Isolation and Selection of Actinobacteria Against Pathogenic Bacteria From Shrimp Pond Water on Duyen Hai District, Tra Vinh Province, Vietnam

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Abstract— *In the shrimp-farming process at Vietnam has used antibiotic mostly, this leads status of antibiotic resistant bacteria and product do not qualified to the market. Bacteria, especially actinobacteria, had resistant ability to human pathogenic bacteria in water and they have an important role in sustainable aquaculture. This study aimed to isolate and select good actinobacterial strains against pathogenic bacteria, from 8 samples of shrimp-pond water at 3 sites Ngu Lac, Phuoc Tri and Long Toan of Duyen Hai district, Tra Vinh province on Gause-1 agar medium. Fifty-three actinobacterial isolates were isolated in which 23 isolates resistant to at least one of pathogenic bacteria by well-diffusion method. Among them, 15 isolates were identified as resistant to Bacillus cereus, 12 to Staphylococcus aureus, 11 to Escherichia coli and 18 to Vibrio parahaemolyticus. There were 7 isolates had good resistance to select for PCR technique and sequencing and the result were determined 7 these strains: NL1-1.9, NL1-18a, NL2-2.1b1, NL2-2.2, PT1-1.7a, PT2-2.8a, LT1-1.3 belonged to three genere: Streptomyces, Nocardioides, and Glutamicibacter.*

Keywords— actinobacteria, antimicrobial, schrimp-pond water, Vibrio parahaemolyticus.

I. INTRODUCTION

Vietnam has a suitable location and condition for fishery and aquaculture with shoreline length 3,260 km, together with abundant river canal system, Aquaculture is one an important export of agricultural production among shrimp cultivation jas large contribution in general report of Vietnam agriculture. Shrimp cultivation has been faced with pathogenic bacterial infections, such as luminous vibriosis and acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio harveyi* and *Vibrio parahaemolyticus*, respectively [1]. Shrimp production in Southeast Asia steadily averaged 6.0% annual growth from 2008 to 2011; however, the production declined from 3.45 million metric tons (MMT) to 3.25 MMT in 2012 (down 5.8%) and to 3.21 MMT in 2013 (down 1.1%) due to the impact of early mortality syndrome (EMS) in China, Thailand, Vietnam and Malaysia [2]. To solve these problems, shrimp farmers normally use antibiotics to eliminate the pathogenic bacteria; however, antibiotics can be harmful to consumer health. In addition, long term use of antibiotics leads to residual compounds in sediment and water, and the bacteria can adapt themselves by selection for antibiotic resistant genes [3]. For sustainable shrimp cultivation, probiotics and/or their anti-vibrio compounds as biocontrol agents have been explored to control vibriosis in shrimp farming. Gram-positive actinobacteria have been identified as potential probiotics for aquaculture, with effects against various pathogens [4]. The objective of this work was to isolate and select together with identify bacterial isolates having good resistance to *V. parahaemolyticus* and human pathogenic bacteria in water of shrimp ponds on Duyen Hai district, Tra Vinh province, Vietnam in order to product a probiotic for shrimp cultivation sustainably.

II. MATERIALS AND METHODS

2.1 Materials

Water samples were collected at the depth of 0.2 m and distance shore 4 m, from 8 shrimp-ponds of 3 villages (Long Toan, Ngu Lac, Dinh An) (9°64’37” to 9°67’88” East and 106°45’29” to 106°51’73” North) of Duyen Hai district, Tra Vinh province, Vietnam, they stored in an ice box and transferred to Can Tho University laboratory, stored -4°C in refrigator until to analysis.

Vibrio parahaemolyticus provided from Department of Aquaculture Pathology, College of Aquaculture, Can Tho University.

Nutrient Agar (Difco) medium supplemented with Aginalxic (10 µg/l) and Nystatin (25 µg/l) into medium after autoclaving; Luria Bertani medium [5].

