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Isolation and Selection of Biological Nitrogen Fixing and indole-3-Acetic Acid Synthesizing Bacteria from Different Cropping Systems in Soc Trang province, Vietnam

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Abstract: *Indigenous microorganism (IMO) has great potential in agricultural uses since they have high ability in biodegradation, nitrogen fixation, phosphate solubilization, plant growth hormone production as well as bio-control. However, deeply scientific knowledge about IMO has been limited and should be elucidated. The aim of this study was to isolate bacteria having both functions in biological nitrogen fixation and indole-3-acetic acid synthesis from IMO in different farming ecosystems in Soc Trang province, Vietnam. Nitrogen fixing bacteria were isolated on free N element Burk agar medium.* NH_4^+ *concentration in Burk liquid medium was determined by nitroprusside reagent method at a wavelength of 650 nm for total nitrogen and 636 nm for available ammonium. In addition, nifH functional gene involving in nitrogen fixation was also detected by specific polF/polR primers for some best isolates. The IAA synthesizing capacity of bacteria was assessed quantitatively by Salkowski's reagent method at a wavelength of 530 nm. The results showed that from 19 different indigenous microbial communities 83 isolates in total able to fix nitrogen were obtained. Quantitative tests showed that bacteria fixed total nitrogen and IAA synthesis varied between 0.58 and 22.55* $\mu g.mL^{-1}$ *and between 0.85 and 58.04* $\mu g.mL^{-1}$*. Among them seven isolates labeled as TP-1.3, TP-1.4, MQ-2.1, MQ-2.5, MT-16.5, OM-17.2 and OM-17.5 were identified as highest nitrogen fixers having also other function of IAA synthesis with amount of 0.86, 4.23, 1.37, 0,42, 1.15, 1.76 and 1.83* $\mu g.mL^{-1}$*, respectively for available ammonium and 27.54, 26.98, 23.99, 35.32, 58.04, 41.87 and 29.44* $\mu g.mL^{-1}$*respectively for IAA. Particularly, all twenty-two tested bacteria showed a present of functional nifH gene for biological nitrogen fixation in their genetic structure. Results of 16S rARN ribosomes sequences of seven best bacteria indicated that TP-1.3 had 99% identification index with Paraburkholderia tropica, TP-1.4 and MQ-2.1 had highly similarity (99%) with the strain of Paenibacillus cineris and Bacillus aryabhattai while MQ-2.5 and OM-17.5 were highly homologous with Bacillus megaterium and MT-16.5 and OM-17.2 had a highly homologous sequence as Klebsiella pneumoniae. These nitrogen fixing bacteria have other function in IAA synthesis can be used for sustainable agricultural cultivation in Soc Trang province.*

Keywords: *Bacillus megaterium, IAA synthesizing, Klebsiella pneumoniae, nifH gene, nitrogen fixation, Paenibacillus cineris, Paraburkholderia tropica.*

1. INTRODUCTION

Fertilizers, particularly chemical nitrogen fertilizers have contributed significantly to increasing crop yields. However, abuse of chemical fertilizers, especially nitrogen fertilizer, will lead to excess nitrogen in the soil or in plants. This causes a harmfulness to the environment and human health because the excess of nitrogen in agricultural products especially nitrate (NO_3^-) and nitrite (NO_2^-) produced in the process of denitrification, causing direct poison to aquatic animals and indirectly to terrestrial animals when using contaminated water sources [1]. Moreover, for crop products, especially when vegetables have very high nitrate content, it will cause some dangerous diseases for humans. Thus, sustainable agriculture, eco-friendly farming methods and climate change adaptation are topics given priority today modern agriculture. Utilization of biological fertilizers containing native and beneficial microorganisms for plants is considered as one of the most feasible approaches to replace or reduce partly the amount of inorganic nitrogen fertilizer, avoiding the impact to environment and saving input costs in agricultural activities. Therefore, research for new methods in agriculture to improve the quality of food, eco-friendly and soil fertility has been taken place over the past decades. Using the indigenous microorganisms (IMO) in the natural farming system was studied and developed by Cho Han Kyu in the 1960s. This technology in using indigenous microorganisms (IMO) was an innovative method in crop cultivation, helping plants to produce maximum productivity instead of human intervention [2]. Since 1997, many farms and farmers around the world have adopted with this new agricultural technique in natural agriculture by using the natural origin of IMO to protect crops and to improve soil fertility, health and quality because IMO itself has many functions such as nitrogen fixation, phosphorus solubilization, phytohormone synthesis, etc [3]. Although IMO has been produced in a simple process, it has brought to many efficacies in agricultural cultivation. However, studies on beneficial bacteria for plants especially bacteria which have two

functions in nitrogen fixation and IAA synthesis in IMO have been limited. Therefore, the aim of this study was to isolate and to select some of indigenous bacteria from different IMO having nitrogen fixing and IAA synthesizing capacities for agricultural cultivation in Soc Trang province, Vietnam.

