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Isolation and identification of bioflocculant-producing bacteria, heterotrophic nitrogen removal bacteria and poly-phosphate bacteria in wastewater from "My Tho rice noodle" factories, Tien Giang province, Vietnam

Le Thi Loan[1], Tran Vu Phuong[2], Cao Ngoc Diep[3]
[1] *Dept. Educational high school, Dept. Education - Training, Tien Giang province, Vietnam*
[2,3]*Dept. Microbiology Biotechnology, Biotechnology R&D Institute, Can Tho University, Can Tho City, Vietnam*

Abstract- A total of 42 bioflocculant-producing bacterial isolates (BPB), 59 heterotrophic nitrogen removal (HNR) bacterial isolates and 8 poly-Phosphate bacterial isolates were isolated from wastewater of 8 rice noodle factories of My Tho city, Tien Giang province, Mekong Delta, Vietnam. Bioflocculant-producing bacterial isolates were divided two groups: protein and polysaccharide, HNR bacterial isolates were classified in four kinds of heterotrophic ammonia-oxidizing bacteria (15 isolates), nitrite-oxidizing bacteria (15 isolates), nitrate-oxidizing bacteria (18 isolates) and heterotrophic nitrifying and denitrifying bacteria (11 isolates). The virtually complete 16S rRNA gene was PCR amplified and sequenced. The sequences from the selected BPB, HNR and poly-P bacterial isolates showed high degrees of similarity to those of the GenBank references strains (between 97% and 99%). Phylogenetic trees based on the 16S rDNA sequences displayed high consistency, with nodes supported by high bootstrap (1000) values. These presumptive HNRB isolates were divided two groups that included members of genera belonged to Gram-positive bacteria phylum and Proteobacteria phylum while BPB and poly-P bacteria strains belonged to bacilli.
Keywords: 16S rDNA Gene Sequence, Bioflocculant-producing bacteria, Heterotrophic Nitrogen Removal bacteria, poly-Phosphate Bacteria, wastewater of rice noodle factory

I. INTRODUCTION

Since ancient times, noodles in various formulations and shapes have been used as staple food in many parts of Asia [1-2]. This is evident from the literature that Chinese were the first to introduce noodles to the world, rather than Arabs or Italians. Noodles were very popular during the era of Han dynasty (25-220 AD) in China. During the early 20th century, alkaline noodles were introduced in Yokahama city of Japan by Chinese immigrants. Later on, Japanese develop instant noodles by a different process, which became the most popular food not only in Asia but all over the world [1].

Rice is a main staple in Asian diet that has many unique attributes, including ease of digestion, a mild flavor and hypoallergenic properties (3). China is the largest producer and the consumer of rice in the world, with an average the capita comsumtion of 102 kg of rice-based food products (4). The main rice variety used for rice noodle is *indica*, which is widely cultivated in the south China for its high yield and adaptibility to local weather condithions. Indica is more suitable for rice noodle applications than japonica because it contains more amylose (5).

Both China and Japan did a lot of innovation and employed modern technology in noodle processing that substantially increased the consumption and acceptance of noodles outside Asia also. Noodles experienced substantial evolution and migration in spite of its origin due to the increase in globalization (1, 6). Rice noodles are the most consumed form of rice product next to cooked rice grain in Asia (7). Noodles may either be served by frying and mixing with vegetables and meats or served as a soup noodle by boiling in a broth. Rice protein lack gluten; hence lack the functionality of continuous visco-elastic dough.

My Tho is capital of Tien Giang province in the Mekong Delta, Vietnam [106^{o}33'96" to 106^{o}33'27" E and 10^{o}36'51" to 10^{o}36'27 N] (Figure 1).

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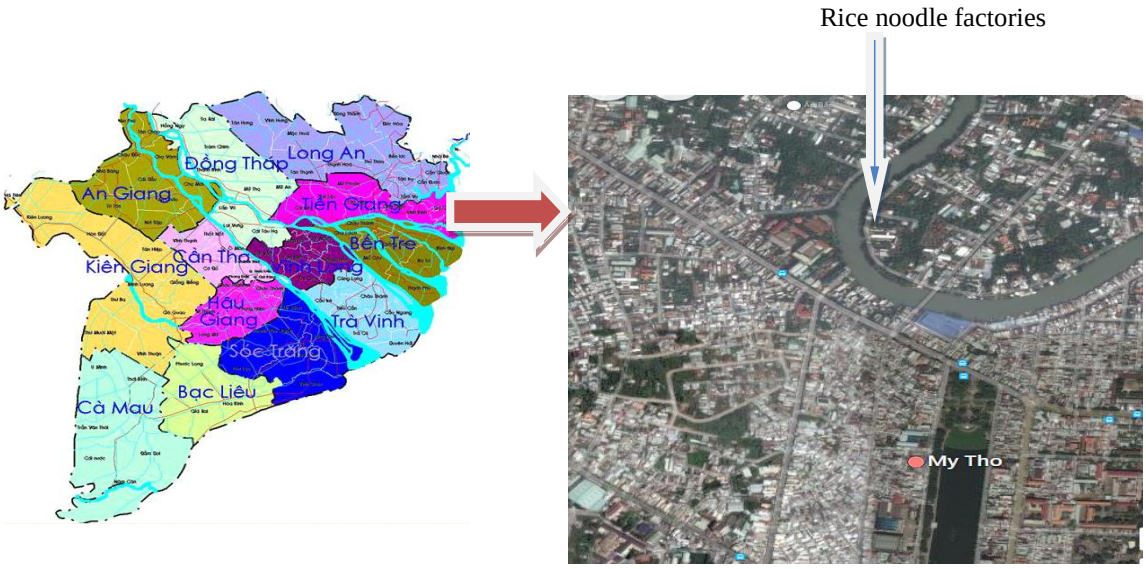

Figure 1. Rice noodle factories at My Phong village, My Tho city, Tien Giang province, Vietnam (from Google map)

My Tho city has many traditional technologies and "My Tho rice noodle" is among famous technology for along time. Rice noodle has been produced from special local rice vriety and there are seven manufacture factories have gatherd in a cooperative and they have a "HU TIEU MY THO" trademark. However these factories work in the traditional condition therefore they have no wastewater treatment plant and wastewater have irrigated to canal or river directly. Especially wastewater from rice noodle factories contain many toxic elements as ammonium, orthophosphate,…and high TN, TP, BOD_5, TSS concentrations (Table 1).

The characteristics and volume of wastewater discharged from food processing factories vary with the products and production procedures. In factories like accompanying dishes makers and beverage makers, due to changes of products and/or production the wastewater fluctuates in characteristics and volume. Starch making factories in Hokkaido and sake breweries produce for a specified period of the year and only generate wastewater then.

