-11; NCBI: National Center for Biotechnology Information; BLASTn: Basic Local Allignment Search Tools Nucleotida

Running title: isolation and molecular identification of halotolerant diazotrophic bacteria

INTRODUCTION

National rice production is faced with the problem of increasingly narrowing productive fields. The rate of land conversion every five years shows a relatively high number, reaching 650,000 ha for various purposes such as housing, infrastructure, and industrial area development. (Firmansyah et al., 2021). Furthermore, the north coast of Java is a national rice barn but cannot be separated from the shrinking of productive land for other purposes. Zuhri (2018) reported that the rate of land use change on the northern coast of Central Java reaches 0.31% per year. Efforts to maintain national rice production can be made by expanding the area. Since fertility is very low, the alternative is to use marginal land with the potential for rice cultivation. Land with saline is one of the potentials for the development of new paddy fields. The total area of saline land in Indonesia reaches 440,300 ha, with the criteria of slightly and totally saline reaching 304,000 ha and 140,300 ha (Karolinoerita & Yusuf, 2020).

The North Coast of Pemalang is the center of Central Java's rice production. However, its location causes seawater intrusion into the rice fields, causing saline conditions in the paddy fields. Salinity occurs when the concentration of soluble salts is high in the soil and the electrical conductivity is above 4 dS m^{-1} or 40 mM NaCl (Abbas et al., 2019;

Karolinoerita & Yusuf, 2020). According to Karolinoerita & Yusuf (2020), the use of irrigation water with high salt content can cause accumulation in the root areas of plants and negatively influence growth. The negative effect of salinity stress affects plants, especially in decreasing the rate of photosynthesis and accumulation of plant biomass (Yan et al., 2013). Suryaman et al. (2021) reported that salinity decreases photosynthetic activity, both stomata and non-stomata factors, decreased chlorophyll content, leaf area, plant height, and biomass of mung bean plants. Soils with high salt content cause ion toxicity, nutritional deficiencies, oxidative and osmotic stress, limiting water uptake, disrupting nutrient balance and affecting plant nutrient absorption (Abbas et al., 2019). The high salt content in the form of Na, Ca, and Mg as chloride or sulfate causes plants to be deficient in nutrients N, P, Mn, Fe, and osmotic stress (Shrivastava & Kumar, 2015; Subowo, 2015; Sulasih & Widawati, 2016).

The biological technology approach can be carried out using tolerant varieties and beneficial microbes or Plant Growth Promotion Rhizobacteria (PGPR). Furthermore, the utilization can stimulate plant growth and increase the availability of nutrients. PGPR interacts with plants through the colonization of root areas and stimulates growth through direct and indirect effects (Shrivastava & Kumar, 2015). The direct and indirect effects improve plant growth and increase tolerance to saline stress (Abbas et al., 2019). Agustiyani et al. (2021) reported the isolation of N-fixing bacteria from various ecosystems with the genus *Sinomonas* strain and *Arthrobacter*. Furthermore, Sultana et al. (2021) stated that the bacteria *A. denitrificans*, *B. aryabhattai*, and *Pseudomonas* spp. were tolerant to salinity and could produce siderophores.

It is imperative to study the diversity of nitrogen-fixing bacteria in saline paddy fields on the Northern Coast of Pemalang. The utilization of saline-tolerant nitrogen-fixing bacteria is significant for developing biofertilizers to support plant growth and yield in saline soils. Tirry et al. (2021) reported that the application of saline-tolerant bacteria such as *Pseudomonas putida*, *Alcaligenes* sp., *Klebsiella* sp., and *Pseudomonas cedrina* could reduce the negative impact of stress caused by NaCl, increasing chlorophyll content compared to uninoculated plants. This study aims to isolate and analyze the diversity of N_2-fixing bacteria from saline rice fields.

This research is essential in providing information about nitrogen-fixing bacteria in coastal ecosystems. The nitrogen-fixing bacteria found can be an important source of inoculants in supporting the growth and development of plants in coastal areas, both for food and conservation needs. Based on the previous research, it was possible to find bacteria that can fix free nitrogen in the Northern Coastal Area, Pemalang Regency, Central Java Province.