2. MATERIAL AND METHODS

2.1 Collection and cultivation of IMO

IMO were collected from different cropping systems including bamboo, crop rotation (corn-watermelon-courgette), banana, shallot, salad, rice, watermelon, grassland, maize, vegetables, oranges, grapefruit, guava, sugarcane, water spinach, ceylon spinach, chili guinea arrowroot and cilantro within Soc Trang province, Vietnam by following the method described by Kyu and Koyama [4] (Figure 1).

Fig-1: Procedure of IMO collection

Sites of sample collection are presented in Table 1. At each sampling site, three plastic baskets with a size (25x15x8 cm) were used, corresponding to 3 replicates. Each basket was filled with 1 kg of steamed rice and covered on the top of the basket with cloth and waist belt. The baskets were buried under ground at each sampling site and covered the top of baskets with leaf litters for four days. After four days of incubation, all fermented rice colonized by indigenous microorganisms were harvested, put into a glass jar and carried to the laboratory. This source of microorganisms was called IMO1. Collected IMO1 was mixed with brown sugar with a ratio of 1:1 (w/w) until the mixed material became gooey. This mixed material was stored in the ceramic pot in a cool area and away from direct sunlight for seven days for another fermentation time. After seven days of fermentation, this source of microorganisms was called IMO2 and the IMO2 was kept in the refrigerator at 4^0C for further studies.

Table-1: The located of nineteen collected IMO samples in Soc Trang province

Code	Origin of IMO (Farming system)	Located in Soc Trang province
TP-1	Bamboo	Phu Tam commune, Chau Thanh district
MQ-2	Crop rotation	5 Ward, Soc Trang city
CP-3	Banana	7 Ward , Soc Trang city
HV-4	Shallot	Vinh Phuoc commune, Vinh Chau district
RP-5	Lecttuce	3 Ward, Soc Trang city
LM-6	Rice	My Xuyen town, My Xuyen district
DL-7	Watermelon	Truong Khanh commune, Long Phu district
CL-8	Grassland	Truong Khanh commune, Long Phu district
BT-9	Maize	Thanh Tri commune, Thanh Tri district
RM-10	Vegetables	Thanh Quoi commune, My Xuyen district
CK-11	Oranges	Xuan Hoa commune, Ke Sach district
BK-12	Grapefruit	Xuan Hoa commune, Ke Sach district
OK-13	Guava	Xuan Hoa commune, Ke Sach district
MC-14	Sugarcane	Đai An II commune, Cu Lao Dung district
MP-15	Water spinach	8 Ward, Soc Trang city
MT-16	Ceylon spinach	Vien Binh commune, Tran De district
OM-17	Chili	An Ninh commune, My Tu district
CP-18	Guinea arrowroot	7 Ward, Soc Trang city
NM-19	Cilantro	An Ninh commune, My Tu district

2.2 Isolation of nitrogen fixing bacteria

An aliquot of 10 grams of each IMO was put into 250 mL glass bottle containing 90 mL sterilized distilled water. The samples were put on a shaker at a speed of 150 rpm for an hour and left standing for 5 minutes after shaking. Prepared a series dilution with a factor of 10 and aliquot 50 µL of each dilution was spread on Burk agar medium. Samples were incubated for 5-7 days at room temperature. Observe the appearance of bacterial colonies on the agar surface. Bacteria with different morphological characteristics of colonies including size, shape, color form, elevation and magrin were selected and purified on Burk agar medium continuously for 5 times. After purification, biomass of colonies was stored in 30% glycerol solution for further studies.

2.2.1 Colonial morphology of bacteria

The colonies of isolates were described morphologically through the following criteria: size (mm), color, shape, form, elevation and magrin. For smaller colony bacteria, the description of colonial morphology was performed under a stereo microscope.

2.2.2 Cell morphology of bacteria

Using a clean sterilized bamboo toothpick or inoculation loop to take a small biomass of bacterial colonies, spread the biomass on Lame, dropped some drops of sterile distilled water and mixed well the

biomass with water. The sample was passed through the flame to immobilize the bacterial cell, then a drop of 0.5% Fuchsin dye was applied on the position of the fixed bacteria, using Lamella to cover bacteria and dye. The shape and other bacterial cell morphologies were observed and recorded under an optical microscope.

2.2.3 Test gram of bacterial strains

Gram reaction was tested by mixing a loop of bacterial colonies with 1 drop of 3% KOH on a microscope slide by a toothpick. Bacterial strain was considered as Gram negative when a thread was formed between the toothpick and the microscope slide [5].

2.3 Quantification of nitrogen fixation capacity of isolates

2.3.1 Total nitrogen

A loop of bacteria well developed on Burk's agar medium was transferred to a 100 mL Erlenmeyer flask containing 50 mL N-free Burk's liquid medium for 5 days as the enriched generation culture solution. The composition of N-free Burks liquid medium ($g.L^{-1}$) was sucrose (10 g), $K_2HPO_4.4H_2O$ (0.41 g), KH_2PO_4 (1.05 g), $CaCl_2.2H_2O$ (0.1 g), $MgSO_4.7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.015 g), H_3BO_3 (0.0025 g), Mo (0.0025 g). The samples were put on the orbital shaker at a speed of 90 rpm in the dark under the laboratory conditions for five days. After five days of incubation, the total nitrogen content biologically fixed by bacteria in Burk's liquid medium was determined after 0, 1, 2, 3, 4 and 5 days of incubation by a method described by Keeney and Nelson [6]. Briefly described as follows: The microbial cultures were first digested with 5 mL of H_2SO_4 in the digestion chamber at 360°C for two hours. The color change was monitored for the completion of the digestion process when color changed from dark brown to greenish white by adding some drops of hydrogen peroxide. The samples were left for cooling down and transferred the whole amount of sample into 50 mL volumetric flask and filled distilled water up to 50 mL volume. This solution was standed for 20 hours at room temperature. Optical density was measured at 650 nm with the help of spectrophotometer (Spectrometer Thermo Scientific, Multiskan Spectrum).

