J. Viet. Env. **2020**, 12(2):162-168, Special Issue DAW2020
DOI: 10.13141/jve.vol12.no2.pp162-168

RESEARCH ARTICLE

Selection of nitrogen fixation and phosphate solubilizing bacteria from cultivating soil samples of Hung Yen province in Vietnam

Tuyển chọn các chủng vi khuẩn cố định nitơ và phân giải phosphate từ các mẫu đất trồng của tỉnh Hưng Yên, Việt Nam

HA, Thi Quyen[1]*; CHU, Thi Thu Ha[2,3]

[1]VNU University of Engineering and Technology, 144 Xuan Thuy Road, Cau Giay, Hanoi, Vietnam; [2]Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Hanoi, Vietnam; [3]Graduate University of Science and Technology, VAST, 18 Hoang Quoc Viet, Hanoi, Vietnam.

The nitrogen fixing bacteria (NFB) and phosphate solubilizing bacteria (PSB) are widely used for microbiological fertilizers production. This study aims to seek nitrogen-fixing and phosphate-solubilizing bacteria strains to add to the collection of candidates for producing single and multi-function microbiological fertilizers. From 40 soil samples taken from 8 cultivating fields (rice,medicinal plants and vegetables) 15 NFB strains and 12 PSB strains were isolated to determine the ability of fixing nitrogen and solubilizing inorganic phosphate compounds through creation of NH_4^+ and PO_4^- in culture medium. Among the 15 NFB strains, the fixing nitrogen activities of 7 strains were much higher than the remaining ones, including NFBR3, NFBV2, NFBM5, NFBM3, NFBM1, NFBV5 and NFBR2 with variable NH_4^+ concentrations (18.85 mg/l, 18.41 mg/l, 17.32 mg/l, 16.19 mg/l, 15.49 mg/l, 12.83 mg/l and 12.57 mg/l, respectively). Among the 12 PSB strains, the ability of solubilizing phosphate of 5 strains were higher than the others, including PSBM2, PSBR1, PSBV1, PSBR5 and PSBR3 (with PO_4^- concentrations 14.49 mg/l, 11.83 mg/l, 11.33 mg/l, 10.65 mg/l, 10.37 mg/l, respectively). Three NFB strains (NFBR3, NFBV2, NFBM5) and three PSB strains (PSBM2, PSBR1, PSBV1) with higher activity were identified by 16S-r DNA sequence analysis and compared to some homologous sequences in genbank. The results identified NFBR3 as *Azotobacter vinelandii*, NFBV2 as *Azopirillum brasilense*, NFBM5 as *Azotobacter chroococum,* PSBM2 and PSBV1 as *Pseudomonas fluorescens* and PSBR1 as *Bacillus subtilis*.

Vi khuẩn cố định nitơ (NFB) và vi khuẩn phân giải phosphate (PSB) được sử dụng rộng rãi trong sản xuất phân bón vi sinh. Nghiên cứu này nhằm mục đích tìm kiếm các chủng vi khuẩn cố định nitơ và hòa tan phosphate, bổ sung vào bộ sưu tập các chủng dự tuyển cho sản xuất phân bón vi sinh đơn và đa chức năng. Từ 40 mẫu đất của 8 ruộng trồng lúa, cây dược liệu và rau màu, 15 chủng NFB và 12 chủng PSB đã được phân lập và xác định khả năng cố định nitơ và phân giải phosphate vô cơ thông qua sự tạo thành NH4+ và PO4- trong môi trường nuôi cấy. Trong số 15 chủng NFB, có 7 chủng có hoạt tính cố định nitơ cao hơn những chủng còn lại, bao gồm các chủng NFBR3, NFBV2, NFBM5, NFBM3, NFBM1, NFBV5 và NFBR2 với nồng độ NH4+ lần lượt là 18.85mg/l, 18.41 mg/l, 17.32 mg/l, 16.19 mg/l, 15.49 mg/l, 12.83 mg/l và 12.5 7mg/l. Trong số 12, có 5 chủng có khả năng phân giải phosphate cao hơn những chủng khác, bao gồm chủng PSBM2, PSBR1, PSBV1, PSBR5 và PSBR3 với nồng độ PO4- lần lượt là 14.49 mg/l, 11.83 mg/l, 11.33 mg/l, 10.65 mg/l và 10.37 mg/l. Các chủng NFB và PSB này đều xuất hiện ơ các mẫu đất trồng lúa, đất trồng cây dược liệu và đất trồng rau màu. 3 chủng NFB và 3 chủng PSB với hoạt tính cố định nitơ và phân giải phosphate cao hơn được định loại bằng phân tích trình tự gen 16S-rDNA và so sánh với một số trình tự tương đồng trong genebank. Kết quả chỉ ra rằng chủng NFBR3 được định danh là Azotobacter vinelandii, chủng NFBV2 là Azopirillum brasilense, chủng NFBM5 là Azotobacter chroococum, chủng PSBM2 và chủng PSBV1 là Pseudomonas fluorescens và chủng PSBR1 là Bacillus subtilis.

