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Heterotrophic nitrogen removal bacteria in piggery wastes in the Mekong Delta, Vietnam

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Abstract: A total of 2318 heterotrophic nitrogen removal (HNR) bacteria isolated from piggery wastes (after biogas container) were classified in four kinds of heterotrophic ammonia-oxidizing bacteria (569 isolates), nitrite-oxidizing bacteria (580 isolates), nitrate-oxidizing bacteria (600 isolates) and heterotrophic nitrifying and denitrifying bacteria (569 isolates). The virtually complete 16S rRNA gene was PCR amplified and sequenced. The sequences from the selected HNR bacteria showed high degrees of similarity to those of the GenBank references strains (between 97% and 99.8%). Phylogenetic trees based on the 16S rDNA sequences displayed high consistency, with nodes supported by high bootstrap (500) values. These presumptive HNR isolates were divided four groups that included members of genera *Arthrobacter, Corynebacterium, Rhodococcus* (high G+C content gram-positive bacteria), *Staphylococcus, Bacillus* (low G+C content gram-positive bacteria) and *Klebsiella* (gram-negative bacteria). Based on Pi value (nucleotide diversity), heterotrophic ammonium-oxidizing bacteria group had highest values and heterotrophic nitrifying-denitrifying bacteria group had the lowest values and Theta values (per sequence) from S of SNP for DNA polymorphism showed that heterotrophic nitrate-oxidizing bacteria group had the highest theta values in comparison of three groups. The present study, the HNR bacteria from piggery wastes, showed a very diverse community of HNR bacteria with a relatively high number of species involved in solid-wastewater samples and many isolates have nitrogen utilization ability at high concentration (800 – 1200 mM) and high G+C gram-positive bacteria strain occupied higher than low G+C gram-positive bacteria strain.

Keywords: Heterotrophic Nitrogen Removal, 16S RNA Gene Sequence, Biologic Nitrogen Removal, Piggery Waste, Gram-Positive Bacteria

1. Introduction

Environmental pollution has become one of the world problems and water pollution caused by husbandry farms seriously. Pig farms with ten to several hundreds of animals are in many developing countries popularly as Vietnam with a little of adequate systems for waste treatment and disposal as biogas containers. However sewage from biogas containers contains the high organic matter concentration especially the presence of nitrogen in wastewater discharge is underable for several reasons. 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 [1]. 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 [2][3] and they has usually been reported as the result of simultaneous heterotrophic nitrification and aerobic denitrification [4]. 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* [5], *Bacillus* sp. [6], *Acinetobacter calcoaceticus* [7] and they were isolated from piggery wastes.

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 and drop plate count method [8] to enumerate HNR bacteria in sludge

of piggery from pig farmers (wastes released to biogas containers) in the Mekong Delta, Vietnam. The aims of this study were to quantify HNR bacteria populations and identify their diversity in sludge of waste (from biogas containers) and isolates were identified gram staining, population and selected representative strains were identified at the molecular level using 16S rRNA sequence analysis.

2. Material and Methods

2.1. Site description

The Mekong Delta is western basin of Southern Vietnam, it occupies over 4 million ha which is the most important rice area of Vietnam. The Mekong river flows into the basin with two big rivers and run to sea with nine gates and they provide the big quantity of sediment for this basin every year (Figure 1). Besides that, many big pig farms have developed along the rivers/canals and wastewater can be released to rivers directly and this has led water pollution.

Figure 1. *Samples were collected at biogas containers of pig farms of thirteen city/provinces in the Mekong Delta, Vietnam.*

2.2. Sample collection

Sewage samples were collected from wastes (after biogas containers) of big pig farms of 12 provinces and Can Tho city in 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 refrigerator.

2.3. Media

Media were used in this study [7](Zhao et al., 2010) 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 1). Each basal medium was autoclaved for 15 min at 110^{o}C. The chemicals were purchased from Merck.

Table 1. *List of nitrogen and carbon amount added in the basal medium (per 100 ml).*

Component	Nitrogen and carbon amount			
	A	B	C	D
[a]NH_4Cl solution (ml)	12			4
[b]$NaNO_2$ solution (ml)		4		4
[c]$NaNO_3$ 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*

2.4. Count and Isolation of Bacteria in the Mineral

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 dis-

tilled 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°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). Simultaneously, 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°C for long-term storage.

