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# Isolation and Potency Test of Endophytic Bacteria as Nitrogen Fixer from Mangrove Plant in Lamongan

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## ABSTRACT

Endophytic bacteria are microorganisms that live in plant tissues and some of them contribute to nitrogen fixation for plants. This study aimed to isolate and identify endophytic bacteria from mangroves of Kutang Beach, Lamongan, which potentially as nitrogen fixing bacteria. Bacterial Isolates were used as candidates for biofertilizers. Leaves samples were taken from 10 sampling points. Bacterial isolation was initiated by sterilizing the surface of the leaves sample and grinding it aseptically. Isolation was carried out with a pour plate method on Nutrient Agar medium. Screening for endophytic bacteria's potential as N-fixing agent was carried out by growing the bacterial isolates on a semi-solid Nitrogen Free Bromothymol Blue (NFB) medium. The isolates that produced a positive reaction with a change in the color of the medium to blue were then subjected to macroscopic (shape, color, elevation, and the edge of the colony) and microscopic observations (Gram stain and bacterial cell measurements). The isolates showed the fastest change in the color of the medium were identified by the molecular marker of the 16S rRNA gene. The data obtained were analyzed descriptively. As many as 20 isolates were obtained from the mangroves of Kutang Lamongan Beach, and ten isolates of twenty potentially as nitrogen-fixing bacteria. The ten nitrogen-fixing bacteria isolates had varying macroscopic characteristics. The microscopic characteristics showed that eight isolates had Gram-positive bacilli, and two isolates were Gram-negative with varying bacterial sizes. Based on the 16S rRNA gene sequence, the most potential of nitrogen-fixing bacteria was LMG II-14 isolate and identified as *Paenibacillus alvei* LMG II-14 with 99.36% similarity to *Paenibacillus alvei* strain DSM 29 based on the NCBI database. The ten nitrogen fixing isolates that have been obtained can later be used as candidates for biofertilizer composition, especially *Paenibacillus alvei* LMG II-14.

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## INTRODUCTION

Indonesia is an agricultural country where agriculture plays a vital role in the national economy. Indonesia faces agricultural issues almost every year, such as declining crop yields, pest attacks, yellowing of plant leaves, and others. Nitrogen (N) is an essential element for living organisms, especially plants. Element Nitrogen is one of the constituents of proteins and plays a role in the photosynthetic process (Leghari et al., 2016).

The nitrogen content in the atmosphere is very high at about 78%, but nitrogen is in the form of molecules, most of which are non-reactive and are not directly absorbed by plants. Therefore, it is necessary to convert nitrogen in the air into a molecular form that plants can absorb. Nitrogen is only absorbed by + plants in the form of ammonium ions (NH) or nitrate ions (No). Atmospheric dinitrogen gas is converted to ammonia (NH) and fixed to the soil by a fixing process (Martinez-Dalmau et al., 2021).

Chemical nitrogen fertilizers help meet the needs for nitrogen. However, excessive and inappropriate use will decrease soil fertility and soil fertilizing microorganisms, and it causes changes in soil ecosystems (Sulistiyani and Lisdiyanti, 2016). These negative impacts can occur because some inorganic fertilizers contain heavy metals (such as cadmium and chromium) and high concentrations of radionuclides (Savei, 2012). To overcome these problems, farmers are trying to find other alternatives, including using organic fertilizers such as compost (manure or microbial-based fertilizers) known as biological fertilizers or biofertilizers.