MATERIALS AND METHODS

Sampling Area

Soil samples were taken from Nyamplung Sari Village, Petarukan District, and Pemalang Regency rice fields about 500 meters from the north coast of the Java Sea. Furthermore, the sampling location has a pH and EC above 7 and 4 mS/cm. The samples were obtained from the roots of rice plants in the maximum vegetative phase. The soil from the rice roots was excavated and taken with the plant roots, put into a plastic bag, placed in a cooler box at a temperature of 4 ^{0}C and brought to the laboratory for further isolation and characterization.

Isolation and Purification

A total of 10 g of soil sample was mixed with 90 mL of sterile distilled water, and graded dilution was conducted by taking 1 mL mixed with 9 mL of sterile distilled water to obtain a dilution of 10^{-2}. The graded dilutions were carried out up to 10^{-6}, and a total of 0.1 mL of the last two dilutions were inoculated on Nfb medium (the composition of Nfb medium : malic acid 5.0 g/L; K_2HPO_4 0.5 g/L; $MgSO_4 \cdot 7H_2O$ 0.2 g/L; NaCl 0.1 g/L; $CaCl_2 \cdot 2H_2O$ 0.02 g/L; KOH 4.5 g/L, NH_4Cl 1 g/L; FeEDTA 4 mL (solution 16.4 g/L); micronutrient solution 2 mL ($CuSO_4 \cdot 5H_2O$ 0.04 g/L; $ZnSO_4 \cdot 7H_2O$ 0.12 g/L; H_3BO_3 1.40 g/L; $Na_2MoO_4 \cdot 2H_2O$ 1.0 g/L; $MnSO_4 \cdot H_2O$ 1.175 g/L); vitamin solution 1 mL (biotin 100 mg/L; pyridoxal-HCl 200 mg/L); pH to 6.5, without the addition of bromothymol blue). Each medium was incubated at room temperature for 2-4 x 24 hours. Meanwhile, the isolates that grew and differed were purified and stored in a slanted medium.

IAA Production Capability Test

The ability to produce IAA was tested using the Salkowski reagent (Agustiyani et al., 2021). About fifty milliliters of NB medium (for 1L, beef extract 0.3g, peptone 0.5g, sodium chloride 0.5g, and distilled water 1L) containing 0.1% DL-Tryptophan was inoculated with 500 mL of bacterial culture aged 24 hours with a population density above 10^7. Subsequently, the bacterial culture was shaken at 180 rpm for 48 hours in the dark and centrifuged at 10,000 rpm for

ten minutes. About one milliliter of supernatant was added to the test tube with 4 mL of Salkowski reagent and incubated for 30 minutes until a pink color appeared. The absorbance was measured using a spectrophotometer at 535 nm, and IAA production was calculated by a standard curve regression equation expressed in ppm.

N_2 Fixation Assay

N_2 fixing ability was tested on 9 isolates using the Kjeldahl method (Oedjijono et al., 2014). Each isolate was grown in a semisolid Nfb medium and incubated for 20 days at room temperature. The bacterial culture was put into a Kjeldahl flask containing a mixture of salts in a ratio of 40:2.5:1.5 of K_2SO_4, $CuSO_4$ and metallic selenium, and 3 mL of concentrated sulfuric acid was added. The flask was heated in a fume hood in the Digester at 420^0C for 20 minutes and was cooled by adding distilled water to reach a final volume of 50 mL. A total of 20 mL of the digestion sample was poured into a distillation tube and inserted into the apparatus. A 250 mL Erlenmeyer flask containing 20 mL of 4% boric acid and 6 drops of conway reagent (1000 mg methyl red, 150 mg bromcresol green, 200 mL 96% ethanol) was placed under the condenser of the distillation apparatus, and the outlet of the condenser was under the solution. Distillation was performed using a semi-automatic distillation unit. Meanwhile, 30 mL of 40% NaOH and 100 mL of distilled water were automatically poured through the apparatus in the distiller. The solution containing NH_3, boric acid and mixed indicator was titrated with 0.05 N HCl using an autotitrator. The following formula calculated the N2 value fixed by each isolate:

$$N2\ (ppm) = \frac{sample\ titer - blank\ titer\ x\ normality\ of HCl\ x\ 14\ x\ 1000000}{sample\ weight\ (g) x\ 1000}$$