Table -1 pH and physical and chemical characteristic of wastewater from 8 rice noodle factories of My Tho city, Tien Giang province, Vietnam

Factory No	pH	TN (mg/L)	Orthophosphate (PO_4^{3-}) (mg/L)	TSS (g/L)	BOD_5 (mg O_2/L)
1	4.65	1456.00	176.59	0.518	>1500
2	3.76	114.80	60.52	11.474	>1500
3	3.58	333.20	57.33	15.856	>1500
4	3.52	224.00	58.78	9.210	>1500
5	3.54	299.60	62.08	13.130	>1500
6	3.51	310.80	61.77	14.620	>1500
7	3.51	294.20	60.86	11.340	>1500
8	3.58	260.40	143.07	13.130	>1500
Average	3.71	411.62	85.12	11.16	>1500
VN Standard 40:2011/BTNMT	low	Over 10 times	Over 21 times	low	Over 37 times
A level B level	6 – 9 5.5 - 9	20 40	4 6	50 100	20 40

Origin: Advanced Lad., Can Tho University, 2015

Table -2 pH and physical and chemical characteristic of wastewater from rice noodle factories of My Tho city, Tien Giang province, Vietnam

Treatment	**2013***	**2014***	**2015****
pH	7.53	4.35	3.71
TN (mg/L)	7.85	121.1	411.6
Orthophosphate (mg/L)	-	-	85.1
TP (mg/L)	1.51	14.1	-

TSS (Total of suspended solid)	49	354.8	11.1
BOD5 (biological oxy demand)	60	1200	>1500
Coliforms (MPN/100 mL)	7.10^3	$9.2.10^5$	-

*Samples were measured at centre of technique and biotechnology, Dept. Science and Technoly, Tien Giang province in 2013 and 2014
** samples were measure at Advanced Lab. of Can Tho University in 2015

Rapid development of industrialization and human activities has lead to increase the discharge of waste and wastewater containing organic and inorganic pollutants. A lot of effort have been tried to search for effective and economic techniques removal of pollutants from wastewater. Flocculation is an essential process in the treatment of wastewater, tap water production and dredging or downstream processing techniques in a variety of industrial fields (8). Flocculants are divided into inorganic flocculants such as aluminium sulfate (Alum) and polyaluminium chloride (PAC), organic synthetic high polymer flocculants such as polyacrylamide (PAA) derivatives, polyacrylic acids and polyethylene imine and naturally occurring bio-polymers flocculants such as chitosan, sodium alginate and microbial flocculants or bioflocculant (9,10,11). Bioflocculant is a kind of biodegradable macromolecular flocculantsecreted by microorganisms. Because of their biodegradability, harmlessness and lack of secondary pollution, bioflocculants have gained much wider attention and research to date (12). Most of research focused on screening for microorganisms, culture conditions, mechanism of flocculation, chemical structure, and so on (13,14,11,15,16,17). The actual application of this bioflocculant in rice noodle wastewater dewatering were investigated under a variety of conditions. Furthermore, based on the performance in the flocculation of kaolin clay suspension (4.0 g L^{-1}).

Free ammonia is toxic to fish and many aquatic organisms, both ammonium ion and ammonia are oxygen-consuming compounds which deplete dissolved oxygen in receiving water (18). Nitrogen removal (i.e., the conversion of ammonium and organic nitrogen to nitrogen gas forms) by heterotrophic microorganisms has attached increasing interest recently in wastewater treatment (19,20) and they has usually been reported as the result of simultaneous heterotrophic nitrification and aerobic denitrification (21). Specifically, the pathway has been widely accepted as the removal of NH_4^+ to NO_2^- or NO_3^- (heterotrophic nitrification) and simultaneously aerobic conversion of the NO_3^- or NO_2^- to N_2O and/or N_2 (aerobic denitrification) as *Alcaligenes faecalis* (22), *Bacillus* sp. (23), *Acinetobacter calcoaceticus* [24] and they were isolated from wastewaters. Polyphosphate accumulating organisms (PAOs) is known as the microorganisms to absorb free phosphate in the environment and assimilate them as intracellular polyphosphate (poly-P) particles. This process was viewed as enhanced biological phosphorus removal (EBPR) in wastewater treatment systems (25, 26, 27). One of the methods to identify bacteria that are able to accumulate poly-P more or less is qualitative and quantitative analysis of intracellular poly-P particles. Several techniques can be used as methyl blue staining of poly-P (25). Buzoleva et al., (28) demonstrated that most of the bacterial poly-P is alkali-soluble, especially at the preliminary stage of cell growth (28). Eixler et al., (29) using several different methods for extraction of intracellular poly-P of *Chlorella vulgaris* and *Synechocystic* sp. The results clearly indicate that NaOH and hot water treatment followed by filtration of the extracts are suitable to obtain a cell free suspension of intact poly-P granules without hydrolysing the polymers (29).

At present, molecular methods based on 16S rRNA has been used widely to study the population structure of bacteria domain. In this study, molecular methods based on 16S rRNA was used to identify the population composition of heterotrophic nitrogen removal (HNR) bacteria, poly-P bacteria and bioflocullant-producing bacteria (BPB) and drop plate count method (30) to enumerate HNR bacteria, poly-P bacteria and FPB in wastewater from 8 rice noodle factories in the Tien Giang, the Mekong Delta, Vietnam.

II. MATERIALS AND METHODS

2.1 Isolation and screening of bioflocculant-producing bacterial [31]

Polyglutamic Acid (PGA) was used as a medium in culturing biopolymer-producing bacterial.

Compositions of the medium were as follows: 20 g/L glucose, 0.5 g/L yeast extract, 50 g/L L-glutamic acid and 0.5 g/L $MgSO_4.7H_2O$ and the medium was adjusted to pH 7 (32). The culture were incubated at $30^oC – 50^oC$ for 43 – 77 hours in order to investigate the effect of temperature and incubation period on flocculating activity of bacterial growth on PGA medium for protein-flocculating bacteria. With polysaccharide-flocculating bacteria, the culture medium consisted of (g/l): glucose, 10; yeast extract, 0.5; carbamide 0.5 g; K_2HPO_4, 5 ; KH_2PO_4, 0.2; agar, 20. The initial pH of media was adjusted to 7.0–7.2 (33). After 43-77 hours incubation period, the flocculating activities for each culture were determined using the procedures that were modified from methods as described by Kurane et al. (34) and Suh et al. (11). Kaolin clay suspension was used as a test material in this flocculating activity measurement. The kaolin clay was suspended in distilled water at 5000 mg/L concentration (Kaolin suspension). In a 100 ml beaker, 45 ml kaolin suspension was added and was mixed with 4.5 ml of 1% $CaCl_2$ solution for about 30 seconds. 0.5 ml of the test culture broth was added into the kaolin and $CaCl_2$ solution followed by mixing for about 1 minute

on stirring hot plate. pH of the solution adjusted to pH 7. The mixture then was allowed to stand for 5 minutes (35) at room temperature to allow the flocculation of particles in kaolin clay suspension by the bioflocculant produced. Flocculation was determined by measuring the absorbance of the upper phase of suspension at 550nm. A control experiment using 0.5 ml of distilled water was used instead of the cultures broth was added to the suspension performed in the same manner and the absorbance was measured. Determination of the flocculating activity and flocculation rate was determined using following formula (31).