Bacteria that are beneficial to such plants are a type of bacteria that provide many benefits to the host plant and help to withstand a variety of biological and abiotic stresses that can affect growth. These bacteria can live both outside and inside the host plant. Bacteria that live outside the host plant are either epiphytes that live on the surface of the leaves of the plant or root spheres that live in the roots of plants in the soil. Bacteria that live and reproduce in host plants are called endogenous bacteria, but all of these classes of bacteria share many of the essential properties that promote the growth of host plants (Afzal et al., 2019). In addition, it is necessary to understand the nature of the microorganisms before their use as biofertilizers in order to utilize only microorganisms safe for human health; this includes not only the consumer or end user but also those handling the biofertilizers during their production. Strains belonging to the genera *Azospirillum*, *Gluconacetobacter*, *Bacillusor Azotobacter*, among others, are currently commercialized as biofertilizers for non legumes without any adverse effects being reported to date (Celador-Lera et al., 2018). N-fixing bacteria are often called diazotrophic bacteria. These bacteria live freely in the root area and plant tissue. They can use air N as a source of N for their growth. The role of bacteria in fixing air nitrogen influences the economic value of agricultural land (Ristiati *et al.*, 2008).

Alfaro & Ullrich (2014) have successfully isolated the endophytic bacteria (*Marinobacterium mangorovicola*) from mangrove roots as nitrogen-fixing plants. Castro *et al.* (2018) have successfully isolated endophytic bacteria as nitrogen-fixing bacteria from Brazilian mangrove forests. The results showed that 38 isolates from 115 isolates as nitrogen-fixing plants. Salsabila (2020) has also found six isolates of nitrogen-fixing bacteria from 20 isolates from the soil of the Jenu Tuban Mangrove Center.

Kutang Lamongan Beach diverse mangrove species. Based on the literature search, no information has been found regarding the exploration of nitrogen-fixing endophytic bacteria from the mangroves of Kutang Lamongan Beach. Therefore, this study was focused on isolating endophytic bacteria from the mangroves of Kutang Lamongan Beach, then testing the potential of these isolates in fixing nitrogen. Endophytic bacteria with nitrogen-fixing ability can be used as biofertilizer candidates. Hopefully, it can solve the problem of soil quality degradation due to chemical fertilizer application.

## MATERIALS AND METHODS

### Sample Collection

The sample used in this study was mangrove leaves at Kutang Beach, Lamongan. Sampling was carried out in a coastal area near the mainland with a random sampling method. The samples were put in clean labelled plastics and stored in a cool box. Further, sample was carried to the Microbiology Laboratory of Faculty of Science and Technology, Universitas Airlangga,

### Endophytic Bacteria Isolation

The samples were cleaned with running water and weighed (20g). Next, the sample was immersed in 70% ethanol for one minute and 0.01% HgCl2 solution for five minutes to kill microbes on the leaf surface. The samples were rinsed twice with sterile distilled water for one minute of each. The leaf samples were ground using a mortar aseptically and mixed with 2 mL of sterile distilled water. One ml of the grinded sample was taken and put into a sterile petri dish. All processes were carried out aseptically in Laminar airflow (LAF) (Purwanto *et al.*, 2014; Anjum and Chandra, 2015).

### Bacterial Isolate Purification

The bacterial colonies with different morphologies and colors were taken to be purified.

The purification of bacterial isolates was carried out using the streak plate method. Each bacterial colony was taken using a sterile ose and streaked on the NA medium. Purification was repeated until pure cultures were obtained. Bacterial isolates were maintained periodically by transferring pure cultures to slant NA medium (Ed-har et al., 2017). Bacterial preservation can be done by preserving bacteria in 10% glycerol and stored at -80°C.

