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Screening and identification of Free Nitrogen-Fixing Bacteria from Rhizosphere of Mangrove Jenu, Tuban

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ABSTRACT
This study aimed to examine the nitrogen-fixing potential of bacterial isolates from Mangrove Center Jenu, Tuban soil. Nitrogen-fixing bacterial isolates were filtered qualitatively using a selective nitrogen-free bromothymol blue (NFB) medium. Screening results showed that six bacterial isolates from Jenu Tuban mangrove soil had potential as nitrogen-fixing bacteria, namely isolates 1, 8, 10, 15, and 16. The six nitrogen-fixing bacterial isolates had varied macroscopic characteristics and were gram-positive. Based on the identification of the 16S rRNA, it was found that the six isolates were detected as a *Bacillus* group: *Bacillus cereus* with 99% query cover and 99.58% identity; *B. toyonensis* with identical coverage of 99% and 99.79% identity; *B. altitudinis* with 99% query cover and 99.72% identity; *B. subtilis* with 99% query cover and 99.66% identity; *B. megaterium* with 99% query cover and 99.79% identity; and *B. aerius* with 98% query cover and 99.86% identity.

1. Introduction

Currently, agriculture in Indonesia still uses inorganic fertilizers to meet market demand related to increasing agricultural products. This can lead to farmers' dependence on the excessive use of inorganic fertilizers, resulting in several adverse effects on soil conditions, including the soil hardening quickly so that the soil's ability to store water decreases, the ground becomes acidic, which can reduce crop productivity, drop the organic matter, and result in land degradation both physically, chemically and biologically [1, 2]. Thus, a more environmentally friendly alternative is needed using biological fertilizers.

Biofertilizer is a biological fertilizer made from active living organisms that can help increase soil nutrient availability for plants. It has the advantage of being cost-effective and environmentally friendly, and the microbes contained in biological fertilizers have an essential role in soil fertility, especially in the root system area and nutrients that plants need [3]. Microorganisms contained in biofertilizers are generally groups of microorganisms that fix N, phosphate solvents, break down organic matter, and produce growth hormones such as gibberellins, cytokinins, and IAA (Indole Acetic Acid) [2]. These microorganisms can be isolated from rhizosphere soil, one of which is mangrove forest rhizosphere soil.

Nitrogen is the most important element for plant growth and development. Nitrogen can be regarded as one of the nutrients needed by plants that need the help of microbial activity to anchor and change the form of nitrogen so that it can be absorbed and used directly in plant growth [4]. Commonly in soil or aquatic systems, chemical nitrogen fixation occurs when

atmospheric nitrogen (N_2) is converted into ammonia (NH_3) or other compounds [5]. Mangrove forests function as a barrier between land and sea, which causes environmental factors such as salinity and nutrient availability to be varied to provide unique and specific characteristics. Therefore, various groups of bacteria, such as nitrogen fixers, phosphate solvents, cellulose decomposers, nitrifiers, denitrifiers, sulfur oxidizers, iron oxidizers, and iron reducers, are found in mangrove environments [6].

Tuban has an area of 119.98 hectares of mangrove forest, located in five sub-districts, one of which is the Jenu sub-district, namely the Mangrove Center Tuban (MCT). Pradipta (2016), in his research, stated that the Jenu Tuban mangrove forest area has a fairly high nitrogen nutrient content. The high levels of nitrogen-fixing are associated with the decomposition of mangrove leaves and mangrove rhizosphere soils [8]. Some bacterial genera that have been found in mangrove rhizosphere soil are *Aeromonas, Arthrobacter, Azospirillum, Azotobacter, Clostridium, Corynebacterium, Klebsiella, Oceanimonas, Paracoccus, Phyllobacterium, Pseudomonas, Rhizobium,* and *Vibrio* [9] as well as other nitrogen-fixing bacteria found by Liu et al. (2012) including *Brevundimonas, Stenotrophomonas, Paenibacillus,* and *Bacillus.*

Several studies on MCT about microorganisms in the rhizosphere soil have been conducted, including the isolation and potential testing of cellulolytic and phosphate solvent yeasts [10], organic matter decomposer bacteria [11], actinomycetes [12, 13], and mold potential test [14]. Based on these studies, it can be said that MCT rhizosphere soil contains a lot of microorganisms and can be considered fertile soil. Hence, the area supports the presence of nitrogen-fixing bacteria that can be candidates for the formulation of biofertilizer formulas and the creation of the University of Airlangga Culture Collection (UACC). Therefore, in this study, screening and identification of nitrogen-fixing bacteria isolates from MCT rhizosphere soils were carried out.