Pathogenicity test

A pathogenicity test was performed on tobacco plants (Zuraidah, 2013), and bacterial isolates were propagated using NB media. They were incubated for 24 hours in a shaker at 120 rpm until the population reached above 10^7 cfu/mL. Furthermore, 1 mL of inoculum was injected using a sterile syringe without a needle on the back of a healthy tobacco leaf. Sterile distilled water was used as a negative control, and tobacco leaves were labelled according to the injected isolate. Plant response was observed within 24-48 hours to observe the level of necrosis on tobacco leaves.

Salinity Tolerance Test

A tolerance test for salinity was carried out using NB media with varying concentrations of NaCl (Shultana et al., 2020) at 3, 5, and 7%. Furthermore, 20 mL of bacterial culture was inoculated on NB media with NaCl concentrations of 3%, 5%, and 7% and incubated for 24 hours in a shaker at 120 rpm. The population was measured using a 10-fold dilution and total plate count (TPC) technique.

Molecular Indentification

Isolation of bacterial genomic DNA followed the protocol of the TIANamp Bacteria kit (Tiangen). The results were visualized with 1% agarose electrophoresis gel, followed by visualization under UV light. Meanwhile, absorbance measurements were performed at a wavelength of 260nm with a UV-Vis (Nano Drop) spectrophotometer to determine the concentration of genomic DNA.

DNA concentration and purity were determined according to the protocol (Green & Sambrook, 2012) using an Ultra Violet (UV) spectrophotometer at 260 nm. The amount of radiation absorbed is directly proportional to the concentration of DNA, i.e. A = 1 is proportional to 50 g of double-stranded DNA per mL. A comparison of absorbance at wavelengths of 260 and 280 nm indicates the purity of DNA. The 16S rDNA sequence amplification reaction was conducted using universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1429R (GGTTACCTTGTTACGACTT). The amplification reaction was carried out with the following reaction composition:

Table 1. The reaction composition used for the 16S rRNA. sequence amplification reaction

Composition	Volume	Keterangan
DNA template	>50 ng	The volume is adjusted according to the concentration of each genomic DNA isolate
Primer F (IDT)	1 μL	Concentration 10 μM
Primer R (IDT)	1 μL	Concentration 10 μM
MasterMix PCR (Thermo Scientific)	12,5 μL	Half of the total reaction
Nuclease-free water (Thermo Scientific)	Adjusted	Added up to a total volume of 25 μL
Total Reaction	25 μL	

The 16S sequence amplification reaction followed the pre-denaturation protocol at 95oC for 4 minutes, then by 35 repeated cycles consisting of denaturation at 95 ^{0}C for 40 seconds, annealing at 54 ^{0}C for 30 seconds, and extension (elongation) at 72 ^{0}C for 1 minute, followed by the final extension stage at 72 ^{0}C for 10 minutes. Furthermore, the amplification results were visualized by agarose gel electrophoresis with a concentration of 2% and observed with a UV transilluminator (UV light). The 16S bands are located at the base length of about 1500 bp, and the PCR products were sent to 1st Base (Singapore) for the rRNA sequencing determination. The sequence was determined using a two-way reaction with DNA primers forward 27F and reverse 1429R. Furthermore, 16S rRNA sequences from bidirectional sequencing reactions (with forward and reverse primers) were assembled using BioEdit 7.2 software (Hall, 1999). Bacterial species were identified based on nucleotide sequences using BLASTn (Basic Local Alignment Search Tool) analysis at *https://blast.ncbi.nlm.nih.gov/Blast.cgi*.