**Ability testing of bacteria to fix nitrogen**

The test was started by inoculating endophytic bacterial isolates into Nutrient broth (NB) medium for 24 hours. After that, it was taken using a skewer and inserted into the semi-solid NFB medium in a test tube. Semi-solid NFB medium was prepared for 1,000 mL of distilled water consisted of 5 g of malic acid; 0.5 g $K_2HPO_4$; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.02g $CaCl_2.2H_2O$; 2 mL micronutrient solution (0.04 g $CuSO_4.5H_2O$; 0.12g $ZnSO_4.7H_2O$; 1.40g $H_3BO_3$; 1.0g $Na_2MoO_4.2H_2O$; 1.175g $MnSO_4.H_2O$ dissolved in 1000 mL distilled water); 2 mL bromothymol blue; 0.05g $FeSO_4.7H_2O$; 1.8g Ag; 4.5g KOH. The components of these materials were dissolved in distilled water, heated on a hot plate and stirred until completely dissolved with a pH of 6.5. After the material was dissolved, the medium was poured into 6 mL test tubes. A sterile semi-solid NFB medium was used to test the potential of bacterial isolates in fixing nitrogen. Furthermore, the bacterial isolates in NFB were incubated at room temperature of 30°C for ten days and observed every day. The positive result was indicated by a color change in the NFB medium from a greenish-yellow color to a bluish color, and a pellicle was formed on the surface of the medium (Baldani *et al*, 2014; Quintana *et al*, 2020; Rilling *et al*, 2018).

**Macroscopic and Microscopic Characterization of Nitrogen-fixing bacteria**

Isolates of endophytic bacteria with nitrogen-fixing ability were characterized macroscopically by observing the bacterial colonie shape, color, elevation, and edge. Microscopic characterization was carried out by a Gram stain test, cell shape observation, and cell measurement (Kaburuan *et al*, 2014).

**Identification of Bacteria using the 16S rRNA gene**

DNA isolation of bacterial isolates was carried out according to the Promega Genomic DNA Purification Kit Wizard procedures. The results of genomic DNA could be seen on 1% agarose gel. Band was obtained, indicating the DNA isolation was worked out.

The isolated DNA was measured using a spectrophotometer at 260 nm and 280 nm to determine the level of DNA. Measurement of isolated DNA was carried out at 260 nm. In comparison, protein measurements were carried out at 280 nm. Then, the 16S rRNA gene PCR was performed using PCR Kit from Promega with the following composition, 25 µl PCR mix, universal primers 27F and 1492 R each 2 µl, 2 µl DNA template, and 19 µl nuclease free water with total volume 50 µl (Wardani *et al*, 2017). The amplification process using the PCR technique was carried out using universal primers 27 F (5' AGA GTT TGA TCM TGG GTC AG 3') and 1492 R (5' GGT TACCTT GTT ACG ACT T 3') at denaturation conditions of 92°C for five minutes and 95°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for one minute, and post extension for seven minutes.

The result of the 16S rRNA gene DNA sequence was trimmed to remove low-quality DNA sections (<15%) and remove gaps using the Bioedit program. Furthermore, the DNA sequences of the bacterial samples were matched with the data on GeneBank using blast N via the NCBI (National Center for Biotechnology Information) website to determine the degree of similarity of the sample nucleotide base sequences to the closest bacterial species.

## RESULTS & DISCUSSION

**Endophytic Bacterial Isolates from Mangrove Leaves in Kutang Beach, Lamongan**

In this study, 20 isolates of endophytic bacteria from mangrove plants from Kutang Beach, Lamongan, were coded as LMG II-1 to LMG II-20 isolates. Ten of the 20 bacterial isolates were potential being nitrogen-fixing bacteria. This was indicated by a change in the color of the semi-solid NFB medium from yellow to greenish to blue, and a pellicle formed (Figure 1). The screening results can be seen in Table 1.

Furthermore, the endophytic bacterial isolates that had the potential of being nitrogen-fixing bacteria were observed macroscopically, namely size, shape, color, margin, and colony elevation, as well as microscopic character observations, namely the results of Gram staining, shape, and size of bacterial cells (Table 2; Figure 2, 3).