2. Materials and methods

This research was conducted at the Microbiology Laboratory, Department of Biology, Faculty of Science and Technology, and the PUI-PT ITD Laboratory, Airlangga University, Surabaya, in April–August 2020.

2.1.Screening Nitrogen-fixing Bacteria Isolates

Twenty pure bacterial isolates from MCT [15] were rejuvenated in NA media and then inoculated into semisolid (1,75% bacterial agar powder) NFB media and incubated for ten days. A positive result was indicated by a change in the color of the NFB medium from a greenish yellow to a blue color and a pellicle that formed on the surface of the medium. The isolates were then observed microscopically and macroscopically.

2.2.DNA Extraction of Nitrogen-fixing Bacteria

Nitrogen-fixing bacterial isolates are grown in nutrient broth (NB) media and incubated for 24 hours at room temperature. DNA isolation was carried out using the Wizard Genomic DNA Purification Kit Promega [16].

2.3.DNA Quantitative and Qualitative Test

The bacterial DNA samples were measured quantitatively using a spectrophotometer and qualitatively by electrophoresis of a 1% agarose gel containing Ethidium Bromide. The agarose gel was put into the electrophoresis chamber, and 1 x TAE buffer was added until the gel was submerged. DNA samples mixed with loading dye were then added and fed into each well. Running is done at 100 volts for 20 minutes. Visualization of electrophoresis results observed under a UV transilluminator [17].

2.4.PCR

The 16S rRNA gene of the nitrogen-fixing bacteria isolates was amplified by PCR using universal primers 27F (5' – AGA GTT TGA TC TGG CTC AG – 3') and 1492R (5' – CGG TTA CCT TGT TAC GAC TT – 3') [18]. Amplification was carried out for 30 cycles. First, initial denaturation was carried out at 94 C for 2 minutes. Then, the denaturation of the DNA template was carried out at 94 C for 30 seconds, continued annealing at 55 C for 30 seconds, followed by extension at 72 C for 30 seconds, and final extension at 72 C for 7 minutes. The PCR product was electrophoresed on a 1% agarose gel in 1x TAE buffer and then sequenced at 1st Base Company in Singapore.

2.5.DNA Sequencing and Phylogenetic Analysis

Analysis was carried out using the Bioedit program and then identified by aligning the nucleotide sequences of the 16S rRNA gene of the studied isolates with the 16S rRNA genes of other bacteria found in the GenBank database. to determine the level of similarity of the nucleotide base sequences of samples with several genera using the BLAST N program through the NCBI website [19]. If the bacterial isolates produced have >97%–99% sequence similarity with homologous bacterial isolates, they can represent the same species. The phylogenetic tree was constructed using MEGA XI.

3. Results and discussion

3.1.Screening of Nitrogen-Fixing Bacteria Isolates

The results showed that six bacterial isolates (1, 8, 10, 15, 16, and 18) were able to fix nitrogen (Figure 1), with a change in color on the NFB medium from yellow to yellowish green to blue (Figure 2). in contrast to another 14 isolates that did not show any color change in NFB media so it was assumed that these isolates did not have the same potential as nitrogen fixers. Morphological observations of the six isolates of nitrogen-fixing bacteria were carried out macroscopically and microscopically (Figure 3 and 4). The morphological characteristics of nitrogen-fixing bacteria are represented in Table 1.

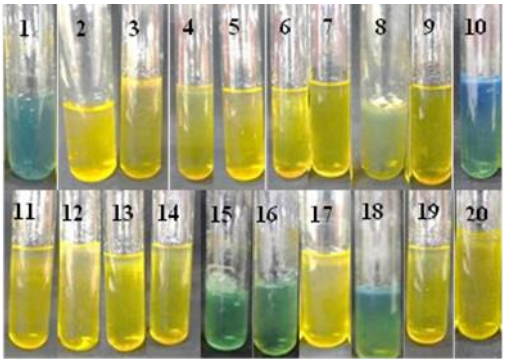

Figure 1. Screening results of nitrogen-fixing bacterial isolates

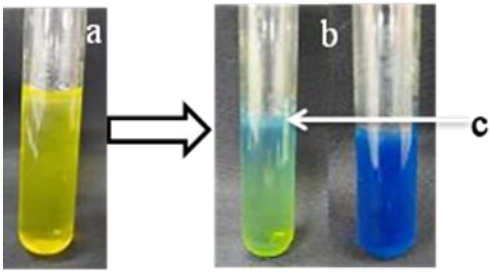

Figure 2. Positive reaction of nitrogen-fixing bacteria on semisolid NFB media a) first incubation of NFB media (yellow), b) Change in color of NFB media due to the activity of nitrogen-fixing bacteria, yellow becomes blue-green, c) White pellicle formed on the surface of the media.

