Book Chapter

Isolation of *Pseudomonas stutzeri* and its application in agricultural production in the Mekong Delta, Vietnam

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Abstract

Three hundred and one (301) isolates were isolated onto the Minimal medium, 202/301 isolates (67.11%) were identified with universal primers (*Pseudomonas* sp.) and 185/202 isolates (91.58%) were determined with the specific primers (*Pseudomonas stutzeri*). Therefore, the identification of *Ps. stutzeri* with 2 pairs of primers ensured certainly. Many strains had the good ability of ammonium oxidation (*amo* genes), many strains had the good ability in oxidized nitrite and nitrate, they has been applied in wastewater treatment as water in TRA-fish ponds infecting ammonium and piggery wastewater. Besides,

many *Pseudomonas stutzeri* strains were discovered the presence of *nif* genes and they were used as biofertilizer for rice cultivation, some *Pseudomonas stutzeri* strains had *phyt* gene in their genome and they were used as source of supplementary for pigs, chickens and TRA-fish feeds. The diversity of *Pseudomonas stutzeri* in soils and water in the Mekong Delta, Vietnam is plentiful and their applications were applied in many aspects as wastewater treatment, biofertilizers production and supplementary feeds.

Keywords

Isolation, Mekong Delta, Nitrogen Fixation, *Pseudomonas stutzeri*, Wastewater Treatment

Introduction

The genus *Pseudomonas* is one of the most diverse and ecologically significant groups of bacteria on the planet [1], playing an especially important role in the carbon and nitrogen cycles. *Pseudomonas stutzeri* is a remarkable member of this genus, with exceptional physiological capacities, being able to metabolize a wide range of organic substrates. Members of the species mineralize organic contaminants aerobically and anaerobically as denitrifiers. *Ps. stutzeri* strains are not only able to denitrify—some strains are also able to fix dinitrogen. The species is well defined phenotypically by means of few biochemical tests that discriminate *P. stutzeri* from other species of *Pseudomonas*, but additional biochemical properties are very diverse [2]. *P. stutzeri* is ecologically relevant, occupying many niches and being commonly isolated from environmental and clinical samples [3,4].

Pseudomonas stutzeri was first described by Burri and Stutzer in 1895 [5]. van Niel and Allen, in 1952 [6], precisely defined its phenotypic features and discussed its definitive designation as *Pseudomonas stutzeri* by Lehmann and Neumann [7]. In spite of marked differences from the type strain of the genus, the sequence similarities of the rRNAs, demonstrated initially by DNA-rRNA hybridization, show the legitimacy of the inclusion

of *P. stutzeri* in the genus *Pseudomonas*. Strains of the species have been identified among denitrifiers found in natural materials. *P. stutzeri* is distributed widely in the environment, occupying diverse ecological niches, and has also been isolated as an opportunistic pathogen from humans [8].

According to Lalucat et al. [8], *Pseudomonas stutzeri* is a member of the genus *Pseudomonas* sensu stricto. It is in group I of Palleroni's DNA-rRNA homology group within the phylum *Proteobacteria* [2,9]. *P. stutzeri* is now recognized as belonging to the class *Gammaproteobacteria*. Phylogenetic studies of *P. stutzeri* strains' 16S rRNA sequences and other phylogenetic markers demonstrate that they belong to the same branch, together with related species within the genus, such as *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. balearica*. Typically, cells are rod shaped, 1 to 3 μ m in length and 0.5 μ m in width, and have a single polar flagellum. Under certain conditions, one or two lateral flagella with a short wavelength may be produced. Phenotypic traits of the genus include a negative Gram stain, positive catalase and oxidase tests, and a strictly respiratory metabolism. In addition, *P. stutzeri* strains are defined as denitrifiers. They can grow on starch and maltose and have a negative reaction in arginine dihydrolase and glycogen hydrolysis tests. The G_C content of their genomic DNA lies between 60 and 66 mol%. DNA-DNA hybridizations enable at least 17 genomic groups, called genomovars, to be distinguished. Members of the same genomovar have more than 70% similarity in DNA-DNA hybridizations. Members of different genomovars usually have similarity values below 50%..

Pseudomonas stutzeri is a Plant Growth Promoting Rhizobacteria (PGPR) to use as Plant Promoters as nitrogen fixation and phosphate solubilization for crop cultivation (biofertilizer) and many strains of the species have been identified as denitrifiers for wastewater treatment

Due to the diverse of *Pseudomonas stutzeri* in the natural environment, the aims of this study were (i) isolate *Pseudomonas stutzeri* from many different sources as soils, biowastes,

wastewater....in the Mekong Delta, Vietnam and (ii) its application in agricultural production as biofertilizer production and wastewater treatment.

Isolation of *Pseudomonas stutzeri*

Materials and Methods

Materials

- Samples were collected from cultivated soil, wastewater and wastes from fish-ponds, shrimp-ponds, pig-farms... from the provinces of the Mekong Delta, Vietnam. Samples were kept in sterile bottle (-250-ml) or polyethylene bags in freeze-box with ices (5°C) and they were transported to Lab of Can Tho University as soon as in day in 2008. Soil samples were kept in the sterile polyethylene bags; water samples were kept in sterile PET bottles and they were stored in refrigerators until isolation.