Phylogenetic Tree Construction

The obtained sequences were analyzed using Molecular Evolutionary Genetic Analysis-11 (MEGA-11) software downloaded at http://www.megasoftware.net (Felsenstein, 1985; Tamura et al., 2021). The assembly of 16S rRNA sequences was carried out using BioEdit software (Hall, 1999). Furthermore, the nucleotide sequence of each isolate was aligned (multiple sequence alignment was performed) with the MUSCLE program on MEGA 11 software. The phylogenetic tree construction was performed using the Maximum-Likelihood statistical method and was tested with 1000 times bootstrap replication. The nucleotide substitution model follows the Tamura & Nei (1993) model, and this analysis involved 9 isolated bacterial nucleotide sequences downloaded from the National Center for Biotechnology Information (NCBI) website, as well as 1 (one) outgroup sequence from the 18S rRNA genetic material of maize (*Zea mays*). There were 1441 positions for the 16S rRNA gene used as the final dataset.

RESULTS AND DISCUSSION

Nitrogen Fixation Capacity And IAA Production

The exploration of diazotrophic bacteria from saline fields on the north coast of Pemalang obtained 9 isolates with the highest nitrogen-fixing ability. The nitrogen-fixing ability ranged from 17.85 ppm to 29.05 ppm (Table 2), and Jn3 has the highest ability to fix nitrogen, reaching 29.05 ppm. The results showed that all isolates from saline rice fields could fix nitrogen, and the fixation ability varies greatly due to environmental conditions. Diazotrophic bacteria were isolated from the rhizosphere of rice plants, and the plant root environment was very dominant by diazotrophs. Purwanto et al. (2017) reported that the presence of nitrogen-fixing bacteria is more dominant in the plant rhizosphere of the total microflora and a flooded environment strongly supports the fixation activity.

Nitrogen-fixing bacteria play a vital role in maintaining nitrogen availability in the soil. The contribution of the bacteria to nitrogen availability varies greatly depending on several factors, including environmental variability, management, cropping patterns, and genotype differences (Aasfar et al., 2021). The variations reflect the diversity of microorganisms resistant or tolerant to saline environmental conditions. Saline conditions are negatively correlated with the abundance and diversity of diazotrophic bacteria (Barua et al., 2011).

Limited isolates of diazotrophic bacteria can produce phytohormones, especially indole acetic acid (IAA). Table 2 showed that the ability to produce IAA was only on isolates Jn3, J, J12, J5, Kn1 and A3 at 2.00, 2.69, 2.22, 1.76, 3.47, and 1.79 ppm, respectively. The highest IAA production capability by isolate Kn1 was 3.47 ppm (Table 2), and diazotrophic bacteria that can produce this compound are potential Plant Growth Promotion Rhizobacteria. Oedjiono et al. (2014) stated that the ability of rhizobacteria to produce phytohormones is one of the principal mechanisms as candidates for PGPR. Diazotrophic bacteria capable of producing IAA can convert L-tryptophan as the primary precursor in synthesizing the compound. Goswami et al. (2014) noted that IAA is a product of L-tryptophan in the metabolism of microorganisms, hence some PGPRs can produce the compound depending on the availability of L-tryptophan in the media. The level of L-tryptophan in the medium was 0.1%, but some isolates could not synthesize IAA, especially Jn1, Jn, and K3. The ability to produce IAA by microorganisms varies widely between species. Meanwhile, culture conditions and substrate strongly influence the inability to produce IAA (Yousef, 2018).

Table 2. Nitrogen fixation capacity and IAA production of diazotrophic bacteria from The Northern Coastal of Pemalang

Isolate	Fixation of N_2 (ppm)	IAA (ppm)

Jn3	29.05	2.00
Jn1	23.10	-
J	22.75	2.69
J12	20.30	2.22
J5	19.95	1.76
Kn1	18.90	3.47
A3	18.55	1.79
Jn	17.85	-
K3	17.85	-

Salinity Tolerance

The results showed that all diazotrophs could live in media with high salt concentrations of up to 7%. At this concentration, almost all isolates experienced a population decline, but Ju1, J12, Kn1, and A3 remain high reaching 1.13 x 10^8, 7.10 x 10^8, 6, 00 x 10^8, and 4.30 x 10^8 cfu/mL (Figure 1). The lowest population was at 7% salt concentration, namely isolate Jn1 at 3.80 x 10^5 cfu/mL.