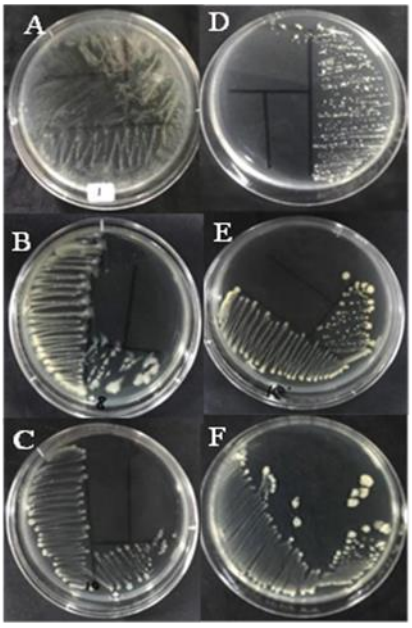

Figure 3. Colony morphology of nitrogen-fixing bacteria from the rhizosphere of mangrove Jenu Tuban. A:1, B:8, C:10, D:15, E:16, F:18.

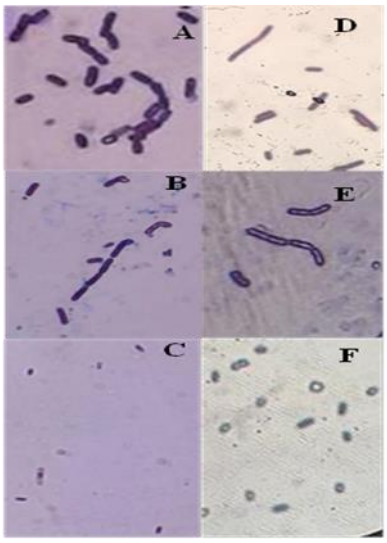

Figure 4. Morphology of nitrogen-fixing bacteria cells from the rhizosphere of mangrove Jenu Tuban. A:1, B:8, C:10, D:15, E:16, F:18.

Table 1. Morphology Characteristics of nitrogen-fixing bacteria from the rhizosphere of mangrove Jenu Tuban.

Isolate Code	Macroscopic Characteristics				
	Gram Stain	Color	Shape	Margin	Elevation
1	Bacilli +	White	Filamentous	Filamentous	Flat
8	Bacilli +	White	Irregular	Serrate	Flat
10	Bacilli +	White	Circular	Lobate	Flat raised
15	Bacilli +	White	Rhizoid	Serrate	Umbonate
16	Bacilli +	Cream	Circular	Entire	Flat
18	Bacilli +	White	Circular	Entire	Raised

Based on the results of macroscopic observations, each isolate had different colony characters, whereas in microscopic observations, all isolates had bacilli and gram-positive cells.

3.2.DNA Extraction of Nitrogen-fixing Bacteria

The results of genomic DNA analysis by 1% agarose gel electrophoresis (Figure 5) in the form of the appearance of bands indicate that all DNA samples of nitrogen-fixing bacterial isolates have been successfully isolated. DNA quantitative testing using a nanodrop spectrophotometer at a wavelength of 260 nm and 280 nm so that the level of purity and concentration of DNA can be determined (Table 2).

Table 2. Test results for the DNA quantity of nitrogen-fixing bacteria from the rhizosphere of mangrove Jenu Tuban

Isolate	**Abs 260 nm**	**Abs 280 nm**	**DNA Purity (260/280nm)**	**DNA Concentration (ng/ µl)**
1	6.51	3.5	1.85	325.41
8	29.6	14.1	2.4	1479.3
10	1.23	0.85	1.51	64.09
15	8.01	5.17	1.57	400.66
16	0.45	0.27	1.74	22.76
18	29.3	16.1	1.82	1463.9

In terms of quantity shown in Table 2, the level of DNA purity of nitrogen-fixing bacterial isolates ranges from 1.51 to 2.04 with relatively varied concentrations. A DNA purity level of less than 1.8 indicates that the DNA is still contaminated by phenol and residual isolation solvents; if it is more than 2.0, then the DNA is contaminated with protein.

3.3.Molecular identification of nitrogen-fixing bacteria with the 16S rRNA

PCR results by electrophoresis (Figure 6) showed that the DNA coding for 16S rRNA was successfully amplified with a PCR product of around 1500 bp. The results of six isolates of 16S rRNA gene sequencing were analyzed by BioEdit and matched to the Gene Bank database via the BLASTn program at the NCBI site (Table 3). The results of constructing the phylogenetic tree of free nitrogen-fixing bacteria and *Bacillus* sp. on GenBank are shown in Figure 7.