- Minimal medium ([4]; Lorenz and Wackernagel, 1991) were used to isolate, identify and culture ...the bacterial isolates; LB medium (Luria Bertani) [10] for DNA extraction; - primers: 16f27: 5'- AGA GTT TGA TCM TGG CTC AG- 3' (M = A or T) 16r1488: 5'- CGG TTA CCT TGT TAG GAC TTC ACC- 3' [3]- specific primers: fps158: 5'- GTG GGG GAC AAC GTT TC - 3' rps743: 5'- CGA TTA TGA CTG TGA CTC CAC - 3' [11] Besides, to identify the ability of nitrogen removal, the strains were tested with primers.

Methods

Each soil sample (~10 g) was thoroughly mixed in 90 mL volumes of sterile distilled water in sterile conical flasks. A decimal dilution series was prepared in sterile distilled water up to $1 - 10^5$ mL⁻¹. From each dilution, 0.1 mL aliquots were streaked onto minimal medium supplemented with 50 mg/L of cycloheximide. The plates were then incubated at 28°C for 1 week. The different colonies were picked and streaked twice to obtain pure cultures. Isolates were then maintained on Minimal media agar slants at 4°C and subcultured at 2 month intervals. For long-term storage, the isolates were maintained at -80°C in a 20% glycerol solution. Wastewater samples were also done as soil samples.

Chromosomal DNA of the isolate was extracted by a slightly modified procedure of Wilson [12]. The 16S rDNA was amplified using PCR by Bennasar et al., [3] with Taq polymerase (Fermentas) and the universal primer pair of 27f and 1488r [3], PCR reactions were performed in a volume of 25 mL containing 25 ng of genomic DNA and 0.15 mmol/L concentrations of each primer described above. The amplification program included an initial denaturation of 4 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 55°C, 90 s at 72°C, and a final extension step of 7 min at 72°C, in a C1000 Thermal Cycler (Bio-Rad). Following amplification, the PCR products were electrophoresed on 1% agarose gels buffered with 0.5 TBE (4.5 mmol/L Tris, 4.5 mmol/L boric acid, and 1 mmol/L EDTA, pH 8), and stained with ethidium bromide. The PCR products were excised from the agarose gels and the DNA was purified with the Gene-Clean II Kit System (Q-Biogene, Carlsbad, California) according to the manufacturer's specifications. Partial 16S rRNA gene of selected isolates in each 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>);

The isolates were continued to amplified using PCR with specific primers: fps158 and rps743 to determine *Ps. stutzeri* strains. The cycling programme was 5 min at 94°C, 40 cycles of 2 min at 94°C, 2 min at 57°C, 3 min at 72°C and a final step of 10 min at 72°C. PCR reaction products were run and visualized on a 1.2% agarose gel electrophoresis and ethidium bromide staining. *Pseudomonas stutzeri* ATCC 14405 (*Ps. stutzeri* ZoBell) as reference bacteria (kindly received by Prof. Dr. Dirk Springael in Leuven University, Belgium).

After isolation and determination by PCR technique with universal primers and specific primers, 1/ The strains were tested to select the good denitrifiers for wastewater treatment as follows: Spread onto 3 kinds of medium containing 10 mM NH_4^+ , 10 mM NO_3^- and 10 mM NO_2^- to compare their growth and chose some good strains - Medium for evaluation of NH_4^+ (10 mM) oxidation composed: sodium succinate (8.g/l); KH_2PO_4

(340 mg/l); K_2HPO_4 (435 mg/l); trace elements (1 ml/l); NH_4Cl (0.53 g/l); 100 mM SW (consisting of $NaHCO_3$ [0.11 g/l], $MgSO_4 \cdot 7H_2O$ [10.5 g/l], $NaCl$ [24 g/l]); KOH 4M (50 ml/l); pH 6,8 adjusted by KOH 0.1M; agar (2%).- Medium for evaluation of oxidized NO_2^- (10 mM) composed: sodium succinate (8.1 g/l); KH_2PO_4 (340 mg/l); K_2HPO_4 (435 mg/l); trace element (1 ml/l); KNO_2 (0.85 g/l); 100 mM SW (consisting of $NaHCO_3$ [0.11 g/l], $MgSO_4 \cdot 7H_2O$ [10.5 g/l], $NaCl$ [24 g/l]); KOH 4M (50 ml/l); pH 6,8 adjusted by KOH 0.1M; agar (2%).

- Medium for evaluation of oxidized NO_3^- (10 mM) composed: sodium succinate (8.1 g/l); KH_2PO_4 (340 mg/l); K_2HPO_4 (435 mg/l); trace element (1 ml/l); $NaNO_3$ (0.85g/l); 100 mM SW (consisting of $NaHCO_3$ [0.11 g/l], $MgSO_4 \cdot 7H_2O$ [10.5 g/l], $NaCl$ [24g/l]); KOH 4M (50 ml/l); pH 6,8 adjusted by KOH 0.1M; agar (2 %).