2.3.2 Available NH_4^+ content in liquid medium

A loop of bacteria well developed on Burk's agar medium was transferred to a 100 mL Erlenmeyer flask containing 50 mL N-free Burk's liquid medium for 5 days as the enriched generation culture solution. An aliquot of 100 µL enriched generation culture solution was transferred to 100 mL Erlenmeyer flask containing 30 mL fresh N-free Burks liquid medium and each strain was repeated 3 times. The composition of N-free Burks liquid medium ($g.L^{-1}$) was sucrose (10 g), $K_2HPO_4.4H_2O$ (0.41 g), KH_2PO_4 (1.05 g), Na_2SO_4 (0.05 g), $CaCl_2.2H_2O$ (0.1 g), $MgSO_4.7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.05 g), Mo (0.0025 g). The samples were left in the dark under the laboratory conditions for five days. The content of available ammonium in liquid medium which was biologically fixed by bacteria was determined after zero, one, two, three, four and five days of incubation by indophenol method [6]. The method to analyze the available NH_4^+ in liquid medium is described as follows: An aliquot of 1mL of culture solution was transferred to Eppendorf 2mL, then centrifuge 13,000 in the rpm for 10 minutes. Transfer 0.5 mL of centrifuged bacteria into a test tube. Continue to add tubes of 2.0 mL H_2O, 0.5 mL of EDTA 6% solution, 1 mL of phenol sodium nitroprusside solution and 2 mL of sodium hypochloride, respectively. Vortex solution, and waited for 20 minutes for the reaction to complete. The composition of 100 mL phenol nitroprusside reagent contained 7g phenol and 34 mg nitroprusside, hypocloride buffer included (g/100 mL) 1.48 g NaOH, 4.98 g Na_2HPO_4 and 20 mL NaClO. Optical density was taken at 636 nm with the help of spectrophotometer (Spectrometer Thermo Scientific, Multiskan Spectrum). The concentration of NH_4^+ produced was measured with the help of standard graph of NH_4^+ obtained in the range of 0-1 $mg.L^{-1}$.

2.4 Detection of the presence of functional *nifH* gene indicating for biological nitrogen fixation of 22 isolated strains

Firstly, DNA of each selected isolate was extracted by MO BIO kit (MOBIO Laboratories, a QIAGEN Company, Valencia, CA), then, polF/polR primers [7] were used for PCR reaction to amplify 360 bp sequences of *nifH* gene. The volume of 25 µL of PCR reaction included 12.5 µL Green mix (2X), 2 µL polF primer (10 µM), 2 µL polR primer (10 µM), 2 µL of pure DNA and 6.5 µL deionized water. The reactions were carried out as follows: 5-min initial denaturation of DNA at 94°C, followed by 35 cycles of 1-minute denaturation at 94°C, 1-minute primer annealing 57°C and 1-minute extension at 72°C. Amplification was completed by a final extension step at 72°C for 10 minutes. To visualize the PCR products, 5 µL of the reactions were loaded into 2% of agarose gel, 5 µL of 100 bp ladder was also loaded into gel as a molecular weight marker. Gels ran for 30 minutes at 150 volts and 500 milliamps and were then visualized and photographed by UV light from Gel Logic 1500 (Kodak) to find target sequences with the size of 360 bp.

2.5 IAA synthesizing ability of selected isolates

An aliquot of 100 µL bacteria solution (prepared in section 2.3.1) was transferred to 100 mL Erlenmeyer flask containing 30 mL fresh N-free Burks liquid medium with 100 $mg.L^{-1}$ tryptophan (pH = 7). Three replicates were repeated for each strain. The samples were put in dark and under laboratory conditions for ten days. The IAA concentration synthesized by bacteria in the liquid medium was determined after 0, 2, 4, 6, 8 and 10 days of incubation by Salkowski's reagent method at a wavelength of 636 nm followed by the modified method described by Brick *et al*. [8]. To

measure IAA concentration, one mL culture solution was centrifuged at 12,000 rpm for 10 minutes. The supernatant (1 mL) was mixed with two 2 mL of the Salkowski's reagent (one liter of Salkowski'sreagent consist of 50 mL H_2SO_4 98% and 4.5 g $FeCl_3$). Development of pink color indicates for IAA production. Optical density was taken at 530 nm with the help of spectrophotometer. Concentration of IAA produced by each isolate was measured with the help of standard curve of IAA obtained in the range of 0-100 $mg.mL^{-1}$.