Keywords: nitrogen fixing bacteria, phosphate solubilizing bacteria, soil microorganism

1. Introduction

Microorganisms in the soil help improve soil structure, through the resolution of organic compounds such as cellulose and protein, into organic humus. Humus and secretions of micro-organisms bond soil particles together to create soil structure, making the soil fertile and improving soil texture. Microorganisms resolve organic matters of fertilizer into mineral form and convert indigestible inorganic form into digestible forms. Soil microorganisms have the ability of fixating nitrogen in the air converting nitrogen into NH_4^+ and NO_3^- forms and releasing minerals that are locked in the soil such as

* Corresponding author
Email: quyenhathi76@gmail.com

http://dx.doi.org/10.13141/JVE
ISSN 2193-6471

sulphur, iron, potassium, phosphorus among others for being absorbed easily by the plants. In addition, the rhizosphere microorganisms also use secretions of plant as nutrients and provide nutrients to plants through their resolution, secreting vitamins and stimulants (Jacoby, R. et al, 2017).

Nitrogen is an important nutrient factor for plants. Every year, the crops take away hundreds of millions of tons of nitrogen from the soil. By fertilization, humans return to the soil >40% of the lost nitrogen source; the remaining nitrogen deficit is replenished by living activity of microorganisms. Nitrogen-fixing bacteria (NFB) groups such as *Azotobacter, Azospirillum* and *Rhizobium* appear frequently in the soil and play an important role in agricultural production. The free-living nitrogen-fixing bacteria group, *Azotobacter,* is not only providing nitrogen nutrients but also stimulating germination by producing plant growth stimulants; the commensalism living nitrogen fixation bacteria group, *Azospirillum,* is in the roots of herbaceous plants, cotton and vegetables. A symbiotic living nitrogen fixation bacteria group, *Rhizobium,* lives in the roots of legumes and creates nodules. The group of *Rhizobium* plays the most important and popular role for N_2 fixation. The presence of nitrogen fixing bacteria in soil is considered to be an indicator for assessing soil quality (Ridvan Kizilkaya, 2009).

Phosphorus, one of the major nutrients for the plants, is required in optimum amount for proper plant growth. Phosphorus is known involving in many functions of plant growth and metabolism. When applying phosphate fertilizer, only about 25% of it is available for the crops while the rest become unavailable due to chemical fixation with aluminum and iron in acidic soils, turning indigestible form for the plants (T. Baliah, et al., 2016). In Vietnam, most of the cultivating soils are poor in phosphate. Phosphate compounds in soil barely exist in water-soluble form; they mainly exist in the form of iron phosphate and precipitated aluminum phosphate. However, these inorganic phosphate compounds, which are difficult to dissolve, are converted into soluble phosphate by solubilizing microorganisms. Phosphate solubilizing microorganisms are found in all soils but their number varies according climate. The phosphate solubilizing microorganisms can help in metabolization, providing about 20-25% of the plant's phosphate requirement (A. Dave, H.H. Patel, 2003). Nitrogen fixing bacteria (NFB) and phosphate solubilizing bacteria (PSB) are widely used for microbiological fertilizers production. Usage of microbial fertilizers, containing nitrogen fixing bacteria, can replace inorganic nitrogen fertilizer (Nguyen Huu Hiep, 2009; Jace Natzke et al, 2018). This study aims to select nitrogen fixing and phosphate solubilizing bacteria in soils from different crops, in communes of Hung Yen province in Vietnam, that are capable of being applied for multifunction microbial fertilizer production.

2. Materials and methods

2.1. Materials

2.1.1 Sample sources

Forty soil samples were collected from 8 fields of cultivating rice, medicinal plants and vegetables from 3 communes in Hung Yen province, Vietnam (see table 1). Soil samples (0-20 cm) from the surface layers were taken according to perpendicular lines (Vietnamese standard TCVN 4046: 1985). Each field was sampled at 5 points.

2.1.2 Bacteria culture medium

Ashby medium for the free-living nitrogen fixing bacteria; YMA supplemented with 0.5% congo red reagent for the symbiotic and commensalism of living nitrogen fixing bacteria; NBRIP medium for phosphate solubilizing bacteria.