3.4.Discussion

The rhizosphere soil contains many microorganisms that play an important role in soil fertility and plant growth and play a role in the function and maintenance of ecosystems, especially in terms of the nitrogen cycle [20]. In the rhizosphere, nitrogen-fixing activity is carried out by bacteria to meet their needs in the formation of bacterial nucleic acids. When the rhizosphere bacteria's need for nitrogen is met, the bacteria will release excess nitrogen into the soil so plants can use it to meet their nitrogen needs through absorption by roots [21]. Nitrogen-fixing bacteria play an important role in terms of nutrition in plants, where nitrogen

is an essential element to support plant growth [22]. The presence of nitrogen-fixing bacteria in the soil is more common in the rhizosphere than in the non-rhizosphere; this is due to the availability of carbon compounds needed by bacteria in the rhizosphere environment in sufficient quantities to maximize bacterial activity in carrying out N-fixing [23, 24].

Screening of nitrogen-fixing bacteria was carried out on 20 bacterial isolates that had been successfully isolated by previous researchers from the soil at the Jenu Tuban Mangrove Center. Qualitative screening results using semi-solid NfB media (Figure 1) showed that there were six bacterial isolates (1, 8, 10, 15, 16, and 18) that were capable of fixing nitrogen; this was shown by a positive reaction in the form of a color change on the NfB medium from yellow to yellowish green to blue (Figure 2). Based on the macroscopic observations (figure 3), each isolate had different colony characters, while the microscopic observation results (figure 4) showed that all isolates have gram-positive bacilli.

NFB media is a selective medium that does not contain nitrogen in the material composition, so bacterial isolates that can grow on it have the ability to fix free nitrogen. The color change that occurs in NFB media to green or blue is caused by oxidation in a medium containing malate, which is called an alkalinization process [25]. This color change also occurs due to nitrogenase activity, which causes the pH to be higher and the media to turn blue due to the bromthymol blue indicator in the composition of the NFB media [26]. Nitrogenase activity can also be indicated by the formation of a white pellicle near the surface of the medium, which will then move to the surface of the medium when the amount of nitrogen in the medium has accumulated [19]. This is because the bacteria in NFB media have aerotactic abilities, making them motile and able to make movements to seek a balance of oxygen diffusion. Excess oxygen in the media can inhibit nitrogenase activity [27].

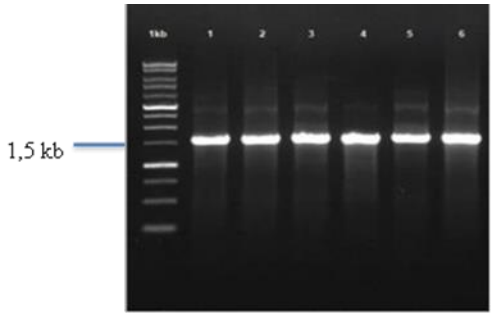

Figure 6. Electrophoresis results of DNA PCR products of nitrogen-fixing bacterial isolates with universal primers. 1) Isolate 1, 2) Isolate 8, 3) Isolate 10, 4) Isolate 15, 5) Isolate 16, 6) Isolate 18.

The genomic DNA was measured for concentration and purity (Table 2). These aims are to make sure the DNA to be amplified by the PCR method has a concentration of appropriate purity [28]. The purity of the DNA was influenced by technical factors during the DNA extraction, which occurred when the supernatant containing DNA was transferred after incubation. This was done carefully so that the destroyed tissues under the tube were not removed. Likewise, during the drying process from ethanol, if it is not completely dry, it can be one of the causes of contaminants and reduce the purity value of DNA when measured using a spectrophotometer [29]. PCR results (Figure 4.6) showed that the DNA coding for 16S rRNA was successfully amplified with PCR products ranging from 1500 bp. Insulation 1, 8, 10, 15, 16, and 18 each have sequences with lengths of 1452 bp, 1455 bp, 1450 bp, 1454 bp, 1455 bp, and 1453 bp. The basic principle of the PCR method is the repeated multiplication of DNA fragments using polymerase enzymes at high temperatures.

Table 3. Identification results of 16SrRNA gene of nitrogen-fixing bacteria isolates with BLASTn

Isolates code	Length (bp)	BLASTn Results			
		Species	Query cover *(%)*	Identity (%)	Access Code
1	1452	*Bacillus wiedmanni* FSL W8-0169	99	99.58	NR_152692.1
		Bacillus proteolyticus MCCC 1A00365	99	99.58	NR_157735.1
		Bacillus albus MCCC 1A02146	99	99.58	NR_157729.1
8	1455	*Bacillus toyonensis* BCT-7112	99	99.79	NR_121761.1
10	1450	*Bacillus altitudinis* 41KF2b	99	99.72	NR_042337.1
15	1454	*Bacillus subtilis* JCM 1465	99	99.66	NR_113265.1
16	1455	*Priestia megaterium* (*Bacillus megaterium)* ATCC 14581	99	99.79	NR_112636.1
18	1453	*Bacillus aerius* 24K	98	99.86	NR_118439.1