2.6 16S rARN gene sequence of seven best isolates

Seven bacterial isolates labeled as TP-1.3, TP-1.4, MQ-2.1, MQ-2.5, MT-16.5, OM-17.2, and OM-17.5 showed their highest nitrogen fixation and strong IAA synthesis were selected to do 16S rARN gene sequence. The results of 16S rARN gene sequence were compared with available database of gene bank to identify bacterial species of selected isolates by using the BLAST program (GenBank). Combining the morphological, cell and homologous morphological characteristics of BLASTN to determine the species names of seven nitrogen fixing bacteria.

3. RESULTS AND DISCUSSION

3.1 Isolation of biological nitrogen fixing bacteria from different IMO

Characteristics of colonial and cell morphologies as well as bacterial Gram are important characteristics in microbiological isolation step. Based on these characteristics, eighty three nitrogen fixing bacteria were isolated from 19 collected IMO samples. The results showed that there was a very high diversity of colonial morphology of bacteria in color, size, shape, surface and bacterial cell shapes (Figure 2A, 2B, 2C and 2D). Among them, the bacteria with circular colonies, milky and smooth-face were predominant. Specifically, the size of bacterial colonies developed on Burk's agar medium after 5 days ranged from 1 to 7 mm, circular colonies accounted for 75%, white milky surface colonies accounted for 56.3% and 82.5% of the convex colonies.

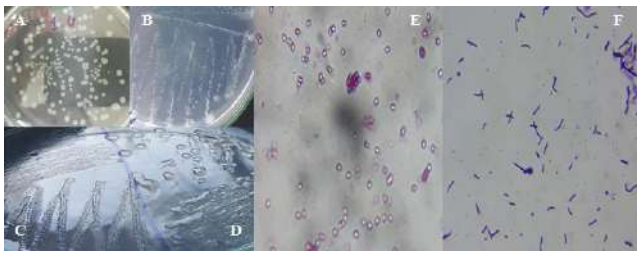

Figure-2: Colony morphology and bacterial cell shape of nitrogen fixing bacteria grew on Burk's agar medium
**note: A: large-sized milky colony, B: small-sized milky colonies, C: small white colonies and D: large white colonie , E: round shape (100X) and F: rod shape (40X)*

For cell observations under the microscope, the results showed that majority types of bacterial cell shape were rod-shaped and spherical (Figure 2E and 2F). Specifically, 51 bacterial isolates were rod shape and accounting for 61.4%, including short and long rods while 32 isolates were globular shape and accounted for 38.6%. For Gram determination, among them, 40 isolates were gram-negative bacteria, accounted for 48.2% and 43 isolates were gram-positive bacteria and accounted for 51.8% of the total.

3.2 Quantification of nitrogen fixing capacity of isolates

3.2.1 Total nitrogen content

The result of nitrogen fixing capacity of 83 isolates is presented in Table 1. It can be seen that there was a significant difference among bacterial isolates in total nitrogen content in liquid medium. It was also recorded that the nitrogen fixation ability of each strain was not only varied among the isolates strains but also dramatically fluctuated during the time period of incubation. In this present study, thirty-seven isolated fixed nitrogen biologically over 10 mg $N.L^{-1}$ and maximum amount of nitrogen fixed in the liquid medium was 22.55 mg $N.L^{-1}$ by TP-1.3 from bamboo farm after two days of incubation, followed by MP-15.1 and TP-1.7 with the highest amount of ammonium was 20.81 and 18.35 mg $N.L^{-1}$, respectively. Forty-two isolates fixed the highest amount of nitrogen ranged from 1.02 to 9.76 mg $N.L^{-1}$. Particularly, the amount of nitrogen fixed by CP-3.6, RP-5.1, CK-11.4 and MC-14.3 were relatively low and the highest amount of fixed nitrogen of these isolates was lower than 1 mg $N.L^{-1}$.

3.1.2 Available ammonium content in liquid medium

The results obtained from the determination of available NH_4^+ content in Burk's liquid medium of 83 bacterial isolates after 5 days of inoculation is presented in Table 2. Unlike total nitrogen, the amount of available ammonium in Burks liquid medium was very low and varied greatly. The concentration of NH_4^+ was produced in liquid culture media by 83 isolates was found to be different with each other. A number of 20 isolates showed their highly biological nitrogen fixation with NH_4^+ concentration ranged from 1.00 to 4.23 mg $NH_4^+.L^{-1}$. Among them, TP-1.4 fixed the highest NH_4^+ level after 3 days of culture (4.23 mg $NH_4^+.L^{-1}$) and followed by TP-1.2 and RP-5.6. The highest NH_4^+ concentration of these two isolates found at 2.79 mg $NH_4^+.L^{-1}$ and 2.43 mg $NH_4^+.L^{-1}$ after 1 day of incubation. In addition, CP-3.1, OK-13.2, BT-9.1 and CL-8.4 also fixed NH_4^+ with an amount up to 1 $mg.L^{-1}$. The other sixty-five isolates were also able to fix nitrogen biologically, but the amount of available nitrogen was less than 1 mg $NH_4^+.L^{-1}$ and ranged from 0.1 to 0.99 mg $NH_4^+.L^{-1}$. In short, TP-1.2, TP-1.4 and RP-5.6 were identified as the three best nitrogen fixing bacteria in Burk's liquid medium among 83 isolates.