2.5. DNA extraction, PCR Amplification and 16S rRNA Gene Sequence Analysis

DNA was extracted from a bacterial suspension (1 ml from a TSB medium at 30oC and 120 rpm for 24 h) to DNA protocol of Neumann et al. (1992)[9]. Primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3')[10] 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 biH2O. The standard thermal profile used for amplification of the 16S rRNA sequence was as follows: 5 min at 95°C; then 30 cycles consisting of 30 s at 94°C (denaturation), 30 s at 53°C (annealing), and 90 s at 72°C (elongation) and a final cycle of consisting of 10 min at 72°C. Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of selected isolates in each nitrogen group was 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 13 isolates of 13 sites 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 [11](Tamura et al., 2011) and phylogenetic tree were constructed by the neighbor-joining method based on 500 bootstraps.

2.6. SNPs Discovery

The sequence data from 64 nitrogen removal bacterial isolates were analysed with SeqScape@ Software (Applied Biosystem, Foster City, CA, USA). SeqScape is a sequence comparison tool for variant identification, SNP discovery and validation. It considers alignment depth, the base calls in each of the sequences and the associated base quality values. Putative SNPs were accepted as true sequence variants if the quality value exceeded 20. It mens a 1% chance basecall is incorrect.

Nucleotide Diversity (Θ)

Nucleotide diversity (Θ) was calculated by the method described by Halushka et al. (1999)[12].

$$\Theta = K/aL \qquad a = \sum_{i=2}^{n} 1/(i - 1)$$

where K is the number of SNPs identified in an alignment length, n is alleles and L is the total length of sequence (bp).

3. Results and Discussion

Pork is an important source of protein for human meal in Vietnam and pig farms with hundreds to several thousands of animals are in operation in the Mekong Delta, Vietnam without adequat systems for waste treatment and disposal and pig manure has considerable amounts of non-stabilized organic matter and high concentration of ammonia [13](Bernet et al., 1996). Due to high organic matter concentration of the piggery wastes, anaerobic digestion has been recommended as a primary step of treatment as Anaerobic Fixed Bed Reactors (AFBR) and these models have been employed in the treatment of agricultural and industrial wastewaters with success partly. However the suspended solid wastewaters have high concentration of ammonia and pH varied from 3.53 to 8.02 (Table 2) especially pH of solid wastewater from biogas containers related with nitrogen removal bacterial population closely (Figure 2).

From 170 sewage samples, 2318 heterotrophic nitrogen removal bacterial isolates were isolated with 569 heterotrophic ammonia-oxidizing bacteria (HAOB) isolates, 580 heterotrophic nitrite-oxidizing bacteria (HNiOB) isolates, 600 heterotrophic nitrate-oxidizing bacteria (HNaOB) isolates and 569 heterotrophic nitrifying and denitrifying bacteria (HNDB) isolates (Table 3) especially many isolates can utilize at high concentration (800 – 1200 mM)(ammonium, nitrite or nitrate) in each group however the bacterial isolates having three kinds of nitrogen ability only grew on 300 mM (ammonium+nitrite+nitrate) media.

Their colonies have round-shaped or not identified; milky; light brown, pink yellow; entire or lobate margin (Figure 3) and all of them are Gram-positive by Gram stain.

Table 2. *pH and nitrogen concentrations in piggery sewage of 13 city/provinces in the Mekong Delta, Vietnam.*

Location	pH	NH_4^+ concentration in sewage (mg/wet litre)	
		Dry season	Wet season
AN GIANG	3.53 – 7.52	105 - 557	2110 – 2430
BẠC LIÊU	4.86 – 7.10	1226 - 1710	132 – 1596
BẾN TRE	4.63 – 7.08	1036 - 2018	122 – 458
CÀ MAU	3.73 – 7.33	521 - 584	127 – 386
CẦN THƠ	4.32 – 7.11	68 - 113	59 – 68
ĐỒNG THÁP	5.55 – 7.05	3000 - 3331	30 – 53
HẬU GIANG	4.03 – 7.58	185 - 4270	42 – 4056
LONG AN	4.95 – 7.49	1313 - 1877	963 – 1071
KIÊN GIANG	4.01 – 7.33	448 - 755	125 – 154
SÓC TRĂNG	6.51 – 8.02	18 - 23	370 – 401
TIỀN GIANG	5.71 – 7.41	616 - 837	854 – 903
TRÀ VINH	5.61 – 7.93	657 - 679	92 – 152
VĨNH LONG	5.56 – 7.44	1313 - 1877	42 - 4056

