# Isolation, Selection and Identification of Nitrogen Fixation Rhizospheric and Endophytic Bacteria from Maize (*Zea Mays* L.) Grown on the Soil

Thai Thanh Duoc[1*], Nguyen Huu Hiep[2]

Biotechnology R&D Institute, Can Tho University, Can Tho City, Vietnam
*Corresponding Author

Received:- 04 December 2021/ Revised:- 11 December 2021/ Accepted:- 18 December 2021/ Published: 31-12-2021


***Abstract***— *N-free medium (Nfb, LGI and Burks) were used to isolate bacteria having nitrogen fixation characteristics. Quantitative measurement by colorimetric methods helped to select the best isolates of nitrogen-fixing and IAA biosynthesis. The result of isolation was a total of 30 rhizopheric and 150 endophytic isolates having the both abilities. Sixty isolates having good biofertilizer activity were chosen to study. Isolates strains have both nitrogen fixation produced indole-3-acetic acid (IAA) in vitro. Six selected isolates had 16S rDNA sequences similarities with bacterial strains in data of GenBank with the values ranging between 94% and 99% of similarity in which includes strains of Enterobacter ludwigii DNL14, Enterobacter kobei DNT5, Bacillus pumilus DBT4, Klebsiella pneumoniae DNR5, Lactobacillus plantarum DLR6, Pseudomonas nitroreducens DND5. Especially, strains of endophytic isolates root maize origin as Klebsiella pneumoniae DNR5 biofertilizer activities synthesized average highest as NH4 (5.64 mg/L) and IAA (5.29mg/L) which can be exploited for enhancing soil fertility and plant growth.*

***Keywords— Bacillus sp, Endophytic, Klebsiella sp, Maize, Nitrogen fixing bacteria, Rhizosphere.***

## I. INTRODUCTION

Maize (*Zea may* L) is an important food crop in the world economy and Vietnam. Maize kernels are used as human food, animal feed and raw materials for industry. Maize needs to absorb a large amount of fertilizers to grow and develop [1]. However, chemical fertilizer applied to maize too much will cause environmental pollution, harmful effects on human and animal health. High chemical nitrogen fertilization increases investment costs, causes imbalances in natural ecosystems such as soil erosion, increases in nitrate concentrations in surface water, groundwater and discharges nitrous oxide during denitrification [2]. In today's agricultural production, improving soil fertility, reducing the amount of chemical fertilizers, increasing biological fertilizers and including N-fixing bacteria are necessary to develop sustainable agriculture. Nitrogen-fixing bacteria convert the free nitrogen of the biosphere to $NH_3$ by ATP energy and catalysis enzyme nitrogenase under normal conditions [3]. Plants absorb nitrogen to synthesize protein for growth and development.

Most microorganisms present in the rhizosphere play important roles in the growth and in the ecological fitness of their plant host [4]. Many studies showed that biofertilizer containing nitrogen fixing bacteria promoted plant growth and high yield could be achieved [5]. Several Plant Growth Promoting Rhizobia (PGPR) were isolated from maize grown in field soils such as *Azospirillum lipoferum*, *Bacillus polymixa*, *Pseudomonas putida* [6], [7]. Inoculated maize with *Azospirillum* sp. could support growth parameters including plant height. Nitrogen fixing bacteria such as Pseudomonas, Klebsiella, Enterrobacter, could use as growth promoters of maize plants [8]. Bacterial strains isolated from soil samples and maize roots include dominant bacterial genera identified were Klebsiella and Burksholderia potential as plant growth-promoting on maize plants [9]. Only inoculation of Azotobacter was done just a few hours before seed sowing. It helps on growth and yield of maize increasing 15 to 35% grain yield over non-inoculated treatments [10]. The results showed that strain AC had the highest nitrogen and phosphatase activity and it helped increase plant biomass up to 39%. In Vietnam, the application of PGPR for maize grown in the field was limited [11].

Dong Thap is a province in the Mekong River Delta, within the limit of 10°07'-10°58' North latitude and 105°12'-105°56' East. The area has alluvial soil with a pH range of 5.76-7.02 and freshwater where crops grow well. This province has a large maize growing area of 4,800 hectares, identified as the second-largest maize area in the Mekong Delta. However, Farmers often provide maize with a big amount of chemical fertilizers (180 kg N+135 kg P2O5+90 kg K2O/1ha) to get high grain yield (6-8 tons/ha). The fields must be irrigated, sprayed with herbicides, pesticides and especially urea with high prices, so investment costs are high and incomes are low.

The application of native, adapted microorganisms might improve yields by direct plant growth promotion and increasing grain yield, decreasing cost in maize cultivation in order to enhance income for the farmers. The aims of this study were (i) isolation and characterization of rhizospheric and endophytic bacteria (ii) studying characteristic such as nitrogen fixation and IAA production, (iii) the genetic diversity of isolated strains from maize plant and soil was evaluated in order to identify an efficient growth promotion strains that can be also improve the growth of maize plant as biofertilizer.

## II. MATERIALS AND METHODS

### 2.1 Materials

Maize rhizosphere soil samples and plant samples of maize were collected from in 8 sites villages Tan Thuan Tay, Tinh Thoi, Tan Binh Thuong, Tan Long, Bình Thanh, Binh Hoa, Long Khanh, Tan Thuan) of four as Lap Vo, Cao Lanh, Thanh Binh, Hong Ngu districts, Dong Thap province, Vietnam (Figure 1) from 10°34'12" to 10° 80'83" East and 105°28'74'' to 105°67'48" North. The maize plants were sampled at the stage plant growth stage from 30 to 45 days from the fields.