Consequently, these strains need to be identified *nif* genes:

The strains were tested to select the good nitrogen fixation and phosphate-solubilization for biofertilizer production as follows:- They were selected on the Burk-N free medium agar, the strains developed very well on the Burk N-free mefium agar and quantitative of high N concentration (NH_4^+) by colormetric method with Nitroprusside.

- PCR amplification of *nif* genes

NifH gene amplification for sequencing was performed using the PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCCRCCGGA) primers using previously described methods [13]. Briefly, PCR mixtures contained 1x green buffer (Fermentas), 1.8 mM $MgCl_2$ (Fermentas), 0.5 mM each deoxynucleoside triphosphate (Fermentas), 500 nM primer (IDT), 0.1 mg/ml bovine serum albumin (NEB), 2.5 U of Taq polymerase (Fermentas) and 1 ng/ul template DNA in a 20-ul reaction volume. Amplifications were performed for 3 min at 95°C, 30 cycles of 45 s at 94°C, 45 s at 61°C, and 1 min at 72°C, followed by a final extension for 7 min at 72°C.

PCR amplification of *amo* genes

From the results of the ability of oxidized ammonium of *Ps. stutzeri* strains, *amo1* primers and *amo2* primers were done by DNAMAN programme based on putative ammonia monooxygenase (CP30004.1) gene of *Pseudomonas stutzeri* A1501 reported on database Gene (NCBI database).

Using *amo1* primers amplified 690 bp of *amo* gene; *amo1* with sequence and PCR as follows:

- *amo1* sequence

Forward: 5' GCG AGA ATC ACC AGA AAC AG 3'

Reverse: 5' GCT GAT CGG CAC CTT CAT CC 3' 95°C in 5' 35 cycle (95°C in 45'' 53°C in 45'; 72°C in 60'') 72°C in 10' Using *amo2* primers amplified 1023 bp of *amo* gene. *amo2* with sequence and PCR as follows:

- *amo2* sequence :

Forward: 5' CTA GCC TGC GCG GCG CTG CCA G 3'

Reverse: 5' ATG CGC CAG CGG CTG CCG GCC TG 3' 95°C in 5' 35 cycle (95°C in 45'' 65°C in 45'; 72°C in 90'') 72°C in 10'

- PCR amplification of *phyt* gene

Using P588F and P1321R primers amplified

Forward : 5' - CTA CGT CAA GGA GAA CG - 3'

Reverse : 5' - ACA TGC ACA GGC CGT AGA TA - 3' 95°C/3'; 40 cycle: (95°C/30'', 56°C/40'', 72°C/1'); 72°C/5'.

Results and Discussion

Isolaton, Identification and Selection

***Pseudomonas stutzeri* from Wastewater Samples (Tra-Fish Ponds, Shrimp Ponds and Piggery Wastewater)**

From sludge and water samples of Tra fish ponds, shrimp ponds and piggery wastewater of Tien Giang, Ben Tre, Vinh Long, Dong Thap, Can Tho, An Giang, Kien Giang and Bạc Lieu, (in the Mekong Delta) 301 isolates were isolated onto the Minimal medium. 202/301 isolates (67.11%) were identified with universal primers (*Pseudomonas* sp.) and 185/202 isolates (91.58%) were determined with the specific primers (*Pseudomonas stutzeri*). Therefore, the identification of *Ps. stutzeri* with 2 pairs of primers ensured certainly.

The colonies appeared onto minimal medium after 36 to 48 h incubation at 30°C, almost their colonies have round-shaped; milky, white clear and yellow, entire or lobate margin; diameter size of these colonies varied from 0.2 to 3.0 mm (Figure 1) and all of them have Gram-negative and they are motile (Figure 2a, 2b, 2c, 2d, 2e, 2f,,).

One hundred-eighty five strains were identified by PCR technique with two primers:

- 16f27 và 16r148 primers and
- fps158 và rps743 primers (Figure 3)

Selection of Good Strains (Good Denitrifiers) or Nitrogen Removal

Selection from *Ps. stutzeri* originated from Water and Sludge of TRA Fish Ponds

In order to separate between strains originated from water and sludge of TRA fish ponds and shrimp ponds. One hundred-sixty-two isolates (including 193/285 isolates identified at universal primers and 162/193 isolates determined at specific primers) were selected by they were spreaded onto in three kinds of ammonium, nitrite and nitrate with 10 mM (with 2 replications) and this concentration increased dramatically, these strains developed at high concentration which selected to DNA sequence. The result showed that there was a little of strain

developed in 10 mM NO_2^- but there were a lot of strains developed very well in 10 mM NH_4^+ and 10 mM NO_3^- at day 2 (table 1 and table 2); 32/162 strains were selected to select in the next experiment.

