Characterization and identification of nitrogen-fixing bacteria isolated from agricultural soil

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Abstract:

To isolate and characterise free nitrogen-fixing bacteria, we collected randomly soil samples from different areas of Ha Noi. Nitrogen-fixing bacteria were isolated using Burk's medium without nitrogen mineral supplement. The ammonia (NH_4^+) synthesis of these bacterial strains after biomass production was determined by means of Nessler reagent. Based on the results of isolation, we observed and evaluated colony and cellular morphology, pigment production, and metabolic activities of twenty-five isolates. Among the isolated bacteria, two bacterial strains (6.2 and 8.2) with high NH_4^+ concentration in the cultural medium were selected as the best strains for nitrogen-fixing ability. The optimal pH and temperature for their growth and nitrogen fixation are 7.0 and 30°C, respectively. Growth is best favored in the presence of sucrose. We sequenced the 16S rRNA gene of selected strains and compared the homology of them in GenBank using BLAST search. The result of the comparison shows that the 6.2 and 8.2 strains have 99% and 100% 16S rRNA-sequence similarity with *Pseudomonas* sp. and *Bacillus* sp., respectively.

Keywords: **biological nitrogen fixation, nitrogen-fixing bacteria, 16S rRNA.**

Classification numbers: **3.1, 3.4**

Introduction

Nitrogen is an important element of all organisms because it is an essential constituent of proteins, nucleic acids, amino acids, chlorophyll, and other organic substances. In addition, nitrogen is one of the most important nutrients for plant growth and the plant metabolic system. Consequently, nitrogen plays a key role in agriculture by increasing of crop yield. The element is present in the soil in small amounts, in both inorganic and organic forms. Most of nitrogen in the soil exists in organic form, and inorganic nitrogen constitutes only a small fraction of total soil nitrogen. The total amount of nitrogen in the mineral soil surface normally ranges between 0.05 and 0.2% and is directly available to plants, principally as nitrate (NO_3^-) and NH_4^+. The organic nitrogen slowly becomes available through mineralisation [1].

Although nitrogen gas (N_2) accounts for approximately 78% of the atmosphere, it cannot be directly used by plants; therefore, N_2 must be transformed into a form such as ammonia before being consumed through biological nitrogen fixation (BNF), chemical nitrogen fixation, and atmospheric addition. Of these methods, BNF by micro-organisms is the best way to make nitrogen fertiliser. In addition, the contribution of the BNF method leads to reduced use of chemical nitrogen fertiliser, thereby preventing soil erosion and reducing environmental pollution. Nitrogen-fixing micro-organisms are commonly found in the plant rhizosphere. By releasing exudates, plants can exhibit higher nitrogen-fixation activity in the soil [2]. Free-living nitrogen-fixing microorganisms have generally been reported to be plant growth promoters [3, 4]. The primary objective of this study is to isolate and characterise nitrogen-fixing bacteria from different agricultural soils, and, hence, to identify the strains with the greatest nitrogen-fixing ability by means of

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16S rRNA gene-sequence analysis.

Materials and methods

Soil sampling

Soil samples were collected from agricultural lands in Ha Noi. A 2 mm sieve was used to remove stones and plant debris from the samples.

Isolation of nitrogen-fixing bacteria

Individual samples of 1 g each were dissolved in 10 ml sterile distilled water and its 0.1 ml soil suspension was inoculated on Burk's solid medium (sucrose 20.0 g, K_2HPO_4 0.64 g, KH_2PO_4 0.16 g, $MgSO_4.7H_2O$ 0.20 g, NaCl 0.20 g, $CaSO_4.2H_2O$ 0.05 g, $Na_2MoO_4.2H_2O$ (0.05%) 5.0 ml, $FeSO_4.7H_2O$ (0.3%) 5.0 ml, 15 g agar 1,000 ml, pH=7) at 30˚C for 2 days.