The results of identification based on BLAST in Table 3 provide information in the form of query cover and identity, where query cover is the percentage of base length that is aligned with the BLAST database, and identity is the percentage of identity or similarity between the sequences analyzed and the aligned database sequences. Isolate 1 has similarities with three sequences of bacterial species, namely *Bacillus wiedmannii, Bacillus proteolyticus, and Bacillus albus,* which have the same percentage of query coverage and identity at 99% and 99.58%, respectively. *B. wiedmannii, B. proteolyticus, and B. albus* are bacterial species belonging to the *B. cereus* group. Group *B. cereus species* have facultative anaerobic properties and have potential as probiotics for plant growth (biofertilizers) [30-32]. Some studies have proven that *B. cereus* isolated from the rhizosphere can act as a PGPR (Plant Growth Promoting Rhizobacteria), which helps the growth of legumes [33]. In isolate 8, *Bacillus toyonensis* was identified with 99% query cover and 99.79% identity. This is consistent with research that states that *B. toyonensis* isolated from the rhizosphere of ferns, besides being able to produce ammonia, also acts as a producer of IAA hormones and phosphate solvents, which are important biochemical aspects for plant growth [34].

Isolates 10 and 15 were identified as *Bacillus altitudinis* and *Bacillus subtilis*, with the same percentage of query cover (99%) and identities (99.72 and 99.66%, respectively). *Bacillus altitudinis* and *Bacillus subtilis,* isolated from mangrove rhizosphere, are bacterial strains that are capable of carrying out nitrogen fixation, where the source of nitrogen comes from the decomposition of mangrove forest leaves. Twenty-two isolates of nitrogen-fixing and phosphate-solubilizing bacteria were isolated from the mangrove rhizosphere in the Mekong River, Thailand, where 70% of these isolates belonged to the genus *Bacillus* [35]. *B. subtilis* has a nitrogenase complex, so it can fix nitrogen from the air [19]. *B. altitudinis,* isolated from the soil around mangrove plants on the Egyptian Red Sea Coast, has the ability to produce exopolysaccharides, which aim to support the existence of the bacteria itself in extreme habitats [36].

Isolates 16 and 18 were identified as *Priestia megaterium* (98% query cover and 99.86% identity) and *Bacillus aerius* (98% query cover and 99.86% identity), respectively. Based on research conducted by Aslam et al. (2016), *Priestia megaterium* (*B. megaterium*) isolated from soil polluted by chromium waste can fix nitrogen and detoxify chromium-contaminated soil, so *B. megaterium* has the potential as a biofertilizer and bioremediator. *B. aerius* also has the

potential to fix nitrogen in the air, as reported by Yousuf et al. (2020), who succeeded in obtaining eight isolates of nitrogen-fixing gram-positive bacteria from the coast of the Arabian Sea, one of which was identified as *B. aerius.*