2.2 Methods

2.2.1 Isolation and culture

Water samples were serially diluted with sterile saline water (0.01%). Hundred microlitres of the suspensions were spreaded onto NB agar medium. All plates were inoculated at room temperature for 24 h; the disjointed colonies recorded and re-streaded to obtain pure culture. The colonies bearing distinct morphological characteristics were picked up and transferred to freshly prepared media until pure cultures were obtained.

2.2.2 Screening assays for antibacterial activity

The liquid cultures were grown with shaking at 150 rpm for 1 day depending on their growth rate at 30°C. The broth was centrifuged in 50 mL falcon tubes (5000 rpm, 15 min at room temperature; Megafuge 1.0R, Heraeus) and the supernatant was stored at 4°C. The bacterial test organisms were plated in LB medium. Antimicrobial extract was added to the wells, the plates were incubated at 4°C for 2 h for diffusion of antimicrobial extract and observed for the zones of inhibition at 28°C after 48 h incubation.

2.2.3 The Agar well diffusion method

The active isolates were cultured by the method given in the previous step. The supernatants were used for testing extracellular antimicrobial activity by agar well diffusion method. By using a sterile cork borer, wells were punctured in appropriate agar medium previously seeded with one of the test organisms. One hundred microlitre of the culture supernatants were added to each well. The plates were then incubated at 4^{o}C for at least 2 h to allow the diffusion of crude extracts followed by incubation for 24 h at 37^{o}C for bacteria and 48 h at 28^{o}C for yeast. The diameters of inhibition zones were monitored and measured [6] and positive control was penicillin.

2.2.4 Genomic DNA Extraction

Bacterial cells from these cultures were collected by centrifugation and genomic DNA was extracted [5].

2.2.5 PCR Amplification and Sequencing of 16S rDNA

PCR was used with primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') [7] and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') [8] Cyclying condition were as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, and a final extension of 5 min for 72°C.

2.2.6 Sequence Analysis

The 16S rRNA gene sequences were compared with those from the type strains available in NCBI (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) [9].

For phylogenetic analysis, multiple sequence alignment was performed using CLUSTALX, version 1.81. Phylogenetic tree was constructed using Mega 7.0 [10]. The consistency of the trees was verified by bootstrapping (1000 replicates) for maximum likelihood.

2.2.7 Data analyses

The experimental results were analysed as a two-way ANOVA with the isolates and with levels of diameters of inhibition zones. All analyses were conducted using the programme MSTATC, Minitab 16. The data were considered significantly different at $P<0.01$. Duncan test at $P=0.01$ was used to differentiate between statistically.