Determination of nitrogen-fixation capacity of isolated bacteria using Nessler's reagent

The bacteria were cultured in Burk's liquid medium, shaken at 180 rpm, at 30°C. After 48 hours of incubation, the broth was centrifuged at 10,000 rpm for 2 min at 4°C, and the supernatant was reserved. NH_4^+ concentration was determined by the Nessler method. The reaction between Nessler's reagent and NH_3 can be shown as:

$2K_2[HgI_4] + NH_3 + 3KOH \rightarrow I\text{-}Hg\text{-}O\text{-}Hg\text{-}NH_2 + 7KI + 2H_2O$

After treatment with Nessler's reagent, amount of the sample develops a yellowish-brown colour. The colour intensity of solution corresponds to the amount of ammonia originally present. The standard curve was generated to determine the concentration of ammonia produced in the reaction.

Biological characterisation of selected bacteria

The isolates showing high nitrogen fixation, namely 6.2 and 8.2, were selected for further study. The morphology, colour, and size of the colonies on Burk's solid medium were recorded.

The effects of temperature, pH, carbon sources, and incubation time on the growth and development of the two selected strains were determined.

The bacteria were grown in Burk's broth, shaken at 180 rpm at temperatures ranging from 25-45˚C to study the effect of temperature on the growth and nitrogen-fixing capability of the soil isolates. The concentration of ammonia was determined colorimetrically with Nessler's reagent at the wavelength of 420 nm.

The influence of pH on the nitrogen-fixing activity of bacteria was studied by inoculating the bacteria in Burk's broth, shaking at 180 rpm, with pH ranging from 4.0 to 10.0. The concentration of ammonia was calculated by colourimetry with Nessler reagent at 420 nm.

The bacteria were cultured in liquid Burk's medium, shaking at 180 rpm, with different carbon sources (20 g/l): glucose, sucrose, maltose, and mannitol in order to study the effect of these on the growth and nitrogen-fixation of soil isolates. The concentration of ammonia was determined colorimetrically with Nessler's reagent at the wavelength of 420 nm.

To test the effect of incubation time on the nitrogen-fixation capacity of bacteria, the bacteria were cultured in liquid Burk's medium, shaking at 180 rpm, with the optimal temperature, pH, and carbon source conditions. Samples were taken at intervals of every 24 hours. The concentration of ammonia was calculated by colorimetry with Nessler's reagent at 420 nm.

Identification of selected bacteria

Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3') are used to amplify the 16S rRNA sequences from the DNA of the two selected bacterial strains. Aliquots (5 µl) of PCR products were electrophoresed in 1% agarose gel using standard electrophoresis procedures. 16S rRNA gene of selected isolates was sequenced by 1st BASE company (Malaysia). Finally, sequences of the bacteria with the highest ability to fix nitrogen were compared to sequences from GenBank based on the 16S rRNA sequences to ascertain the relationships between the endophytic strains [5] and phylogenetic trees were thereby constructed by the neighbour-joining method using MEGA software version 6.06 based on 1,000 bootstraps.

Results and discussion

Isolation of nitrogen-fixing bacteria

Nitrogen-fixing micro-organisms were isolated on Burk's medium. These isolates use atmospheric N_2 to synthesise their cell proteins. The cell proteins are then mineralised in soil after tcell death, thereby contributing towards nitrogen availability for plant growth. Burk's