Table 3. *Nitrogen removal bacterial isolates isolated from sewage of 170 biogas container samples in 13 city/provinces of the Mekong Delta, Vietnam.*

No	City /province	HAOB	HNiOB	HNaOB	Combination*	Total **
01	An Giang	31	23	27	28	109
02	Bac Lieu	37	57	71	40	205
03	Ben Tre	46	59	79	54	238
04	Ca Mau	71	73	69	68	281
05	Can Tho	48	49	46	48	191
06	Dong Thap	39	37	40	34	150
07	Hau Giang	54	62	48	57	221
08	Kien Giang	49	45	37	34	165
09	Long An	34	30	38	38	140
10	Soc Trang	74	70	74	77	295
11	Tien Giang	37	18	19	29	103
12	Tra Vinh	33	31	31	40	135
13	Vinh Long	16	26	21	22	85
	Total	569	580	600	569	2318

HAOB: Heterotrophic Ammonium Oxidation Bacteria

HNiOB: Heterotrophic Nitrite Oxidation Bacteria

HNaOB: Heterotrophic Nitrate Oxidation Bacteria

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

*** total of four groups*

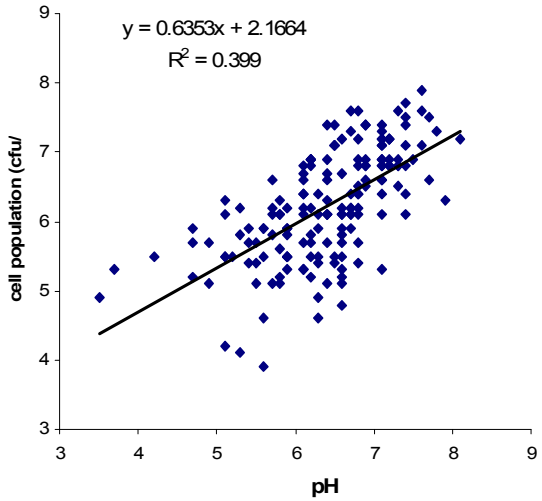

Figure 2. *Correlation between pH and nitrogen removal bacterial population in solid-wastewater samples.*

The cells were observed by SEM and appeared as short rods and most of them are motility (Figure 4).

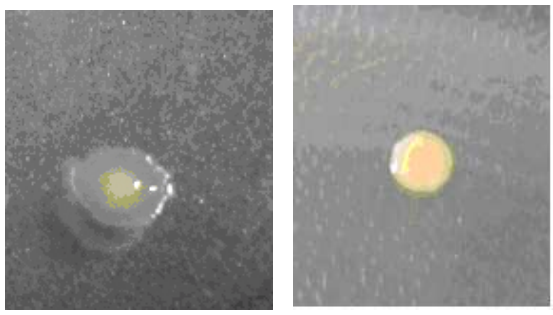

Figure 3. *The colonies of several nitrogen removal bacterial isolates.*

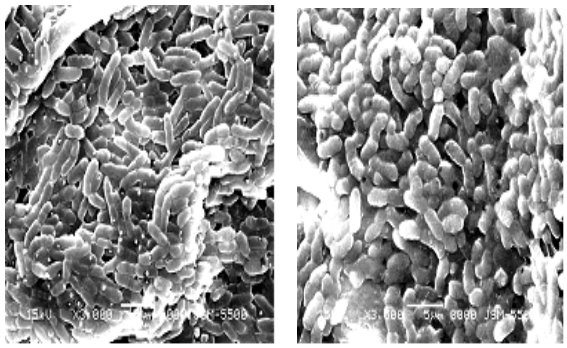

Figure 4. *Scanning electron micrograph of two nitrogen removal bacterial isolates.*