Flocculating activity = (1/A -1/B) (1)

Flocculation rate (%) = B – A x 100/B (2)

A = absorbance of the samples

B = absorbance of the control

2.2. Isolation of Heterotrophic Nitrogen Removal Bacteria (HNRB)

The sources for isolating micro-organisms were wastewater of 7 rice noodle factories in the My Tho city, Tien Giang province, the Mekong Delta (Figure 1), samples were stored at 15-20^{o}C in plastic containers and they were moved to laboratory to stored in the refregirator.

2.2.1 Media

Media were used in this study (24) with a standard medium was prepared for enrichment and isolation of bacteria by dissolving 10 g of peptone, 10 g of beef extract, and 5 g of NaCl in distilled water (per liter). This standard medium was autoclaved for 30 min at 121^{o}C.

The ingredients of a basal medium in 100 ml distilled water (pH 8) were as follows: 0.4 g of NaCl, 2.15 g of Na_2HPO_4, 0.09 g of KH_2PO_4 and 3 ml of trace elements solution. The trace elements solution contained 0.3 g of $MgSO_4.7H_2O$, 0.1 g of $MnSO_4$, 0.112 g of H_3BO_3, 0.03 g of $FeSO_4.7H_2O$ and 0.06 g of $CaCl_2$ (per liter). Different amounts of nitrogen and organic carbon sources were added to basal medium for groups of nitrifiers or denitrifiers (Table 3). Each basal medium was autoclaved for 15 min at 110^{o}C. The chemicals were purchased from Merck.

Table 3- List of nitrogen and carbon adding in the basal medium per 100 ml

	Nitrogen and carbon amount			
Component	A	B	C	D
NH_4Cl solution (ml)	12			4
$NaNO_2$ solution (ml)		4		4
$NaNO_3$ solution (ml)			4	4
Glucose (g)	0.3	0.1	0.1	0.3

A contained 1 mg/ml of NH_4^+ -N

B contained 1 mg/ml of NO_2- -N

C contained 1 mg/ml of NO_3^- -N

2.2.2. Count and isolation of bacteria in the material

The samples were agitated to obtain homogeneous suspensions between sewage (water) and sludge (solid) in sterile distilled water. Suspended liquid (100 µl) was piped into a tube (10 ml) that contained the standard medium. After 48 h of aerobic incubation at 30^{o}C and 120 rpm, 1 ml suspended liquid were suspended in 90 ml of sterile distilled water in flask-250 mL for 10 min a shaker [New Brunicks, USA]. The supernatant was appropriately diluted using sterile distilled water with 10^{-2}, 10^{-3}….dilution. Five drops put on the media A (for ammonium), the media B (for nitrite), the media C (for nitrate) and the media D (combination of ammonium, nitrite, nitrate) with each dilution and they were incubated in 30^{o}C. After 24 or 48 h, generated colonies were counted for calculating colony-forming units per 1 ml or 1 g of dry matter (CFU g^{-1} DM). Simulteneously, each isolate was cultivated in each medium to detect the ability of ammonium, nitrite, nitrate or combination of three kinds of above nitrogen. Purified isolates were obtained by repeated streaking on fresh agar plates. A bacterium with high nitrogen removal efficiency was obtained and named and they were suspended in 20% glycerol solution at -80^{o}C for long-term storage.

2.3. Isolation of poly-phosphate bacteria (poly-P)

2.3.1. Media

The media used for isolating micro-organisms are composed of the agar-based culture medium and agar-based subculture medium. Components for the agar-based culture medium (g/l): 5.0 Acetate, 0.5 Glucose, 0.5 Succinate, 0.02 NH_4Cl, 0.088 KH_2PO_4, 0.5 Pepton, 0.01 $MgSO_4.7H_2O$, 0.005 $CaCl_2$ and 0.5 ml of a trace element solution (36). The trace element solution contained (g/l): 1.5 $FeCl_3$. $6H_2O$, 0.15 H_3BO_3, 0.03 $CuSO_4$. $5H_2O$, 0.18 KI, 0.12 $MnCl_2$. $4H_2O$, 0.06 N.

2.3.2. Isolation of Bacteria

Cultivation-based techniques were used to gain insight into the abundance and species composition of bacterial communities, and to reveal the poly-P accumulation of bacteria. Serial dilutions (10^{-2} to 10^{-4}) of composite samples were prepared. Dilutions (0.05 ml) were aseptically plated on the agar-based culture medium. Plates were incubated at 30^{o}C for 5 days. Bacterial colonies were differentiated on the basis of colony morphology and pigmentation. Colonies were subculture on the agar-based subculture medium plates by striking technique and re-incubated at 30^{o}C for 5 days. This isolation process carries out in shifts of the agar-based culture medium to the agar-based subculture medium until monocultures were obtained. Monocultures were culture on the agar-based culture medium slant in the test-tube (12 ml) and incubated at 30^{o}C for 4 days following by stored 10^{o}C in refrigerator.

2.3.3. Culture Medium and Enrichment Culture for poly-P

Phosphate Uptake Studies Inoculums were prepared by plated onto the agar-based culture medium with a isolate stored and incubated at 30^{o}C for 6 days. Inoculums were picked and inoculated to 4 ml of broth medium tube sterilization at 30^{0}C for 6 days (on shaker at 160 r min-1). The following medium was used throughout this study: 5 g/l sodium acetate, 0.5 g/l $MgSO_4$, $7H_2O$, 0.18g/l KNO_3, 0.25 g/l KH_2PO_4 (36) with supplemented components as 0.5 g/l pepton, 0.5 g/l yeast extract and 0.5 ml/l of a trace elements solution containing (37).

2.3.4. Extraction of Poly-P

Extraction of intracellular poly-P was carried out according to the method of Eixler et al., (29). Cell suspension (4 ml) was centrifuged (10,000 rpm; 10 min), the supernatant discarded and the pellet re-suspended in 4 ml NaOH 0.2 N at 30^{o}C (on shaker at 160 r min^{-1}) for 20 hours in order to extracting poly-P. All extracts were filtered (Sartorius Stedim CE, Cat. No. 17598 and 0.45 µm) for reject cells.

2.3.5. Quantification of Intracellular Poly-P

Subsamples of this suspension were used for chemical analysis. The supernatant was dividing into two parts. The first fraction was measured as soluble reactive phosphorus with the molybdenum blue method (38). This fraction was named pp1 in the following. The second fraction was hydrolyzed in the present of 1 M HCl for 10 min at 100^{o}C. Amount of labile phosphorus in all poly-P fractions was determined again as described above. This fraction was named pp2 (total extractable cellular phosphorus). The difference between pp2 and pp1 should represent the content of poly-P.