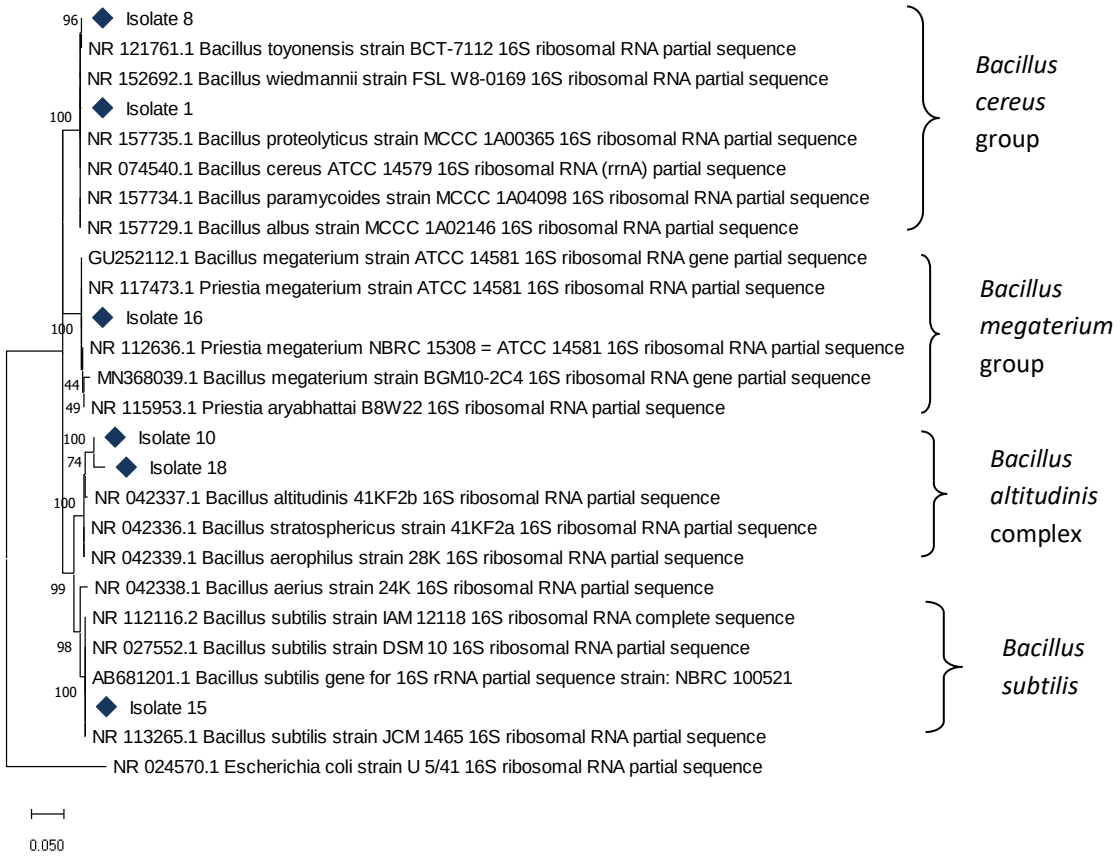

Figure 7. Phylogenetic tree based on 16s rRNA gene sequences showing the position of six free nitrogen-fixing bacteria within genus *Bacillus* using Neighbor-Joining method and model of Maximum-likelihood.

Based on the phylogeny tree in Figure 7, it is known that isolates 1 and 8 are in the same clade where isolate 8 identified as *Bacillus toyonensis* is part of the *Bacillus cereus* group [38]. Isolates 15 and 16 are closely related to the *Bacillus subtilis* group and the *Priestia megaterium* group (*Bacillus megaterium)*, respectively. The kinship of isolate 18 is close to isolate 10, which was identified as *Bacillus altitudinis* 41KF2b, in contrast to the identification results based on the NCBI GenBank as *Bacillus aerius* 24K. Strains 41KF2b and 24K were part of 4 novel bacteria isolated from cryogenic tubes and identified as the genus *Bacillus*. Phylogeny analysis based on the 16S rRNA gene showed that strains 24K and 41KF2b have a close relationship where strain 24K is closer to *Bacillus licheniformis* while strain 41KF2b is closer to *Bacillus pumilus* [39]. However, Liu et al. (2015) reported that the 16S rRNA gene sequence of *B. aerius* is identical to *B. altitudinis*. Strain 24K also has similarities in housekeeping gene sequences, namely the *gyrB, rpoB, and pycA* gene sequences are 100% identical to *B. altitudinis* 41KF2b. Therefore, strain 24K should be affiliated with *B. altitudinis* 41KF2b.

4. Conclusions

Of the 20 bacterial isolates isolated from the soil of the Jenu Tuban Mangrove Center, six of them (1,8,10,15,16 dan 18) had potential as nitrogen fixers. The sixth nitrogen-fixing bacterial species from Jenu Tuban mangrove soil had varied macroscopic characteristics and Gram-positive bacilli. The results of the identification of nitrogen-fixing bacterial isolates based on the molecular

markers of the 16S rRNA gene were that isolate 1 was identified as *B. cereus* group with 99% query cover and 99.58% identical, isolate 8 identified as *B. toyonensis* with 99% query cover and 99.79% identity, isolate 10 as *B. altitudinis* with query cover 99% and 99.72% identity, isolate 15 as *B. subtilis* with query cover 99% and identical 99.66%, isolate 16 as *B. megaterium* with query cover 99% and 99.79% identity, and isolate 18 as *B. aerius* with 98% query cover and 99.86% identity.