Table 1. Soil samples collected from Hung Yen province, Vietnam

Field samples	No. of samples	Location (commune, district)	Cultivating plants
RGP	5	Giai Pham, Yen My	Rice
MGP	5	Giai Pham, Yen My	medicinal plant
RNL1	5	Ngoc Long, Yen My	Rice
RNL2	5	Ngoc Long, Yen My	Rice
MTuD	5	Tu Dan, Khoai Chau	medicinal plant
VTuD	5	Tu Dan, Khoai Chau	Vegetable
VTaD1	5	Tan Dan, Khoai Chau	vegetable
VTaD2	5	Tan Dan, Khoai Chau	vegetable

2.2. Methods

2.2.1 Isolation of nitrogen fixing bacteria (NFB)

Ten grams of each soil sample were homogenized in a 90 ml sterilized saline, diluting the samples to reach the concentration of $10^{-1} \div 10^{-6}$. A volume of 100 µl of each concentration was spread directly onto the surface of respective culture medium plates. The incubation of these plates was settled at 30ºC within 3 days. The nitrogen fixing strains were identified by colony characteristics. Colonies of *Azotobacter* are mucous, elastic, convex,

sometimes wrinkled; old colonies are yellow-green, pink or dark brown. Colonies of *Azospirillum* are glossy, light pink to dark pink; Colonies of *Rhizobium* do not catch Congo red, are clear white or milky white.

2.2.2 Isolation of phosphate solubilizing bacteria (PSB)

Ten grams of each soil sample were homogenized in a 90 ml sterilized saline, diluting the samples to reach the concentration of $10^{-1} \div 10^{-6}$. A volume of 100 µl of each concentration was spread directly onto the surface of respective culture medium plates. Incubation of these plates at was settled at 35 ± 2ºC for seven days. At the end of incubation, PSB colonies were visually identified by the clear zone around the bacterial colony.

2.2.3 The ability of fixing nitrogen of bacteria

The NFB strains were cultured in liquid Ashby medium at 30°C in 3 days, centrifuged to obtain cell free supernatant. The ability of fix nitrogen of strains was determined based on the concentration of NH_4^+ (mg/l) in cell free supernatant by a color comparison method using Nessler reagent (K_2HgI_4). For making standard line, NH_4Cl was used.

2.2.4 The ability of phosphate solubilizing bacteria

The PSB strains were cultured in a liquid NBRIP medium at 35 ± 2ºC for seven days, centrifuged to obtain cell free supernatant. The phosphate solubilizing ability of strains was determined based on the concentration of PO_4^- (mg/l) in cell free supernatant by a color comparison method using the amonmolybdate ($(NH4)_2MoO_4$)). For making standard line KH_2PO_4 was used.

2.2.5 Classification of NFB and PSB strains by genetic tests

Sequence analysis of 16S-rDNA were used to classify and identify the NFB and PSB isolates. A PCR was carried out by using primers:

- 16SF: 5'-AGAGTTTGATCCTGGCTCAG-3'
- 16SR: 5'-TACGGTTACCTT GTTACGACTT-3'

The components of PCR are: Buffer for Taq polymerase 10x: 5µl; dNTPs 10 mM: 2µl; Dream Taq polymerase 5000U/ml: 0,3µl; Primer 16SF 10pmol: 1µl; Primer 16SR 10pmol: 1µl; ADN template 20ng: 2 µl; DI water: 38,7 µl.

The process of PCR was: set up at 95°C in 3 minutes.; 95°C in 1 minute; 55°C in 1 minute; 68°C in 1 minute 15 seconds; 70°C in 7 minutes; keeping at 4 °C; repeating 30 cycles. PCR products were checked by agarose gel electrophoresis and purified by Kit GeneJET™ Gel Extraction (Fermentas, Canada). PCR products were sequenced by ABI-377 Perkin Elmer machine.

The software ClustalX2.1 and MEGA version 6.0 were used to determined phylogenetic relationships of strains. The experiments and sample analysis were performed at the laboratory of University of Engineering and Technology (VNU) and the laboratory of Institute of Biotechnology (VAST).

3. Results and discussions

3.1 Isolation of nitrogen fixing and phosphate solubilizing bacteria

The isolates that have ability of nitrogen fixing were identified by colony characteristics as follows: Colonies of *Azotobacter* are mucous, elastic, convex, sometimes wrinkled, yellow-green, pink or dark brown; Colonies of *Azospirillum* are glossy, light pink to dark pink; Colonies of *Rhizobium* do not catch Congo red so they are clear white or milky white (N. J. Hahn, 1966). Isolates that have the ability of solubilizing phosphate were identified by a clear zone around the bacterial colony.