2.4. DNA extraction, PCR amplification and 16S rRNA gene sequence analysis

2.4.1. Bioflocculating bacteria (BPB)

Genolic DNA was extracted from the cultures grown to the protocol Neumann et al. (39)

Bacteria universal primers, the forward primer 37F and the verse primer 1479R (40) were used to amplified partial length of 16S rRNA gene sequence. The 16S rRNA gene quence was amplified in a PCR mixture, composed with 1 mol l^{-1} of each primer (2 µl/primer); 200 µmol l^{-1} of each dNTP (8 µl), 1.5 mol l^{-1} $MgCl_2$ (7 µl) in 10 m mol l^{-1} Tris/HCl pH 8.3) buffer, BSA (0.5 µl). DNA (0.1 µg) (8 µl) and 2.0 U Taq DNA polymerase [Fermentas](0.5 µl) were added in 50 µl PCR mixture. PCR amplications were performed at 95^{o}C for 4 min, 35 cycles of denaturation at 95^{o}C for 1 min, annealing at 56^{o}C for 56 s and extension at 72^{o}C for 120 s; and a final extension at 72^{o}C for 10 min. Partial 16S rRNA genes of some good bacterial strains was sequenced by MACROGEN, Republic of Korea (dna.macrogen.com) and they were chosen to sequence, the results were compared to sequences of GenBank based on partial 16S rRNA sequence to show relationships between other bacterial strains (41) and the phylogenetic analysis was constructed by the Maximum Likelihood method based on 1.000 bootstraps.

2.4.2. Heterophic Nitrogen Removal Bacteria (HNRB)

DNA was extracted from a bacterial suspension (1 ml from a TSB medium at 30^{o}C and 120 rpm for 24h) to DNA protocol of Neumann et al. (39). Primers 8F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (42) were used to amplify 16S rRNA gene by a PCR protocol. Amplification was performed in a total volume of 50 µl in 0.2 ml Eppendorf tubes using a DNA thermocycle (BioRAD). The reaction mix was prepared using the following: 1 x PCR buffer (20 mM Tris-HCl-NH_4SO_4) with 5 µl, 4 µl dNTP (20 nmol of each deoxynucleoside triphosphate), 2 µl primer 8F; 2 µl primer 1492R (30 pmol of each primer), 0.5 µl BSA (100 µg of bovine albumin per ml), 2 µl of template DNA and 2.5 U of *Taq* DNA polymerase (Fermentas, Singapore) and 24 µl biH_2O. The standard thermal profile used for amplification of the 16S rRNA sequence was as follows: 5 min at 95^{o}C; then 30 cycles consisting of 30 s at 94^{o}C (denaturation), 30 s at 53^{o}C

(annealing), and 90 s at 72^{o}C (elongation) and a final cycle of consisting of 10 min at 72^{o}C. Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of selectived isolates in each nitrogen group were sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by way BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi); In each site (city or province) and the best isolate (bacterial isolate can utilize the highest nitrogen concentration) in every group (bacteria utilize ammonia, nitrite, nitrate or combination of three nitrogen kinds) and 11 isolates were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between heterotrophic nitrogen strains (41) and phylogenetic tree were constructed by the maximum-likelihood method based on 1000 bootstraps.

2.4.3. PAOs bacteria

A whole cell direct lysis was used to extract DNA as described previously (43). Universal eubacterial primers 27F and 1492R (44) were used for PCR to amplify 16S rRNA gene from the genomic DNA. The PCR was carried out according to Ivanov et al., (45). in a 25µl (total volume) reaction mixture containing 100 ng purified template DNA, 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM each of dNTP, 0.2 µM each of forward and reverse primers and 1U Taq polymerase. After initial denaturation at 95^{o}C for 5 min, followed by 30 cycles of thermal cycling (denaturation at 95^{o}C for 1 min, primer annealing at 57^{o}C for 0.45 min, extension at 72^{o}C for 1.5 min), a final extension at 72^{o}C for 7 min. PCR products were run on 1,2% agarose gels with 1X TAE buffer at 90 V for 1.5 h, and bands visualized with ethidium bromide. Gels were viewed and photographed with Bio-Rad Universal HoodII. PCR amplicons were purified with Qiagen PCR purification kits. For the sequencing of 16S rRNA gene, the nucleotide sequence of each amplicon was determined using the dideoxy chain termination chemistry and the ABI model 310A sequencer (Applied Biosystems, USA). For each determined sequence, the Basic Local Alignment Search Tool (BLAST) search program was queried for initial determination of the nearest phylogenetic neighbour joining sequences in the database maintained by National Centre for Biotechnology Information (NCBI) (National Institutes of Health, USA).

The reference sequences were chosen from representatives of the nearest neighbour groups, as well as the sequences from taxa representing several different bacteria phyla. All sequences were multi-aligned by CLUSTALW (1.6) program. The phylogenetic tree were constructed from evolutionary distances using MEGA7.0 program (41). Bootstrap confidence values were obtained with 1000 resamplings. The bootstrap values indicated the resampling percentage that supported a specific branching pattern. The identification of stains was based on closest species on the phylogenetic tree and classified to various classes, families according to Bergey's Manual of Systematic Bacteriology, 2nd edition (New York: Springer) (46).

2.5. Statistical Analysis

The experiment was analyzed as a two-way ANOVA with the isolates and with levels of N as nitrite, nitrate, ammonium and combination. All analyses were conducted using the programme MSTATC, Minitab 16. The data were considered significantly different at $P<0.01$.

III. RESULTS AND DISCUSSION

3.1. Population of Bioflocculant-producing bacteria (BPB), Heterophic Nitrogen Removal Bacteria (HNRB) and poly-Phosphate (PAOs) Bacteria

From eight samples of wastewater from 8 rice noodle factories at My Tho city, population of Bioflocculant-producing bacteria (BPB), Heterophic Nitrogen Removal Bacteria (HNRB) and poly-Phosphate (PAOs) Bacteria was presented in Table 4.

Table 4- Population of Bioflocculant-producing bacteria (BPB), Heterophic Nitrogen Removal Bacteria (HNRB) and poly-Phosphate (PAOs) Bacteria in wastewater from 8 rice noodle factories of My Tho city

Factory Number	pH	Bacterial population in the media (log_{10}CFU/ml)						
		$NaNO_2$	$NaNO_3$	NH_4Cl	Combination (3 kinds of N)*	Bioflocculant protein	Bioflocculant polysaccharide	PAO's
1	4.65	0	0	0	0	4.12	4.31	4.11
2	3.76	0	0	0	0	4.18	3.11	4.91

3	3.58	4.35	4.42	4.27	4.27	4.26	4.33	4.27
4	3.52	3.94	4.07	4.16	3.94	4.21	4.71	5.14
5	3.54	3.97	4.18	4.31	4.26	4.34	4.39	5.65
6	3.51	0	0	0	0	4.19	4.76	5.41
7	3.51	4.65	4.81	4.65	4.81	4.29	4.24	4.57
8	3.58	0	0	0	0	4.18	4.28	4.57

*bacterial isolates utilize three kinds of N (ammonium, nitrite and nitrate)

The results of Table 2 showed that low pH of wastewater varied from 3.51 to 4.65, there were 4/8 factories did not find out heterotrophic nitrogen removal bacteria and 4/8 factories had low population of HNRB in the wastewater ($<10^5$ cells/ml) while both bioflocculant-producing bacteria (protein and polysaccharide) had low population ($<10^5$ cells/ml). In the contrary, population of PAO's bacteria always appeared in the 8 wastwater samples with high population in comparison to HNRB and BPB.

