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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

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The nitrogen fixing bacteria (NFB) and phosphate solubilizing bacteria (PSB) are used widely for producing of microbiological fertilizers. This study aims to seek nitrogen-fixing and phosphate-solubilizing bacteria strains to add to the collection of candidate strains for producing single and multi-function microbiological fertilizers. From 40 soil samples of 8 fields for cultivating rice, medicinal plants and vegetables, 15 NFB strains and 12 PSB strains were isolated and determined the ability of fixing nitrogen and solubilizing inorganic phosphate compound through creation of NH_4^+ and PO_4^- in culture medium. Among 15 NFB strains, the fixing nitrogen activities of 7 strains were much higher than the remaining strains, including NFBR3, NFBV2, NFBM5, NFBM3, NFBM1, NFBV5 and NFBR2 with NH_4^+ concentration 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 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^- concentration 14.49 mg/l, 11.83 mg/l, 11.33 mg/l, 10.65 mg/l, 10.37 mg/l, respectively.. 3 NFB strains (NFBR3, NFBV2, NFBM5) and 3 PSB strains (PSBM2, PSBR1, PSBV1) with higher activity were identified by 16S-rDNA sequence analysis and comparing to some homologous sequences in genbank. The results showed that NFBR3 was identified 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, protein,... into organic humus. Humus and secretions of micro-organisms link soil particles together to create soil structure, make the fertile soil, improve soil texture. Microorganisms resolve organic matters of fertilizer into mineral form and convert indigestible inorganic form into digestible form. Soil microorganisms have the ability of fixation nitrogen in the air, convert nitrogen into NH_4^+ and NO_3^- forms, releasing minerals that are locked in the soil such as sulphur, iron,

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potassium, phosphorus etc. 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 hundred million tons of nitrogen from the soil. By fertilizing, humans return to the soil about >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, producing plant growth stimulants; the commensalism living nitrogen fixation bacteria, group of *Azospirillum,* is in the roots of herbaceous plants, cotton and vegetables. And symbiotic living nitrogen fixation bacteria, group of *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 fertiliser, only about 25% of them is available for the crops and the rest become unavailable due to chemical fixation with aluminium and iron in acidic soils, becomes the indigestible form for the plants (T. Baliah, et al., 2016). In Vietnam, most of the cultivating soil is poor in phosphate. The phosphate compounds in soil exist very little in water-soluble form, mainly exist in the form of iron phosphate, precipitated aluminium phosphate. However, these inorganic phosphate compounds, which are difficult to dissolve, are converted into soluble phosphate by phosphate solubilizing microorganisms. Phosphate solubilizing microorganisms are found in all soils but their number varies with soil climate. The phosphate solubilizing microorganisms can help metabolize and provide about 20-25% of the plant's phosphate requirement (A. Dave, H.H. Patel, 2003).

Through their roles, nitrogen fixing bacteria (NFB) and phosphate solubilizing bacteria (PSB) are widely used for producing of microbiological fertilizers. Especially, by using microbial fertilizers containing nitrogen fixing bacteria can replace inorganic nitrogen fertilizer (Nguyen Huu Hiep, 2009; Jace Natzke et al, 2018).

This study aims to selection nitrogen fixing and phosphate solubilizing bacteria in soils under different crops from some communes of Hung Yen province in Vietnam, that are capable of applying for producing multifunction microbial fertilizer.

2. Materials and methods

2.1. Materials

2.1.1 Sample sources

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

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

2.1.2 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 living nitrogen fixing bacteria; NBRIP medium for phosphate solubilizing bacteria.

2.2. Methods

2.2.1 Isolation of nitrogen fixing bacteria (NFB)

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

10g of each soil sample were homogenized in 90 ml sterilized saline, diluting the samples to reach the concentration of $10^{-1} \div 10^{-6}$. 100µl of each concentration was spread directly onto the surface of respective culture medium plates. Incubation of these plates 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 30ºC 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 color comparison method using Nessler reagent (K_2HgI_4). NH_4Cl was used for making standard line.

2.2.4 The ability of phosphate solubilizing bacteria

The PSB strains were cultured in 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 color comparison method using the amonmolybdate ($(NH4)_2MoO_4$)). KH_2PO_4 was used for making standard line.

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. PCR was carried out by using primers:

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

The components of PCR: 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: set up at 95ºC in 3 minutes.; 95ºC in 1 minute; 55ºC in 1 minute; 68ºC in 1 minute 15 seconds; 70ºC in 7 minutes; keeping at 4 ºC; 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). The isolates that have ability of phosphate solubilizing were identified by clear zone around the bacterial colony. A collection including 15 NFB strains and 12 PSB strains was isolated from the soil samples in different communes of Hung Yen province (Table 2).