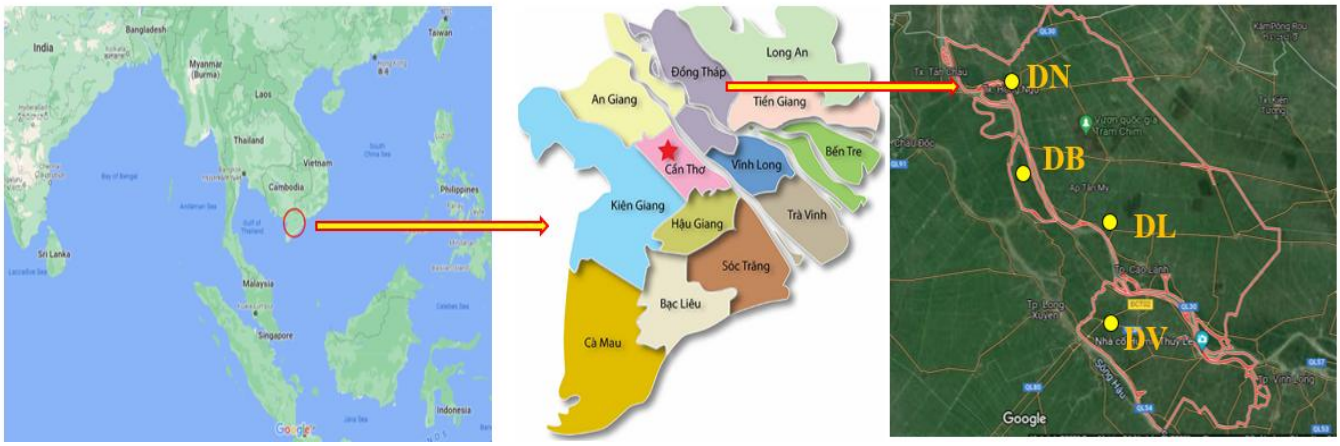


**FIGURE 1: The geographic map and location of maize samples and soil were collected at the these sites four districts, Dong thap Province**

**** Note* district of s*ymbol DL (Cao Lanh), DB (Thanh Binh), DV (Lap Vo) and DN (Hong Ngu)***

### 2.2 Methods

#### 2.2.1 Sample collect operation

Samples were taken whole plant with leaf, stem, root (10-20 cm depth) together with soil which around maize plants roots. Collected only samples that were free from pests and diseases. Each site selected 5 maize (4 trees at the base and 1 tree in the middle of the field). Samples were obtained whole plant after that soil rhizosphere was separated for further experiments. Lightly separating muddy soil around the maize roots into plastic bags, labeled (approximately 500 g/sample). Samples were kept in 18°C plastic box before tranferred to laboratory in Can Tho University. Rhizosphere soil around maize plants and leaf, stem, root were of maize plant will be used in an experiment, kept in the refrigerator (5°C), and brought for isolation.

#### 2.2.2 Isolation and culture

Weighed 10 g of soil samples, added 90 mL of sterile distilled water, put into sterile flasks, samples were stirred by magnetic stirrer for 2 hours, let stand for 1 hour, then diluted into decimal range $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$… Pipetted 50 µl of samples (in each concentration) using a micropipette, dripped on agar plates containing medium without nitrogen mineral (each concentration 3 plates). There are three different types of isolation media: Nfb [12], Burks N-free [13] and LGI [14]. Using a

sterile glass rod spread the sample drops on the surface of medium, covered the plate and standed for a few minutes then turning the disk down, incubated cultural plates at 30$^{o}$C in incubator.

Samples were obtained whole plant after that soil rhizosphere and separated for further experiments. Maize roots were washed with tap water to remove attached clay; maize leaf, stem and root were cut separately. Subsequently, the stems and roots were immersed in 70% ethanol in 3 min, washed with fresh sodium hypochlorite solution (2.5% available CT) for 5 min, rinsed with 70% ethanol for 30 s and finally washed five times with sterile distilled water. To confirm that the sterilization process was successful, the aliquots of the sterile distilled water used in the final rinse were set on tryptone-yeast extract-glucose agar medium plates. Bacterial growth were examined after incubation at 28$^{o}$C for 3 days. Maize leafs, stems and roots samples that were not contaminated as detected by culture-dependent sterility test were used for further analysis. Samples (leafs or stems or roots) were cut to 1-2 cm pieces and macerated with a sterile mortar and pestle; tissue extracts were then serially (tenfold dilution) in sterile water, 200 µl-aliquot samples were used to inoculate in (in triplicate) Nitrogen-free semisolid Nfb, LGI, Burks in 5 mL tubes. After 48-72 h of incubation, bacteria growing in tubes as a white or yellow pellicle at a depth of 1 to 4 mm were streaked on Nfb, LGI, Burks agar plates, cultures were streaked on media to obtain single colonies.

Bacterial colonies were differentiated on the basis of colony morphology and pigmentation. This isolation process carries out in shifts of the agar-based culture medium to the agar-based subculture medium until monocultures were obtained. Monocultures were cultured on the agar-based culture medium slant in the test-tube (12 mL) and incubated at 30$^{o}$C for 4 days following by stored 10$^{o}$C in refrigerator.

### 2.2.3 Colony Characteristic and Microscopic Examination

The characteristics of colony such as size, color, shape etc. were presented in each group, cell morphologies of the isolates were observed using an optical microscope.

### 2.2.4 Screening for Biofertilizer Activities

Nitrogen-fixing bacteria can thrive on cultural medium without nitrogen mineral due to their ability to synthesize ammonium from atmosphere nitrogen. The bacterial strains with capability of growing well were selected and subcultured in liquid Nfb or LGI or Burks medium to measure levels of ammonium in cultural medium by Indophenol Blue method [15] after 2,4 and 6 day inoculation (DAI).

The qualitative detection of indole-3-acetic acid (IAA) production was carried out based on the colorimetric method [16]. Precultures were grown in Nfb or LGI or Burks's N free (100 mL) with 100 mg/L tryptophan in 250mL-flask at 30$^{o}$C on a rotary shaker at 100 rpm and samples were taken from at 2, 4 and 6 DAI, cell free supernatants were mixed 2:1 with Salkowki reagent (0.01 M FeCl3 in 35% perchloric acid) and incubated in the dark for 20 min at RT. IAA-containing solutions were indicated by reddish color with an absorption peak at 530 nm on Genesys 10uv Thermo Scientific spectrophotometer.

### 2.2.5 16S rDNA Gene Amplification and Sequencing

Bacterial DNA was isolated following published protocols [17]**.** Amplification of 16S rDNA of rhizosphere soil bacteria by PCR was carried out using the universal primers with primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') [18] and 1492R (5'- TACGGTTACCTTGTTACGACTT-3'). [19] Cyclying condition were as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1.5 min, and a final extension of 5 min for 72°C.

Amplification of 16S rDNA of endophytic bacteria by PCR was carried out using primers:

p515FPL (5'-GTGCCAGCAGCCGCGGTAA- 3') [20], p13B (5'-AGGCCCGGGAACGTATTCAC-3',

PCR-1 5' AGTTTGATCCTGGCTCAGGA-3') [21]. The thermocycling profide was carried out with an initial denaturation at 94$^{o}$C (3 min) fol owed by 30 cycles of denaturation at 94$^{o}$C (1 min), annealing at 57$^{o}$C (1 min), extension at 72$^{o}$C (2 min) and a final polymerization step 72$^{o}$C (4 min) in C1000 Thermal Cycler (Bio-Rad). Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures.