Table 2: The maximum total nitrogen concentration and available NH_4^+ of 83 isolates in Burk liquid medium

No.	*Name*	*Total Nitrogen ($mg.L^{-1}$)*	*Available NH_4^+ ($mg.L^{-1}$)*	*No.*	*Name*	*Total Nitrogen ($mg.L^{-1}$)*	*Available NH_4^+ ($mg.L^{-1}$)*
1	TP-1.1	11.30	0.55	43	CL-8.2	2.99	1.12
2	TP-1.8	16.47	0.11	44	CL-8.3	12.27	0.14
3	TP-1.2	9.46	2.79	45	CL-8.4	8.58	1.27
4	TP-1.3	22.55	0.86	46	BT-9.1	16.23	1.01
5	TP-1.6	2.07	0.28	47	BT-9.2	8.27	0.93
6	TP-1.4	14.55	4.23	48	BT-9.3	4.63	0.68
7	TP-1.5	16.89	0.43	49	RM-10.1	6.00	0.33
8	TP-1.7	18.35	0.20	50	RM-10.2	14.23	0.35
9	MQ-2.1	9.70	1.37	51	RM-10.3	5.87	1.00
10	MQ-2.5	10.71	0.42	52	CK-11.1	10.82	0.49
11	MQ-2.3	15.48	0.27	53	CK-11.2	8.75	0.33
12	MQ-2.4	8.82	0.12	54	CK-11.3	9.09	0.15
13	MQ-2.2	8.89	0.12	55	CK-11.4	0.58	0.27
14	MQ-2.6	3.37	0.99	56	CK-11.5	6.53	1.06
15	MQ-2.7	9.33	0.01	57	BK-12.1	8.52	0.87
16	CP-3.1	5.93	1.28	58	BK-12.2	10.86	0.32
17	CP-3.2	8.41	0.30	59	BK-12.3	10.70	0.29
18	CP-3.3	4.28	0.16	60	OK-13.1	7.21	0.15
19	CP-3.4	9.50	0.00	61	OK-13.2	14.10	1.23
20	CP-3.5	12.10	0.15	62	OK-13.3	13.07	0.79
21	CP-3.6	5.89	0.51	63	OK-13.4	12.93	0.12
22	CP-3.7	2.58	1.19	64	OK-13.5	7.70	0.90
23	HV-4.1	2.51	0.06	65	OK-13.6	16.24	0.07
24	HV-4.2	4.77	0.63	66	MC-14.1	9.65	0.24
25	HV-4.3	3.58	1.00	67	MC-14.2	12.64	0.10
26	RP-5.1	0.80	0.08	68	MC-14.3	0.83	0.00
27	RP-5.2	10.86	0.24	69	MC-14.4	17.62	0.16
28	RP-5.3	8.51	0.60	70	MP-15.1	20.81	0.27
29	RP-5.4	8.52	0.87	71	MT-16.1	7.61	0.46
30	RP-5.5	1.02	0.00	72	MT-16.2	10.10	0.23
31	RP-5.6	10.23	2.43	73	MT-16.3	9.20	0.22
32	LM-6.1	9.25	0.30	74	MT-16.4	3.87	1.15
33	LM-6.2	10.28	0.21	75	MT-16.5	4.40	1.15
34	LM-6.3	10.83	0.30	76	OM-17.1	8.35	0.05
35	LM-6.4	11.06	0.08	77	OM-17.2	12.67	1.76
36	DL-7.1	15.35	0.61	78	OM-17.3	5.57	1.09
37	DL-7.2	13.32	1.20	79	OM-17.4	8.58	0.85
38	DL-7.3	14.77	0.22	80	OM-17.5	15.19	1.83
39	DL-7.4	9.29	0.40	81	CP-18.1	9.76	0.75
40	DL-7.5	10.02	0.20	82	CP-18.2	12.38	0.23
41	DL-7.6	2.90	1.01	83	NM-19.1	17.42	0.37
42	CL-8.1	11.53	0.42				

In a comparison with other previous studies, it was clear that the amount of available nitrogen (NH_4^+) in liquid medium of some isolates was much higher than those of isolates from the previous studies. Davis *et al*. [9] reported that the *Pseudomonas methanitrificans* could utilize methane as a sole source of energy and

could fix 70 mg.L^{-1} nitrogen in a period of two months. In larger scale experiments, the maximum fixed nitrogen was 53 mg N.L^{-1} and the higher nitrogen fixed observed in their study might be due to the autolysis process of cells when staying in a longer incubation period. However, this study showed similar results with the study of Thavasi *et al*. [10] revealed that *Azotobacter chroococcum* isolated from crude oil contaminated marine environment could fix 4.2 mg N.L^{-1} in 96 hours. Mazumdar and Deka [11] estimated that the amount of nitrogen fixed by free-living nitrogen fixing bacteria isolated from crude oil contaminated soil was recorded with a range of 9.74 mg N.L^{-1} and 17.45 mg N.L^{-1} over a time period of two months. Similarly, Smita and Goyal [12] estimated that the amount of nitrogen fixed by free-living nitrogen fixing bacteria from alkaline soils was found to be highest after 9 – 12 days of incubation and with an amount ranged from 14.44 mg N.L^{-1} to 18.73 mg N.L^{-1} as total nitrogen content.