Table 2. NFB and PSB strains isolated from soil samples

NFB strains	Samplae source	NFB strains	Samplae source	PSB strains	Samplae source	PSB strains	Samplae 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						

Among 27 strains of NFB and PSB, there are 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.

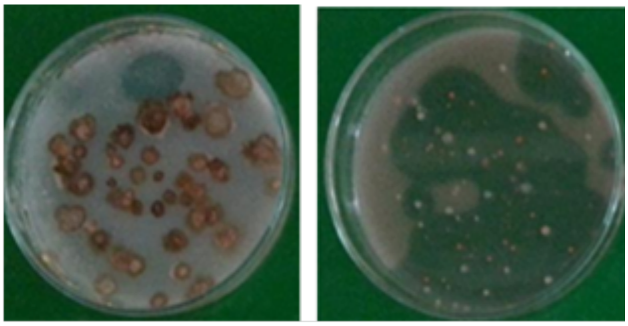

Figure 1. Colonies of NFB strains on Ashby and YMA medium

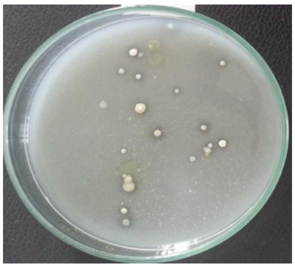

Figure 2. Colonies of PSB strains on NBRIP medium

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).

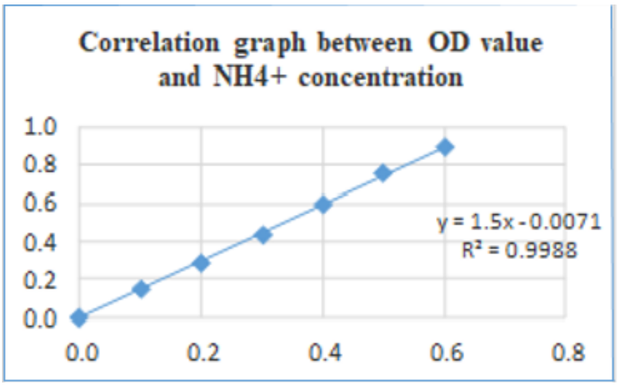

Figure 3. Correlation graph between OD value and NH_4^+ concentration

The correlation equation is y=1.5x – 0.0071. In which, y is OD value and x is NH_4^+ concentration. NH_4^+ concentration was calculated based on this correlation equation (Table 3).

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

Strains	NH_4^+ concentration (mg/l)	Strains	NH_4^+ 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		

NH_4^+ content varied from 5.22mg/l to 18.85mg/l. Among 15 NFB strains, 7 strains had NH_4^+ 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. NH_4^+ content of 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).

Pham Thi Ngoc Lan et al. (2017) isolated 10 NFB strains from vegetable land in Thua Thien Hue province. In which, 4 strains (named N49, N128, N161 and N184) had capacity of fixation nitrogen very high based on producing NH_4^+ content after culturing in liquid Ashby medium (NH_4^+ 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 NH_4^+ under 7.8mg/l. N49 and N161 strains were identified as *Stenotrophomonas maltophilia* and *Paenibacillus mucilaginosus* by 16s-rDNA sequences.

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 NH_4^+ content was 8.09mg/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, Ben Tre in Vietnam. Among these strains, Az 07 strain had NH_4^+ concentration gaining 164.27mg/l in optimal culture conditions.

3.3 The ability of phosphate solubilizing of PSB strains

12 PSB strains were cultured in NBRIP medium for 5 days. In order to assess phosphate solubilizing activity of these isolates, PO_4^- concentration was checked by using molybdate blue method with amonimolypdate reagent $(NH4)_2MoO_4$). 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 690nm. 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 PO_4^- concentration. NH_4^+ concentration was calculated based on this correlation equation (Table 4).

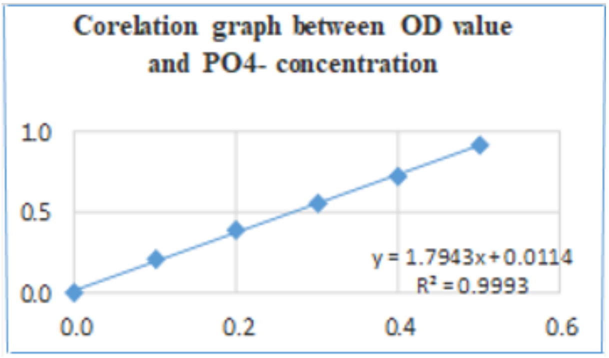

Figure 4. Correlation graph between OD value and PO_4^- concentration

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 7 remaining strains. PO_4^- concentration measured in culture solution of PSBM2 strain was the highest (14.49 mg/l), next was 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 of 5 above PBS strains, there 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 had selected and added to the collection of candidate strains as raw material resource for researching and developing multi-functional microbiological fertilizers. NFB strains were selected including NFBR2, NFBR3, NFBM1, NFBM3, NFBM5, NFBV2, NFBV4. And PSB strains were selected including 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 were also isolated from soil around rice root in Hai Duong province (Vietnam) by Nguyen Thu Huong et al. (2018). The activity of resolution phosphate were determined by 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) was checked 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 the number of organic phosphate degradable bacteria were dominant over the inorganic phosphate degradable bacteria and appeared in 13/15 surveyed soil samples. $Ca_3(PO_4)_2$ 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 0.0mg PO_4^-/l to 1.83 mg PO_4^-/l. In general, the phosphate degradation capacity of the above PSB bacteria was much lower than that of PSB strains that we were isolated.

