Isolation, characterization and identification of nitrogen fixing bacteria with organic fertilizer applications in paddy soil

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Abstract. The high organic matter content allows soil microbial activity to recycle organic matter, which is essential for life, such as the nitrogen cycle. The process of biological nitrogen fixation by microorganisms is crucial for nitrogen entry into the nitrogen cycle. This study aims to isolate and characterize nitrogen-fixing bacteria in paddy soil using organic fertilization applications. This study was conducted in Salassae Village, Bulukumba, South Sulawesi, Laboratory of Microbiology, Soil Science Department, Hasanuddi University. This research method is a descriptive study using morphological Characterization, biochemical tests, and molecular identification. This study succeeded in isolating ten nitrogen-fixing bacterial isolates from wetland soil samples using organic fertilizers. Morphological characterization results showed quite different results in terms of color, size, shape, and level. The highest nitrogen-fixing ability test was obtained in isolates L1.P, where the highest nitrogen fixation ability was 0.26%. Biochemical Characterization using VITEK®. 02 shows that almost all biochemical tests show a positive reaction. The identification of Nitrogen-fixing bacteria species using 16S rRNA gene sequencing showed that L1.P was identified with 99% similarity to Bacillus subtilis subsp. subtilis str. 168.

1. Introduction
The use of nitrogen fertilizers, especially in the form of urea, is still relatively high Based on data from the Indonesian Fertilizer Producers Association (APPI), throughout 2018, urea consumption grew 5%
from 5.97 million tonnes in 2017 to 6.27 million tonnes, while NPK consumption rose 7.88% from 2.60 million tonnes to 2.80 million tonnes. The increase was seen in phosphate, ZA, and organic fertilizers [1]. Nitrogen was needed by plants in large quantities, including as a building block for protein. Nevertheless, the composition of nitrogen as much as 78% in the atmosphere cannot be utilized directly by plants. The accomplishment of nitrogen supply in the soil can be done by fertilizing or naturally with microorganisms' help. Biological nitrogen fixation is an essential process in nature carried out by nitrogen-fixing bacteria in converting free nitrogen gas in the atmosphere to ammonium. Ammonium is an essential nitrogen source in the ecosystem [2].

Urea fertilizer efficiency is very low, often only 30-40%, even lower in some cases [3]. Besides, some of the urea fertilizer nitrogen is lost through several mechanisms, including ammonia volatilization, denitrification, and leaching, resulting in pollution problems to the environment [4]. The use of biologically nitrogen-fixing (BNF) technology can reduce the use of urea as a nitrogen source, prevent the decrease in soil organic matter and reduce pollution to the environment [5]. According to [6], biologically nitrogen fixation optimization is a feasible alternative to reducing synthetic urea special chemical fertilizers.

Nitrogen due to the biological fixation of nitrogen by microorganisms is crucial for nitrogen entry into the nitrogen cycle. Several soils and water microorganisms can fix atmospheric nitrogen into ammonium. According to the way nitrogen-fixing is carried out, two groups of microorganisms: (1) non-symbiotic nitrogen-fixing; (2) Symbiotic nitrogen-fixing. The non-symbiotic group is divided into two, namely, aerobic and anaerobic organisms [7].

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This study aims to isolate, characterize and identify nitrogen-fixing bacteria in rice fields in Sallasae Village, Bulukumba Regency that can fix nitrogen. This research expected to get information on the species of nitrogen-fixing bacteria in paddy fields

2. Materials And methods
2.1. Soil sampling and isolation of nitrogen-fixing Bacteria
Soil samples were taken from Salassae, Bulukumba Regency, South Sulawesi in the rice plants' rhizosphere area with organic fertilizers. Samples were taken from the plant's rhizosphere at a depth of 30 cm from the soil surface, then put into a sterile sample bag and taken to the Soil Biotechnology Laboratory of the Hasanuddin University Soil Science Department. The soil sample was then filtered with a 2.00 mm mesh sieve. One gram of each soil sample is put into a 250 ml Erlenmeyer flask containing 100 ml of nitrogen-free medium, namely Burk's medium [8] (Sucrose, 20.0 g; K2HPO4, 0.64 g; KH2PO4, 0.16 g; MgSO4.7H2O, 0.20 g; NaCl, 0.20 g; CaSO4.2H2O, 0.05; Na2MoO4.2H2O, (0.05%) 5.0 ml; FeSO4.7H2O, (0.3%) 5.0 ml; 15 g agar and aquadest, 1000 ml) (Jack et al 1953) and Ashby's medium (mannitol, 20 g; K2HPO4, 0.2 g; MgSO4.7H2O, 0.2 g; NaCl, 0.2 g; K2SO4, 0.1 g and CaCO3, 0.5 g; aquadest, 1000 ml [9], then incubated for 14 days at 27 °C. One mL of medium The liquid was taken aseptically, then diluted to 10⁻³ dilution, then inoculated on Burk's and Ashby's solid medium using the scatter plate method, then incubated for seven days at 27°C. Separate colonies were purified three times by the streak method. The different groups were then characterized morphologically according to the procedure described. Isolate those characterized were then stored in an agar slant for further analysis.