The fragments of 1485 bp 16S rRNA were obtained from PCR 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 Firmicutes phylum. A neighbor-joining phylogenetic tree in HAOB group showing the two clusters: big cluster with two clusters as cluster A11 *Arthrobacter* with CANTHO.27, VINHLONG..03, LONGAN.03, BENTRE.01, SOCTRANG.01, HAUGIANG..02, HAUGIANG..09, TRAVINH.01, CAMAU.02, LONGAN.12 isolates and cluster

A12 with TIENGIANG.18, CAMAU.01, TRAVINH.02, DONGTHAP.49, KIENGIANG.06 were classified as *Corynebacterium*. Cluster A21 with BACLIEU.06 was identified as *Staphylococcus* and BACLIEU.08 and SOCTRANG.02 were identified as *Bacillus* (low G+C Firmicutes phylum) in Cluster A22 and small cluster (cluster B) with ANGIANG.12 isolate was belong to *Klebsiella* (Gram-negative bacteria)(Figure 5). The results showed that HAOB group had relationship closely even through they were isolated from various sites in the Mekong Delta.

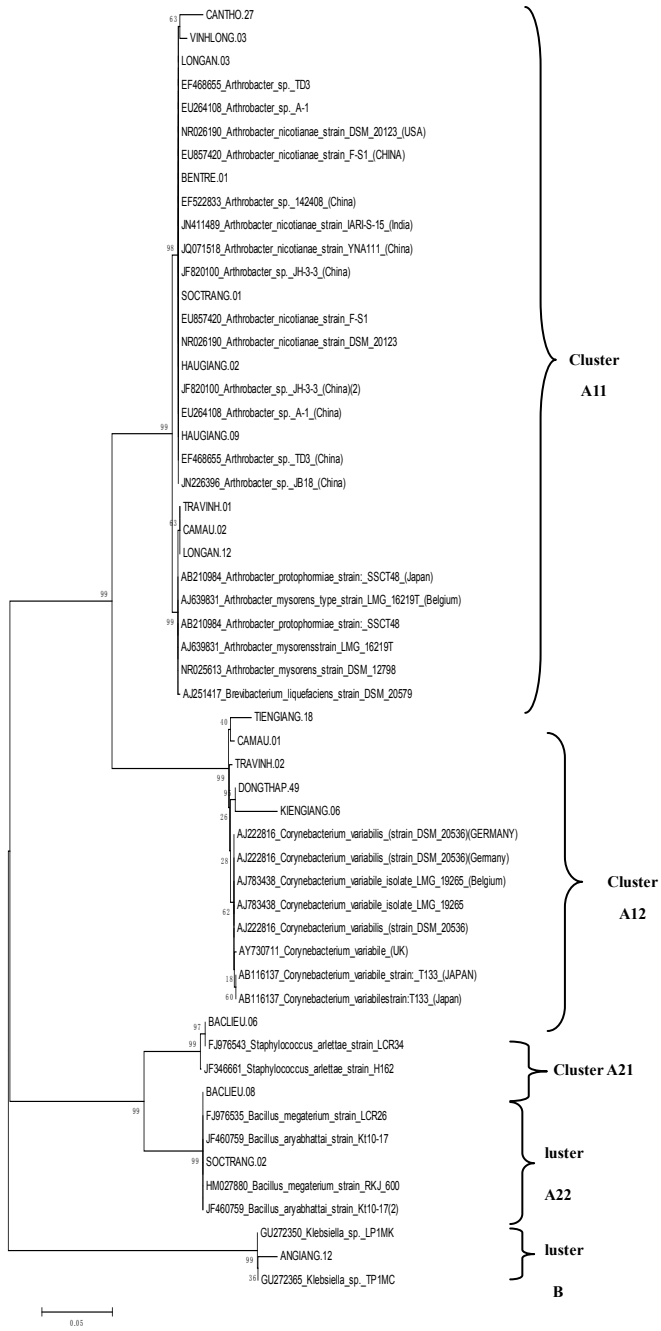

Figure 5. *Phylogenetic tree showing the relative positions of HAOB (ammonia utilization) by the neighbor-joining method of complete 16S rRNA sequences. Bootstrap values of 500 replicates are shown at the nodes of the trees.*

In HNiOB group, phylogenetic tree showed that two clusters with genus *Arthrobacter,* (cluster A1) and *Corynebacterium* (high G+C gram-positive bacteria) in cluster A2 and *Bacillus* (low G+C gram-positive bacteria) in cluster B (Figure 6).