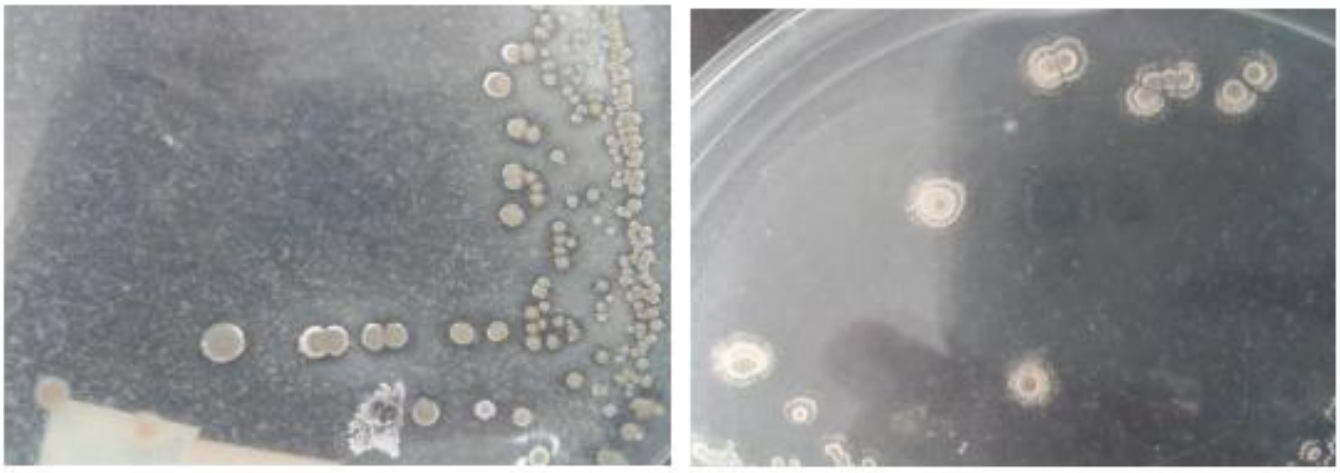
III. RESULTS AND DISCUSSION

3.1 Isolation of bacteria

A total of 53 isolates of bacteria was purified from 8 water samples collected at 3 sites (Long Toan, Phuoc An and Ngu Lac) (Table 1). Almost their colonies have round-shaped; milky, white clear and yellow, entire or loabate margin; diameter size of these colonies varied from 0.2 to 3.0 mm (Figure 1) and all of them have Gram-positive.

TABLE 1
ACTINOBACYERIAL ISOLATES ISOLATED AT THREE SITES ON GAUSE-1 MEDIUM

No	Site	Samples	Isolated Bacterial Isolates
1	Long toan	3	14
2	Phuoc Tri	3	22
3	Ngu Lac	2	17
Total		**8**	**53**