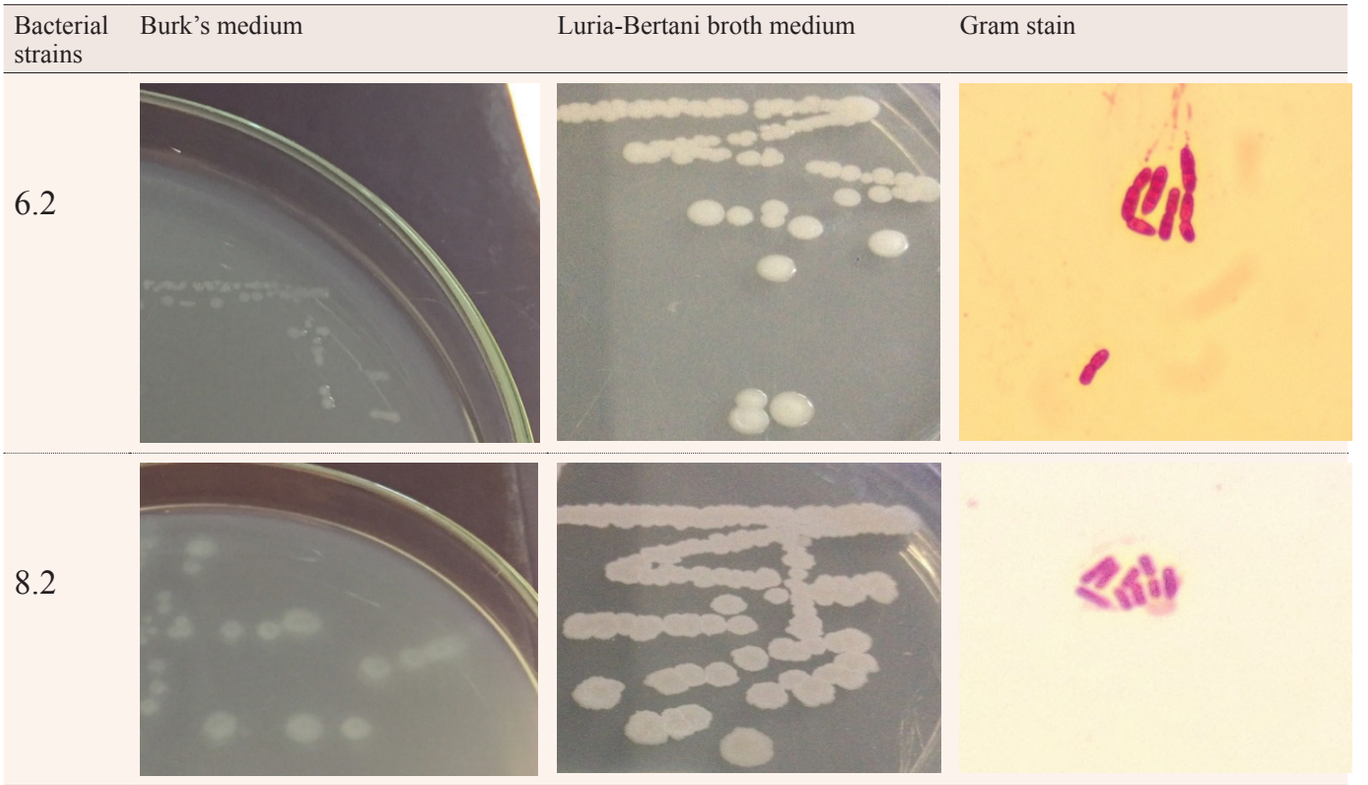

Fig. 1. Morphology and gram stain of the two isolated strains.

medium contains inorganic salts and carbohydrate sources but lacks a nitrogen source. Nitrogen-fixing bacteria can fix atmospheric nitrogen, thus they can live and grow in this nitrogen-free medium. Twenty-six isolates were obtained after two days of incubation on Burk's medium. Morphologically, most isolated bacterial colonies are a whitish cream colour, smooth, irregular, and shiny. Almost all the isolates were gram-positive and gram-negative by gram stain. Of the twenty-six isolated bacterial strains, 65.38% were rod-shaped and 34.62% spherical (Fig. 1).

Determination of nitrogen-fixing capacity of isolated bacteria

Construction of calibration curve: a standard curve was generated specifically to determine the concentration of ammonia in samples by means of colourimetry with Nessler's reagent at 420 nm. Using this method, we can evaluate the nitrogen-fixation capacity of the isolated bacteria.

We used the absolute values of blank and standards measured at a wavelength of 420 nm to generate a calibration curve, and measured absolute as a function of the ammona concentration (Fig. 2).