Partial 16S rRNA genes of selected isolates in each site were sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by way

BLAST. Among the best isolates (high ability of nitrogen fixation, phosphate solubilization and IAA synthesis) of 8 sites, 6 isolates were chosen to sequence and were compared to results with sequences of GenBank based on partial 16S rRNA sequences to show relationships between endophytic and rhizosphere strains and phylogenetic tree were constructed by the neighbor-joining method using the MEGA X software version [22] based on 1000 boot straps.

## III. RESULTS AND DISCUSSION

### 3.1 Bacterial Isolation and Colony Characteristic

From 40 maize plant samples and 8 rhizosphere soil samples which were collected from 4 districts in Dong Thap provinces on 3 kinds of medium (Nfb, LGI and Burks). A total of 180 bacterial strains were isolated (Table 1). Seventy bacterial strains were isolated from Nfb medium, 70 ones on LGI and 40 ones on Burks medium. Thirty three strains were isolated from roots (18.33%), 51 isolates from stems (28.33%), 66 isolates from leaf (36.67%) and 30 isolates from soil (16.67%)

**TABLE 1**
**SUMMARY OF BACTERIAL STRAINS RHIZOSPHERE SOIL AND ENDOPHYTE ISOLATED FROM MAIZ**

| No | Site (districts) | Samples | | Number of strains | Symbol of the sample group |
|---|---|---|---|---|---|
| | | Maize | Soil | | |
| 1 | Cao Lanh | 10 | 2 | 40 | DL |
| 2 | Thanh Binh | 10 | 2 | 49 | DB |
| 3 | Lap Vo | 10 | 2 | 40 | BV |
| 4 | Hong Ngu | 10 | 2 | 51 | DN |
| **Total** | | **40** | **8** | **180** | |

On semi-solid, isolates of endophytic bacteria all grew and developed under microaerobic conditions and formed an opaque white pellicle far from the medium surface 1- 4 mm after 48 hours).They developed very well on these media from 24 h at 30$^{o}$C. Colonies had various colors on 3 kinds of medium. Colors of colony as dark white or light white 147/180 colonies, light yellow, pink. Their colonies had round-shape 167/180 colonies, entire 145/180 colonies, emerge 122/180 colonies and glossy surfaced 155/180 colonies, raised on medium (Figure 2). Diameter size of these colonies varied from 0.3 to 4.0 mm after 48 hours (Figure 3). The results of the study demonstrated that endogenous bacteria on maize plants all form opaque white pellicle rings in the culture medium. Nfb medium, after culturing the bacteria for 4 DAI, the growth of endophytic bacteria causes the medium to completely turn blue, light or dark depending on the strain [23].

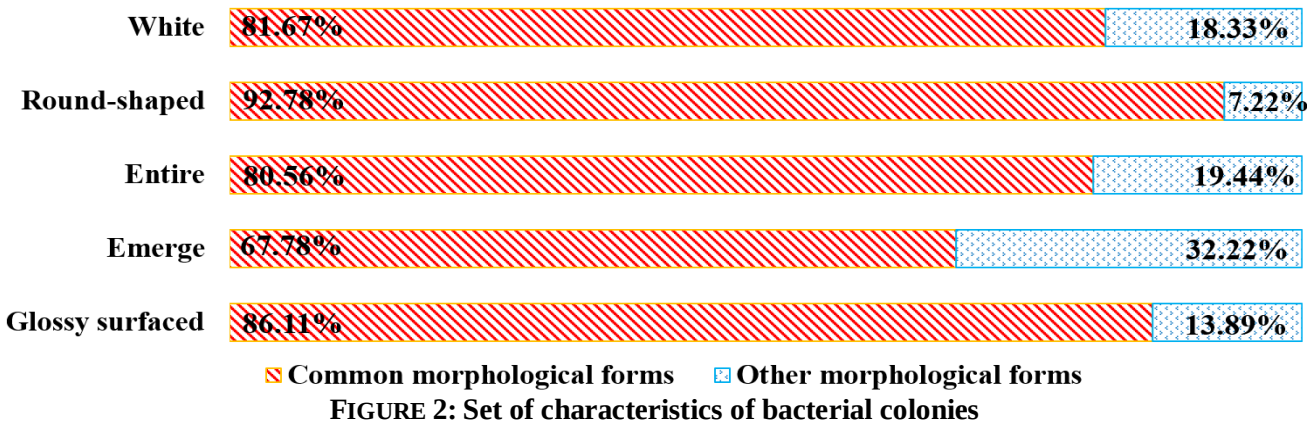


**FIGURE 2: Set of characteristics of bacterial colonies**

The cells were observed on 3 kinds of medium had short rod, few of long rod and round (Table 2), most of them have motility and Gram- negative by Gram stain.

**TABLE 2**
**SET OF CHARACTERISTICS OF BACTERIAL CELLS**

| No | Characteristics of cell bacterial | Maize sample | Rate (%) |
|---|---|---|---|
| 1 | Cell shape | Rod-shaped | 76.11 |
| | | Round | 23.89 |
| 2 | Motile | Fast | 42.22 |
| | | Slow | 32.78 |
| | | Not motile | 25.00 |
| 3 | Gram reaction | Positive | 37.22 |
| | | Negative | 62.78 |

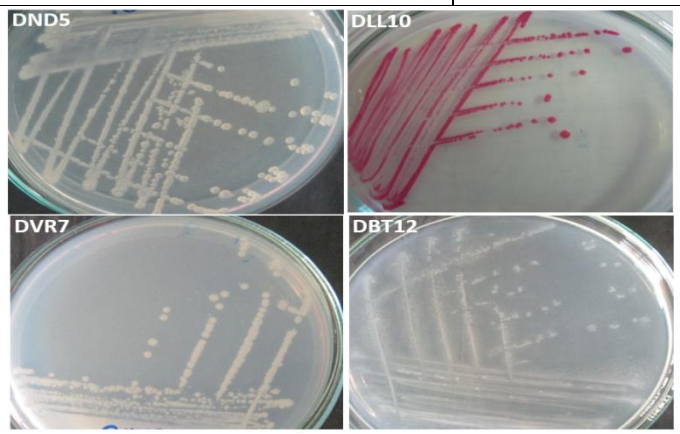


**FIGURE 3: The colonies of several rhizosphere soil and endophyte isolate from maize**

The results of previous studies reported that rhizosphere soil and endophyte include genus *Azospirillum*, *Pseudomonas*, *Burksholderia*, *Herbaspirillum*, *Gluconacetobacter*, *Enterobacter*, *Klebsiella*,... of Gram-negative, Bacillus of Gram-positive. The cells were observed had short rod, few of long rod *(Bacillus)* and round, have flagella, most of them are motile. These bacteria have also been investigated for their ability to fix nitrogen, dissolve insoluble phosphorus, decompose potassium and some other benefits to plants [24].