In the next step, 32 isolates were continuously tested on three kinds of medium (ammonium, nitrite and nitrate) with concentration increasing 300, 400 and 500 mM NH_4^+ and NO_3^- (NO_2^- at 50 mM) with the results there were 32/162 isolates were chosen to test in the Petri dishes with higher concentration, and 14/32 grew very well after 2 days incubation.

The results from Table 3 showed that many strains grew very well in medium supplement 500 mM ammonium as AG3, VL2, DT1, BT1, BT2, BT3, BT4 and TG12 (Figure 4); two strains: TG9 and CT1 strains developed very well in medium supplement 400 mM nitrate in day 1 while many strains: grew very well on medium supplement 500 mM ammonium in day 2. In day 2, there were two strains developed very well in the medium supplement 300 mM nitrate. There were several strains developed on the medium supplement 50 mM nitrite and 500 mM nitrate and 400 mM ammonium) and these strains will be evaluated in the wastewater (Figure 5).

To study diversity of *Pseudomonas stutzeri* in the water of TRA fish ponds in the Mekong Delta, Vietnam

A neighbor-joining tree was constructed to analyse their relationship in the water. This phylogenetic tree had 2 big clusters; cluster A composed of 2 clusters: cluster A1 had 2 small clusters: cluster A11 had 8 strains having high similarity as TF01, CT01, ST01 and two strains: AG02 and KG01 together with TG13 and ST02 with ST03; cluster A2 had a similar result of cluster A11 with TG07, TG09 together with TG08. Three strains as BT01, BT03 and TG03 had a relationship very closely, In cluster A2 also had 2 small clusters; cluster A21 had 4 strains as DT01, ST04, AG01 and CT02 had close relationship, this result showed that 4 strains originated from 4 different sites but they had close genetic relationship. Cluster A22 divided two groups, group 1 had 4 strains as ST5, TG12, TG5 together with

CT02 and group 2 had 3 strains as TG02, TG06 and KG02. Both two groups had close relationship in each group

Cluster B has 2 clusters, in cluster B1 had 3 strains as KG03, KG04 and K6 while cluster B2 divided two groups: group 1 with TG10, TG11 and BT04 and group 2 with 3 strains with VL1, VL2 and TG02, cluster B2 showed that the strains had relationship very closely or genetic similarity very high. (Figure 6)

In general, the *Pseudomonas stutzeri* strains had a relationship very closely even though they were isolated at different sites far from over 100 km and they originated from water in TRA fish pond or piggery wastewater. The couple strain as ST02 and ST03; BT01 and BT03; TG5 and TG12; AG02 and AG04; TG10 and TG11, VL01 and VL02, they were seen as one strain because they had high similarity and originated from the same site.

Selection of *Ps. stutzeri* originated from Water and Sludge of Shrimp Ponds

From 69 *Ps. stutzeri* isolates isolated from water and sludge of shrimp ponds at 2 provinces: BAC LIEU and KIEN GIANG. They were spreaded onto in three kinds of ammonium, nitrite and nitrate with 10 mM (with 2 replications) and this concentration increased dramatically, these strains developed at high concentration which selected to DNA sequence. The result showed that there was a little of strain developed in medium with 10 mM NO_2^- but there were a lot of strains developed very well in 10 mM NH_4^+ and 10 mM NO_3^- at day 2 (Table 3); 21/69 strains were chosen to select in the next experiment (Figure 7).

In the next step, 21 isolates were continuously tested on three kinds of medium (ammonium, nitrite and nitrate) with concentration increasing 300, 400 and 500 mM NH_4^+ and NO_3^- (NO_2^- at 50 mM) with the results there were 7/21 isolates were chosen to test in the Petri dishes with higher concentration, and 7/21 developed very well at medium supplement 500 mM NH_4^+ ,

100 mM NO_2^- and 500 mM NO_3^- after 2 days incubation (Figure 8).

In Figure 9 presented phylogentic tree of 11 *Ps. stutzeri* strain and *Ps. stutzeri* A1501, showed that had 2 clusters: cluster A had 2 small clusters: cluster A1 composed of 2 sluters, cluster A11 had 3 strains from water in shrimp ponds at Kien Luong, Kien Giang (KG1, KG1a and KG3), and three these strains had a relationship together with *Ps. stutzeri* A1501 while cluster A12 had 4 strains and 3 in 4 strains originated from water in shrimp ponds at Kien Luong, Kien Giang (KG2, KG5, and KG4), they related with BL15 strain (isolated from water in shrimp ponds at Bac Lieu province) closely, this showed that these strains had a genetic relationship even through they had originated far from more 200 km; cluster A2 only one strain (KG8) and cluster B had 2 strains KG7 and KG6, therefore in this phylogenetic tree presented the relationship of the strains *Ps. stutzeri* originated from water in shrimp ponds at Kien Luong, Kien Giang however they also had the differiations about gene sequences and they made a genetic diversity, they had high effectiveness (Figure 10).