2.2. Ammonium Concentration Measurement
The ammonium excretion test was carried out on two growth mediums, namely Burks and Ashby medium through pure isolate in a test tube suspended with 5 mL of the liquid medium then 1 mL was taken then inoculated into Erlenmeyer containing 29 mL of the liquid medium then incubated through a shaker for 24 hours at 27°C. This incubation time is when the bacterial cell was predicted to be in an
exponential growth phase entering a stationary phase. After 24 hours of incubation, samples were then taken and centrifuged at 13,000 rpm for 15 minutes. Three mL of the supernatant was taken and adjusted to pH 11 with the addition of 1N NaOH, then added 0.07 ml of EDTA, 0.07 ml of sodium potassium tartrate, and 0.13 ml of Nessler’s reagent [9] then homogenized and incubated for 30 minutes at 25°C. The absorbance was measured at a wavelength of 435 nm using a spectrophotometer [10].

2.3. Morphological and Biochemical Characterization of Nitrogen-Fixing Bacteria Isolates
Morphological analysis is macroscopic with direct observation to observe bacterial isolates from shape, elevation, color, size, and edge. Microscopic observation of bacterial isolate with gram stain and spore observation. Biochemical Characterization of nitrogen-fixing bacteria using automatic biochemical methods using the VITEK® 2 program [11].

2.4. Molecular Identification
Molecular identification of nitrogen-fixing bacteria from selected isolates was sequenced at First Base Laboratories Sdn Bhd, Syah Alam, Selangor, Malaysia, to determine the sequence of DNA bases. The results are then aligned with the Gen Bank data (http://www.NCBI.nlm.nih.gov), using the BLAST-N program [11], to find out the similarities of the selected species.

3. Results and discussion

3.1. Isolation and Characterization of Nitrogen-Fixing bacteria
Based on the results of the initial isolation of Nitrogen-fixing bacteria from the rhizosphere rice plant, locations on paddy soil with organic fertilizer application, ten selected Nitrogen-fixing bacteria isolates that we can grow well on the selection media, namely Ashby and Burks media, and then characterized Morphological (table. 1)

| Isolat  | Color         | Size    | Edge   | Evilest | Shape    |
|---------|---------------|---------|--------|---------|----------|
| LK.2.C  | Whitish Yellow| Moderate| Smoot  | Convex  | Smoot    |
| LK.2.F  | Milky White   | Moderate| Rhizoid| Raised  | Rhizoid  |
| LK.2.G  | Milky White   | Small   | Smoot  | Raised  | Smoot    |
| LK.2.H  | Milky White   | Moderate| Rhizoid| Flat    | Rhizoid  |
| LK.1. K | Milky White   | Moderate| Irregular| Flat   | Irregular|
| LK.2.N  | Milky White   | Small   | Irregular| Flat   | Irregular|
| LK.1. O | Kuning Keputahan| Moderate| Irregular| Raised | Irregular|
| LK.1.P  | Milky White   | Moderate| Lobate | Raised  | Lobate   |
| LK.1.V  | Milky White   | Moderate| Lobate | Flat    | Lobate   |
| LK.1.X  | Milky White   | Small   | Flamencos| Raised | Flamencos|

Based on (table 1) the morphological Characterization of Nitrogen-fixing bacteria isolates, most isolates were milky white, except for L.1., O isolates, and L.2.C isolates which were whitish-yellow. All isolates were almost irregular in shape, but some were rhizoid. The colony elevation was varied from flat to raised, with more varied edges. Bacterial colony characters that showed a high enough diversity among the selected isolates were from the colony's edges. The variations in Nitrogen-fixing bacteria isolates' morphological Characterization indicated the diversity of nitrogen-fixing bacteria in morphology [13].

3.2. Ammonium Concentration Measurement
After testing the nitrogen-fixing ability of ten bacterial isolates, almost all isolates could fix nitrogen because they could grow on nitrogen-fixing selection media. The highest nitrogen-fixing abilities were obtained in LK.1.P isolates, where the highest nitrogen ability was 0.26%
(figure 1). The process of nitrogen fixation by bacteria will produce ammonia (NH3). The presence of hydrogen ions will form ammonium (NH4 +) [14]. The bacterial isolate with the code L.1.P will be continued for further molecular identification to be identified to the species level.