### 3.2 Screening for Biofertilizer Activities

#### 3.2.1 Testing the $NH_4^+$ synthetic ability of isolated bacterial strains

Among 180 isolated strains, 60 isolates grew strongly on Nfb, LGI, Burks's N-free agar after 24, 48 and 72 hours at 30°C. Include, 20 isolated bacterial strains from Nfb medium, 20 isolated bacterial strains from LGI medium, 20 isolated bacterial strains from Burks medium. The result of the study on $NH_4^+$ synthesis ability of bacterial strains from different area ecosystem habitats soil and sample maize on 3 type Nfb, LGI, Burks was presented in Table 2. All 60 isolates have produced $NH_4^+$ and all of them can be amount of $NH_4^+$ produced from different habitats varied significantly over the time period and was sigNfbicantly different when compared with each other. The $NH_4^+$ producing capacity of bacterial strains was synthesized very early even after one day of incubation.

Based on the survey results on the ability to synthesize $NH_4^+$ of 20 bacterial strains isolated on Nfb medium, indicating that all 20 bacterial strains on Nfb medium were capable of synthesizing $NH_4^+$ (Table 3). By the 2nd day, the $NH_4^+$ content of all bacterial strains ranged from 0.96-3.61 mg/L. Only 3 DNR5, DLD1, DBT5 bacterial strains had the synthesized $NH_4^+$ content higher than 3 mg/L, respectively 3.61 mg/L: 3.41 mg/L: 3.32 mg/L, and had a statistically significantly difference from other strains. Bacterial strains produced highly $NH_4^+$ content at the 4th day and reached the highest level of DNR5 (7.52 mg/L). By the 6th day, the $NH_4^+$ content of all bacterial strains ranged from 0.88 to 5.78 mg/L. The average results of $NH_4^+$ concentration synthesized over days 2, 4 and 6 of 20 bacterial strains in Nfb environment ranged from 0.88 to 5.64 mg/L. Two strains, DNR5 (5.64 mg/L) and DBT4 (4.90 mg/L), which had high amount of $NH_4^+$ were selected for gene sequencing.

**TABLE 3**
**TOTAL $NH_4^+$ CONCENTRATION IN NFB LIQUID MEDIUM OF 20 ISOLATES WITHIN SIX DAYS OF INCUBATION (n=3 AND STANDARD DEVIATION)**

| No | Sample | Bacterial isolates | Day 2 (mg/L) | Day 4 (mg/L) | Day 6 (mg/L) | Average value (mg/L) | Site |
|---|---|---|---|---|---|---|---|
| 1 | Leaf | DLL1 | 2.02 e | 1.99 h | 2.18 d | 2.06 | Cao Lanh |
| 2 | | DBL7 | 1.53 h | 1.73 i | 0.93 i | 1.40 | Thanh Binh |
| 3 | | DVL2 | 1.31 i | 3.53 d | 1.72 g | 2.19 | Lap Vo |
| 4 | | DNL3 | 1.36 i | 1.58 i | 1.02 i | 1.32 | Hong Ngu |
| 5 | | DNL5 | 0.96 j | 0.60 k | 1.07 hi | 0.88 | Hong Ngu |
| 6 | | DNL6 | 1.59 h | 1.98 h | 1.26 h | 1.61 | Hong Ngu |
| 7 | Stem | DLT1 | 1.65 h | 2.62 fg | 2.08 de | 2.12 | Cao Lanh |
| 8 | | DBT4 | 2.24 d | 7.43 a | 5.05 b | 4.90 | Thanh Binh |
| 9 | | DBT5 | 3.32 b | 3.84 c | 1.90 efg | 3.02 | Thanh Binh |
| 10 | | DVT1 | 1.89 efg | 2.01 h | 2.14 d | 2.02 | Lap Vo |
| 11 | | DNT3 | 1.52 h | 1.69 i | 1.26 h | 1.49 | Hong Ngu |
| 12 | Root | DLR1 | 0.98 j | 1.15 j | 0.88 i | 1.00 | Cao Lanh |
| 13 | | DBR2 | 1.61 h | 2.64 fg | 2.20 d | 2.15 | Thanh Binh |
| 14 | | DVR2 | 2.62 c | 1.69 i | 2.03 def | 2.11 | Lap Vo |
| 15 | | DNR5 | 3.61 a | 7.52 a | 5.78 a | 5.64 | Hong Ngu |
| 16 | | DNR6 | 1.99 ef | 2.55 g | 2.18 d | 2.24 | Hong Ngu |
| 17 | | DNR7 | 1.86 fg | 2.78 ef | 1.82 g | 2.15 | Hong Ngu |
| 18 | Soil | DLD1 | 3.41 b | 4.61 b | 2.79 c | 3.60 | Cao Lanh |
| 19 | | DVD1 | 1.82 f | 2.87 e | 1.82 g | 2.17 | Lap Vo |
| 20 | | DND1 | 2.02 g | 2.44 g | 1.84 fg | 2.10 | Hong Ngu |
| **CV (%)** | | | **3.29** | **3.36** | **4.42** | | |

***Means within a column followed by the same letter/s are not significantly different at p < 0.01***

Twenty bacterial strains isolated on LGI medium were able to synthesize $NH4^+$ but lower than bacterial strains isolated on Nfb medium (Table 4). On the 2nd day, the $NH_4^+$ content of all bacterial strains ranged from 0.54 to 3.78 mg/L. On the 4th day, the $NH_4^+$ content of all bacterial strains ranged from 0.46 to 6.00 mg/L. In those strains, 2 strains DNL14 (6.00 mg/L) and DNT5 (5.07 mg/L), had a high $NH_4^+$ content and a statistically significant difference from the rest of the strains. Similarly, DNL14 and DNT5 with a high $NH_4^+$ content had a statistically significant difference from the rest of bacterial strains at the 6 day. The average results of $NH4^+$ content synthesized over days 2, 4, and 6 of 20 bacterial strains in LGI medium ranged from 0.78 to 4.47 mg/L. Selecting 2 strains, DNL14 (4.47 mg/L) and DNT5 (4.23 mg/L) with a high amount of $NH_4^+$, were selected for gene sequencing.