3.2. Bioflocculant-producing bacteria

Total of 41 isolates of bioflocculanting-producing bacteria were isolated on two media with isolates 23 isolates (on protein medium) and 19 isolates (on polysaccharide medium) (Table 5)

Table 5 – Bioflocculant-producing bacterial isolates isolated from wastewater of 8 rice noodle factories and flocculant rate (in kaolin clay)

No	Protein-producing bacteria	Flocculation rate (%)*	Polysaccharide-producing bacteria	Flocculation rate (%)*
01	PRO.01C-	74.01 a	PO.01C-	62.69 cd
02	PRO.03B-	63.01 cd	PO.01B	43.87 gh
03	PRO.03A-	45.57 fg	PO.03A-	55.96 e
04	PRO.05.01-	48.49 f	PO.03.A1	44.75 gh
05	PRO.05.02-	71.56 ab	PO.03.05-	66.36 a
06	PRO.06.01-	51.99 e	PO.04C	49.54 f
07	PRO.06.03-	35.17 ij	PO.05.01	42.21 h
08	PRO.08A-	59.37 d	PO.07.02-	48.93 f
09	PRO.01B-	46.18 f	PO.08.01-	32.42 i
10	PRO.01.02-	37.31 i	PO.02A	40.01 h
11	PRO.02.01-	37.62 i	PO.02C	33.98 i
12	PRO.02A-	41.91 h	PO.03.A3	30.98 i
13	PRO.07F-	13.76 k	PO.03.01-	59.02 d
14	PRO.04.03-	50.46 e	PO.05.02	34.76 i
15	PRO.04.06-	47.11 f	PO.06.03-	45.26 g
16	PRO.06V-	51.99 e	PO.07.01	50.18 ef
17	PRO.02.02-	44.04 g	PO.07.03-	57.19 de
18	PRO.07B-	39.45 hi	PO.07.04	47.72 g
19	PRO.07D-	47.41 f	PO.08A-	63.31 b
20	PRO.07E-	50.46 e		
21	PRO.07.04-	71.56 ab		
22	PRO.08C-	58.72 d		
23	PRO.04.01-	67.28 b		
	C.V (%)	16.11%	C.V (%)	12.12%

*Flocculating rate in kaolin clay

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

Their colonies have round-shaped or not identified; milky; viscosity; entire or lobate margin (Figure 2) and all of them are Gram-positive or Gram-negative by Gram stain. The cells were observed by SEM and appeared as short rods and most of them have motility very fastly.

Figure 2 – The colonies of flocculating-producing bacterial isolates (PRO.08A and PO.03.05)

3.3. Heterotrophic Nitrogen Removal Bacteria (HNRB)

Total of 59 heterotrophic nitrogen removal bacterial isolates were isolated from 8 wastewater samples of 8 rice noodle factories at My Tho city, Tien Giang province including of 15 heterotrophic nitrite oxidation bacterial isolates (HNiOB), 18 heterotrophic nitrate oxidation bacterial (HNaOB) isolates, 15 heterotrophic ammonium oxidation bacterial (HAmOB) isolates and 11 combination isolates (combination of three kind of N = bacterial isolates utilize three kinds of nitrite, nitrate and ammonium) (Table 6).

The result from table 6 showed that the isolates as HNi.03D, HNa.03A, HAm.06Dr, COM.02F and COM.05A were the isolates grew on the media nitrite (60 mM), nitrate (400 mM), Ammonium (800 mM) and combination (300 mM), respectively.

Table 6. Fifty-nine heterotrophic nitrogen removal bacterial isolates were isolated from wastewater of 8 rice noodle factories and the they grew on media with different nitrite, nitrate, ammonium concentrations.

Isolate name HNiOB	The best Nitrite conc.	Isolate name HNaOB	The best Nitrate cone.	Isolate name HAmOB	The best ammonium Conc.	Isolate name Combina.	The best Conc.
HNi.04A-	30	HNa.01A-	400	HAm.01B-	800 mM	COM.07C-	300 mM
HNi.03B-	60 mM	HNa.04B-	600	HAm.02A	200	COM.02E	100
HNi.03Dm	30	HNa.01B	300	HAm.04A	100	COM.07B	200
HNi.03Cr-	60	HNa.03A-	500	HAm.06B-	600	COM.03D	100
HNi.03D-	40	HNa.07A	300	HAm.06C1-	400	COM.05C-	300 mM
HNi.03DL-	60 mM	HNa.03G	200	HAm.03D-	500	COM.06A	200
HNi.08C-	60 mM	HNa.03F	100	HAm.05G	200	COM.02F-	200
HNi.08E-	30	HNa.06C	200	HAm.06Dr-	800 mM	COM.02D1	300 mM
HNi.08A-	30	HNa.05B-	500	HAm.05F	100	COM.02A-	200
HNi.03A	30	HNa.05A1-	800 mM	HAm.07A	400	COM.02C-	200
HNi.04B-	60 mM	HNa.07C	300	HAm.06D-	800 mM	COM.02Dn	100
HNi.03F1	30	HNa.03C-	800 mM	HAm.03B-	500		
HNi.02D-	40	HNa.07B-	800 mM	HAm.06C2-	700		
HNi.05A2-	40	HNa.03D-	600	HAm.05C-	800 mM		
HNi.05A-	40	HNa.06B	300	HAm.06C-	800 mM		
		HNa.05A2	300				
		HNa.01D	100				
		HNa.05A	300				

Their colonies have round-shaped or not identified; milky; yellow; entire or lobate margin (Figure 3) and all of them are Gram-positive or Gram-negative by Gram stain. The cells were observed by SEM and appeared as short rods and most of them have motility.