A neibor-joining phylogenetic tree was construted to analyse the relationship of 17 *Pseudomonas stutzeri* strains isolated from water in shrimp ponds of 2 provinces (Bac Lieu and Kien Giang) (Figure 11) showed that tree had 2 clusters. Cluster A with 2 clusters: cluster A1 had 2 small clusters which cluster A11 composing of 7 strains among the strains: BL02, BL3 and BL6 having the high similarity with BL13 strain, therefore these strains had a close genetic relationship; Similarly two strains BL8 and KG3 had the high similarity with strain BL9, this result showed that BL8 and KG3 strains had genetic relationship very closely even through they originated from far from more 200 km.

Cluster A12 had 3 strains: BL11, KG4 and BL5 with relationship closely; cluster A2 had 4 strains (BL12, BL14, BL10 and BL15) had close relationship because of they originating from one site (Bac Lieu). Cluster B had 3 strains: BL1, BL4 and BL7 and they had close relationship.

In general, 17 *Pseudomonas stutzeri* strains isolated from water in shrimp ponds from 2 provinces. Bac lieu province locates at the seaside of Eastern Sea and Kien Giang locates at the seaside of Western Sea (Thailand Bay) but they had a genetic relationship very closely perhaps they live in saline water and two sites are only far from more 200 km but the seawater of this region connects each other through the small rivers and canals.

On the otherhand, *Pseudomonas stutzeri* strains has the genes as *amo* gene (transfer ammonia to nitrite and nitrate) for nitrogen removal, *phyt* gene (phytase biosynthesis) for transfer phytate to P, Ca and protein and *nif* gene (nitrogen fixation) for biofertilizer production (Figure 12).

Detection of *amo* Gene in *Pseudomonas stutzeri* Strains

The strains had the good ability of ammonia oxidation which were identified by PCR technique with *amo1* primers and sequencing.

The PCR results showed that reference bacterial strains as ATCC14405 and A1501 with *amo1* primers presented band at position 690 bp and the isolated *Pseudomonas stutzeri* having the ability of ammonia oxydation were selected to control *amo 1* gene, the result presented in Figure 13 showed that all of *Ps. stutzeri* strains had bands at position 690 bp with *amo 1* primers, this demonstrated they had putative ammonia monooxygenase gene. *Pseudomonas stutzeri* A1501 discovered the presence of *gen amo1* for detection of enzyme ammonia monooxygenase and this gene participated into ammonia oxidation process [14].

A neighbor-joining phylogenetic tree was constructed to analyse the relationship of 12 strains were isolated in the water of TRA-fisj ponds and shrimp ponds in the Mekong Delta, this tree had 2 clusters, cluster A composed of 2 small clusters. Cluster A1 had 2 groups, group 1 had 3 strains among 2 strains (KG4 and BL5) had high similarity and both had close relationship with KG5 while group 2: KG2 strain related reference strain (ATCC 14405) closely. Cluster A2 had 4 strains KG1a and KG03 with high relationship together with KG1 and KG8 (Figure 14).

Cluster B only had 2 strains: KG6 and KG7 with high relationship.

From the above result, almost strains of *Pseudomonas stutzeri* had *amo* gene and they oxidized ammonia strongly.

According to Lalucat et al., [8] A total of about 50 genes are needed in a single denitrifying bacterium to encode the denitrification apparatus'core structures [15]. The genes contain structural information for the nitrogen oxide reductases and functions for metal processing, cofactor synthesis, electron donation, assembly processes, protein maturation, and regulation. These denitrification genes have a chromosomal location in *P. stutzeri*. In *P. stutzeri*, the genes coding for the dissimilatory reductases, the *nar* genes, are not linked to the denitrification genes sensu stricto (which include the *nir-nor* loci and the *nos* genes). Instead, they form a separate locus in this bacterium [16,17].

After years of controversy, it now seems that several strains that are unambiguously classified as true *Pseudomonas* species can be added to the list of nitrogen fixers, on the basis of physiological properties, nitrogen assays, phylogenetic studies, and the detection of *nifH* by hybridization or PCR amplification and sequencing [18]. All well-classified nitrogen-fixing *Pseudomonas* strains described in the literature are members of *P. stutzeri* [19].

Pseudomonas stutzeri A15 is a member of genomovar 1. It is a particularly predominant diazotrophic strain. It was isolated from the rice paddy rhizosphere and is widely used as a rice inoculant in China [20-23]. Previously identified as an *Alcaligenes faecalis* strain, it has been widely studied physiologically, biochemically, and genomically. Strain A15 is able to colonize and infect rice roots and to grow endophytically [19].

Detection of *phyt* Gene in *Pseudomonas stutzeri* Strains

Besides, *Pseudomonas stutzeri* is phosphate-solubilizing bacteria, they have the ability of dissolve phytate, a kind of

organic phosphate presences in the legume seeds, cereals as rice, wheat, corn..and in the compost. Phytate reacts with phytase releasing 6 atoms P, 2 atoms Ca and 1 protein, supports animals one-stomach digest and absorb phospho very well.