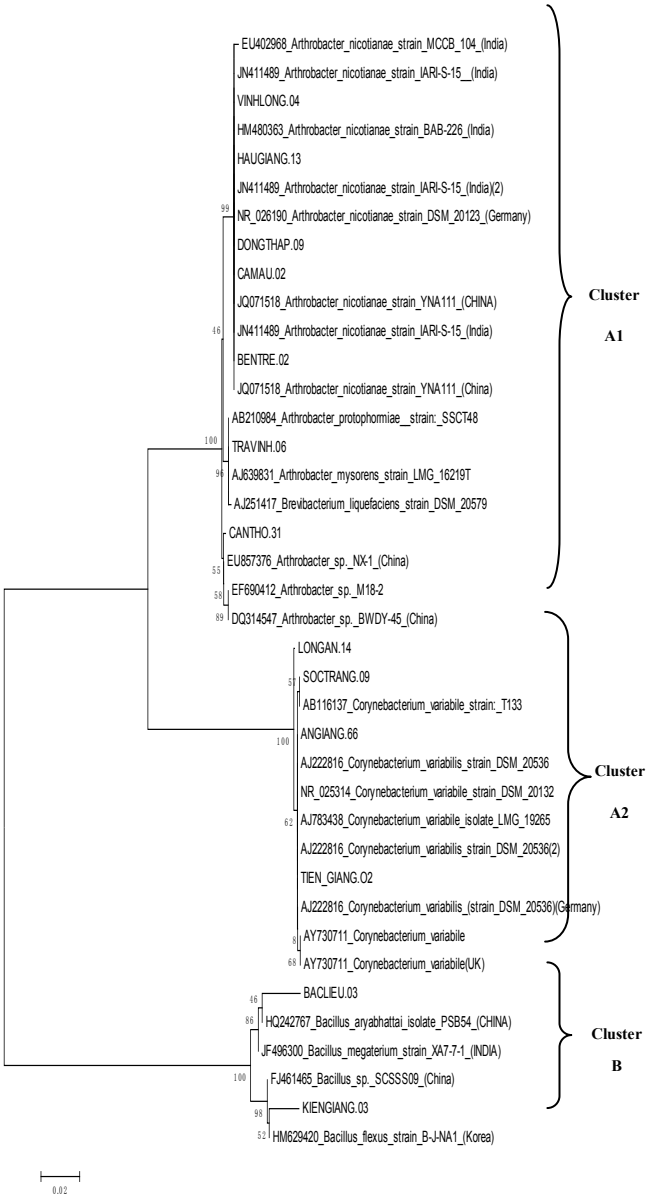

Figure 6. *Phylogenetic tree showing the relative positions of HNiOB (ntrite utilization) by the neighbor-joining method of complete 16S rRNA sequences. Bootstrap values of 500 replicates are shown at the nodes of the trees.*

While HNaOB group, phylogenetic tree distributed as HNiOB group with Cluster A1 as Arthrobacter (LONGAN.15, HAUGIANG.12, DONGTHAP.40, BACLIEU.10, TRAVINH.06) and Cluster A2 as Rhodococcus and Corynebacterium (TIENGIANG.12, ANGIANG.88, SOCTRANG.15, BENTRE.01)(high G+C gram-positive bacteria)(Figure 7).

With HNDB group, phylogenetic tree showed that two clusters: cluster A composed of cluster A11 with HAUGIANG.14, LONGAN.03, TIENGIANG.04, DONG-

THAP.25, LONGAN.11, SOCTRANG.15, TRAVINH.09, CANTHO.26, CAMAU.01, CAMAU.02 isolates in genera Arthrobacter and cluster A12 with TRAVINH.13, BAC-LIEU.06, BACLIEU.08 and ANGIANG.07 isolates were genus Rhodococcus and VINHLONG.03, BENTRE.03, KIENGIANG.01 and SOCTRANG.16 isolates were classified in genus Bacillus. Cluster B with HAUGIANG.03 isolate was determined in genera Enterobacter (gram-negative bacteria)(Figure 8).