3.3 Functional *nifH* gene detection

The result of polymerase chain reaction showed that the primer used in this study was successful to amplify *nifH* gene from DNA of all selected isolates and there was an obvious variation in the size of *nifH* gene products between 22 tested isolates (Figure 3). The size of target sequences of *nifH* gene was approximate 360 bp which matches with the earlier study of Poly *et al*. [7][13] . When these studies used polF/polR primers to detect functional *nifH* gene and it also showed that these primers were sensitive with 5 referencing N2-fixing strains such as *Azospirillum brasilense, Azospirillum lipoferum, Rhizobium leguminosarum, Sinorhizobium meliloti* and *Frankia alu* and 19 isolates from soil as well. It is also important to suggest that all tested isolates showed a great potential and function for biological nitrogen fixation and no matter what species they were. This result is consistent with the above quantitative results. It is obvious that bacteria can grow in condition of nitrogen lacking because they owned nitrogen fixing function genes so that they could fix nitrogen from the air for their using.

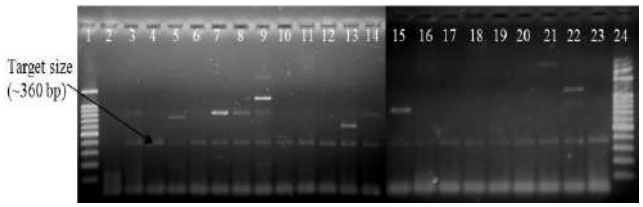

Figure-3: Functional *nifH* gene PCR products by polF/polR primer amplification of 22 selected isolates

**Note: Lane 1: 100 bp standard ladder; lane 2: negative control (H2O); lane 3: TP-1.2; lane 4: TP-1.3; lane 5: TP-1.4; lane 6: TP-1.5; lane 7: MQ-2.1; lane 8: MQ-2.5; lane 9: CP3.1; lane 10: CL-8.2; lane 11: DL-7.2; lane 12: DL-7.3; lane 13: OM-17.2; lane 14: OM-17.5; lane 15: MT-16.5; lane 16: CK-11.1; lane 17:RP-5.4; Lane 18: BK-12.3; lane 19: MC-14.4; lane 20: CP-18.2; lane 21: BT-9.1; lane 22:CL-8.4; Lane 23: HV-4.3; lane 24: 100 bp standard ladder*

3.4 IAA synthesizing production

IAA concentrations synthesized by 50 selected isolates from different ecosystem habitats are presented in Table 3 and it can be seen that the amount of IAA produced by different isolates was significant difference when compared with each other and varied significantly over the time period (data not showed). There were forty-six out of fifty tested strains capable of synthesizing IAA in the Burk's liqiud medium with 100 mg.L^{-1} tryptophan. The synthesized IAA content of 50 strains varied largely from 0.85 to 58.04 mg.L^{-1}. The IAA production of some isolates was synthesized very early even after two day of incubation and the period to reach the highest IAA production was very much different from each other. Around 18 isolates synthesized over 20 mg.L^{-1} IAA in liquid medium.

Table-3: Highest concentrations of IAA synthesized by 50 isolates in Burk's liquid medium supplemented with tryptophan (100 mg.L^{-1})

No.	Isolate	Highest IAA content (mg.L^{-1})	No.	Isolate	Highest IAA content (mg.L^{-1})
1	TP-1.1	4.42	26	CL-8.2	24.37
2	TP-1.8	0.00	27	CL-8.3	1.90
3	TP-1.2	0.00	28	CL-8.4	12.72
4	TP-1.3	27.54	29	BT-9.1	5.64
5	TP-1.4	26.98	30	RM-10.2	0.85
6	TP-1.5	27.61	31	RM-10.3	9.65
7	TP-1.7	12.87	32	CK-11.1	18.16
8	MQ-2.1	23.99	33	CK-11.5	3.22
9	MQ-2.5	35.32	34	BK-12.2	0.00
10	MQ-2.3	31.24	35	BK-12.3	31.23
11	CP-3.1	0.00	36	OK-13.2	1.14
12	CP-3.5	2.05	37	OK-13.3	16.02
13	CP-3.7	6.11	38	OK-13.4	4.21
14	HV-4.3	6.58	39	OK-13.6	15.61
15	RP-5.2	17.31	40	MC-14.4	16.14
16	RP-5.6	0.00	41	MC-14.5	9.42
17	LM-6.2	21.35	42	MP-15.1	3.54
18	LM-6.3	3.51	43	MT-16.2	28.92
19	LM-6.4	4.30	44	MT-16.3	27.54
20	DL-7.1	1.67	45	MT-16.5	58.04
21	DL-7.2	4.01	46	OM-17.2	41.87
22	DL-7.3	39.30	47	OM-17.3	32.63
23	DL-7.5	2.92	48	OM-17.5	29.44
24	DL-7.6	4.50	49	CP-18.2	34.62
25	CL-8.1	20.26	50	NM-19.1	7.81