Firstly, 147 isolates were tested on medium supplement phytic acid and there were 38/147 isolates made a halo (Figure 15)

These 38 isolates were tested the ability of phosphate solubilization and 18/38 isolates had the high effectiveness but 7/18 isolates had the highest effectiveness which were selected to identify by PCR technique and sequencing; consequently a phylogenetic tree constructed to find the relationship between 6 strains and reference bacteria (*Pseudomonas stutzeri* A1501) (Figure 16).

In neighbor-joining tree analysis 5 strains and reference strain (A1501) had 2 clusters: Cluster A had 4 strains and BT5 and AG1 strains had a relationship closely (2 strains had high similarity) together with reference strain (A1501) and 3 strains had a relationship close with KG9 (Figure 17) and cluster B had 2 strains (BT6 and TG14). In this figure, based on the sequences of the strains had *phyt* gene, they had a relationship closely especially 2 strains BT5 and AG1 strains.

Application of *Pseudomonas stutzeri* in agricultural production in the Mekong Delta, Vietnam

Introduction

The rapid development of catfish aquaculture in the Mekong Delta has caused water pollution through increased ammonia, organic matter, and hydrogen sulfide from waste water disposal. High N (especially ammonia) in fish-pond water may be toxic to fish and it may cause eutrophication in canals, streams, small rivers. It threatens the quality of water used for household purposes by many farm-families. Nitrogen removal from wastewater is generally achieved by a combination of nitrification and denitrification processes. *Pseudomonas stutzeri* has been shown to be involved in nitrification and denitrification

processes as well as in the degradation of environmental pollutants. *Pseudomonas stutzeri* strains to treat wastewater have been used successfully in many developed countries [24,25]. In this project, the bacteria was isolated from wastewater in the Mekong Delta and evaluated for removal of nitrogen from the water.

Application of Good Strains to Treat Ammonia in the Water of TRA-Fish Ponds

The aim was to use selected *Pseudomonas stutzeri* strains in wastewater to treat ammonia in waste in TRA-fish-ponds.

Selected strains to remove ammonia in the water with 50-ml falcon, container: 1-L, 10-L, 50-L, 500-L, 5,000-L.

The results showed that pH of water in TRA-fish ponds decreased to the time while pH of water applied *Ps. stutzeri* strains reduced at Day 1 but pH increased over 7.0 at Day 2 (Figure 18a, 18b). Bacterial population in water of control treatment decreased from Day 0 to Day 2 and Day 3 (from 3.0 log₁₀ to >1.0 log₁₀) while bacterial population in water applied *Ps. stutzeri* strains increased at Day 1 but they reduced at Day 2 after that bacterial population were stable (>3.0 log₁₀) especially pH water of treatment (TG4+TG9b) increased over >5.0 log₁₀/ml and this led ammonia concentration in water decreased very low after 3 days applying *Ps. stutzeri* strains (Table 5).

Especially, nitrite and nitrate concentration in water of *Ps. stutzeri* strains treatment reduced very low and reached to 0 mg/l while in the control treatment, nitrite concentration enhanced very high and nitrite did not transfer to nitrate therefore nitrate concentration in water was low (Table 6).

In the experiment with bigger volume (10-L), application of *Ps. stutzeri* strains into (0.1%) water in TRA-fish ponds showed that pH of water were stable after 3 days especially TG5a+TG13a treatment increased pH from 7.5 over >8.0 after 2 days after that decreased 7.7, all of *Ps. stutzeri* treatments had pH >7.5 after 3 days while pH water of control treatment and BKC treatment

(chemical for water treatment) reduced from 7.5 to 6.4 or 6.8 after 3 days, respectively. (Figure19). In Figure 20 showed that population of *Ps. stutzeri* strains in water of TRA-fish ponds had big variation among population in TG4+TG9b treatment was the highest in compare to others (including control and BKC treatments) (Figure 20). This results affected on ammonium concentration in water of TRA-fish ponds (Table 4) among two treatments **TG5a +TG13a** and **TG4 + TG9a** treatments removed ammonia in water completely with efficiency 100%. in experiment 1-L.

In the third experimentm, bigger container was used (10-L) and the treatments were selected as TG4, TG9a, TG4+TG13a, TG11a+TG13a, the results showed that application of TG4 strain increased pH of water over 8.0 during 4 days, three treatments of *Ps. stutzeri* also increased pH water. On the contrary, two treatments (control and BKC) decreased pH water (<7.0) (Figure 21).

Application of *Ps. stutzeri* strains into water increased bacterial population ($\log_{10}/\text{ml}$) in comparison to control and BKC treatments (Figure 22), this led reduced ammonium concentration in water (Table 6) and the highest efficiency was TG11a+TG13a treatment, this treatment had the highest bacterial population, this result demonstrated bacterial population was negative correlation with ammonium concentration in water and the effect of *Ps. stutzeri* strain in removal nitrogen in water (Table 7 and Figure 23).