The high salt content decreased the diazotrophic bacteria population in all isolates. Salinity is one of the stress factors that affect the decrease in bacterial diversity and controls the abundance, composition and function due to dissolved Na^+ ions greater than 0.15 M. In addition, it causes hyperosmotic conditions and diffusion of water out of the microbial cells. (Moradi et al., 2011). Microorganisms adapt to saline conditions by maintaining internal salt levels at the same environmental conditions, or microorganisms actively secrete NaCl and produce compatible organic osmoregulators such as betaine (Moradi et al., 2011; Otlweska et al., 2020). Based on the experimental results, 9 bacterial isolates can live in high salt concentrations and be categorized as halotolerant. According to (Moradi et al., 2011), microorganisms, based on their optimal growth, can be categorized into non, slight, moderate, and extreme halopiles, which grow best on media with a salt content below 0.2 M (1%), 0.2 – 0.5 M (1-3%), 0.5 – 2.5 M (3-15%), and 2.5-5.2 M (15-32%), respectively.

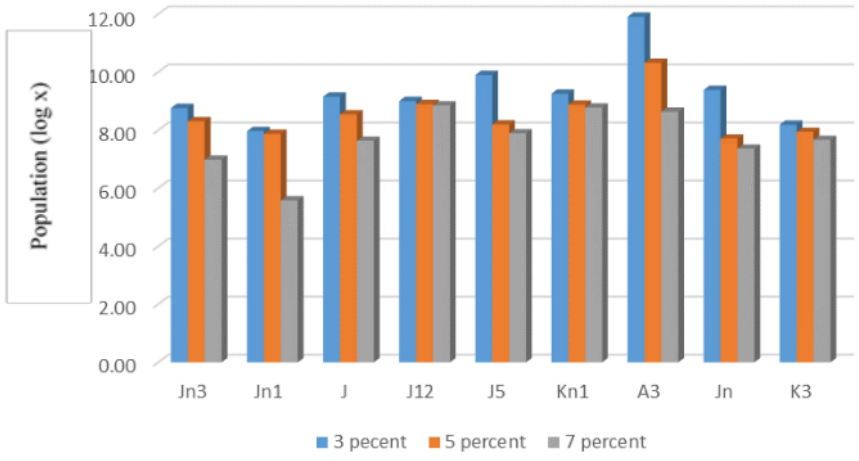

Figure 1. Population of Diazotrophic Bacteria in Different Salt Concentration (data was transformed in Log (x))

Pathogenicity test

Diazotrophic bacteria pathogenicity test was conducted on tobacco plant leaves and observed 48 hours after injection. The experimental results reported that all isolates did not show necrosis symptoms (Figure 2)). Therefore,

the isolates of diazotrophic bacteria are not pathogenic to tobacco plants and do not cause collapse or tissue death symptoms.

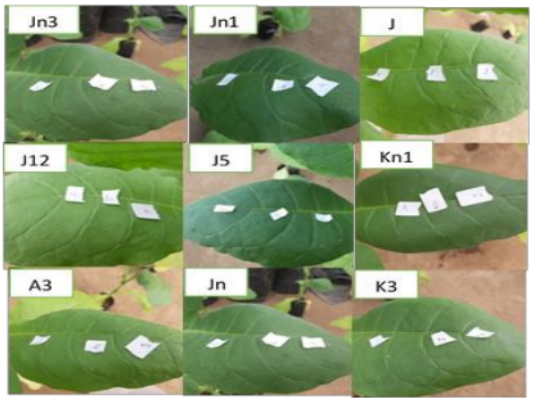

Figure 2. Symptoms of Diazotrophic Bacteria injection on tobacco leaves

Analysis of 16S rRNA

Visualization of the 16S rRNA sequence from 9 (nine) bacteria isolates can be seen in Figure 3 with PCR reaction products along ±1500bp.