A collection, including 15 NFB strains and 12 PSB strains, was isolated from the soil samples from different communes of Hung Yen province (Table 2). Among 27 strains of NFB and PSB, there were 4 NFB and 5 PSB strains from rice cultivating soil; 6 NFB and 4 PSB strains from medicinal plants cultivating soil; 5 NFB and 3 PSB strains from vegetables cultivating soil. Some examples are displayed in Figures 1 and 2.

Table 2. NFB and PSB strains isolated from soil samples

NFB strains	Source	NFB strains	Source	PSB strains	Source	PSB strains	Source
NFBR1	RGP	NFBM5	MTuD	PSBR1	RGP	PSBM2	MTuD
NFBR2	RNL1	NFBM6	MTuD	PSBR2	RNL1	PSBM3	MTuD
NFBR3	RNL1	NFBV1	VTuD	PSBR3	RNL1	PSBM4	MTuD
NFBR4	RNL2	NFBV2	VTuD	PSBR4	RNL2	PSBV1	VTuD
NFBM1	MGP	NFBV3	VTaD1	PSBR5	RNL2	PSBV2	VTaD1
NFBM2	MTuD	NFBV4	VTaD1	PSBM1	MGP	PSBV3	VTaD2
NFBM3	MTuD	NFBV5	VTaD2				
NFBM4	MTuD						

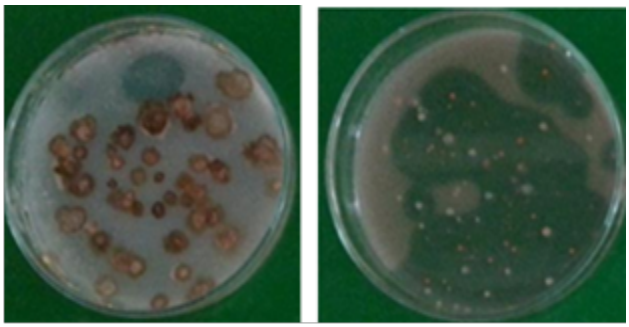

Figure 1. Colonies of NFB strains on Ashby and YMA medium

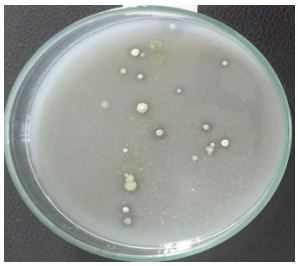

Figure 2. Colonies of PSB strains on NBRIP medium

3.2 The ability of fixing nitrogen of NFB strains

The nitrogen fixation activity of NFB strains was determined through the concentration of NH_4^+ generated in the culture medium. Ammonium in alkaline medium reacted with Nessler reagent (K_2HgI_4) to form a yellow complex ($Hg(HgIONH_2)$) or yellow-brown complex ($Hg(HgI_3NH_2)$) depending on the concentration of NH_4^+. The color from reaction between the Nessler reagent and ammonium has a maximum optical absorption at 420-500 nm wavelength. The correlation graph between OD (optical density) and NH_4^+ concentration is showed in Figure 3 (standard line). The correlation equation is y=1.5x – 0.0071. In which, y is OD value and x is NH4+ concentration. NH4+ concentration was calculated based on this correlation equation (Table 3). The NH4+ content varied from 5.22mg/l to 18.85mg/l.

Among 15 NFB strains, 7 strains had NH4+ concentration higher than the others, including 2 strain isolated from rice soil (NFBR2 and NFBR3); 3 strains isolated from medicinal plant soil (NFBM1, NFBM3 and NFBM5) and 2 strains isolated from vegetable soil (NFBV2 and NFBV4). The highest ability of fixing nitrogen of strains was gained at NFBR3 and NFBV2 (18.85mg/l and 18.41mg/l respectively). The NH4+ content of the 5 remaining strains was 17.32mg/l (NFBM5), 16.19mg/l (NFBM3), 15.49mg/l (NFBM1), 12.83mg/l (NFBV5) and 12.57mg/l (NFBR2).

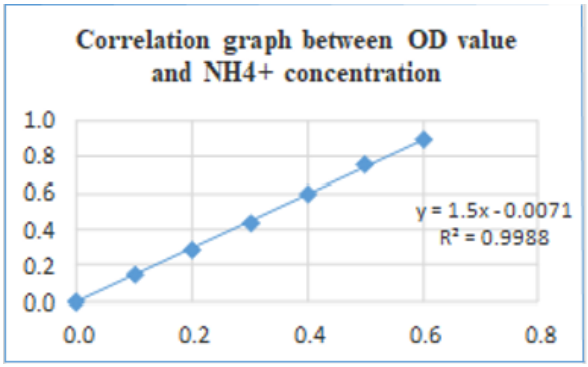

Figure 3. Correlation graph between OD value and NH_4^+ concentration

Pham Thi Ngoc Lan et al. (2017) isolated 10 NFB strains from vegetable land in Thua Thien Hue province. From such study, 4 strains (named N49, N128, N161 and N184) had capacity of fixation nitrogen very high based on producing NH4+ content after culturing in liquid Ashby medium (NH4+ content in the range from 31,23mg/l to 43,41mg/l). The nitrogen fixation capacities of 4 above strains were much higher than the NFB strains of this study. Meanwhile, the remaining strains only had concentration of NH4+ under 7.8mg/l. N49 and N161 strains were identified as Stenotrophomonas maltophilia and Paenibacillus mucilaginosus by 16s-rDNA sequences.