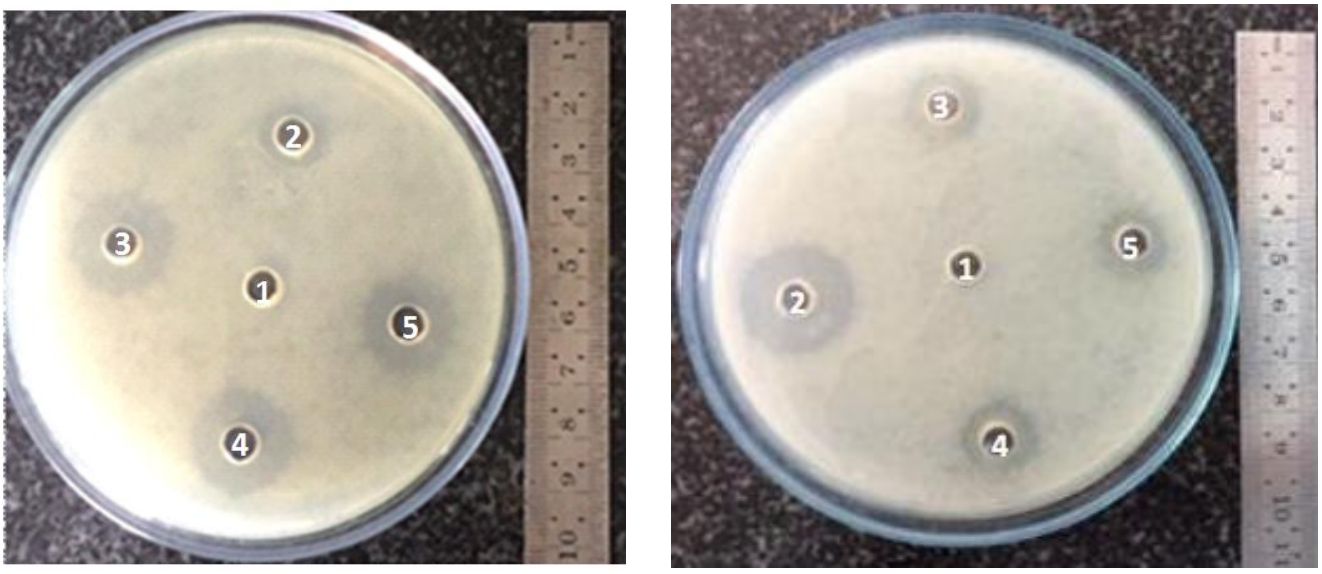


NL1.1.8a **NL1.1.9b**

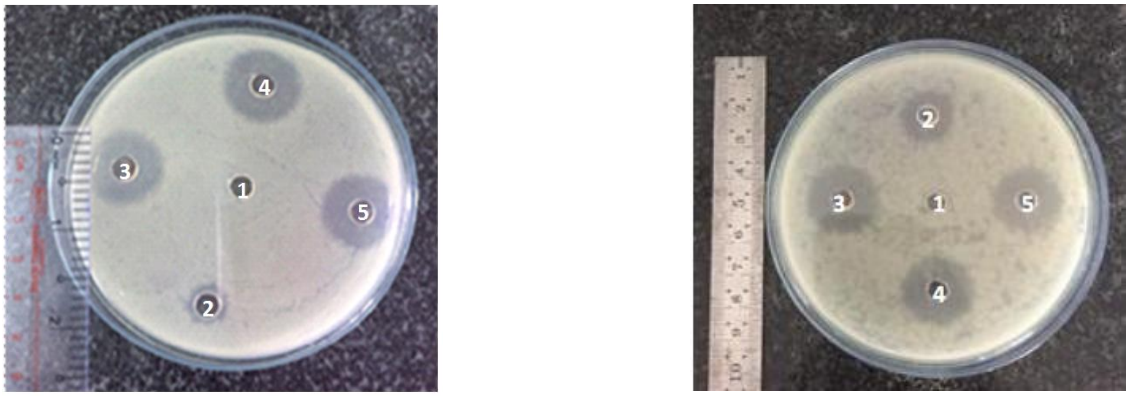
FIGURE 1. Colonies of actinobacterial isolates on Gause-1 medium

3.2 Antimicrobial activity by agar well diffusion method

In 53 isolates, there are 23 isolates produce antimicrobial active metabolites inhibiting at least one of four pathogenic bacteria including *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Vibrio parahaemolyticus* by well-diffusion method (Figure 2) and (Table 2).



(A) (B)



(C) (D)

(1: Negative; 2: Positive with antibiotic; 2,3,4: sample of Actinobacyerial isolates)
(A): Antibacterial activity to Bacillus cereus of NL1-1.9b isolate
(B): Antibacterial activity to Staphylococcus aureus of NL2-2.1b1 isolate
(C): Antibacterial activity to Escherichia coli of NL1-1.8a isolate
(D): Antibacterial activity to Vibrio parahaemolyticus of NL2-2.2 isolate

FIGURE 2. Antibacterial activity to pathogenic bacteria of Actinobacyerial isolates

TABLE 2
ANTIMICROBIAL ACTIVITY OF 23 BACTERRIAL ISOLATES TO PATHOGENIC BACTERIA

STT	Isolates	Antimicrobial activity: inhibition zone diameter [D = d_1 - d_2] (mm)			
		Bacillus cereus	***Staphylococcus aureus***	***Escherichia coli***	***Vibrio parahaemolyticus***
1.	LT1-1.1	-	4.33fg	3.67^{cde}	3.00^{k}
2.	LT1-1.3	8.33^{bc}	-	-	9.67^{cd}
3.	LT1-1.3c	6.00^{fg}	-	-	8.67^{de}
4.	LT1-1.3d	4.67^{gh}	4.33fg	3.00^{e}	6.67^{fgh}
5.	LT2-2.2	-	-	-	7.67^{efg}
6.	LT3-3.3a1	-	-	-	3.33^{k}
7.	LT3-3.3	6.67^{de}	5.67^{ef}	5.00^{cd}	8.00^{def}
8.	LT3-3.3a	3.33^{h}	7.33^{cd}	-	5.33^{hij}
9.	LT3-3.3a2	-	-	-	11.67^{b}
10.	NL1-1.1	6.33^{ef}	6.67^{cde}	3.67^{cde}	-
11.	NL1-1.8a	12.33^{a}	5.67^{ef}	12.33^{a}	11.00^{bc}
12.	NL1-1.9	8.33^{bc}	9.33^{b}	4.33^{cde}	4.00^{jk}
13.	NL1-1.9b	8.33^{bc}	-	9.00^{b}	9.6^{7cd}
14.	NL2-2.1	6.33^{ef}	-	-	-
15.	NL2-2.1b1	-	8.00^{bc}	3.33^{de}	15.00^{a}
16.	NL2-2.2	7.67^{cde}	3.33^{g}	9.00^{b}	4.00^{jk}
17.	NL2-2.2d	4.33^{h}	3.33^{g}	-	-
18.	NL2-2.6a	9.33^{b}	-	-	-
19.	NL2-2.6b	-	8.00^{bc}	-	4.66^{ijk}
20.	PT1-1.7a	6.67^{def}	6.00^{de}	5.33^{c}	6.00^{ghi}
21.	PT1-1.8b	-	-	-	3.33^{k}
22.	PT2-2.8a	-		7.67^{b}	9.33^{cde}
23.	PT3-3.12a	11.33^{a}	-	-	-
control (penicillin)		*8.00^{bcd}*	*11.33^{a}*	*7.33^{b}*	*11.67^{b}*
Total	**23**	**15**	**12**	**11**	**18**
CV (%)		**7.69**	**9.25**	9.45	**7.8**