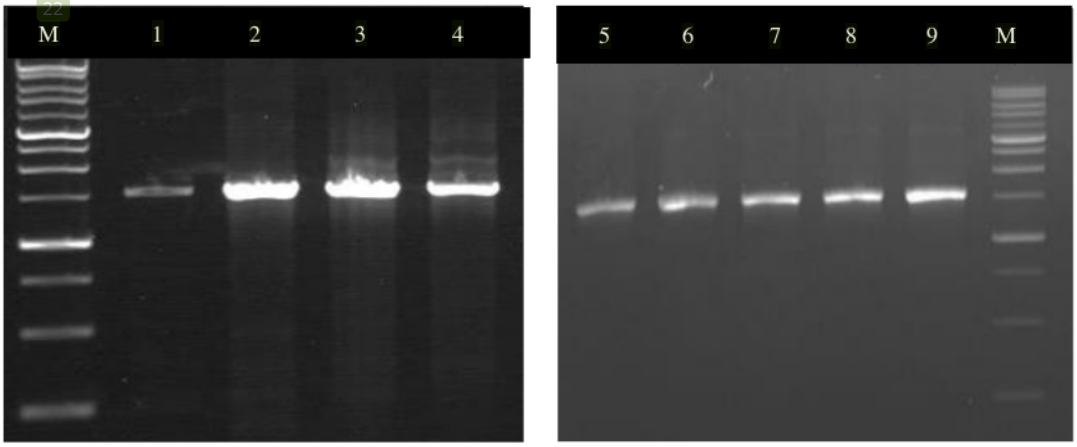

Figure 3. Visualization of the 16S rRNA sequence (1 = J5; 2 = J12; 3 = Jn3; 4 = J; 5 = K3; 6 = Kn1; 7 = A3; 8 = Jn; 9 = Jn1; M = 1Kbp DNA Ladder)

The results of species identification by BLASTn analysis are shown in Table 2.

Table 2. Identification of isolated bacterial species based on 16S rRNA sequences

No. Sumuran	Code of isolates	Description		Scientific Name	Percentage Identification	Accession
1	J5	*Acinetobacter* strain	*baumannii* MS14413	*Acinetobacter baumannii*	99.57%	CP054302.1

		chromosome, complete genome			
2	J12	*Bacillus pumilus* strain MGB1027 16S ribosomal RNA gene, partial sequence	*Bacillus pumilus*	98.69%	MH260924.1
3	Jn3	*Acinetobacter junii* strain WR4 16S ribosomal RNA gene, partial sequence	*Acinetobacter junii*	98.67%	KT886456.1
4	J	*Acinetobacter schindleri* strain HZE30-1 chromosome, complete genome	*Acinetobacter schindleri*	98.57%	CP044483.1
5	K3	*Bacillus subtilis* strain WR10-qm 16S ribosomal RNA gene, partial sequence	*Bacillus subtilis*	100%	OM320447.1
6	Kn1	*Pseudomonas stutzeri* strain SGAir0442 chromosome, complete genome	*Pseudomonas stutzeri*	100%	CP025149.2
7	A3	*Bacillus altitudinis* strain GUCC 1037 16S ribosomal RNA gene, partial sequence	*Bacillus altitudinis*	100%	OM319557.1
8	Jn	*Bacillus cereus* group sp. strain HD1.3 16S ribosomal RNA gene, partial sequence	*Bacillus cereus*	100%	OM368635.1
9	Jn1	*Bacillus tropicus* strain CU96 16S ribosomal RNA gene, partial sequence	*Bacillus tropicus*	100%	OM108144.1

The phylogenetic tree was generated from the analysis using alignment (MUSCLE) and Maximum Likelihood statistical method (Figure 4).