Table 3. NH_4^+ concentration was generated by NFB strains

Strains	NH4+ concentration (mg/l)	Strains	NH4+ concentration (mg/l)
NFBR1	6.90	NFBM5	17.32
NFBR2	12.57	NFBM6	5.22
NFBR3	18.85	NFBV1	6.81
NFBR4	7.52	NFBV2	18.41
NFBM1	15.49	NFBV3	8.67
NFBM2	8.41	NFBV4	12.83
NFBM3	16.19	NFBV5	9.08
NFBM4	7.70		

According to the study of Do Kim Nhung and Vu Thanh Cong (2011), 16 strains belong to *Gluconacetobacter* sp. and *Azospirillum* sp. isolated from sugar cane soil samples. Among them, strain A1 had the ability to fix nitrogen with a NH_4^+ content of 8.09 mg/l after culturing 4 days.

Similarly, Do Hoanh Quan et al. (2011) studied the nitrogen fixation capacity of *Azotobacter* strains isolated from soil samples of Hanoi, Lam Dong, Dong Nai, Long An, Tien Giang and Ben Tre in Vietnam. Among these strains, Az 07 strain had a NH_4^+ concentration gaining 164.27mg/l in optimal culture conditions.

3.3 The ability of phosphate solubilizing of PSB strains

Twelve PSB strains were cultured in NBRIP medium for 5 days. In order to assess phosphate solubilizing activity of these isolates, the PO_4^- concentration was checked by using molybdate blue method with amonimolypdate reagent $(NH4)_2MoO_4$). The PO_4^- reacts with $(NH4)_2MoO_4$ to create a yellow complex ((NH_4) $2PO_4.12MoO_3$). Under acidic conditions and Sn^{2+} ion, the yellow complex will turn into a blue complex ($(NH4)_3$ $(4MoO_2.2MoO_3)$). The blue complex has the maximum absorption wavelength at 690 nm. The greater color absorption, the higher concentration of PO_4^- ions. The correlation graph between OD and PO_4^- concentration is showed in Fig. 4. The correlation equation is y=1.7943x + 0.0114. In which, y is OD value and x is the PO_4^- concentration. The NH_4^+ concentration was calculated based on this correlation equation (Table 4).

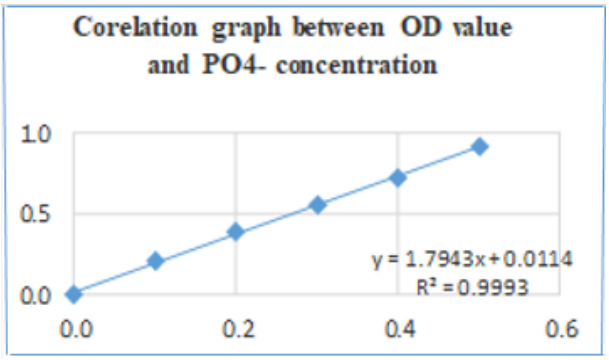

Figure 4. Correlation graph between OD value and PO_4^- concentration

Table 4. PO_4^- concentration was generated by NFB strains

Strains	PO_4^- concentration (mg/l)	Strains	PO_4^- concentration (mg/l)
PSBR1	11.83	PSBM2	14.49
PSBR2	5.28	PSBM3	7.78
PSBR3	10.37	PSBM4	4.56
PSBR4	2.89	PSBV1	11.33
PSBR5	10.65	PSBV2	3.42
PSBM1	5.69	PSBV3	5.83

There were 5 PSB strains with ability of phosphate solubilizing much higher than the 7 remaining strains. The PO_4^- concentration, measured in culture solution of PSBM2 strain, was the highest (14.49 mg/l); the next ones were PSBR1 strain (11.83 mg/l), PSBV1 strain (11.33 mg/l), PSBR5 strain (10.65 mg/l) and PSBR3 strain (10.37 mg/l). Among the 5 PBS strains, 3 strains were derived from rice cultivated soil, one strain from medicinal plant soil and the other from vegetable soil. The strains with high nitrogen fixation and phosphorus resolution activity have been selected and added to the collection of candidate strains as raw material resource for researching and developing multi-functional microbiological fertilizers. The selected NFB strains include NFBR2, NFBR3, NFBM1, NFBM3, NFBM5, NFBV2, NFBV4. The selected PSB strains were PSBR1, PSBR3, PSBR5, PSBM2 and PSBV1. Among of these, strains of NFBR2, NFBR3 and PSBR3 were isolated from the same rice soil sample in Ngoc Long commune, Yen My district, Hung Yen province.