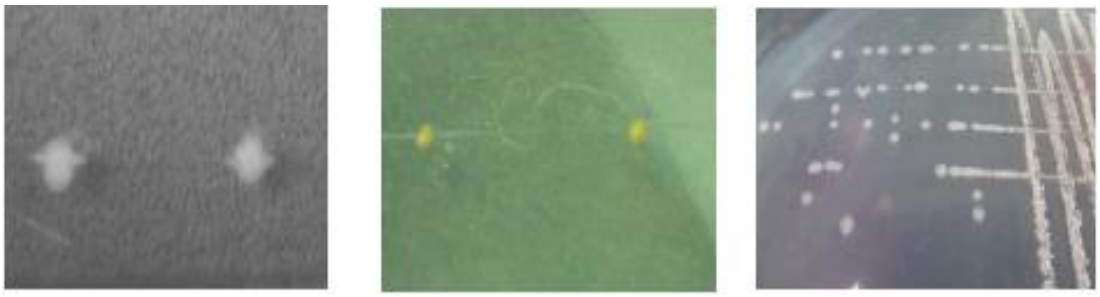

Figure 3. The colonies of heterotrophic nitrogen removal bacterial isolates on different media

3.4. PAOs bacteria

Total of 8 PAO bacterial isolates were isolated from wastewater of rice noodle factories of My Tho city, Tien Giang province (Table 7).

There were three isolates having poly-P accumulation over 1 mg/L (POLYP.01A, POLYP.03C and POLYP.064C). The quantity of isolated PAO bacterial isolates from wastewater of rice noodle factories was low in comparison to HNRB and BPB, this showed that low total of phosphate in wastewater (Table 6) perhaps materials for rice noodle production do not rich phospho and orthophosphate only appeared in rice noodle processing (Table 2).

Table 7 – PAO bacterial isolates were isolated from wastewater of rice noodle factories and poly-P concentration accumulation

Isolate	Poly-P accumulation (mg/L)	Isolate	Poly-P accumulation (mg/L)
POLYP.01A	**9.669 c**	POLYP.05F	7.021 d
POLYP.02B	0.763 f	POLYP.064B	**13.111 a**
POLYP.03C	**11.098 b**	POLYP.078A	1.822 e
POLYP.04D	0.894 f	POLYP.088C	9.552 c

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

Their colonies have round-shaped or not identified; milky; yellow; entire or lobate margin (Figure 4) and all of them are Gram-positive or Gram-negative by Gram stain. The cells were observed by SEM and appeared as short rods and most of them have motility.

Figure 4 – The colonies of PAO bacterial isolates on medium agar

3.5. 16S rRNA gene sequence analysis and phylogenetic tree

3.5.1. Bioflocculant-producing bacteria

From 23 protein-flocculating-bacterial isolates, 4 isolates having the highest floculation rate (PRO.01C, PRO.03B, PRO.0704, PRO.0401) and 2/19 polysaccharide-flocculating bacterial isolates having the the highest flocculation rate (PO.08A, PO.0305) were selected to sequence.

16S rDNA gene amplification and sequencing

The fragments of 1440 bp 16S rRNA were obtained from PCR with 37F and 1479R primers and sequencing. Homology searches of 16S rRNA gene sequence of selected strain in GenBank by BLAST revealved that they had similarity to sequences of Bacilli (6/6 isolates) (Table 6).

Table 8- Phylogenetic affiliation of isolates on the basis of 16S rRNA genes sequences by using BLAST programme in the GenBank database based on sequences similarity

Taxonomic group and strain	Closest species relative	Similarity (%)
Bacilli		
PO 08.A	AB986572 *Bacillus tequilensis*, strain: Cs.10-4.1.9	99
	KX214613 *Bacillus sp*. strain 2N-14	99
PO 03.05	KC172004 *Bacillus methylotrophicus* strain JF29	99
	KU179336 *Bacillus subtilis* strain L23	99
PRO 01.C	FJ392729 *Bacillus subtilis* strain M3-7	99
	KX214613 *Bacillus sp*. strain 2N-14	99
PRO 07.04	AB986572 *Bacillus tequilensis*, strain: Cs.10-4.1.9	99
	LC065158 *Bacillus sp*. NCCP-1131	99
PRO 04.01	MG430224 *Bacillus megaterium* strain CS17	98
	KU991810 *Bacillus aryabhattai* strain J5	98

PRO 03.B	KP735610 *Bacillus subtilis* strain SH23	98
	KY283142 *Bacillus sp*. strain ZJ-1	97

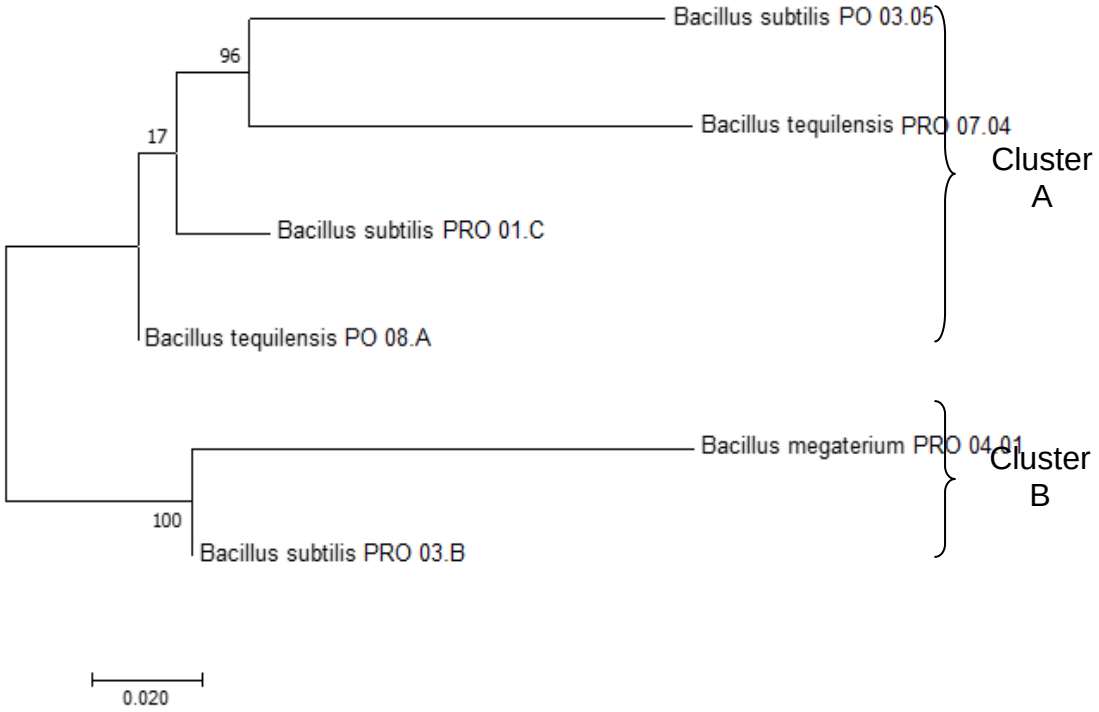

Figure - 4 Phylogenetic tree showing the relative position of bioflocculant-producing bacteria (PPB) by the maximum-likelihood method of complete 16S rRNA sequences. Bootstrap value values of 1000 replicates are shown at the nodes of the trees.