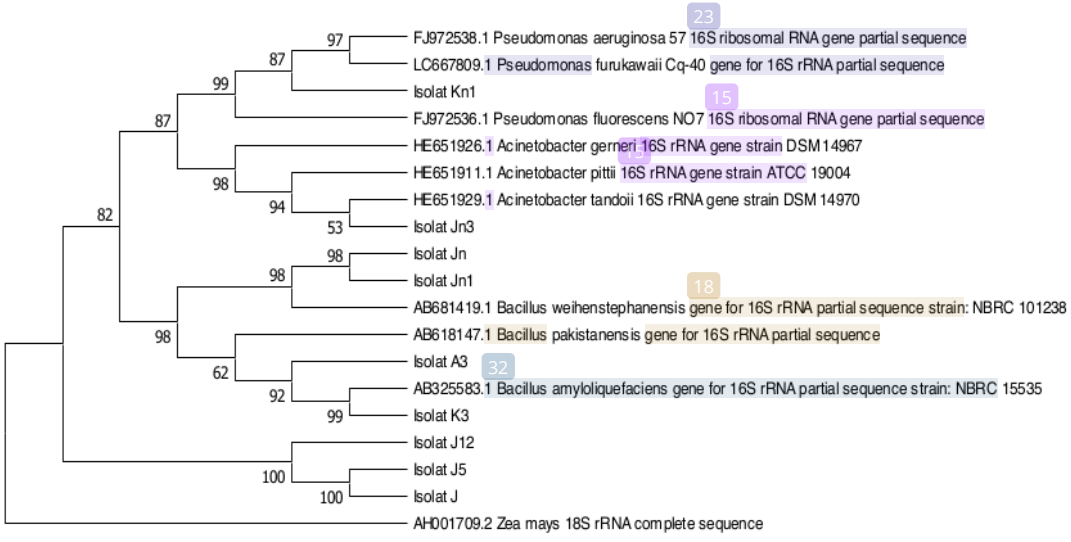

Figure 4. Original tree generated from MEGA11 software

The bacteria identification analysis of 16S rRNA sequences showed that 5 isolates were categorized into the genus *Bacillus*, 3 from the genus *Acinetobacter*, and 1 from *Pseudomonas*. Research on the isolation and identification of nitrogen-fixing bacteria from the rhizosphere of plant roots has been carried out in the root rhizosphere of rice and mangrove plants on saline land in coastal areas.

The isolated bacterial species clustering based on the phylogenetic tree consisted of 3 clusters. The first cluster consisted of Kn1 and Jn3 isolates, identified as *Pseudomonas stutzeri* and *Acinetobacter junii*. The second was Jn, Jn1, A3, and K3 isolates, identified as *Bacillus cereus, Bacillus tropicus, Bacillus altitudinis,* and *Bacillus subtilis*, respectively. Meanwhile, the third cluster, separated from the reference nucleotide sequences, isolates J12, J5, and J, identified as *Bacillus pumilus, Acinetobacter baumanii*, and *Acinetobacter schindleri*, respectively.

The bacterium *Bacillus pumilus* dominated coastal marine habitats and had enzyme activity of amylase, protease, lipase, oxidase, and catalase, showing a positive response to the Voges-Proskauer test (Parvathi et al., 2009). In addition, Faizah et al. (2017) reported the ability to inhibit the growth of the plant pathogen *Xanthomonas campestris* by producing an inhibition zone of 1.84 mm in diameter. There was no report of each species isolated among the various Bacillus species.

The results indicate that in cluster 1, isolate Kn1 was identified as *Pseudomonas stutzeri* isolated from various ecosystems. This is reinforced by the statement of Lalucat et al. (2006) the bacterium is gram-negative with a wide ecosystem. Yan et al. (2008) reported that the *Pseudomonas stutzeri* colonizes the roots of rice and wheat plants. Based on genomic analysis, it has several genes regulating osmotolerance. Furthermore, Li et al. (2017) reported that the bacterial species were isolated from the rhizosphere of sugarcane plants.