The strains of NFBM3, NFBM5 and PSBM2 were isolated from the same sample of medicinal plant soil in Tu Dan commune, Khoai Chau district, Hung Yen province. The strains NFBV2 and PSBV1 were isolated from a sample of vegetable soil in Tu Dan commune, Khoai Chau district, Hung Yen province. There were 5 strains isolated from rice soil; 4 strains from medicinal plant soil and 3 strains from vegetable soil. The PSB bacteria was also isolated from soil around rice roots in Hai Duong province (Vietnam) by Nguyen Thu Huong et al. (2018). The activity of resolution phosphate was determined by the amount of PO_4^- released into the liquid NBRIP culturing medium. The amount of PO_4^- was released by the PSB bacteria in the range from 2.46 mg/l to 14.26 mg/l. Nguyen Tu Diep et al. (2018) verified the ability of solubilizing inorganic and organic phosphate compounds (including $Ca_3(PO_4)_2$ and lecithin) of PSB bacteria isolated from rice growing soil in Red River Delta region. This study showed that the number of organic phosphate degradable bacteria was dominant over the inorganic phosphate degradable bacteria, appearing in 13/15 surveyed soil samples. The $Ca_3(PO_4)_2$ degradation activity of PSB strains ranged from 0.70 mg PO_4^-/l to 5.66 mg PO_4^-/l, while lecithin degradation activity ranged from 0.0 mg to 1.83 mg of PO_4^-/l.

In general, the phosphate degradation capacity of the before mentioned PSB bacteria was much lower than that of PSB strains that were isolated in this work. The PSB isolates of this study were cultured in NBRIP medium containing $Ca_3(PO_4)_2$ - inorganic phosphate source for assessment ability of solubilizing inorganic phosphate compound. However, in the soil, phosphate is also ionized with Al and Fe to form $AlPO_4$ and FePO4. Therefore, it is necessary to test the ability of solubilizing AlPO4 and FePO4 in culture medium of PSB strains for developing microbiological fertilizers.

3.4 Classifying NFB and PSB strains by 16S-rDNA gene

Among the 7 nitrogen fixing strains, there were 3 strains producing higher ammonium than other strains, which were strains of NFBR3, NFBM5 and NFBV2 (NH_4^+ content

was 18.85 mg/l, 17.32 mg/l and 18.41 mg/l respectively). These three strains were classified by 16S-rDNA gene analysis. From the 5 strains with high phosphate solubilizing activity, three strains with higher production of PO_4^- were selected to be classified based on 16S-rDNA gene analysis; those include strains of PSBR1, PSBM2 and PSBV1 (PO_4^- contents generating 11.83 mg/l, 14.49 mg/l and 11.33 mg/l, respectively). The 16S-rDNA gene sequences of NFB and PSB strains were analyzed and compared to some homologous sequences in GENBANK (see accession number of some homologous sequences in GENBANK in table 5 and figure 5). Genetic variation of the 16S-rDNA genes is quantified by the genetic distance between them. Table 5 showed the genetic distance between NFBR3 and LC571924.1, NFBM5 and AB175653.1, NFBV2 and LN874289.1, PSBV1 and LC571926.1, and PSBR1 and AB325584.1 had the same value (zero).