Through 3 experiments (50 ml), 1-L and 10-L, the results demonstrated the ability of removal nitrogen (ammonium oxidation, reduced nitrite and nitrate) of *Ps. stutzeri* strains. However, some *Ps. stutzeri* strains increased BOD₅ (TG9a), TSS (TG4) and all of *Ps. stutzeri* strains increased PO_4^{3-} and TOC concentration in water.

When ammonium concentration in water increased in Day 3, TRA-fish died in water of control treatment (Figure 24a) while TRA-fish alived in the *Ps. stutzeri* treatments (Figure 24b).

Although other parameters as high BOD₅, TOC, PO₄⁻ concentration but low ammonium concentration especially low nitrite and nitrate concentrations in water did not affect to TRA-fish. On the contrary, high ammonium concentration in water of control treatment caused the death of TRA-fish.

To demonstrate the effectiveness of *Ps.sxtutzeri* strains in removal nitrogen, an experiment with bigger volume (50-L) was conducted to evaluate the ability of removal nitrogen of 3 good strains (TG4, TG9a and TG13a) were used in this study, the result in Table 9 showed the initial parameters and after 2 days, three strains had the good ability of removal nitrogen among TG4 strain was the best one, it had the lowest TN concentration.

When bacterial population in water was over 6.0 log₁₀/ml, (Figure 25) nitrogen concentration in water as ammonium and nitrate concentration reduced clearly (Table 9) and finally TN concentration in water of TRA-fish ponds decreased.

In the experiment with container 500-L, two strains were used in this study together with BKC (chemical used to kill pathogenic bacteria in water), the results received as follows (Table 10).

In table 10 showed that the effectiveness of 2 strains of *Ps. stutzeri* in removal nitrogen and their population increased over 6.0 log₁₀/ml from Day 1 to Day 4.

In Day 0, pH of water in TRA-fish ponds was 8.53 and after 4 days, pH water of control treatment and BKC treatment decreased from 8.53 to 7.35 and 7.59, respectively while pH water of 2 inoculated *Ps. stutzeri* strain treatments reduced from 8.53 to 7.95 – 7.87 and nitrogen parameters as TKN, Nitrite and Nitrate concentrations decreased strongly and population of two treatments were always higher than two treatments (control and BKC) with results from experiment 500-L. an experiment 10 m³ was conducted with 3 treatments composing of control, TR13a strain and EM (bio-product using to environment treatment), the results showed that pH water of TG13a strain treatment increased from Day 0 to Day 4 (7.02 to 7.35) while pH water of EM treatment increased slowly but pH water of control treatment

decreased from 7.02 to 6.81 (Day 2) after that pH water enhanced to 7.0 (Figure 27) and ammonium concentration in water of TG13a strain treatment decreased from 6.3 to under 1 mg/L while ammonium in water of control treatment did not decrease and varied from 5.0 to 6.0 mg/L and ammonium concentration of EM treatment varied 5.0 mg/L (Figure 28).

This results led to nitrite (Figure 29), nitrate (Figure 30) and TN concentrations together with bacterial population enhanced (Figure 31 and Figure 32) in water of TRA-fish ponds decreased very low (<0.2 mg/L).

From the results of experiment 50 ml to experiment 10 m³, demonstrated *Pseudomonas stutzeri* strains had *amo* gene in their genomes together with *nir* and *nor*, *nos* genes [8,25] demonstrated *Ps. stutzeri* had *amo* gene to ammonium oxidation and they applied to piggery wastewater in Taiwan.

Recently, the results of Yan et al [14] decoded the entire genome of *Pseudomonas stutzeri* and they identified *amo* gene in genome of this species, we isolated, selected and applied to wastewater treatment by nitrogen removal as ammonium oxidation, oxidized nitrite and nitrate out of the water and nitrogen release into the air.

Application of Good Strains to Treat Ammonia in the Water of Shrimp Ponds

The good *Ps. stutzeri* strains from shrimp ponds were used to treat ammonia in the water of shrimp ponds in the plastic containers 100-mL contained 50 mL water from shrimp ponds with 10 mg/ ammonium, the results presented in table 11 after 1 day inoculation.

After 1 day inoculation, *Ps. stutzeri* strains increased population and reduced ammonium concentration in water however pH water shrimp ponds did not change. BL15 strain enhanced population and decreased ammonium concentration in water.

Application of Good Strains for Piggery Wastewater

All of the good *Ps. stutzeri* strains in the above experiment, was conducted with piggery wastewater with higher ammonium concentration and the results showed that in the treatments having *Ps. stutzeri* strains increased pH of piggery wastewater after 3 days (from 8.0 to 8.5) but control treatment, pH of wastewater reduced from 8.0 down 7.0 (Figure 33) and ammonium concentration decreased during 3 days and reached to 0 while in control treatment, ammonium concentration obviously decreased from 60 mg/L to 50 mg/L after 3 days (Figure 34).

With 2 results of application of good *Ps. stutzeri* strains to wastewater treatment, the results showed that the strong ability of ammonium oxidation of these good *Ps. stutzeri* strains. However, the experiment needs to be conducted to demonstrate this ability (Figure 34).