**TABLE 4**
**TOTAL $NH_4^+$ CONCENTRATION IN LGI LIQUID MEDIUM OF 20 ISOLATES WITHIN SIX DAYS OF INCUBATION (n =3 AND STANDARD DEVIATION)**

| No | Sample | Bacterial isolates | Day 2 (mg/L) | Day 4 (mg/L) | Day 6 (mg/L) | Average value (mg/L) | Site |
|---|---|---|---|---|---|---|---|
| 1 | Leaf | DLL10 | 1.10 ghi | 4.87 c | 2.21 f | 2.73 | Cao Lanh |
| 2 | | DBL15 | 1.26 fg | 1.26 m | 0.73 k | 1.08 | Thanh Binh |
| 3 | | DBL18 | 0.99 hi | 3.31 e | 1.72 g | 2.01 | Thanh Binh |
| 4 | | DVL9 | 2.13 d | 1.98 ij | 2.23 ef | 2.11 | Lap Vo |
| 5 | | DVL11 | 0.96 hi | 2.64 g | 2.43 de | 2.01 | Lap Vo |
| 6 | | DNL12 | 0.72 jk | 3.05 f | 2.70 c | 2.16 | Hong Ngu |
| 7 | | DNL14 | 2.46 b | 6.00 a | 4.93 a | 4.47 | Hong Ngu |
| 8 | | DNL17 | 2.19 cd | 4.31 d | 3.11 b | 3.21 | Hong Ngu |
| 9 | Stem | DLT8 | 0.67 k | 1.57 l | 0.72 k | 0.99 | Cao Lanh |
| 10 | | DBT11 | 0.54 k | 0.75 n | 1.20 ij | 0.83 | Thanh Binh |
| 11 | | DVT4 | 3.78 a | 3.47 e | 2.24 ef | 3.16 | Lap Vo |
| 12 | | DNT5 | 2.34 bc | 5.27 b | 5.07 a | 4.23 | Hong Ngu |
| 13 | | DNT9 | 0.74 jk | 0.46 o | 1.13 j | 0.78 | Hong Ngu |
| 14 | Root | DBR4 | 2.43 b | 4.23 d | 2.56 cd | 3.07 | Thanh Binh |
| 15 | | DBR6 | 1.05 hi | 1.83 jk | 1.48 h | 1.45 | Thanh Binh |
| 16 | | DVR5 | 1.35 f | 1.68 kl | 1.46 h | 1.49 | Lap Vo |
| 17 | | DNR8 | 1.81 e | 2.43 h | 1.32 hij | 1.85 | Hong Ngu |
| 18 | | DNR10 | 1.67 e | 2.01 ij | 1.35 hi | 1.68 | Hong Ngu |
| 19 | Soil | DVD4 | 0.92 ij | 2.06 i | 1.25 ij | 1.41 | Lap Vo |
| 20 | | DND7 | 1.16 fgh | 1.91 ij | 1.20 ij | 1.42 | Hong Ngu |
| **CV (%)** | | | **5.98** | **3.05** | **4.41** | | |

***Means within a column followed by the same letter/s are not significantly different at p < 0.01.***

Twenty bacterial strains isolated in Burks environment were able to synthesize $NH_4^+$ with different amounts. On the 2nd day, the 2nd day, the $NH_4^+$ content of all bacterial strains ranged from 0.40 to 4.25 mg/L (Table 5). On the 2nd day, the $NH_4^+$content of all bacterial strains ranged from 1.03 to 5.76 mg/L. In those, two lines DLR6 (5.76 mg/L) and DND5 (4.87 mg/L) had a high $NH_4^+$ content and had a statistically significant difference from the rest of the strains. Similarly, DLR6 (5.24 mg/L) and DND5 (3.57 mg/L) with a high $NH_4^+$ content had a statistically significant difference from the rest of the strains at the 6th day. The average results of the amount of $NH_4^+$ concentration synthesized over days 2, 4, and 6 of 20 bacterial strains in Burks medium ranged from 0.71 to 4.98 mg/L. Selecting two strains, DLR6 (4.98 mg/L) and DND5 (4.02 mg/L) with produced high amount of $NH_4^+$, were selected for gene sequencing.

**TABLE 5**
**TOTAL $NH_4^+$ CONCENTRATION IN BURKS LIQUID MEDIUM OF 20 ISOLATES WITHIN SIX DAYS OF INCUBATION (n =3 AND STANDARD DEVIATION)**

| No | Sample | Bacterial isolates | Day 2 (mg/L) | Day 4 (mg/L) | Day 6 (mg/L) | Average value (mg/L) | Site |
|---|---|---|---|---|---|---|---|
| 1 | Leaf | DLL15 | 2.46 f | 3.93 c | 3.16 cd | 3.19 | Cao Lanh |
| 2 | | DBL20 | 0.77 k | 1.30 k | 0.88 l | 0.98 | Thanh Binh |
| 3 | | DNL18 | 0.99 j | 1.09 l | 0.76 lm | 0.95 | Hong Ngu |
| 4 | | DNL20 | 0.75 k | 1.18 kl | 0.83 lm | 0.92 | Hong Ngu |
| 5 | Stem | DBT14 | 3.82 c | 3.25 f | 2.99 e | 3.35 | Thanh Binh |
| 6 | | DBT15 | 0.40 m | 1.03 l | 0.69 m | 0.71 | Hong Ngu |
| 7 | | DVT9 | 4.25 a | 3.29 ef | 2.14 h | 3.23 | Hong Ngu |
| 8 | | DVT10 | 0.62 l | 1.90 i | 1.36 j | 1.29 | Lap Vo |
| 9 | | DNT10 | 3.65 d | 3.67 d | 2.21 gh | 3.18 | Hong Ngu |
| 10 | Root | DLR6 | 3.95 b | 5.76 a | 5.24 a | 4.98 | Cao Lanh |
| 11 | | DBR8 | 0.60 l | 1.59 j | 1.24 jk | 1.14 | Thanh Binh |
| 12 | | DVR7 | 3.24 e | 3.43 e | 3.25 c | 3.31 | Lap Vo |
| 13 | | DNR1 | 0.97 j | 1.28 k | 0.80 lm | 1.02 | Hong Ngu |
| 14 | | DNR2 | 3.72 cd | 2.99 g | 2.31 gh | 3.01 | Hong Ngu |
| 15 | | DNR3 | 3.65 d | 2.85 gh | 2.71 f | 3.07 | Hong Ngu |
| 16 | Soil | DBD3 | 1.15 i | 1.49 j | 1.10 k | 1.25 | Thanh Binh |
| 17 | | DBD7 | 1.83 g | 3.85 c | 3.04 de | 2.91 | Thanh Binh |
| 18 | | DBD8 | 1.18 i | 2.80 h | 1.74 i | 1.91 | Thanh Binh |
| 19 | | DND4 | 1.61 h | 2.23 i | 2.33 g | 2.06 | Hong Ngu |
| 20 | | DND5 | 3.61 d | 4.87 b | 3.57 b | 4.02 | Hong Ngu |
| **CV (%)** | | | **2.51** | **2,62** | **3,64** | | |