Table 5. Genetic distance based on sequences of 16S-rDNA genes

	LC571924.1	NFBR3	AB175655.1	AB175653.1	NFBM5	LC571926.1	PSBM2	PSBV1	AJ006110.1	FN395007.1	AB185396.1	NR_044950.1	AB325584.1	PSBR1	LN874289.1	NFBV2
LC571924.1	0.00															
NFBR3	0.00															
AB175655.1	0.03	0.03														
AB175653.1	0.05	0.05	0.04													
NFBM5	0.05	0.06	0.05	0.00												
LC571926.1	0.07	0.08	0.07	0.05	0.05											
PSBM2	0.08	0.08	0.07	0.05	0.05	0.00										
PSBV1	0.07	0.08	0.07	0.05	0.05	0.00	0.01									
AJ006110.1	0.06	0.06	0.06	0.03	0.03	0.02	0.03	0.03								
FN395007.1	0.07	0.07	0.06	0.04	0.04	0.02	0.02	0.02	0.01							
AB185396.1	0.21	0.21	0.20	0.20	0.20	0.20	0.20	0.20	0.19	0.19						
NR_044950.1	0.21	0.22	0.21	0.21	0.22	0.21	0.21	0.21	0.19	0.20	0.02					
AB325584.1	0.26	0.26	0.26	0.26	0.27	0.24	0.25	0.25	0.23	0.25	0.22	0.24				
PSBR1	0.26	0.26	0.26	0.26	0.27	0.24	0.25	0.25	0.23	0.25	0.22	0.24	0.00			
LN874289.1	0.25	0.25	0.25	0.25	0.26	0.23	0.23	0.24	0.22	0.24	0.23	0.24	0.07	0.07		
NFBV2	0.25	0.25	0.26	0.26	0.26	0.24	0.24	0.24	0.23	0.24	0.23	0.24	0.07	0.07	0.00	0.00

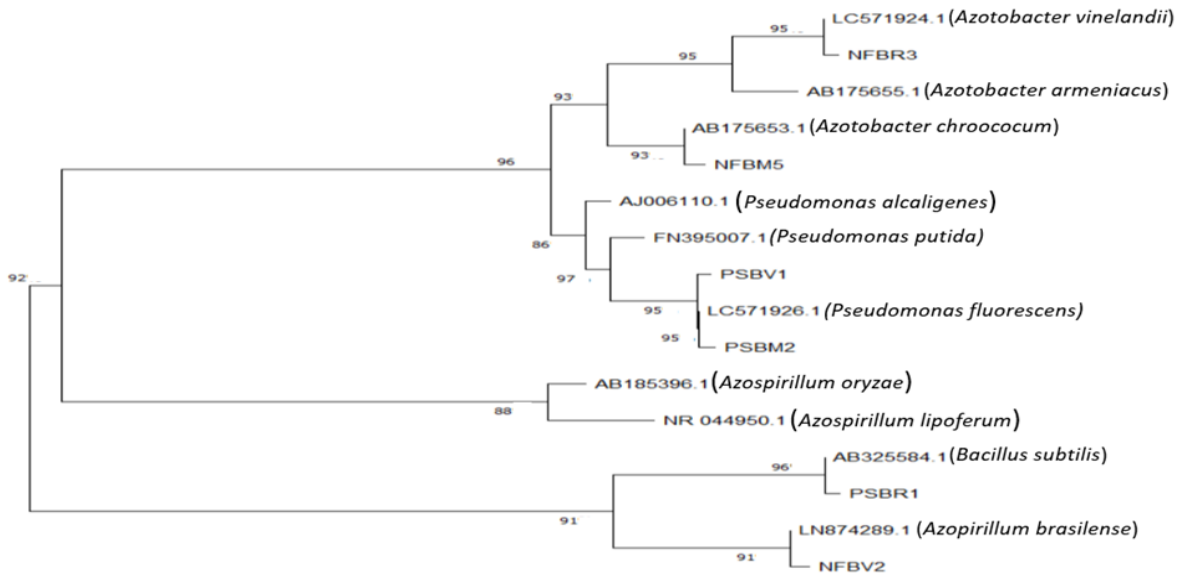

Figure 5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences

Numbers at the root of the branches are the bootstrap value.

Phylogenetic relationships between groups of microorganisms are often presented in a geometric form called "phylogenetic tree". The ends of the branches represent the existing groups of organisms while the length of the branches denotes differentiation of the DNA sequences.The methods of constructing phylogenetic trees from DNA sequence data are based on different evolutionary principles and models described by statistical algorithms. These methods represent the relationship based on calculating the length of branches. We used the neighbor-joining method (NJ) according to the P-distance model for building phylogenetic tree (Tamura K. et al, 2004). The utilized software was MEGA v. 6.0. The phylogenetic tree showed that NFBR3 and *Azotobacter vinelandii* (LC 571924.1) came from the same branch. NBFM5 is closely related to *Azotobacter chroococum* (AB175653.1). NFBV2 and *Azopirillum brasilence* are of same branch. PSBV1 and PSBM2 were closely related to *Pseudomonas fluorescens* (LC571926.1), and PSBR1 was same branch with *Bacillus subtilis*

(AB325584.1). This result of the phylogenetic tree construction was consistent with the result of genetic distance analysis.