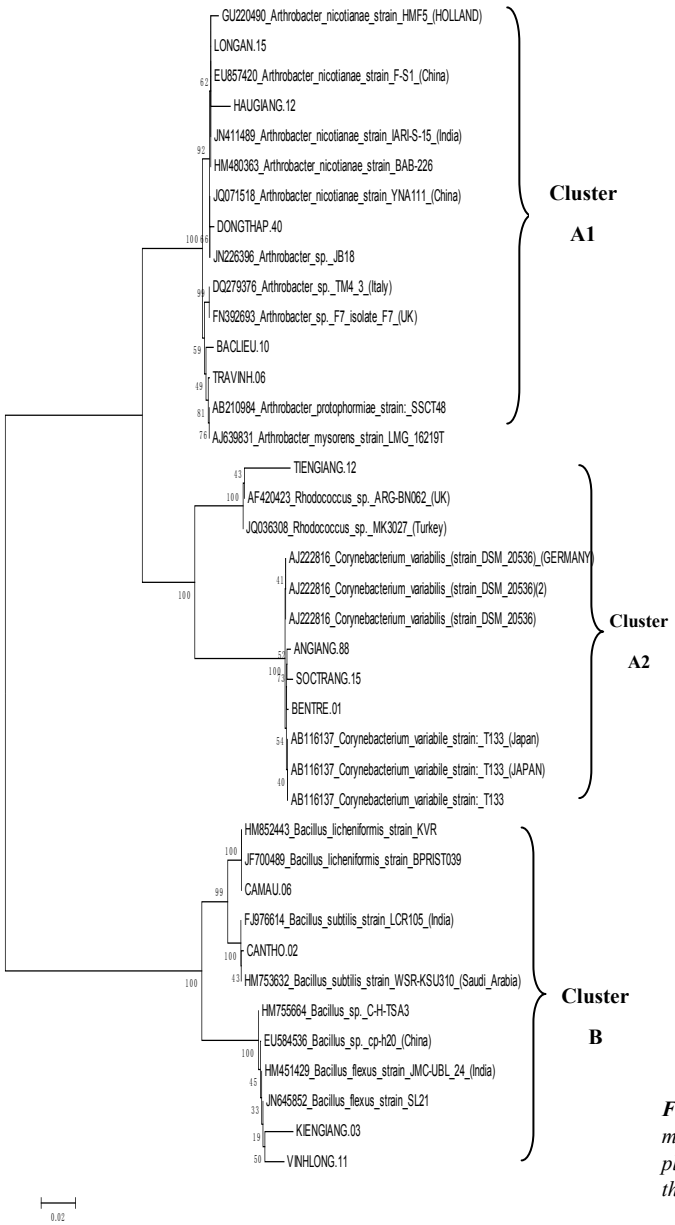

Figure 7. *Phylogenetic tree showing the relative positions of HNaOB (nitrate utilization) by the neighbor-joining method of complete 16S rRNA sequences. Bootstrap values of 500 replicates are shown at the nodes of the trees.*