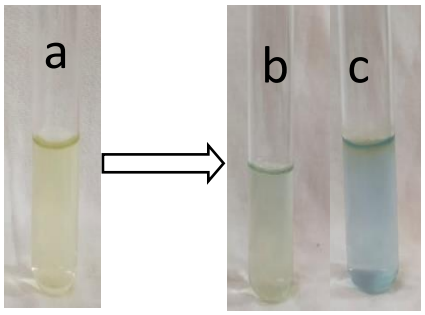


**Figure 1.** The positive reaction of nitrogen-fixing bacteria on semi-solid NFB medium (a). Early incubation of NFB medium (yellow); (b). Color change of NFB medium containing nitrogen-fixing bacteria from yellow to greenish-yellow; (c). The color of the medium changed from greenish-yellow to blue, and a white pellicle formed on the surface of the medium

**Table 1.** Screening results of nitrogen-fixing endophytic bacteria isolates from the Lamongan mangroves (LMG II-1 to LMG II-20).

| Isolat code | Nfb Test | Isolat code | Nfb Test |
|---|---|---|---|
| LMG II-1 | (+) | LMG II-11 | (+) |
| LMG II-2 | (+) | LMG II-12 | (-) |
| LMG II-3 | (-) | LMG II-13 | (+) |
| LMG II-4 | (-) | LMG II-14 | (+) |
| LMG II-5 | (-) | LMG II-15 | (-) |
| LMG II-6 | (-) | LMG II-16 | (-) |
| LMG II-7 | (+) | LMG II-17 | (+) |
| LMG II-8 | (-) | LMG II-18 | (+) |
| LMG II-9 | (+) | LMG II-19 | (+) |
| LMG II-10 | (-) | LMG II-20 | (-) |

**Table 2.** Macroscopic and microscopic characteristics of ten nitrogen fixing bacteria isolated from mangrove plant in Lamongan

| Isolate code | size | shape | color | margin | elevation | Gram stain |
|---|---|---|---|---|---|---|
| LMG II-1 | 2 x <1 | circular | yellow | entire | raised | bacilli + |
| LMG II-2 | 2 x 1 | circular | cream | entire | raised | bacilli + |
| LMG II-7 | 3 x 1 | circular | transparent | entire | raised | Bacilli + |
| LMG II-9 | 2 x <1 | circular | Bone-white | entire | convex | Bacilli + |
| LMG II-11 | 3 x 1 | circular | cream | entire | raised | Bacilli + |
| LMG II-13 | 3 x 1 | circular | white | entire | flat | Bacilli + |
| LMG II-14 | 2 x<1 | circular | Yellowish-white | entire | raised | Bacilli + |
| LMG II-17 | 3 x 1 | circular | white | entire | flat | Bacilli - |
| LMG II-18 | 3 x 1 | irregular | white | undulate | raised | Bacilli - |
| LMG II-19 | 2 x 1 | circular | white | entire | raised | Bacilli + |