**Figure 1.** Test results of nitrogen retaining bacteria ability.

In general, the successful morphological Characterization of nitrogen-fixing bacteria is round and gram-negative (figure 2). The source of N found in various organic compounds and from the air can meet bacteria’s needs for N elements [15]. There are two groups of nitrogen-fixing bacteria: symbiotic and non-symbiotic bacteria, nitrogen-fixing symbiotic bacteria with legumes, while non-symbiotic bacteria live, accessible rhizosphere area [16].

**Figure 2.** Pure isolate code L.K.P.1 (makroskopis dan microskopis).

3.3. Biochemical Characterization of Nitrogen-Fixing Bacteria Isolates

The Characterization of nitrogen-fixing bacteria was carried out by biochemical tests using automatic biochemical methods, namely using the identification program using the VITEK® 2 Compact tool (table 2).
Table 2. Biochemical characterization of nitrogen-fixing bacteria isolates.

| Characterization       | Bacterial isolates |
|------------------------|--------------------|
| Colony forms           | bacilli            |
| Gram staining          | +                  |
| Spores                 | centre             |
| Grows at 380°C         | +                  |
| Grows at pH of 5.5     | +                  |
| Grown at 6.5% NaCL content | +        |
| KOH                    | +                  |
| Catalase               | +                  |
| Motil                  | +                  |
| Urease                 | +                  |
| Novobiocin Resistant   | -                  |
| Bacitracin Resistant   | +                  |

(+) indicates a reaction or positive, (-) indicates no reaction or negative.

The results of the Biochemical Characterization of Nitrogen-Fixing bacteria from the results of gram staining indicated L1.P isolates were gram-positive bacteria with a microscopic form of bacillus, and there were spores located centrally, where these bacteria were able to grow at a temperature of 380°C, pH 5.5, KOH test, Katalese and Motil had positive reactions. The culture and biochemical properties were anaerobic, growing on NaCl 5%, -10%, pH 5.7. In the biochemical examination, selected isolates utilized sodium citrate as a carbon source for metabolism and growth by showing a diffusion pattern from a positive puncture inoculation test indicating bacterial motility. Acid is formed from glucose, sucrose, and maltose, does not form indole, and produces little H₂S, reduces nitrate [17]. Based on the biochemical tests results (table 2), the test results are mostly positive, except for the enzyme resistant novobiocin test, which shows an adverse reaction. In contrast, the enzyme test Resistant Bacitracin and Urease enzymes indicate a positive reaction. The results of several studies show that several bacterial species of Bacillus species have several antibacterial ingredients in supernatants such as polymixin, colistin, circular, and peptide antibiotics such as subtilin, subtilisin-A, Tas-A, and sublation [18].

3.4 Molecular characteristics
Identification of selected isolates of nitrogen-fixing bacteria by using the sequencing method with the 16S rRNA approach to determine what to do can be seen in table 3 below:

Table 3. Results of molecular identification of selected bacterial isolates with the best nitrogen-fixing ability.

| Isolate Code | Genus and species       | Accession no | Nucleotide length | 16S rRNA Identification Results (%) |
|--------------|-------------------------|--------------|-------------------|-------------------------------------|
| LK.1.P       | *Bacillus subtilis*     | CP053102.1   | 4,316,079 bp      | 99                                  |
The results of 16S rRNA gene sequencing were carried out to determine taxonomic strains. It found that it belonged to the *Bacillus subtilis* species with a total genome of 198.89%, where the similarity in the identity of the selected isolates had 99% similarity to *Bacillus subtilis subsp. subtilis* str. 168, reaching an average size of 4,316,079 bp. For this assembly, the average G-C content was: 43.6%, and the total genome size was 4.22 Mb. Therefore, L1.P isolate can be identified with certainty as *Bacillus subtilis subsp. subtilis* str. 168 [16]. The species of *Bacillus subtilis* is one of the potential bacteria that can be fixing nitrogen. The results of research [19] show that *Bacillus subtilis* is biologically capable of binding nitrogen competitively so that it can be used as an alternative in reducing the use of nitrogen fertilizers, where the results of the research [20] show that bacterial inoculants increase the content of N, P, and K total shoots and roots of maize in calcisol soil from 16% to 85% significantly compared to control.

4. Conclusions

Based on the results of the research conducted, it can be concluded were obtained ten isolates. The bacterial colonies’ character showed a high enough diversity among the selected isolates from the colony’s edge. The highest ability to fix nitrogen was obtained in LK.1.P isolates, where the highest nitrogen-fixing ability was 0.26%. Molecular characteristics were carried out to determine taxonomic strains, isolate L1.P, and identify with certainty with 99% similarity as *Bacillus subtilis subsp. subtilis* str. 168.

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