With bigger volume (500 ml) in the flask 1-L and ammonium concentration (100 mg/L) and nitrate concentration (100 mg/L), TG01 strain ($>10^9$ /ml) inoculated into the flasks (1%) and control treatment (no bacteria), after 3 days, the results showed that in control treatment, ammonium concentration (100 mg/L) reduced to 50 mg/L and this level did not change during 3 days while nitrate increased suddenly from 100 mg/L to 300 mg/L in Day 1 and reduced slowly to Day 3 (150 mg/L) and nitrite concentration increased from 0.5 mg/L to 5 mg/L (Figure 35). In TG1 strain, ammonium concentration decreased Day 1 (100 mg/L) to 2 mg/L in Day 3 and nitrate concentration also had same result while nitrate concentration increased in Day 1 (300 mg/L) and reduced 50 mg/L in Day 2 and 25 mg/L in Day 3 and with this concentration, nitrite is toxic for animal (Figure 36).

Application of *Pseudomonas stutzeri* as Nitrogen Fixer

From alluvial soil samples of two districts (Giong Trom and Ba Tri, Ben Tre province), fifteen isolates were isolated on the Pseudomonas Isolation Agar (DIFCO), they grew very well on the Burk' N-free broth and agar, identification of nitrogenase by Acetylene Reduction Assay, all of them grew very well on the

Burk's N-free and nitrogenase biosynthesis. All of them were were *Pseudomonas stutzeri* and they supported the growth rice very good in Leonard model with mineral solution without N; *Pseudomonas stutzeri* strain alone and combination with two strains plus 50 kg N/ha enhanced grain yield as treatment of 100 kg N/ha without inoculation

Fifteen bacterial isolates appeared on *Pseudomonas* isolation agar (DIFCO) after 36 h, Almost their colonies have round-shaped; milky, white clear entire or lobate margin; diameter size of these colonies varied from 0.5 to 2.0 mm and all of them have Gram-negative and they were transferred to Burk's N-free to identify the ability of nitrogen fixation after many times (2, 4, 6 and 8 DAI), amount of ammonium were synthesized.

To demonstrate the ability of biological nitrogen fixation, 15 isolates were measured ARA by HPLC in two methods: bacteria in the Burk' medium broth and bacteria inoculated into rice seedlings planting on Burk's medium agar (Table 14).

From the result of table 14 showed that the isolates had amount of high reduced C_2H_2 (low quantity of C_2H_2) however the isolates did not change quantity of C_2H_2 when measuring in the Burk' N-free broth but high nitrogenase concentration when measuring nitrogenase in the inoculated rice seedlings cultivating the Burk' N-free medium agar, this showed that bacteria need many nutrients from plants (rice) for growth requirements and activated *nif* gene to produce nitrogenase for nitrogen fixation from the air.

Evaluation of the Ability of Bnf on Rice Cultivation

All of 15 strains of were tested on the Leonard model with mineral solution N-free in 21 days to evaluate the effectiveness of 15 strains on the growth of plant height, weight of stem and root length; the results from table 15 showed that reference bacteria was the best strains and it did not differ from positive control.

However many strains also had the good ability of BNF, they showed through the good growth of weight of stem and root length as GT01, BT04, BT06 and BT08 strains.

Two strains *Ps. stutzeri* (BT04 strain and BT08 strain) were chosen to evaluate the ability of BNF on the high-yielding rice in pot-experiment with the alluvial soil of Binh Minh district, Vinh Long province.

The results from table 16 showed that inoculation of BT4 strains or BT8 strain and/or combination BT4 + BT8 strains plus 50 kg N/ha had grain yield and yield component the same as rice applied 100 kg N without inoculation, therefore inoculation of *Ps. stutzeri* saved 50% N/ha or inoculation of *Ps. stutzeri* provided amount of biological N equivalent 50 kg N/ha. Therefore, *Ps. stutzeri* provided amount of biological N equivalent 50 kg N/ha. (Figure 37)

In this study, the discovery of *Ps. stutzeri* strains in the alluvial soil in the Mekong Delta, Vietnam has a high potential in biofertilizer production for rice cultivation, this not only saved amount of chemical nitrogen fertilizer but also limited pollution environment by residue from big amount of nitrate in soil and water. This also is a contribution in the knowledge about *Pseudomonas stutzeri* in alluvial soil on the Mekong Delta, Vietnam.

Conclusion

Discovery of *Pseudomonas stutzeri* has been done by Burri và Stutzer [5], van Niel and Allen, [6] and Lehmann and Neumann [7] however these scientists only *Ps. stutzeri* as bacterial species for nitrogen removal with identify of *amo*, *nir*, *nor*, *nos* genes and some papers studied aerobic catabolism of aromatic compounds in *P. stutzeri* (remove chemicals in environment especially in soils). We studied *Pseudomonas stutzeri* in wastewater, in alluvial soils in the Mekong Delta, Vietnam and using their application in wastewater treatment, biofertilizer production and phytase production for feed for pigs, chickens, and fish especially TRA-fish

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