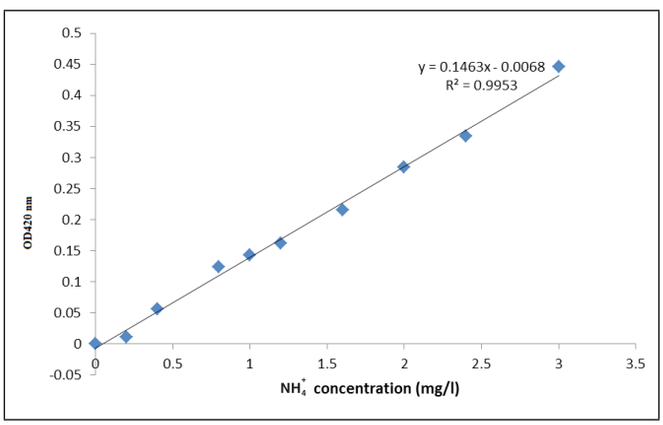

Fig. 2. Calibration curve for ammonia analysis of samples.

Sample analysis: the nitrogen-fixation ability of the twenty-six isolates was measured by Nessler's reagent, allowing us to understand any effective application of this organism by means of studying other attributes in the near future. The bacteria were cultured in Burk's liquid medium. After 2 days of incubation, aliquoted 5 ml of the broth was centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was reserved. The concentration of ammonia was determined colorimetrically with Nessler's reagent and the optical density was measured at 420 nm (Fig. 3).

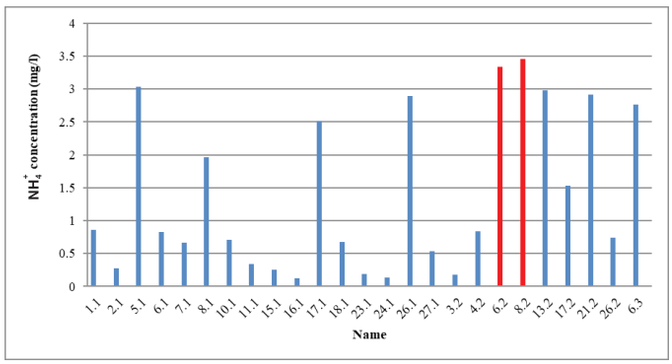

Fig. 3. Ammonium concentration in the medium released by isolates from Trau Quy soil samples.

All twenty-six isolates were able to fix nitrogen. The range of nitrogen-fixation ability ranged from 0.12 to 3.46 mg/l. The 8.2 strain could fix the highest amount of nitrogen (3.46 mg/l); the 16.1 strain fixed the least (0.12 mg/l). Of the twenty-five isolates, two fixed the highest amount of nitrogen, namely 8.2 (3.46 mg/l) and 6.2 (3.34 mg/l). This study shows that the isolates recovered from the soybean fields are of average standard in terms of their nitrogen-fixing potential in the laboratory condition.

Investigating the effect of the cultural conditions on selected bacteria

Investigation of the medium's cultural factors based on the growth and development of two selected strains provided useful information about cultural conditions for further research. The two selected strains (6.2 and 8.2) were cultured in Burk's liquid medium at different temperatures, pH, and with different carbon sources. Observation of the growth and development of these strains is summarised in Table 1.

Table 1. The influence of some environmental conditions on the nitrogen-fixing ability of the two selected strains.

Factor	Optimal Value	
	6.2	*8.2*
Temperature	30°C	35°C
pH	7.0	7.0
Carbon source	Sucrose	Sucrose
Incubation time	3 days	3 days

The results show that the 6.2 and 8.2 strains can fix nitrogen at temperatures of between 20 and 45°C. These strains have the optimal nitrogen capacity at 30-35°C. Both strains can use mantose, mannitol, sucrose, and glucose for growth. However, the maximum ammonia secretion of the two strains was obtained in the presence of sucrose. The maximum nitrogen-fixing ability of the two strains was obtained at pH 7 and after 3 days of incubation.

Physiological studies of the selected strains

The physiological activities of the strains were tested by means of Indole-3-acetic acid (IAA) production, methyl red (MR), acetoin production (Voges-Proskauer, V-P), citrate utilisation, catalase, cellulose hydrolysis, starch hydrolysis, and mobility. The biochemical characteristics of the two selected bacterial strains are shown in Table 2.

Table 2. The physiological activities of the 6.2 and 8.2 strains.