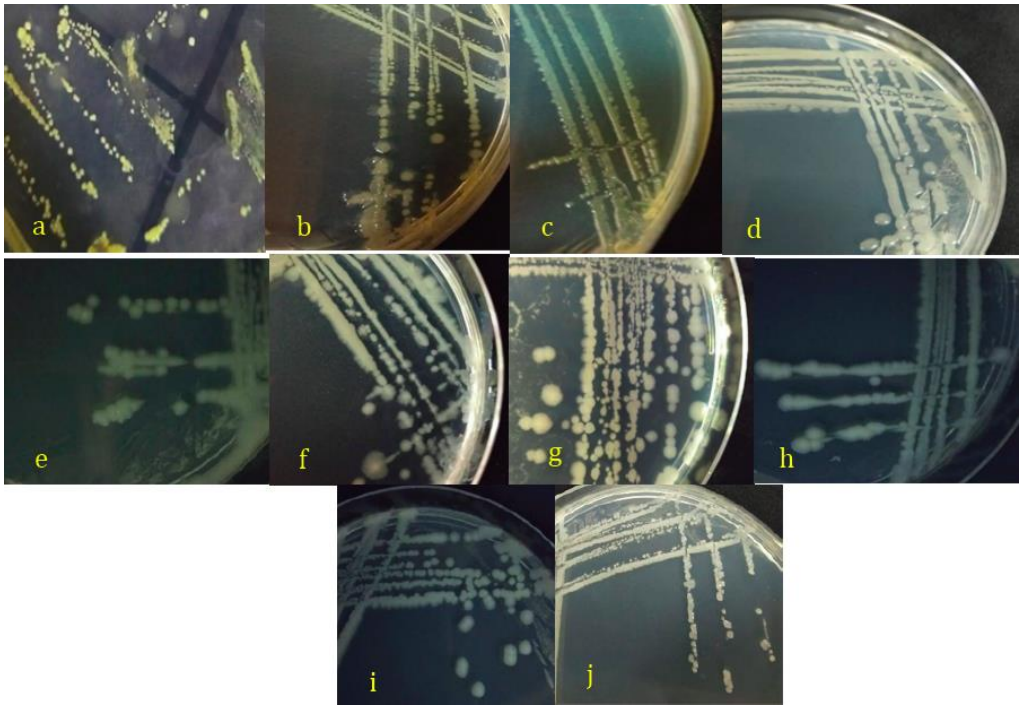


**Figure 2.** Colony morphology of the isolates of nitrogen-fixing bacteria from Mangrove leaves in Lamongan; a: LMG-II 1; b: LMG-II 2; c: LMG- II 7; d: LMG-II 9; e: LMG-II 11; f: LMG-II 13; g: LMG-II 14; h: LMG-II 17; i: LMG-II 18; j: LMG-II 19