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

3.2.1 Antimicrobial activity to *Bacillus cereus*

There are 15 isolates produce antimicrobial active metabolites inhibiting *Bacillus cereus*, (Figure 3)

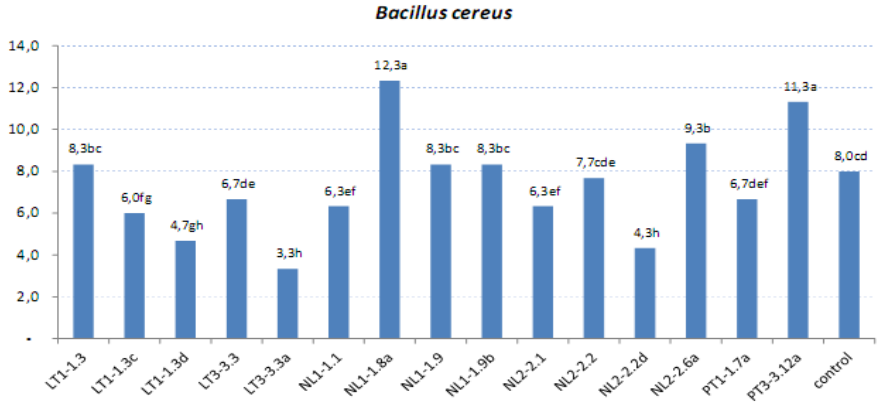


FIGURE 3: Antimicrobial activity of Actinobacyerial isolates to *Bacillus cereus* (inhibition zone diameter -mm)

3.2.2 Antimicrobial activity to *Staphylococcus aureus*

There are 12 isolates produce antimicrobial active metabolites inhibiting *Staphylococcus aureus*, (Figure 4).

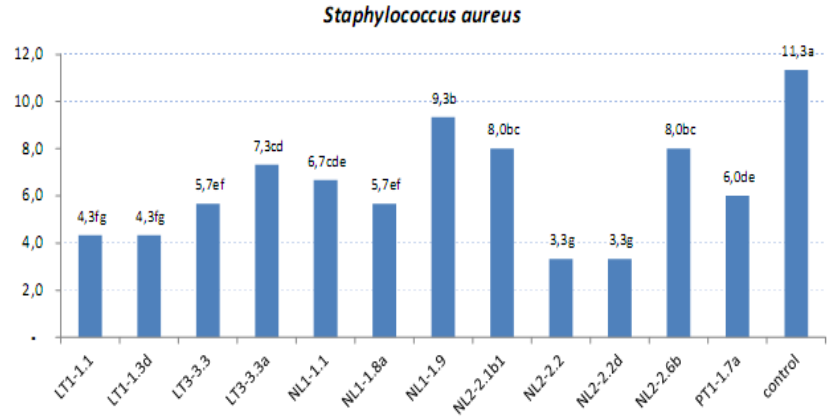


FIGURE 4: Antimicrobial activity of Actinobacyerial isolates to *Staphylococcus aureus* (inhibition zone diameter -mm)

3.2.3 Antimicrobial activity to *Escherichia coli*

There are 11 isolates produce antimicrobial active metabolites inhibiting *Escherichia coli*, (Figure 5).