Especially, the highest amount of IAA was observed after two days of incubation and found for MT-16.5 with the value of 56.58 mg.L^{-1}, followed by OM-17.2 isolated from mono Ceylon spinach cultivated soil with an amount of 41.87 mg.L^{-1} on the second day of incubation. Beside, three others isolates labeled as DL-7.3, BK-12.3 and MT-16.2 produced 39.30, 31.23 and 28.92 mg.L^{-1}, respectively. Only CP-18.2 collected from guinea arrowroot cultivated field reached the highest IAA concentration after 4 days of incubation with

amount of 34.62 $mg.L^{-1}$ whereas four other isolates labeled as TP-1.4, TP-1.5, MQ-2.1 and MQ-2.5 revealed highest IAA productions after six days of inoculation with an amount of 26.98, 27.61, 35.32 and 31.24 $mg.L^{-1}$ IAA. The lowest IAA production was found for OK-13.2, DL-7.1 and CL-8.3 from guava, watermelon and grassland fields with a concentration of 1.14, 1.67 and 1.90 $mg.L^{-1}$ IAA, respectively. Especially, no IAA production was found for TP-1.8, TP-1.2, CP-3.1 RP-5.6 RM-10.2 and BK-12.2. Among isolates able to synthesize IAA tested, isolates labeled as MT-16.5 and OM-17.2 showed their capacity in IAA synthesis early, highly and stably with a very high amount of IAA concentrations throughout the experimental time period surveyed.

The previous study of Ahmad *et al.* [14] tested for the production of IAA in a medium containing 5 $mg.mL^{-1}$ concentrations of tryptophan of 10 strains of *Azotobacter* sp., 11 strains of *Pseudomonas* sp. The result showed that seven *Azotobacter* strains revealed their high production of IAA (7.3 to 32.8 $mg.mL^{-1}$) while the value of IAA varied from 41.0 to 53.2 $mg.mL^{-1}$ for *Pseudomonas* sp. strains. Moreover, Ahmad *et al.* [15] isolated free-living rhizosphere bacteria for their multiple plant growth promoting activities and quantified IAA amounts at concentrations of 500 $\mu g.mL^{-1}$ tryptophan for *Azotobacter* sp., *Pseudomonas* sp. and *Bacillus* sp. Their results showed that these bacteria showed their highest ability in IAA production when the culture medium was added with 500 $\mu g.mL^{-1}$ tryptophan and the amount of IAA produced was ranged from 7.03 to 22.02 $\mu g.mL^{-1}$.

Plant growth is affected by a low amount of auxin provided from outside of the plant by IAA synthesizing soil microorganisms. Many bacterial species were found to be capable of producing phytohormone IAA [16]. The effect of IAA on each plant variety depends on its concentration and at low concentrations, IAA can stimulate the growth of plant, but it can also cause an inhibition at high levels [17]. The numbers of specific bacteria around the roots determined the concentration of auxin. In the same way, different plants respond differently to variable concentrations of auxin [18] and kind of microorganisms [14]. In the soil, the highest level of auxin synthesized by microorganisms was found for IAA and indole acetamide at normal conditions, resulting in maximum growth and yield of wheat [19]. Even slower and more stable IAA-producing strains will also help to improve plant productivity [20].

From the above results, it can be seen that there were eighteen strains having a high capacity not only in nitrogen fixation but also IAA synthesis. Among of them seven isolates showed great potential in nitrogen fixation and stably high IAA product. They were TP-1.3, TP-1.4, MQ-2.1, MQ-2.5, MT-16.5, OM-17.2 and OM-17.5 and these isolates were selected to do 16S rARN sequences.

3.5 16S rARN gene sequencing

Result of 16S rARN gene sequencing of seven selected bacterial isolates is presented in Table 4. When comparing the 16S rARN of seven isolates with the database on the world gene bank BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). Results showed that they were belong to four genera: *Paraburkholderia, Paenibacillus, Bacillus* and *Klebsiella.* Especially, combining with colony morphological characteristics, shape and gram cells, two isolates from bamboo soil (TP-1.3 and TP-1.4) had 99% identification index with *Paraburkholderia tropica* and *Paenibacillus cineris*, respectively. Thus, they were identified as *Paraburkholderia tropica* TP-1.3 and *Paenibacillus cineris* TP-1.4. In addition, MQ-2.5 and OM-17.5 isolated from crop rotation and chili soil were highly homologous with *Bacillus megaterium* strain and they were identified as *Bacillus megaterium* MQ-2.5 and *Bacillus megaterium* OM-17.5. MQ-2.1 had highly genetic relation to *Bacillus aryabhattai*. Therefore, it was named as *Bacillus aryabhattai* MQ-2.1. Furthermore, MT-16.5 and OM-17.2 isolated from Ceylon spinach and chili fields had a highly homologous sequence as *Klebsiella pneumoniae* strain and they were identified as *Klebsiella pneumoniae* MT-16.5 and *Klebsiella pneumoniae* OM-17.2, respectively.