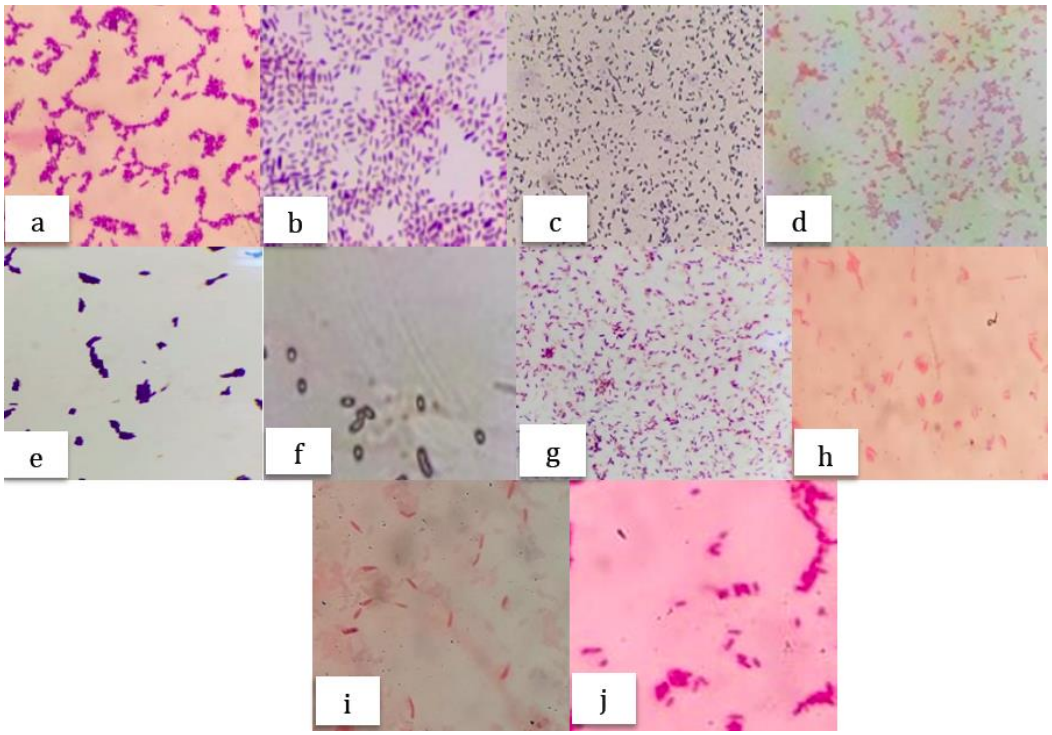


**Figure 3.** Cells morphology of the isolates of nitrogen-fixing bacteria from Mangrove leaves in Lamongan; a: LMG-II 1; b: LMG-II 2; c: LMG- II 7; d: LMG-II 9; e: LMG-II 11; f: LMG-II 13; g: LMG-II 14; h: LMG-II 17; i: LMG-II 18; j: LMG-II 19

**The results of the identification of the most potential isolate (LMG II-14)**

The results of the quantitative DNA test of the LMG II-14 isolate showed an absorbance value of 0.6308 at 260 nm and 0.3555 at a wavelength of 280 nm. Comparison of the two absorbance values of DNA samples showed 1.8 (Abs 260 nm/Abs 280 nm). This indicates that the DNA sample was pure. DNA is declared pure if it is in the range of 1.8-2.0. If the comparison value is less than 1.8, the DNA is still contaminated by phenol and the remaining solvent of DNA isolation. If it is more than 2.0, the DNA is contaminated by protein. The electrophoresis showed that the DNA band was in an area parallel to the ±1500bp marker; this indicated that the 16SrRNA gene was successfully amplified (Figure 4).

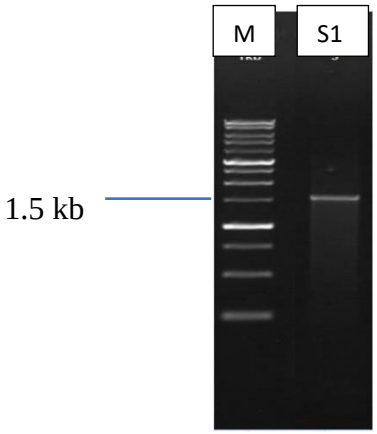


**Figure 4.** Electrophoresis results of 16S rDNA of nitrogen-fixing bacteria. M = Markers; S1 = LMG II-14 Isolate

The results of identifying endophytic bacterial isolates with the most potential of being nitrogen-fixing plants can be seen in Table 3. The identification results based on BLAST also provide information in query cover and identity percentage, where query cover is the percentage of base length aligned with the BLAST database. Whereas, identity percentage is similarity between the analyzed sequences and the aligned database sequences.

The sample used was the fresh part of the leaves. Surface sterilization process aimed to clean microbes on the leaf surface and ensure that only endophytic bacteria will be obtained from the isolation of endophytic bacteria. As many as 20 pure isolates of endophytic bacteria were obtained from mangrove leaves, coded LMG II-1 to LMG II-20. Ten of 20 isolates (with code LMG II-1, LMG II-2, LMG II-7, LMG II-9, LMG II-11, LMG II-13, LMG II-14, LMG II- 17, LMG II-18, and LMG II-19) have the ability to fix nitrogen, indicated by color change in the NFB medium from yellow to yellowish-green to blue. This result was supported by a study conducted by Tam *et al.* (2018), who successfully isolated 86 isolates from mangroves, of which 41 isolates were known to be able to fix nitrogen.

NFB medium is a selective medium that does not contain nitrogen and bacterial isolates that can grow on these mediums and fix free nitrogen (Mallombasi, 2018). Hidayat (2009) stated that the color change in semi-solid NFB medium occurred due to nitrogenase activity which caused a higher pH. The color of the medium turned blue due to these bacteria groups will fix nitrogen and convert it to ammonium (NH4+) which alkaline that causes the bromthymol blue indicator in the NFB medium composition.