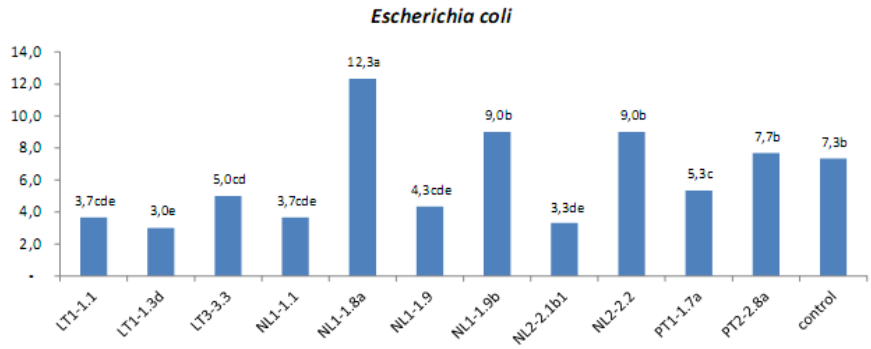


FIGURE 5: Antimicrobial activity of Actinobacyerial isolates to *Escherichia coli* (inhibition zone diameter -mm)

3.2.4 Antimicrobial activity to *Vibrio parahaemolyticus*

There are 18 isolates produce antimicrobial active metabolites inhibiting *Vibrio parahaemolyticus*, (Figure 6).

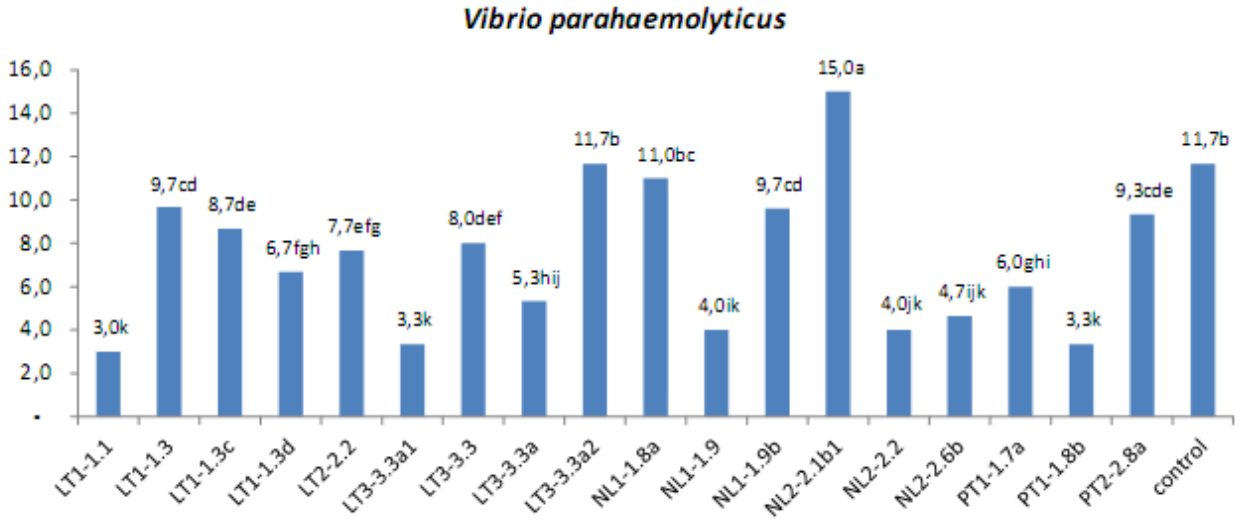


FIGURE 6: Antimicrobial activity of Actinobacyerial isolates to *Vibrio parahaemolyticus* (inhibition zone diameter -mm)

3.3 Identification of bacterial isolates

Seven good bacterial isolates: NL1-1.9, NL1-18a, NL2-2.1b1, NL2-2.2, PT1-1.7a, PT2-2.8a, LT1-1.3 were selected to PCR and sequencing. The result was presented in (Table 3).