Name of the test	Response of strains	
	6.2	*8.2*
IAA production	+	-
MR reaction	+	+
V-P reaction	-	-
Citrate utilisation	-	+
Catalase	+	+
Cellulose hydrolysis	-	-
Starch hydrolysis	+	+
Mobility	+	+

+: positive result; -: negative result.

The two strains were mobility positive, catalase positive, starch hydrolysis positive, and MR positive, but V-P negative and cellulose hydrolysis negative. Strain 6.2 had the property of IAA production, but did not use citrate. In contrast, strain 8.2 can use citrate but cannot produce IAA.

Identification and phylogenetic analysis of selected strains

Molecular tools for the identification of soil bacteria and 16S rRNA gene analysis were used to understand the phylogenetic relationships. The phylogenetic tree was constructed by the neighbour-joining method using MEGA software version 6.06 based on 1,000 bootstraps. According to the genetic analysis, the amplified 16S rRNA sequence of the two selected strains produced 1.5 kb fragments. Homological searches of the 16S rRNA gene sequence of the selected strains in GenBank by means of BLAST revealed that strain 6.2 had sequence similar to *Pseudomonas* sp. and that strain 8.2 belongs to *Bacillus* sp. The phylogenetic trees were constructed as shown in Figs. 4 and 5, respectively. The position of the two selected strains and their relatedness to other bacteria were determined (Figs. 4 and 5).