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^+ contents were 18.85 mg/l, 17.32 mg/l and 18.41 mg/l, respectively). These three strains were classified by 16S-rDNA gene analysis. Out of 5 strains with high phosphate solubilizing activity, three strains with higher production of PO_4^- were selected to classify based on 16S-rDNA gene analysis, including strains of PSBR1, PSBM2 and PSBV1 (PO_4^- contents generating 11.83 mg/l, 14.49 mg/l and 11.33 mg/l, respectively). 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 of NFBR3 and LC571924.1; between NFBM5 and AB175653.1; between NFBV2 and LN874289.1; between PSBM2, PSBV1 and LC571926.1; between PSBR1 and AB325584.1 had the same 0 value.

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

Phylogenetic relationships between groups of microorganisms are often presented in geometric form called phylogenetic tree. The ends of the branches represent existing groups of organisms, 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 P-distance model for building phylogenetic tree (Tamura K. et al, 2004) through MEGA software v. 6.0.

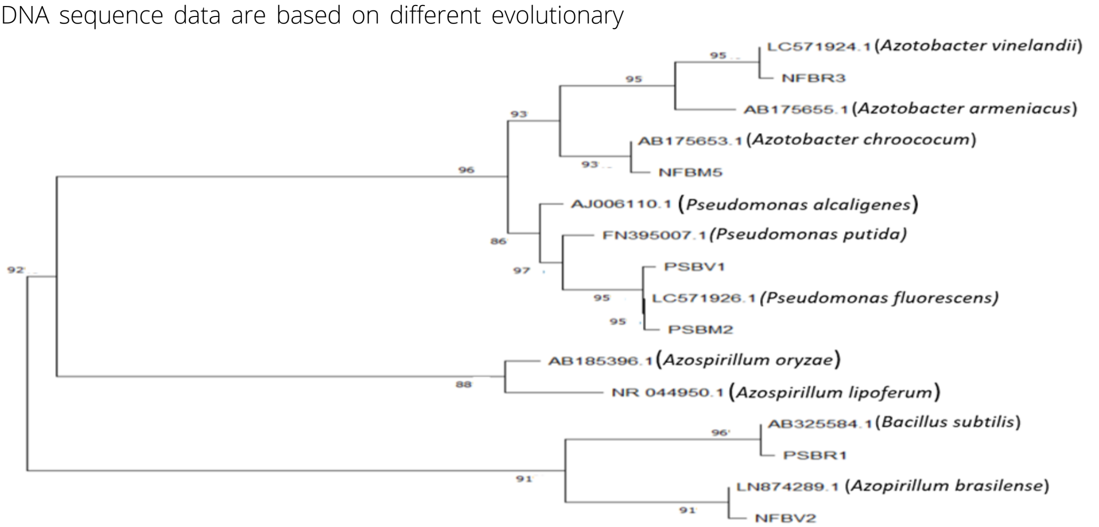

Figure 5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences

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

The phylogenetic tree showed NFBR3 and *Azotobacter vinelandii* (LC 571924.1) came from the same branch. NBFM5 closely related to *Azotobacter chroococum* (AB175653.1). NFBV2 and *Azopirillum brasilence* were 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 of cultivating rice, medicinal plants and vegetable of communes belong to Hung Yen province in Vietnam, 15 NFB and 12 PSB strains were isolated and determined the ability of fixing nitrogen and solubilizing phosphate. Among these strains, 7 NFB strains and 5 PSB strains had high nitrogen fixing and phosphate solubilizing activity were selected as candidate strains for producing microbilogical fertilizer. Those were NFBR2, NFBR3, NFBM1, NFBM3, NFBM5, NFBV2, NFBV4, PSBR1, PSBR3, PSBR5, PSBM2 and PSBV1. In which, NFBR3 was identified as *Azotobacter vinelandii*. NFBM5 was identified as *Azotobacter chrooc
ocum*, NFBV2 as *Azopirillum brasilense*, PSBV1 and PSBM2 as *Pseudomonas fluorescens* (LC571926.1) and PSBR1 as *Bacillus subtilis* (AB325584.1).

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