The first cluster also identified isolate Jn3 as *Acinetobacter junii* bacteria. In the third cluster, isolates J5 and J were identified as *Acinetobacter baumanii* and *Acinetobacter schindleri*. The results of previous studies reported by Sachdev et al. (2010) have successfully isolated the bacterium *Acetinobacter* sp from wheat roots. The most dominant strains were *Ac. calcoaceticus, Ac. baumannii, Ac. lwoffii*, *Ac. Baylyi* and *Ac calcoaceticus*. Rokhbakhsh-Zamin et al. (2011) successfully conducted an isolation from the rhizosphere of *Pennisetum glaucum*. The *genus Acetinobacter* sp. as PGPR has been reported by Padmavathi et al. (2016), where strains of *Acinetobacter junii* have been identified from tomato plants and dual inoculation with mycorrhizae.

Bacillus sp. was reported in this study based on the phylogenetic tree. The second cluster consists of isolates J12, K3, A3, Jn, and Jn1, identified as *Bacillus pumillus* strain MGB1027, *Bacillus subtilis* strain WR10-qm, *Bacillus altitudinis* strain GUCC1037, *Bacillus cereus* strain HD1. 3, and *Bacillus tropicus* strain CU96. *Bacillus* sp is adaptive to marginal environmental conditions and is dominant in various environments, up to 95% of the population of gram-negative bacteria (Miljakovic et al., 2020). According to Kumar et al. (2014), it has a higher tolerance to abiotic stress than Pseudomonas sp. This is due to the ability to form endospore, a dormant resistant form that can survive in unfavorable environmental conditions. Ibarra-Villarreal et al. (2021) explained that *Bacillus* sp can survive in abiotic stress through structural adaptation and has metabolite characteristics such as the production of multi-layered cell wall structures, antibiotic secretion, signal peptide molecules, extracellular enzymes, and formation of cell walls. Meanwhile, several strains of *Bacillus* sp that can fix nitrogen have been previously reported. Antunes et al. (2017) successfully isolated bacteria from the genus *Bacillus* sp originating from the rhizosphere of sugarcane plants with direct and positive influence on plants through their ability to fix nitrogen, solubilize P, and produce various types of hormones. Kumar et al. (2021) successfully isolated strains of *Bacillus pumilus* from saline soil with attributes such as PGPR to produce IAA, PSB, ACC deaminase, ammonia, HCN, siderophore, and EPS production. Ibarra-Villarreal et al. (2021) obtained 10 saline-tolerant *Bacillus* sp strains from the rhizosphere of wheat plants, namely *B. subtilis, B. megaterium, B. cereus, B. amyloliquefaciens, B. pumilus, B. licheniformis, B. endophyticus, B. atrophaeus, B. safeensis, and B. mojavensis*. The highest abundance was dominated by *B. subtilis* and *B. megaterium*

In conclusion, nine isolates of nitrogen-fixing bacteria have been isolated, which can fix N_2. The entire isolates were able to fix N_2, but few could produce IAA. The nitrogen-fixing ability of diazotrophic bacteria ranged from 17.85 ppm to 29.05 ppm. Furthermore, isolate Jn3 has the highest ability to fix nitrogen, reaching 29.05 ppm. The ability to produce IAA was only on Jn3, J, J12, J5, Kn1, and A3, with values of 2.00, 2.69, 2.22, 1.76, 3.47, and 1.79 ppm, respectively. Based on the 16S rRNA analysis and phylogeny construction, the isolated bacteria were identified in 3 clusters. The first cluster was identified as *Pseudomonas stutzeri* and *Acinetobacter junii* (Kn1 & Jn3), while the second was identified as *Bacillus cereus, Bacillus tropicus, Bacillus altitudinis,* and *Bacillus* subtilis (Jn, Jn1, A3, and K3 isolates). Furthermore, the third cluster was identified as *Bacillus pumilus, Acinetobacter baumanii*, and *Acinetobacter schindleri* (J12, J5, and J).

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