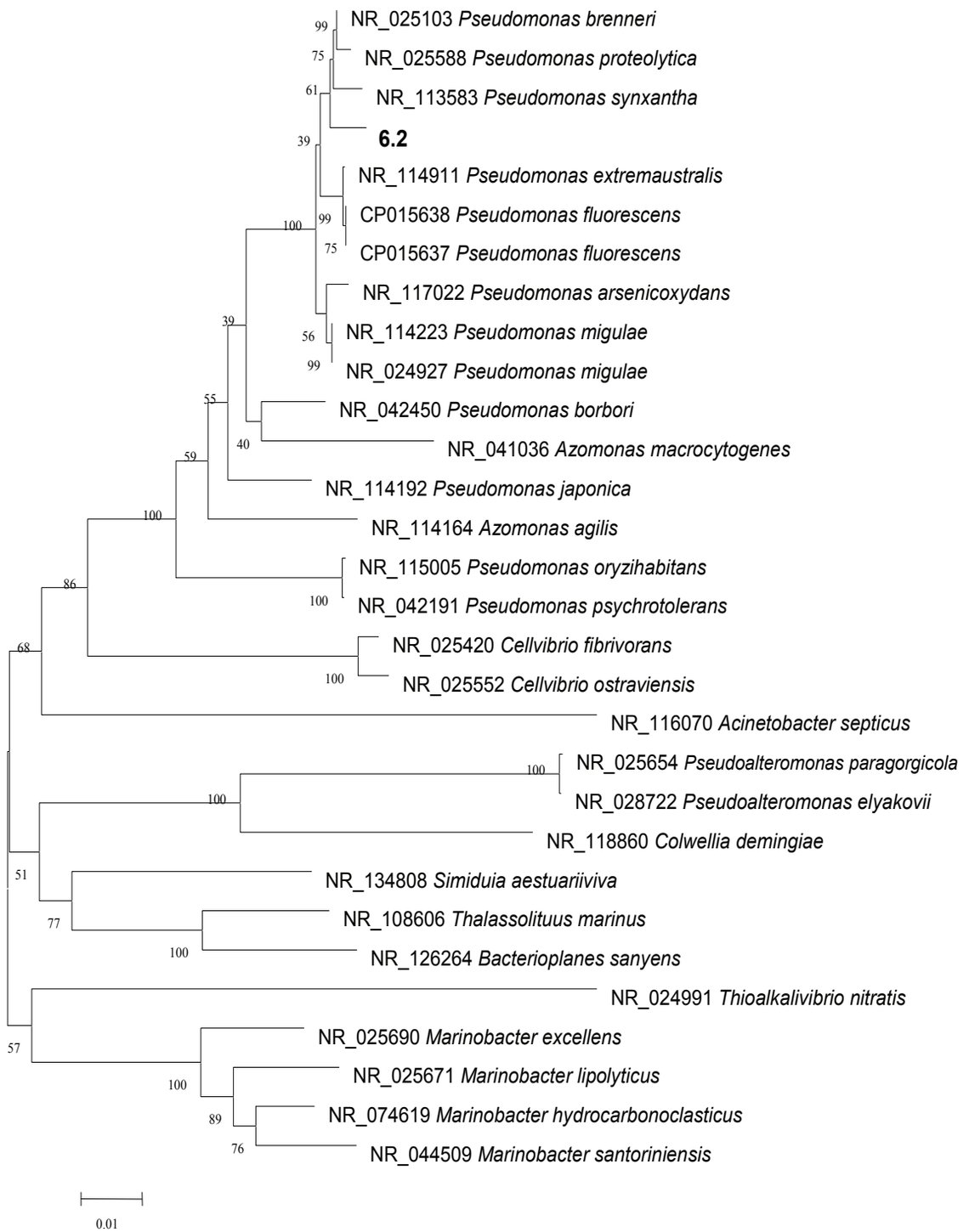

Fig. 4. Phylogenetic tree showing the relative position of the 6.2 strain using the neighbour-joining method of the complete 16S rRNA sequence.