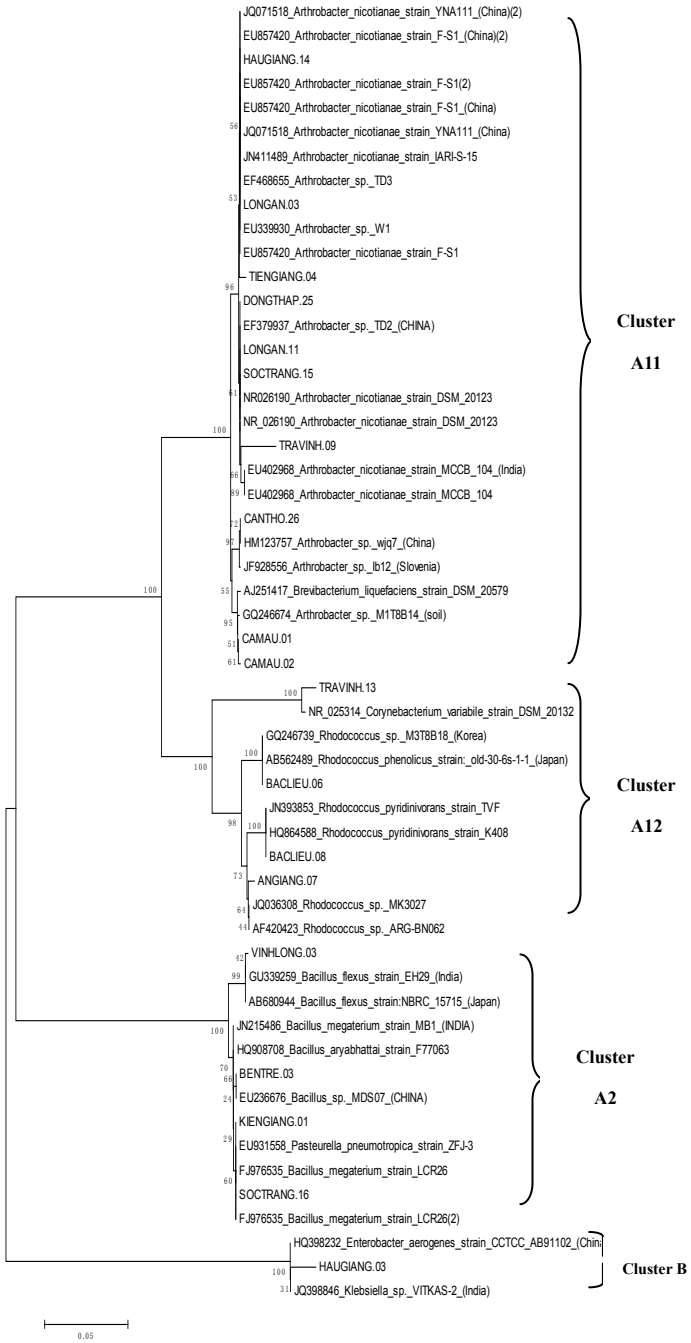

Figure 8*. Phylogenetic tree showing the relative positions of HNDB (ammonia, nitrite, nitrate utilization) by the neighbor-joining method of complete 16S rRNA sequences. Bootstrap values of 500 replicates are shown at the nodes of the trees.*

Some heterotrophic bacteria and fungi can also oxidize ammonia and/or reduced nitrogen from organic compounds tohydroxylamine, nitrite and nitrate. Whilst N oxidation is the only energy-yielding process in autotrophic nitrifiers and nitrification in heterotrophic organisms seemingly does not contribute significantly to their energy metabolism [14]. Heterotrophic nitrifiers are known for their ability to nitrify and denitrify simultaneously [4]. These results showed that

genera *Arthrobacter*, low G+C gram-positive bacteria, presented in four kinds of nitrogen, this desmonstrated that it has an important role in nitrogen removal process.

Nucleotide polymorphism can be measured by many methods, for example, haplotype (gene) diversity, nucleotide diversity, (Pi), (p), Theta (Θ) (per sitr) etc

In this study, nucleotide diversity was estimated as Theta (Θ), the number of segregating sites [15], and its standard deviation (SΘ). These parameters were estimated by DNA Sequence Polymorphism software version 4.0 [16].

Pi value explained nucleotide diversity of sequences for each gene. The higher values, the more diversity. HAOB group had highest values and HNDB group had the lowest values. Theta values (per sequence) from S of SNP for DNA polymorphism were calculated for each group. HNaOB group had the highest theta values in comparison of three groups (Table 4).

Table 4. *Nucleotide diversity (Θ) values of two ESTs using the programme DNASp 4.0 [16].*

ESTs	HAOB	HNiOB	HNaOB	HNDB
Nucleotide diversity (Pi)	0.72447	0.71805	0.72206	0.70353
Theta (per sequence) from S	238.91 ±81.46	293.57 ±110.13	318.70 ±119.53	238.62± 81.37

Primer 8F *5'- AGAGTTTGATCCTGGCTCAG-3'*

Primer1492R *5'-TACGGTTACCTTGTTACGACTT-3'*

4. Conclusions

In general, all of nitrogen removal bacteria strains were isolated from piggery wastes in the Mekong Delta, Vietnam which were identified as Firmicutes phylum among high G+C gram-positive bacteria strain occupied higher than low G+C gram-positive bacteria strain.

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