Total of 6 isolates were identified as 6 bioflocculant-producing bacterial strains and they belonged to Bacilli, genus *Bacillus* with the relationshion in plylogenetic tree (Figure 4). A maximum-likelihood phylogenetic tree in BPB showing the two clusters: In cluster A with 6 strains as: *Bacillus subtillis* PO.03.05, *Bacillus tequilensis* PRO 07.04, *Bacillus subtilis* PRO.01C and *Bacillus tequilensis* PO.08A and cluster B only two strains as: *Bacillus megaterium* PRO.04.01 and *Bacillus subtilis* PRO.03B; this result showed that no difference between protein and polysaccharide group and our result was also similar with result of Deng et al., (33) when he identified *Bacillus mucilaginosus* strain MPFA9 which high viscosity and high flocculation rate (99.6%). Lixi et al., (47) showed that characteristic of bioflocculant-producing bacterial colonies on agar media had high viscosity and smooth colony. Tien and Diep (48) also determined 10/16 bioflocculant-producing bacterial isolatates were isolated from piggery wastewater at 9 provinces of Mekong Delata, Vietnam and they were classified to genus *Bacillus* while Salehizadeh et al. (14) identified polysaccharide-producing bacterila isolates as genus *Bacillus* AS-101. Kaewchai and Prasertsan (32) isoltaed and determined polymer-producing bacterial isolates belonging to genus *Bacillus* as *Bacillus subtilis* WD90 and Bacillus *subtilis* SM 29.

3.5.2. Heterotrophic Nitrogen Removal Bacteria (HNRB)

From 59 HNRB isolates were isolated on different kinds of nitrogen, there were 4 HNi isolates (HNi03B, HNi03DL, HNi08C, HNi04B), 3 HNa isolates (HNa05A1, HNa03C, HNa07B), 5 HAm isolates (HAm01B, HAm06Dr, HAm06D, HAm05C, HAm06C), and 3 COM isolates (COM07C, COM 05C, COM02D1) with the highest nitrite concentrations (60 mM), nitrate (800 mM), Ammonium (800 mM), combination (300 mM), respectively; and 11 isolates (HNi, HNa and HAm) were selected to sequence.

16S rDNA gene amplification and sequencing

The fragments of 1485 bp 16S rRNA were obtained from PCR with primers 8F and 1492R and sequencing. Homology searches of the 16S rRNA gene sequence of selected strain in GenBank by BLAST revealved that they had high similarity to sequences of Gram-positive bacteria phylum and Proteobacteria phylum.

Table 9 - Phylogenetic affiliation of isolates on the basis of 16S rRNA genes sequences by using BLAST programme in the GenBank database based on sequences similarity

Taxonomic group and strain	Closest species relative	Similarity (%)
Firmicutes		
HNi03DL	KP761420 *Bacillus altitudinis* strain SP028	99
	KC915229 *Bacillus pumilus* strain ESR21	99
HNi04B	JX010951 *Rhodococcus erythropolis* strain A6	98
	KM282585 *Rhodococcus* sp. BF-5 (2014)	98
Alphaproteobacteria		
HAm06Dr	KU977111 *Agrobacterium* sp. Strain yangyueN8	98
	KX449275 *Agrobacterium tumefaciens* strain VN2013-48	98
HNi08C	KJ870193 *Sphingobacterium multivorum* strain B13	99
	KJ000804 *Sphingobacterium* sp. SCU-B137	98
HAm36D	NR_114151 *Ochrobactrum oryzae* strain NBRC 102588	98
	AJ920029 *Ochrobactrum shiyianus*, type strain WSH-W04T	98
Betaproteobacteria		
HNa07b	KC775771 *Achromobacter xylosoxidans* strain 7B	99
	GU367388 *Achoromobacter* sp. BIT-56	98
Gammaproteobacteria		
HNi03B	KM893074 *Stenotrophomonas maltophilia* strain LH15	99
	KF516076 *Stenotrophomonas* sp. NTa	99
HNa03C	KX350046 *Stenotrophomonas maltophilia* strain F3-2-41	98
	DQ991144 *Stenotrophomonas* sp. BMC	98
HAm01B	KJ396827 *Stenotrophomonas maltophilia* strain IR50	99
	EU770269 *Stenotrophomonas* sp. A18(2008)	99
HAm05C	KC431781 *Enterobacter hormaechei* strain RB3	99
	KY672864 *Enterobacter* sp. strain BAB-6042	98
HAm06C	MF144477 *Enterobacter cloacea* strain FQ30	98
	KY287933 *Enterobacter kobei* strain BVGN0003	98

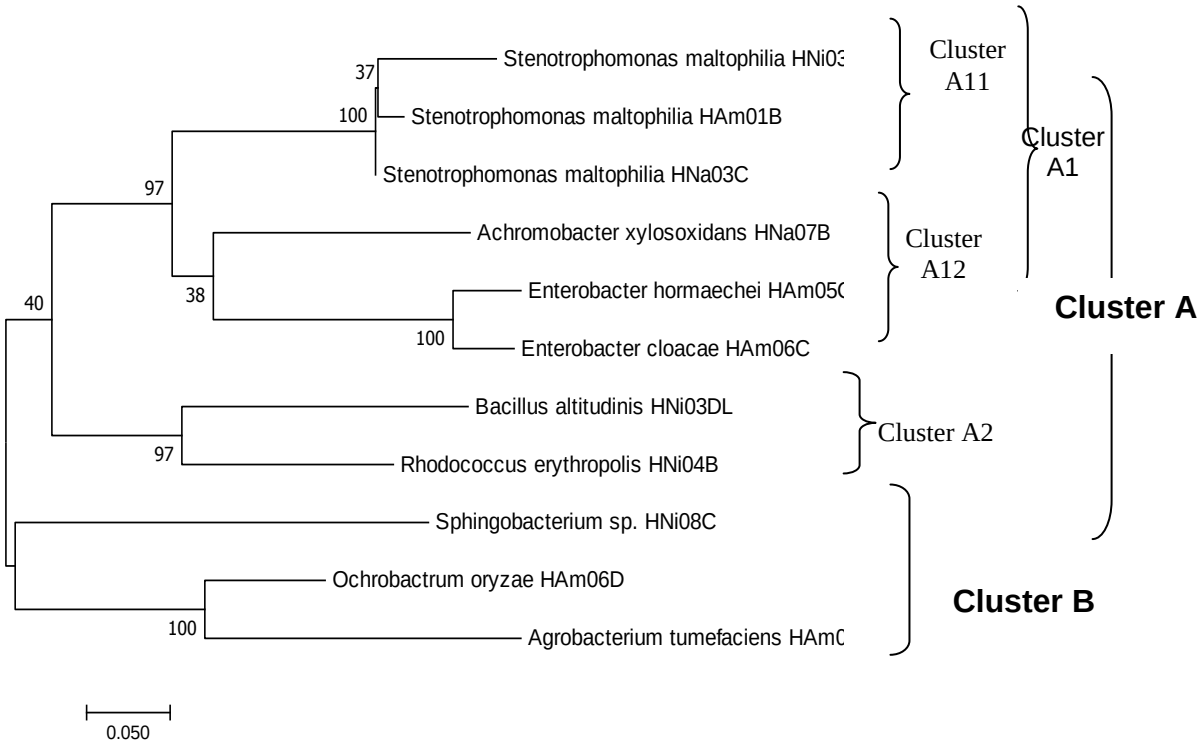

Figure - 5 Phylogenetic tree showing the relative position of heterotrophic nitrogen removal bacteria (HNRB) by the maximum-likelihood method of complete 16S rRNA sequences. Bootstrap value values of 1000 replicates are shown at the nodes of the trees.