TABLE 3
PHYLOGENETIC AFFILIATION OF ISOLATES ON THE BASIS OF 16S rRNA GENE SEQUENCES BY USING BLAST PROGRAMME IN THE GENBANK DATABASE BASED ON SEQUENCES SIMILARITY

Isolate	Closest species relative	Gene length (nu)	Somolarity (%)	NCBI Number
NL1-1.9	*Nocardioides luteus*	1325	99	MH182604.1
NL1-1.8a	*Glutamicibacter uratoxydans*	1281	99	HM625746.1
NL2-2.1b1	*Streptomyces* sp.	1289	99	JF751041.1
NL2-2.2	*Glutamicibacter uratoxydans.*	1280	99	KY938042
PT1-1.7a	*Streptomyces* sp.	1328	99	KJ534269
PT2-2.8a	*Nocardioides luteus*	1326	99	MH182604
LT1-1.3	*Streptomyces cyaneofuscatus*	1328	99	NR_041226

An UPGMA phylogenetic tree (Figure 7) in these strains showing the two clusters: cluster A with 4 strains with 2 strains belonged to genus *Streptomyces* sp. and 2 strains: *Nocardioides luteus* Cluster B had 3 strains among 2 strains: *Glutamicibacter uratoxydans* ans 1 strain *Streptomyces cyaneofuscatus*. Therefore 7 actinobacterial strains belonged to 3 genre as *Streptomyces, Nocardioides* và *Glutamicibacter*.