4. Conclusion

With 40 soil samples were collected from 8 fields (cultivating rice, medicinal plants and vegetables) from communes in the Hung Yen province in Vietnam. Fifteen NFB and twelve PSB strains were isolated by determining their ability of fixing nitrogen and solubilizing phosphate. Among these strains, 7 NFB strains and 5 PSB strains, showing high nitrogen fixing and phosphate solubilizing activity, were selected as candidate strains for producing microbiological fertilizer. Those were NFBR2, NFBR3, NFBM1, NFBM3, NFBM5, NFBV2, NFBV4, PSBR1, PSBR3, PSBR5, PSBM2 and PSBV1. Given the results, NFBR3 was identified as *Azotobacter vinelandii,* NFBM5 as *Azotobacter chroococum*, NFBV2 as *Azopirillum brasilense*, PSBV1 and PSBM2 as *Pseudomonas fluorescens* (LC571926.1) and PSBR1 as *Bacillus subtilis* (AB325584.1).

5. Acknowledgement

This work has been supported by VNU University of Engineering and Technology under project "Selection of microbiological strains for preparation microbial fertilizer" (CN19.12) and by Hung Yen, Department of Science and Technology under the project: "Research on the technical process of planting, methods of processing and preservation of *Angelica acutiloba* and *Achyranthes bidentata* in Hung Yen province".

6. References

[1] Baliah N. Taliah, Pandiarajan G., Makesh Kumar B. (2016). Isolation, identification and characterization of phosphate solubilizing bacteria from different crop soils of Srivilliputtur Taluk, Virudhunagar District, Tamil Nadu. Tropical Ecology, 57(3), 465-474.

[2] Dave A., Patel H.H. (2003). Impact of different carbon and nitrogen sources on phosphate solubilization by Pseudomonas fluorescens. Indian Journal of Microbiology, 43, 33-36.

[3] Do, Kim Nhung, Vu, Thanh Cong (2011). Investigation of the ability to biosynthesize IAA and protein fixation of Gluconacetobacter sp. and Azospirillum sp. isolated from sugar cane. Science Journal, Can Tho University in Vietnam, 161-167.

[4] Do, Hoanh Quan (2011). Isolation, selection and properties of growth and nitrogen fixation of Azotobacter sp.- experiments on the plants in Vietnam (Master). University of natural science, VNU, Ho Chi Minh, Vietnam.

[5] Hahn N. J. (1966). The congo red reaction in bacteria and its usefulness in identification of Rhizobia. Canadian Journal of Microbiology, 12(4), 725-733.

[6] Jace Natzke, Jesse Noar, José M. Bruno-Bárcenaa (2018). Azotobacter vinelandii Nitrogenase Activity, Hydrogen Production, and Response to Oxygen Exposure. Applied and Environmental Microbiology, 84(16), 1208-1218.

[7] Jacoby R., Peukert M., Succurro A., Koprivova A., Kopriva S. (2017). The Role of Soil Microorganisms in Plant Mineral Nutrition-Current Knowledge and Future Directions. Frontiers in plant science, 8, 1617. https://doi.org/10.3389/fpls.2017.01617.

[8] Nguyen, Thu Huong, Tran, Thi Thuy Ha, Nguyen, Van Giang, (2018). Isolation, identification and characterisation of phosphate solubilising bacteria from rice rhizosphere. J. Vietnamese Science and Technology, 60(8), 18-22.

[9] Nguyen Huu Hiep, Ha Danh Duc (2009). Isolation of nitrogen-fixing bacteria and phosphate-solubilizing bacteria for peanut cultivated in Tra Vinh province. Science Journal of Can Tho University, 11b, 123-133.

[10] Nguyễn Tú Điệp1, Cao Kỳ Sơn2 và Đinh Hồng Duyên (2018). Current Status of phosphate solubilizing microorganisms of rice cultivating slob soil types in Red River Delta, Vietnam. Science Journal, Can Tho University in Vietnam, 54(7B), 79-85.

[11] Ridvan Kizilkaya (2009). Nitrogen fixation capacity of Azotobacter spp. strains isolated from soils in different ecosystems and relationship between them and the microbiological properties of soils. J. Environ. Biol., 30(1), 73-82. http: //www.jeb.co.in info@jeb.co.in.

[12] Pham, Thi Ngoc Lan, Nguyen, Thi Viet, Le, Thi Hoa Sen (2017). Isolation and selection of nitrogen fixing bacteria from soils cultivated with vegetables in Thua Thien Hue, Vietnam. In Proceedings of 7th national science conference on ecology and biological resource, Hanoi, Vietnam, 1296-1303.

[13] Tamura K., Nei M., and Kumar S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101, 11030-11035.

[14] Tejera N. C., Lluch M. V., Martìnez-Toledo J. Gonzàlez-López (2005). Isolation and characterization of Azotobacter and Azospirillum strains from the sugarcane rhizosphere. Plant and Soil, 270, 223–232.