***Means within a column followed by the same letter/s are not significantly different at p < 0.01.***

The results of surveying 60 endogenous bacterial strains of the roots, stems, leaves of maize, and the soil of the maize rhizosphere that synthesized the amounts of $NH_4^+$ was similar to the results of previous studies in the world [25][26]. Endogenous bacteria at roots had a higher fixing activity than endogenous bacteria in stems and leaves, and the nitrogen fixation capacity of endophytic bacteria strains in the different plant species was different. Isolated of high nitrogen fixation bacteria strains from alkaline soil (PH=7.45-8.22), amounts of the synthesized organic substances ranged from 0.225-1.1%. *Pseudomonas* sp VS2 was 12.02 ppm/mL after 12 days of incubation, Paenibacillus sp VS3 was 10,635 ppm/mL after 9 days of incubation [27]. Bacteria from the maize soil with the highest ability to synthesize $NH_4^+$ on the 3rd day was 9.30 mg/L, and gradually decreased to 5.70 mg/L on the 5th day [28].

The general trend of bacteria isolated on all 3 medium Nfb, LGI, Burks was that the amount of $NH_4^+$ synthesized gradually increased to the $4^{th}$ day and gradually decreased to the $6^{th}$ day. Because of bacteria growing and increasing biomass over time, the amount of $NH_4^+$ synthesized gradually increased. On the 4th day, the amount of $NH_4^+$ synthesized went high. When the amount of $NH_4^+$ in the medium exceeded the limitation, it inhibited reversedly the bacteria, then the bacteria would use the available $NH_4^+$ in the medium and do not synthesize any more $NH_4^+$, so the amount of $NH_4^+$ surveyed on the $6^{th}$ day decreased [29].

The second reason is that the nutrient content in the medium was gradually depleted over time, which inhibited the growth of bacterial strains and inhibited the synthesis of $NH_4^+$. Isolated nitrogen-fixing bacteria from rhizosphere soil and soybean plant parts in Daknong province [24]. Bacteria strains that were grown on nitrogen-free Burks medium and NBRIP liquid media including strains of *Bacillus subtilis*, *Acinetobacter lwoffii*, *Agrobacterium tumefaciens* synthesized $NH_4^+$ at the highest 3.86 mg/L on the 2nd day after inoculated. Bacterial strains isolated from the rice rhizosphere in Tra Vinh grown on Burks medium synthesized $NH_4^+$ at the highest 4.65 mg/L on the 4th day after inoculation [30]. Similarly, isolated nitrogen-fixing bacteria strains from oil-contaminated soil at Tuticorin harbor. *Azotobacter chroococcum* strains with nitrogen-fixing capacity reached 4.2 mg/L on the 4th day after inoculation [31]. The research result of nitrogen-fixing bacterial strains were isolated from rice rhizosphere soils in the Mekong Delta on nitrogen-free Burks medium. The bacteria strains synthesized the

average $NH_4^+$ over days 2, 4, and 6 including *Stenotrophomonas maltophilia* was 1.76 mg/L, *Serratia marcescens* was 1.87 mg/L, *Bacillus megaterium* was 2.21mg/L, *Ideonella* sp. was 3.52 mg/L. [32].

### 3.2.2 Test of IAA-biosynthesis of bacteria

All 60 isolates have produced IAA from different habitats varied. The IAA producing capacity of bacterial strains was synthesized very early even after one day of incubation. The highest amount of IAA was observed after 6 DAI of incubation. The synthesized IAA content of the bacterial strains varied largely from 0.18 mg/L to 32.64 mg/L mg/L (Table 6). 16/60 isolates had the high IAA biosynthesis than 19 mg/L as DND4 isolate (32.64 mg/L) from Burks medium, DND7 (28.20 mg/L) from LGI medium, DLL1 (27.33 mg/L) from Nfb medium.

**TABLE 6**
**CONCENTRATION OF SYNTHESIZED IAA PRODUCTION OF 60 ISOLATE IN LIQUID MEDIUM WITHIN 6 DAYS OF INCUBATION (n =3, STANDARD DEVIATION)**

| No | Isolate number were isolated from Nfb | | | Isolate number were isolated from LGI medium | | | Isolate number were isolated from Burks | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sample | Isolate | IAA (mg/L) | Sample | Isolate | IAA (mg/L) | Sample | Isolate | IAA (mg/L) |
| 1 | Leaf | DLL1 | 27.33 a | Leaf | DLL10 | 10.70 h | Leaf | DLL15 | 6.13 i |
| 2 | | DBL7 | 21.02 c | | DBL15 | 0.18 n | | DBL20 | 21.18 c |
| 3 | | DVL2 | 3.67 j | | DBL18 | 7.43 i | | DNL18 | 13.35 e |
| 4 | | DNL3 | 19.43 d | | DVL9 | 5.52 jk | | DNL20 | 1.44 l |
| 5 | | DNL5 | 21.28 c | | DVL11 | 6.39 ij | Stem | DBT14 | 16.13 d |
| 6 | | DNL6 | 2.57 k | | DNL12 | 4.32 kl | | DBT15 | 11.31 f |
| 7 | Stem | DLT1 | 1.41 l | | DNL14 | 20.76 d | | DVT9 | 1.70 l |
| 8 | | DBT4 | 19.89 d | | DNL17 | 4.42 kl | | DVT10 | 1.18 l |
| 9 | | DBT5 | 4.81 i | Stem | DLT8 | 3.80 l | | DNT10 | 9.60 g |
| 10 | | DVT1 | 2.35 k | | DBT11 | 12.51 g | Root | DLR6 | 21.18 c |
| 11 | | DNT3 | 2.06 kl | | DVT4 | 5.84 j | | DBR8 | 4.45 j |
| 12 | Root | DLR1 | 5.71 h | | DNT5 | 22.73 c | | DVR7 | 6.42 i |
| 13 | | DBR2 | 18.62 e | | DNT9 | 26.59 b | | DNR1 | 7.46 h |
| 14 | | DVR2 | 18.14 e | Root | DBR4 | 19.17 e | | DNR2 | 2.70 k |
| 15 | | DNR5 | 25.29 b | | DBR6 | 27.01 ab | | DNR3 | 3.74 j |
| 16 | | DNR6 | 5.36 hi | | DVR5 | 5.78 j | Soil | DBD3 | 9.34 g |
| 17 | | DNR7 | 6.91 g | | DNR8 | 0.92 mn | | DBD7 | 7.56 h |
| 18 | Soil | DLD1 | 8.56 f | | DNR10 | 1.63 m | | DBD8 | 2.02 kl |
| 19 | | DVD1 | 7.43 g | Soil | DVD4 | 17.65 f | | DND4 | 32.64 a |
| 20 | | DND1 | 8.43 f | | DND7 | 28.20 a | | DND5 | 25.32 b |
| **CV (%)** | | | **2.62** | | | **5.03** | | | **4.24** |