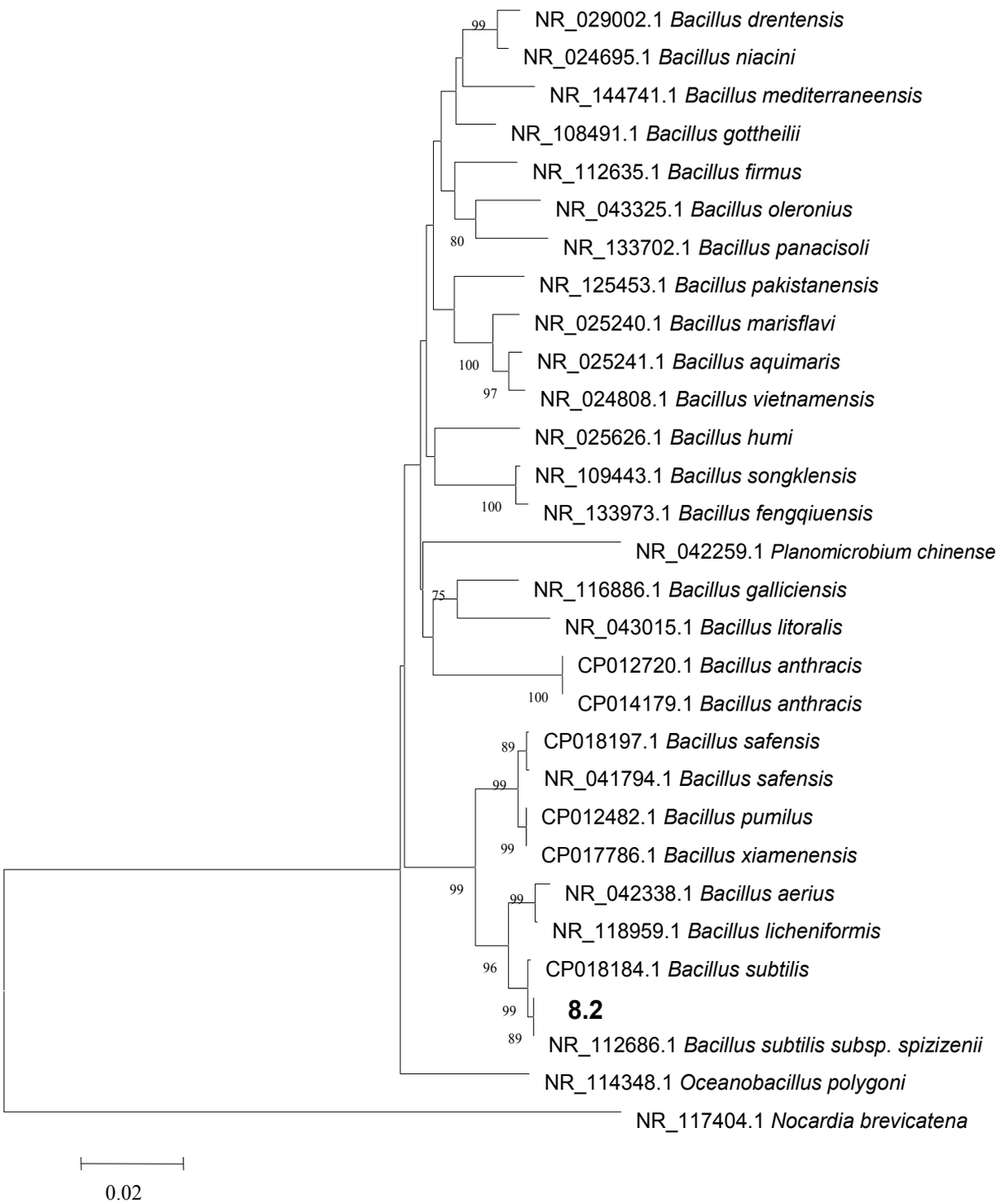

Fig. 5. Phylogenetic tree showing the relative position of the 8.2 strain using the neighbour-joining method of the complete 16S rRNA sequence.

According to *Bargey's Manual of Systemic Bacterilogy*, the biochemical test indicated that the characteristics presented by strains 6.2 and 8.2 are similar to *Pseudomonas* sp. and *Bacillus* sp., respectively. *Bacillus subtilis* sp. and *Pseudomonas* sp. are excellent rhizosphere-colonising bacteria [6]. Strains of *Pseudomonas* and *Bacillus* are among the most efficient plant growth-promoting bacteria and promote growth and yield of a variety of plants [4]. This result is consistent with the results of previous research that indicates that *Bacillus* sp. (*Bacillus subtilis* sp.) and *Pseudomonas* sp. fix nitrogen effectively. *Bacillus subtilis* strains AS-4, OSU-142, UPMB10, and *B. Pumilus* S1r1 [7] have high nitrogen-fixing ability. *Bacillus subtilis* AS-4 could be exploited as a soil inoculant and can be used for nitrogen fixation in soil with a high concentration of salt, which is eco-friendly and cost ineffective in the long run [8]. *B. subtilis* OSU-142 may be used as a substitute for costly N-fertilisers in chickpea production even in cold highland areas such as in Erzurum [9]. Inoculation with *B. pumilus* S1r1 and *B. subtilis* UPMB10 could significantly increase plant N uptake, dry biomass and ear yield of maize. *B. pumilus* S1r1 is able to fix up to 304 mg of fixed plant N_2 [7]. Recent studies have confirmed that some strains belonging to the genus *Pseudomonas sensu stricto*, such as *P. stutzeri* A1501, *P. stutzeri* DSM4166, *P. azotifigens* 6HT33bT, and *Pseudomonas* sp. K1 have the capability to fix nitrogen [10]. The strains CY4 (*P. koreensis*) and CN11 (*P. entomophila*) show *nif*H gene expression in sugarcane (the *nif*H gene has to do with nitrogen fixation). Inoculation of the strains may be an imminent development for biofertiliser application, for sustainable crop production, in reducing environmental pollution, and in biological agri-business [11]. The inoculation of red beets with the nitrogen-fixing bacteria *Pseudomonas putida* 23 increased the activity of nitrogen fixation in the rhizosphere of plants grown in meadow soil in the central part of the Oka River floodplain [12].

Conclusions

Twenty-five bacteria strains capable of nitrogen fixation were isolated. Of these, two strains (6.2 and 8.2) have the best capacity for nitrogen fixation.

The optimal pH and temperature for the growth and nitrogen fixation of the 6.2 and 8.2 strains are 7.0 and 30°C. Growth is best favoured in the presence of sucrose.

Homological searches of 16S rRNA gene sequence of the selected strains in GenBank by BLAST revealed that the 6.2 strain is similar to sequences of *Pseudomonas* sp., and that the 8.2 strain belongs to *Bacillus* sp.

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