Acknowledgments
Not Available

References
[1] S. Parman, "Pengaruh pemberian pupuk organik cair terhadap pertumbuhan dan produksi kentang (Solanum tuberosum L.)," *J Anatomi Fisiologi,* vol. 15, no. 2, pp. 21-31, 2007.
[2] A. Kartikawati, O. Trisilawati, and I. J. J. P. Darwati, "Pemanfaatan pupuk hayati (biofertilizer) pada tanaman rempah dan obat," vol. 16, no. 1, pp. 33-43, 2017.
[3] Chandini, R. Kumar, R. Kumar, and O. Prakash, "The Impact of Chemical Fertilizers on our Environment and Ecosystem," 2019, pp. 69-86.
[4] K. METASARI, "Eksplorasi bakteri penambat nitrogen non simbiosis dari tanah kawasan mangrove Wonorejo Surabaya," UNIVERSITAS AIRLANGGA, 2011.
[5] I. Lawal, "Nitrogen fixing bacteria and their application for heavy metal removal: a mini review," *J Journal of Biochemistry, Microbiology Biotechnology,* vol. 9, no. 2, pp. 43-47, 2021.
[6] B. C. Behera, S. K. Singdevsachan, R. R. Mishra, B. K. Sethi, S. K. Dutta, and H. N. J. W. j. o. a. r. Thatoi, "Phosphate solubilising bacteria from mangrove soils of Mahanadi river delta, Odisha, India," vol. 4, no. 1, pp. 18-23, 2016.
[7] N. PRADIPTA, "Studi Kandungan Nitrogen (N) dan Fosfor (P) Pada Sedimen Mangrove di Wilayah Ekowisata Wonorejo Surabaya dan Pesisir Jenu Kabupaten Tuban," Universitas Airlangga, 2016.
[8] J. Liu, M. Peng, and Y. J. C. j. o. M. Li, "Phylogenetic diversity of nitrogen-fixing bacteria and the nifH gene from mangrove rhizosphere soil," vol. 58, no. 4, pp. 531-539, 2012.
[9] A. L. Flores-Mireles, S. C. Winans, G. J. A. Holguin, and e. microbiology, "Molecular characterization of diazotrophic and denitrifying bacteria associated with mangrove roots," vol. 73, no. 22, pp. 7308-7321, 2007.
[10] P. A. DEWI, "ISOLASI DAN UJI POTENSI YEAST SELULOLITIK DAN PELARUT FOSFAT DARI TANAH MANGROVE CENTER JENU TUBAN," Universitas Airlangga, 2019.
[11] Z. Jannah, "ISOLASI, KARAKTERISASI DAN UJI POTENSI BAKTERI DEKOMPOSER BAHAN ORGANIK DARI TANAH MANGROVE JENU TUBAN," Universitas Airlangga, 2019.
[12] F. Suroiyah, "Isolasi dan Uji Aktivitas Antimikroba Actinomycetes dari Tanah Mangrove Jenu Tuban," Department of Biology, Faculty of Science and Technology, Airlangga University, 2019.
[13] N. SOLIKHA, "ISOLASI DAN UJI POTENSI ACTINOMYCETES PENGHASIL ENZIM SELULASE DARI TANAH MANGROVE JENU TUBAN," Universitas Airlangga, 2019.
[14] S. S. Wulandari, " Isolasi dan Uji Potensi Kapang Penghasil Enzim Selulase dari Tanah Mangrove Jenu Tuban ", Department of Biology, Faculty of Science and Technology, Airlangga University, 2019.
[15] A. Nuraini, "Keanekaragaman dan Dominansi Bakteri dari Tanah Mangrove Jenu Tuban," Master, Department of Biology, Faculty of Science and Technology, Airlangga University, 2019.
[16] Promega, "Technical Manual Wizard Genomic DNA Purification Kit,"
[17] R. T. Fitriya, M. Ibrahim, and L. J. L. Lisdiana, "Keefektifan metode isolasi DNA kit dan CTAB/NaCl yang dimodifikasi pada Staphylococcus aureus dan Shigella dysentriae," vol. 4, no. 1, pp. 87-92, 2015.
[18] B. Dahal, G. NandaKafle, L. Perkins, and V. S. J. M. R. Brözel, "Diversity of free-Living nitrogen fixing Streptomyces in soils of the badlands of South Dakota," vol. 195, pp. 31-39, 2017.