***Means within a column followed by the same letter/s are not significantly different at p < 0.01***

Auxins play a cardinal role in coordination of many growth and behavioral processes in plant life cycles and are essential for plant body development [33]. In addition to endogenous IAA, plant growth is affected by a low amount of auxin outside of the plant from a IAA synthesis of microorganism. Moreover, several recent reports indicate that IAA can also be a signaling molecule in bacteria and therefore can have a direct effect on bacterial physiology [34]. These indigenous colonizers reside in almost all internal tissues/cells of plant ranging from tissues of the roots to stem, leaf, flower, fruit and seed [35]. The fact that bacteria use this phytohormone IAA to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms [36]. Different bacterial isolates biosynthesize IAA at differently quantities [37]. There are many studies on the ability to synthesize IAA of endophytic and rhizobacteria to help plant growth. The presence of endophytic and rhizosphere bacterial strains isolated from IAA-synthetic rice increased the

size and weight of rice roots [38]. IAA promote plant growth through the improved cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients [39]. Over 73% of the bacteria isolated from the rhizosphere are capable to synthesize IAA [40].

All strains of endophytic bacteria of root rice having IAA product. In which, there are 2 strains of *Pseudomonas* sp. RE1 and RE17 produced high amount of IAA. Using ELISA based studies in the presence of maize root exudates in growth chamber study, it was revealed that strain RE1 and RE17 inoculated into germinated maize kernels resulted in an increase in the amount of IAA produced from the roots by 2.8 pmol/mL and 3.4 pmol/mL respectively compared with the uninoculated control by 0.2 pmol/mL [41]. Strains Bacillus sp. (br1, br3, wr2) và Lactobacillus sp. (br2, mr2) were successfully isolated from wheat and maize rhizosphere. The concentration of IAA increased in medium with or without tryptophan Bacillus sp. to 60µg/mL after 120 min of inoculation [42]. The IAA synthesis capacity reached 24.8 mg/L at day 4 after inoculation. Four bacterial strains Ha21, Ha22, Ha23 and Ha30 synthesized high auxin including IAA and IAM, in which Ha22 was the highest at 66.3 mg/kg at normal conditions, resulting in maximum growth and yield of wheat [40]. Bacteria group of innate microbial consortium that inhabits the soil and the surfaces of all living things had the highest ability to synthesize IAA on the 3rd day was 48.45 mg/L, gradually decreased to the 6th day at 23.81 mg/L [28]. The results of the experiment proved that 60 strains of endophytic and rhizosphere bacteria of maize are capable of synthesizing IAA similar to previous studies. In which, 16/60 potential bacterial strains synthesize high amount of IAA.

### 3.3 Identification and construction of the phylogenetic tree bacteria having high ability of NH4+ and IAA biosynthesis.

Six good bacterial isolates: DNL14, DNT5, DBT4, DNR5, DLR6, DND5 were selected to PCR and sequencing. The result was presented in (Table 7).

**TABLE 7**
**PHYLOGENETIC AFFILIATION OF ISOLATES ON THE BASIS OF 16S rRNA GENES SEQUENCES BY USING BLAST PROGRAMME IN THE GENBANK DATABASE BASED ON SEQUENCE SIMILARITY**

| Sample | Symbol of isolate | Closest species relative | Gene length (nu) | Similarity (%) | NCBI Number |
|---|---|---|---|---|---|
| Leaf | DNL14 | *Enterobacter ludwigii strain AA1* | 2235 | 97 | MT613360.1 |
| Stem | DNT5 | *Enterobacter kobei strain BLR45* | 2150 | 97 | MW624688.1 |
| | DBT4 | *Bacillus pumilus strain NSB-10* | 2257 | 97 | KR010180.1 |
| Root | DNR5 | *Klebsiella pneumoniae strain BB-301* | 2230 | 99 | MN844878.1 |
| | DLR6 | *Lactobacillus plantarum* | 1428 | 94 | KJ690749.1 |
| Soil | DND5 | *Pseudomonas nitroreducens* | 2239 | 99 | KY292456.1 |

The result of identifying the nitrogen-fixing bacteria presented in table showed a total of 6 isolated bacteria strains, in which 5 strains were isolated from leaves, stem, maize root, and a strain isolated from maize root-soil. This shows that the ability to isolate many endogenous high nitrogen-fixing bacteria in maize will be higher than bacteria living in the maize root soil (Table 7). On the other hand, Six nitrogen-fixing bacteria strain isolated belong to 2 classes, including Gammaproteobacteria (4 species): *Enterobacter* (2 strains), *Pseudomonas, Klebsiella* and Bacilli (2 species) including *Bacillus* and *Lactobacillus.* They were classified to Bacilli (20%) and Gammaproteobacteria (80%) (Figure 4). This result demonstrates that there are a very high diversity of bacteria isolated from the maize, from the different positions in the root-soil and other parts of maize.