Table-4: Identifying seven bacteria having nitrogen fixation and IAA synthesis based on the similarity of 16S rARN sequences

Isolated Strain	Identification index (%)	Bacteria strain on database	
		Strain	Registration number
TP-1.3	99	*Paraburkholderia tropica*	MK336433
TP-1.4	99	*Paenibacillus cineris*	MF662488.1
MQ-2.1	99	*Bacillus aryabhttai*	MH041178.1
MQ-2.5	99	*Bacillus megaterium*	KF364492.1
MT-16.5	97	*Klebsiella pneumoniae*	FJ608656.1
OM-17.2	99	*Klebsiella pneumoniae*	MK834722
OM-17.5	99	*Bacillus megatrium*	MH196974.1

In comparison with other previous studies, it was found that Ding *et al.* [21] isolated nitrogen fixing bacteria from the rhizospheres of wheat, maize, ryegrass and willow in Bejing and the result showed that two out of seven isolates strains showing their high-nitrogen fixation capacity and belongs to the *Paenibacillus* sp. and three out of them were identified as *Bacillus megaterium* with high homology index of 98% and 100%. Similarly, Rivas *et al*. [22] isolated three bacterial strains capable of great nitrogen

fixation from the rhizosphere of the legume (*Cicer arietinum*) in Argentina. All three strains were belong to the genus *Paenibacillus* and identified them as the *Paenibacillus cineris* LMG 18439T with the similarity index of 99.6%, *Paenibacillus favisporus* LMG 20987T with the similarity index of 99.4% and *Paenibacillus azoreducens* DSM 13822T with the similarity index of 99.4%. Dhara *et al*. [23] isolated *Klebsiella pneumoniae* strains from the rhizospheres of wheat with high ability of IAA synthesis. Particularly, *Klebsiella pneumoniae* K8 could produce maximum IAA at 27.5 $mg.L^{-1}$ in the present of tryptophan ($1mg.mL^{-1}$) at 72 h of incubation with optimum conditions as pH 8.0, 37°C and 0.5% NaCl. In addition, Pankaij *et al*. [24] isolated *Bacillus* sp. BPR7 from rhizosphere of common bean with multifunction. The strain BPR7 produced IAA, siderophore, phytase, organic acid, ACC deaminase, cyanogens, lytic enzymes, oxalate oxidase, cellulase and solubilized various sources of organic and inorganic phosphates as well as potassium and zinc. Strain BPR7 strongly inhibited the growth of several phytopathogens such as *Macrophomina phaseolina, Fusarium oxysporum, F. solani, Sclerotinia sclerotiorum, Rhizoctonia solani* and *Colletotricum* sp. in vitro. Cell-free culture filtrate of strain BPR7 also caused colony growth inhibition of all test pathogens.

The results of genetic correlation analysis of selective bacterial strains by UPGMA method (Fig. 4) also showed that they had a close genetic relationship together. Among them, *Bacillus megaterium* OM-17.5 strain had the most closest relationship with *Paraburkholderia tropica* TP-1.3 and both of them had a close relationship with *Klebsiella pnemoniae* OM-17.2 while *Bacillus aryabhttai* and *Bacillus megaterium* had a very close relationship together and they have genetic relationship with *Klebsiella pneumoniae* MT-16.5 and all these 6 strains have a closely genetic relationship with *Paenibacillus cineris* TP-1.4.

In short, it is obvious to see that IMO samples collected from different farming models have a very high diversity composition of mutual nitrogen fixing and IAA producing IAA bacteria and these seven bacteria have a great potential in plant growth promotion.

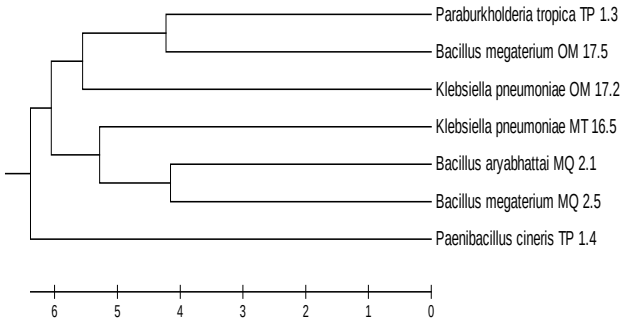

Figure-4: Genetic link between seven selected bacterial strains based on UPGMA method

4. CONCLUSIONS

In general, it was clear that there was a big diversity of bacteria in IMO samples from different cropping systems can fix nitrogen biologically and synthesize IAA. Eighty-three strains of bacteria were isolated from nineteen collected indigenous microorganism from different agri-ecosystem habitats. Twenty-two of them have ability to fix nitrogen and carry the *nifH* functional gene and 18 strains revealed their capacity of good nitrogen fixation and high IAA product. Of these bacteria, seven bacteria labeled as TP-1.3, TP-1.4, MQ-2.1, MQ-2.5, MT-16.5, OM-17.2, and OM-17.5 have highest nitrogen fixation and IAA producing ability and they are identified genetically and diversifyingly as *Paraburkholderia tropica, Paenibacillus cineris, Bacillus aryabhttai, Bacillus megaterium* and *Klebsiella pneumonia,* respectively. They have a great potential in plant growth stimulation for sustainable agricultural cultivation in Soc Trang province, Vietnam.

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