A maximum-likelihood phylogenetic tree in HNRB showing the two clusters: In cluster A with two smaller clusters cluster A1 and cluster A1 had 2 clusters: cluster A11 with 3 species in 3 groups (nitrite, nitrate and ammonium) of genus *Stenotrophomonas*, and cluster A12 with 2 species (nitrate group) of genus *Enterobacter* and *Achromobacter xylosoxilans* HNa07B. Cluster A2 only had 2 species of Gram-positive bacteria: *Bacillus altitidnis* HNi03DL and *Rhodococcus erythropdis* HNi14B, this was 2 species in nitrite group while cluster B included 2 species (ammonium group) *Ochrobactrum oryzae* HAm06D and *Agrobacterium tumefasciens* HAm06Dr had close relationship (100%) and both stayed in cluster with Sphingobacterium sp. HNi08C, all of them belonged to AlphaProteobacteria subphylum.

Almost of nitrogen removal bacteria strains were isolated fromwas found in sedimentary and water of striped cat-fish-ponds (Diep and Cuc, 2013) [49] and in the piggery wastes (Diep and Cuong, 2013) [50] in the Mekong Delta, Vietnam which were identified as Firmicutes phylum among high G+C gram-positive bacteria strain and low G+C gram-positive bacteria strain occupied higher than Proteobacteria phylum. On the contrary, in this study, 9/11 strains (81.81%) were identified as Proteobacteria and Firmicutes only had 2/11 strains (18.19%).

3.5.3. PAO's

Among 8 PAO's isolates were isolated, 3 isolates having the highest P accumulation were chosen to sequence.

The determination of nearest phylogenetic neighbor sequences for 16S rRNA gene sequences of the three isolated by the BLAST search program showed that they grouped into the class Bacilli (Table 10). Their phylogenetic relationship was shown in the phylogenetic tree (Figure 3).

Table 10 - Phylogenetic affiliation of isolates on the basis of 16S rRNA genes sequences by using BLAST programme in the GenBank database based on sequences similarity

Taxonomic group and strain	Closest species relative	Similarity (%)
POLYP.01A	KC456633 *Bacillus subtilis* strain TUST019	98
	KR703626 *Bacillus* sp. JES1	98
POLYP.03C	KU877333 *Bacillus* sp. M29(2016b)	98
	KU179323 *Bacillus subtilis* strain L4	97
POLYP.064B	KU179345 *Bacillus aryabhattai* strain L42	99
	MG430247 *Bacillus megaterium* strain CS40	99

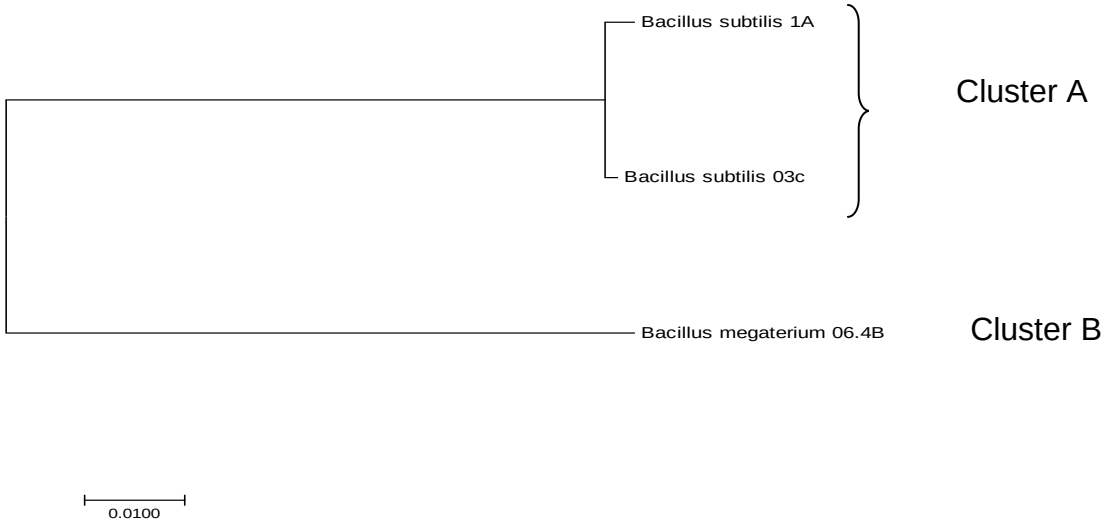

Figure - 6 Phylogenetic tree showing the relative position of poly-P (PAO's) by the neighbor-joining method of complete 16S rRNA sequences. Bootstrap value values of 1000 replicates are shown at the nodes of the trees.

A simple neighbor-joining phylogenetic tree in PAO's showing the two clusters: In cluster A with two strains: *Bacillus subtilis* 01A and *B. subtilis* 03C and cluster B had only one *Bacillus megaterium* 06B strain. Khoi and Diep (2013) [51] isolated twenty-one bacteria isolates from samples of catfish ponds included in four classes: Bacilli, Actinobacteria, Beta-proteobacteria, Gamma-proteobacteria. The majority of the strains showed excess phosphate accumulation. Strains related to *Bacillus* sp. were dominant bacteria group constituted up to 52.4% of all identified isolates, but high phosphate accumulating bacteria are *Burkholderia vietnamiensis* TVT003L within class Beta-proteobacteria, *Acinetobacter radioresistens* TGT013L within Gamma-proteobacteria and *Arthrobacter protophomiae* VLT002L within class Actinobacteria. Our result showed that isolated PAO's identified as genus *Bacillus.*

IV. CONCLUSION

From wastewater samples of eight rice noodle factories in My Tho city of Tien Giang province, Vietnam, 42 BPB, 59 HNRB and 8 PAO's isolates, were isolated on specific media, respectively. Six BPB, 11 HNRB and 3 PAO's isolates were chosen to sequence and relationship. In HNRB, Gram-positive bacteria (2/11 strains, occupied 18.18%) composed of bacili (1/11), actinobacteria (1/11), and Proteobacteria (9/11 strains, occupied 81.12%) composed of Alphaproteobacteria (3/11), Betaproteobacteria (1/11) and Gammaproteobacteria (5/11) while all of BPB and PAO's strains belonged to genus *Bacillus.*

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