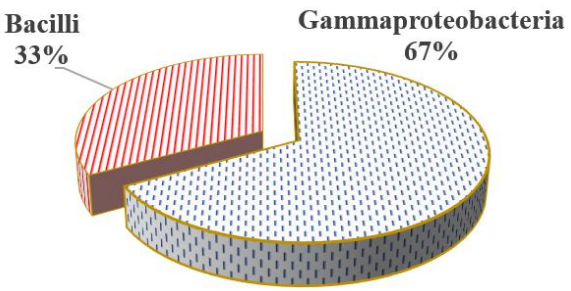


**FIGURE 4: The ratio of 2 classes distributed of nitrogen-fixing bacteria strains**

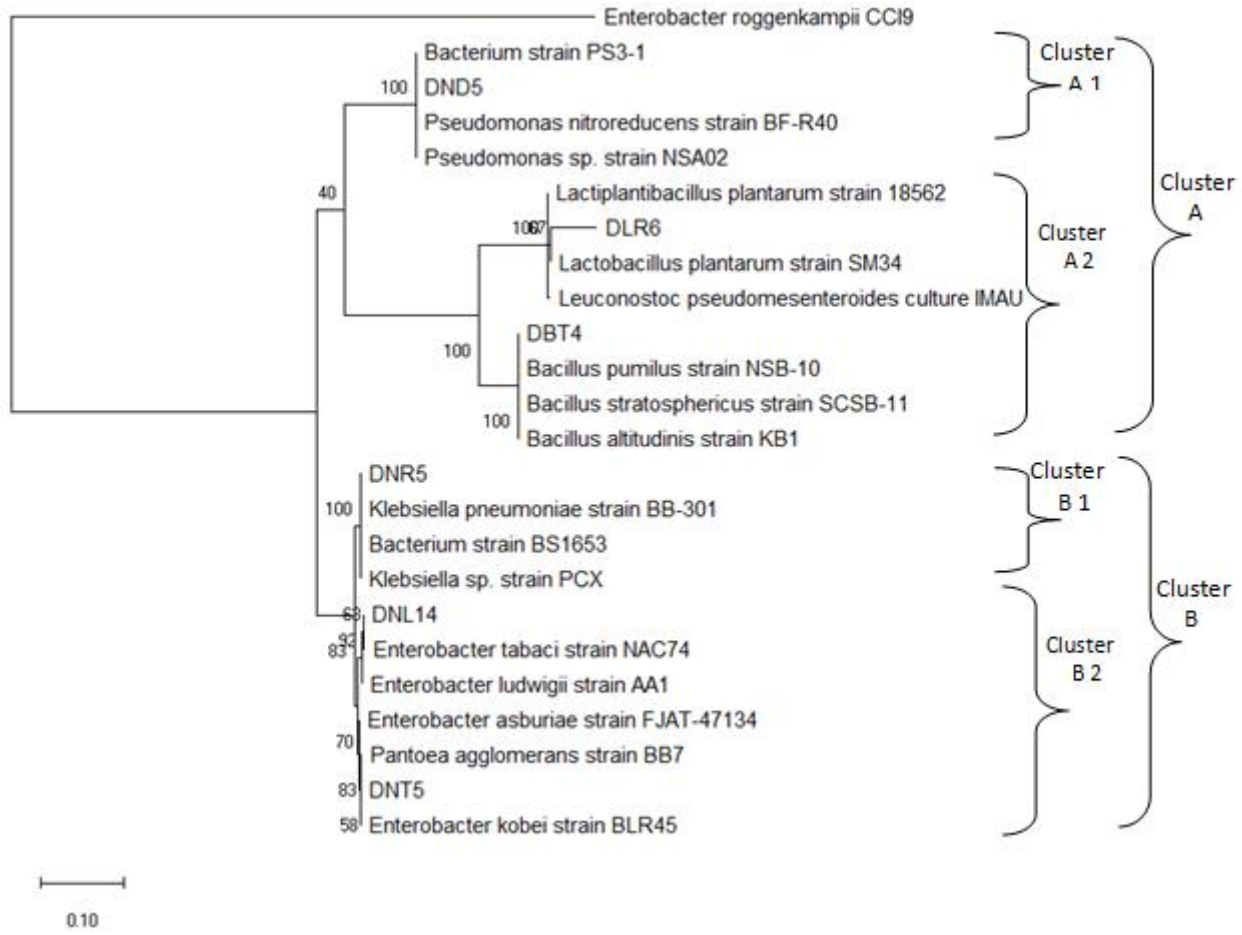


**FIGURE 5: Phylogenetic tree showing the relative position of bacteria by the Maximum Likehood method of complete 16S rRNA sequences**

The determination of nearest phylogenetic neighbor sequences for 16S rRNA gene sequence of the 6 isolates by the BLAST search program showed that they grouped into two clusters (Figure 5). Cluster A composed of two clusters: A1 cluster consisted of the strains DND5 closely related to *Pseudomonas* bacteria. Cluster A2 with strain DLR6 is closely related to *lactobacillus* and DBT4 closely related to *Bacillus* which originated from soil, stem and leaf. This showed that 3 strains had relationship closely eventhrough they were isolated from 3 regions districts far from 90 km. Cluster B is composed of two clusters: B1 cluster consisted of the strains DNR5 closely related to *Klebsiella*. While cluster B2 has consisted of two strains (DNL14, DNT5) closely related to Enterobacter which is endophytic bacteria.

The sequences 16S rRNA of bacterial strains in different plant species are also different for example in rice [26] and maize [43]. In addition, in different parts of the same plant species, the endogenous bacterial species and nitrogen fixation capacity are also different. These bacterial classes showed a fairly good ability of nitrogen fixation, according to previously published studies [44], [45], [46]. *Pseudomonas niethanitrificans* bacteria use the energy source of methane to synthesize $NH_4^+$, averaging to reach 70 mg/L after 2 months of inoculation [47]. *Enterobacter* sp. FD17 fixed nitrogen synthesis $NH_4^+$, synthesizing IAA reaches 12.30 mg/L. The results promote the weight of shoots, roots, amount of leaves, leaf size, and the increase in maize yield by 42% as compared to the non-inoculated control [48]. Bacterial strains isolated from the maize root-soil of transgenic maize and non-transgenic maize grown in the field in South Africa including *Bacillus, Pseudomonas, Aeromonas, Sphingomonas, Burksholderia, Stenotrophomonas, Achromobacter, Ewingella*, both synthesizing ammonium and producing IAA [49]. Similarly, bacterial strains *Klebsiella pneumoniae* 2028 and *Klebsiella pneumoniae* 342 endogenously living in the roots of maize or the maize root-soil do synthesize $NH_4^+$ to supply maize plants [50]. The results of study are similar to the study of endogenous bacteria and rhizosphere soil in the world.

## IV. CONCLUSION

From 40 cultivated maize samples and 8 soil-root samples of districts in Dong Thap Province, Vietnam, 180 isolates were isolated and identified as nitrogen-fixing rhizospheric and endophytic bacteria and 60 isolates having good plant growth

promotion. Six bacteria strains as DNL14, DNT5, DNR5, DND5, DLR6, DBT4, were chosen to analyze their relationship. These strains should be tested in pot and in the field experiments in order to confirm their capacity to improve maize yields and soil fertility of this region.

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