[19] D. N. Susilowati and S. M, "Analisis Aktivitas Nitrogenase dan Gen NIFH Isolat Bakteri Rhizosfer Tanaman Padi dari Lahan Sawah Pesisir Jawa Barat," *AL-KAUNIYAH Journal of Biology,* vol. 9, no. 2, pp. 125-138, 2016.
[20] R. Gothwal, V. Nigam, M. Mohan, D. Sasmal, P. J. A. e. Ghosh, and e. research, "Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants," vol. 6, no. 2, pp. 101-109, 2008.
[21] Santos-Caton and I. R, "Abundance of nifH genes in urban, agricultural, and pristine prairie streams exposed to different levels of nitrogen loading," University Sains Malaysia, 2007.
[22] J. I. Rilling, J. J. Acuña, M. J. Sadowsky, and M. A. Jorquera, "Putative Nitrogen-Fixing Bacteria Associated With the Rhizosphere and Root Endosphere of Wheat Plants Grown in an Andisol From Southern Chile," (in English), Original Research vol. 9, 2018-November-20 2018.
[23] C. Franche, K. Lindström, and C. Elmerich, "Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants," ed: Springer, 2009.
[24] E. Curl and T. Bryan, *The Rhizosphere,* 1 ed. Tokyo: Berlin Heidelberg New York, 1985.
[25] N. R. Krieg and J. G. Holt, *Bergey's manual of systematic bacteriology*. Yi Hsien Publishing Co., 1984.
[26] S. Harran and N. Ansori, *Bioteknologi Pertanian*. Bogor: Pusat Antar Univesitas Bioteknologi, Insititut Pertanian Bogor, 1992.
[27] D. N. Susilowati, S. R., H. R.D., and Y. E, "Peningkatan Serapan N pada Kedelai yang Diinokulasikan Bakteri Diazotrof Endofit di Medium Vermiculit," *Jurnal Tanah Iklim,* vol. 26, pp. 41-46, 2007.
[28] S. D. Sukartiningrum, "Penentuan Pohon Filogenetik Bakteri Xilanolitik Sistem Abdominal Rayap Tanah Berdasarkan 16S rRNA," Department of Chemistry, Faculty of Science and Technology, Airlangga University, 2012.
[29] F. A. Q. Wasdili and G. T., "Penentuan Kualitas Isolasi DNA Salmonella typhimurium dengan Metode Spektrofotometer dan Elektroforesis," *PINLITAMAS 1,* vol. 1, no. 1, pp. 578-582, 2018.
[30] Y. Liu *et al.*, "Proposal of nine novel species of the Bacillus cereus group," vol. 67, no. 8, pp. 2499-2508, 2017.
[31] R. A. Miller *et al.*, "Bacillus wiedmannii sp. nov., a psychrotolerant and cytotoxic Bacillus cereus group species isolated from dairy foods and dairy environments," vol. 66, no. 11, p. 4744, 2016.
[32] Y. Zhao, C. Chen, H.-j. Gu, J. Zhang, L. J. F. i. C. Sun, and I. Microbiology, "Characterization of the genome feature and toxic capacity of a Bacillus wiedmannii isolate from the hydrothermal field in Okinawa Trough," p. 370, 2019.
[33] I. O. Noha and Y. J. A. J. o. M. R. Shixue, "Isolation and characterization of pea plant (Pisum sativum L.) growth-promoting Rhizobacteria," vol. 12, no. 34, pp. 820-828, 2018.
[34] A. Sen, M. K. Bhattacharya, H. K. Prasad, and G. D. Sharma, "Plant growth promoting activities of rhizosphere bacteria from two ferns Pronephrium nudatum (Roxb.) Holttum. and Bolbitis heteroclita (C. Presl) Ching: An analysis of fern-rhizophere relationship," *Indian Journal of Experimental Biology,* vol. 56, pp. 267-273, 04/01 2018.
[35] H. T. Tam, "Isolation and Characterization of Bacteria of Mangrove Rhizosphere in the Mekong Delta , Vietnam," 2017.
[36] S. S. Mohamed, S. K. Amer, M. S. Selim, H. M. J. E. J. o. B. Rifaat, and A. Sciences, "Characterization and applications of exopolysaccharide produced by marine Bacillus altitudinis MSH2014 from Ras Mohamed, Sinai, Egypt," vol. 5, no. 3, pp. 204-209, 2018.
[37] Y. Jesmi, J. Thajudeen, A. Alikunju, D. Sukumaran, and M. H. Abdulla, "Diversity and activity of culturable nitrogen fixing heterotrophic bacteria from estuarine and coastal environments of Southeastern Arabian Sea (SEAS)," *Regional Studies in Marine Science,* 01/13 2020.
[38] G. Jiménez *et al.*, "Description of Bacillus toyonensis sp. nov., a novel species of the Bacillus cereus group, and pairwise genome comparisons of the species of the group by means of ANI calculations," vol. 36, no. 6, pp. 383-391, 2013.
[39] S. Shivaji *et al.*, "Bacillus aerius sp. nov., Bacillus aerophilus sp. nov., Bacillus stratosphericus sp. nov. and Bacillus altitudinis sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes," (in eng), *Int J Syst Evol Microbiol,* vol. 56, no. Pt 7, pp. 1465-1473, Jul 2006.

[40] Y. Liu *et al.*, "Identification of strains Bacillus aerophilus MTCC 7304T as Bacillus altitudinis and Bacillus stratosphericus MTCC 7305T as a Proteus sp. and the status of the species Bacillus aerius Shivaji et al. 2006. Request for an Opinion," vol. 65, no. Pt_9, pp. 3228-3231, 2015.