Based on the results of macroscopic observations, LMG II-18 isolates had different colony shapes from other isolates, which were irregular in shape with undulate edges. Meanwhile, circular bacterial colonies were found in isolates LMG II-1, LMG II-2, LMG II-7, LMG II-9, LMG II-11, LMG II-13, LMG II-14, LMG II- 17, and LMG II-19, which have the entire colony margins. Ten isolates of nitrogen-fixing bacteria had cell sizes range 2-3 µm x <1-1 µm and same cell shape, i.e, bacilli. For gram staining, all isolates are Gram-positive except LMG II-17 and LMG II-18 that were Gram-negative.

Genomic DNA obtained from the isolation process was measured for purity to ensure that the DNA amplified by the PCR method had the appropriate purity (Sukartiningrum, 2012). DNA purity was obtained from the absorbance ratio at wavelengths of 260 nm and 280 nm using a spectrophotometer. The DNA purity limit ranges from 1.8-2.0. Suppose the result of the A260/280 ratio is less than 1.8. In that case, the DNA is still not pure due to contamination by phenol and residual DNA isolation solvents. In contrast, if the A260/A280 ratio value is more than 2.0, then the resulting DNA still contains contaminants in the form of proteins or other compounds (Sambrook and Russell, 2001). The results of the quantitative DNA test of the LMG II-14 isolate, which is the isolate with the most potential to fix nitrogen, showed the absorbance of 260 nm was 0.6308, the absorbance of 280 nm was 0.3555. DNA purity (Abs 260 nm/Abs 280 nm) was 1.8.

**Table 4.** The results of the identification of 16S rRNA nitrogen-fixing bacteria isolate based on the NCBI gene bank database

| Isolate Code | Bp Length | BLAST | | | |
|---|---|---|---|---|---|
| | | Species | Query cover (%) | Identity Percentage (%) | Access Number |
| LMG II-14 | 1173 | *Paenibacillus alvei* | 98 | 99.36 | NR_042091.1. |

PCR results through electrophoresis showed that the DNA encoding 16S rRNA was successfully amplified with PCR products ranging from 1500 bp. Based on the analysis results using BLAST programme, the bacterial isolate LMG II-14 was similar to *Paenibacillus alvei* strain DSM 29 which had a query cover of 98% and a percent identity of 99.36%. Query cover is a presentation of the nucleotide length aligned with the databasecontained in BLAST. At the same time, identity percentage is seen from the maximum identity, which is the highest value of the identity percentage or match between the query sequence and the aligned database sequence (Miller *et al.*, 1990 Kasi *et al.*, 1990). The percentage of acceptable cover queries was at least 95%, and for sequences with lower readings, a minimum of 75% was applied (Narita *et al.*, 2012).

Based on the result study, it was known that *P. alvei* has morphological characteristics of circular form, entire edges, raised elevation, yellowish-white color. While microscopic characteristic was bacilli and purple color of Gram staining result that indicated Gram-positive bacteria. Based on a study conducted by Prihanto *et al.* in 2018, he isolated endophytic bacteria on the leaves of the mangroves (*Sonneratia alba*) from Sendang Biru beach, Malang. The morphological characteristics of the *P. alvei* colonies grown in LBA (Luria Bertani Agar) medium are circular, undulating edges, raised elevation, yellowish-white. Microscopically *P. alvei* are bacillary and purple after Gram staining, classifying them as Gram-positive bacteria. *P. alvei* isolate fixing free nitrogen was a discovery. Thus, it was necessary to conduct further study on the ability of these isolates to produce nitrogenase enzymes that play a role in the free nitrogen fixation process.

## CONCLUSION

The number of endophytic bacteria isolates from the mangroves of Kutang Lamongan Beach was 20 isolates. Ten of them potentially become nitrogen-fixing bacteria. The isolates had different macroscopic and microscopics characteristics. LMG II-14 isolate have the most potential of being nitrogen fixing bacteria and identified as *Paenibacillus alvei* LMG-II 14 based on molecular markers of the 16S rRNA gene.

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