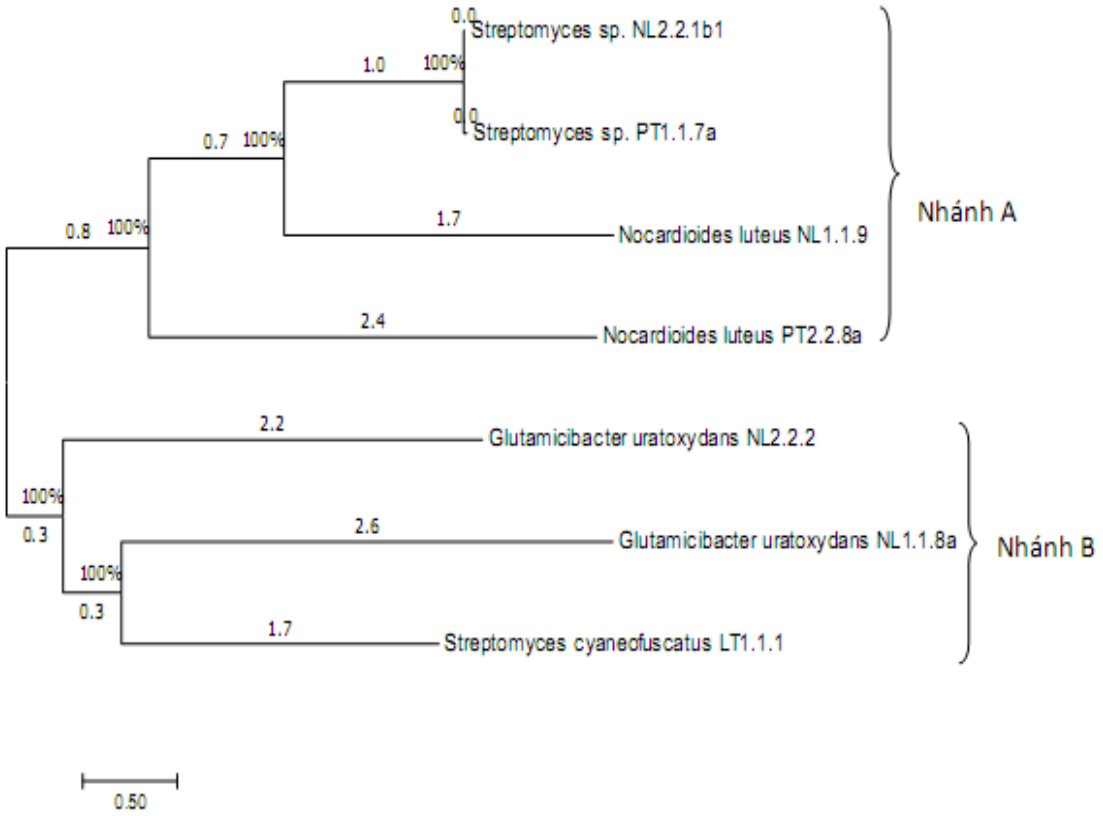


FIGURE 7: UPGMA phylogenetic tree of partial 16S rRNA gene sequences from the bacterial isolates from water pond-shrimp and closely related type strains. Numbers are percentage bootstrap values which were calculated for 1000 replicates. Bar, 0.02 was per nucleotide position.

Previously, the actinobacteria were called actinomycetes, name of greek origin, where aktis means "lightning" and mykes, fungus, or "radgrowth as fungus" that were initially classified as an intermediate group between fungi and bacteria. Investigations with electron microscopy and cytological studies showed that filamentous bacteria are prokaryotic. Since the discovery of actinomycin [11), actinobacteria have been found to produce many commercially bioactive compounds and antitumor agents in addition to enzymes of industrial interest [12]. Approximately, two-thirds of the thousands of naturally occurring antibiotics have been isolated from these organisms [13]. Of them, many have been obtained from Streptomyces [14] and these natural products have been an extraordinary source for lead structures in the development of new drugs [15].

The genus, *Streptomyces*, is responsible for the formation of more than 60% known antibiotics while a further 15% are made by a number of related *Actinomycetes*, *Micromonospora, Actinomadura, Streptoverticillium* and *Thermoactinomycetes* [16]. *Actinomycetes* centered mainly on their ability to form antibiotics.

In fact, the distributions of actinomycetes in the sea remain largely undescribed, and even today, conclusive evidence that these bacteria play important an ecological role in the marine environment has remained elusive. Speculation regarding the existence of indigenous populations of marine actinomycetes arises because these bacteria produce resistant spores that are known to be transported from land into the sea where they can remain viable but dormant for many years [17]. In the recent years, the value of marine sediments as a resource of rich actinobacteria and they produce secondary metabolites [18]. According to [19] cho rằng *Streptomyces* Marine *Streptomyces* occur in different biological sources such as fishes, molluscs, sponges, seaweeds and mangroves, besides seawater and sediments. From the Indian peninsula, 41 species of actinobacteria belonging to 8 genera have been recorded. The genus, *Streptomyces* of marine origin has been more frequently recorded. Of 9 maritime states of India, only 4 have been extensively covered for the study of marine actinobacteria [15]. Bioactive compounds from marine actinobacteria possess distinct chemical structures that may form the basis for synthesis of new drugs that could be used to combat resistant pathogens. With the increasing advancement in science and technology, there would be a greater demand for new bioactive compounds synthesized by actinobacteria from various marine sources in future [20]. In this study, we discovered actinobacteria strain against positiva-gram bacteria and negative-gram bacteria as *Glutamicibacter uratoxydans* strain NL1-1.8a and we will identify structure of bioactive compound by GC-MS method in the future.

IV. CONCLUSION

Fifty-three actinobacteria isolates isolated from 8 water samples of 3 sites of schrimp-pomfs, identying 17 isolates produce antimicrobial active metabolites inhibiting positiva-gram bacteria and negative-gram bacteria. Seven actinobacteria strains as: NL1-1.9, NL1-18a, NL2-2.1b1, NL2-2.2, PT1-1.7a, PT2-2.8a, LT1-1.1 belonged to 3 genera: *Streptomyces, Nocardioides* and *Glutamicibacter.*

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