International Journal of Innovations in Engineering and Technology (IJIET)
http://dx.doi.org/10.21172/ijiet.104.25

Isolation and identification of endophytic bacteria associated with Rhizophora mucronata and Avicennia alba of Nam Can district, Ca Mau Mangrove Ecosystem

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Abstract –A total of 86 endophytic bacterial isolates were isolated from 12 plant samples of two kinds of mangroves at Nam Can district, Ca Mau Peninsula (Ca Mau province, Mekong Delta of Vietnam). All of them showed the potential abilities of ammonium synthesis, phosphate solubilization, and excellent IAA biosynthesis. Especially, while all of the strains tolerated at a concentration of 4% NaCl, 17 bacterial isolates demonstrated a large salt tolerance ranging from 4.5 to 7%. 5 isolates regarding to the best of ability in nitrogen fixation, phosphate solubilization, IAA biosynthesis, siderophores and high salt tolerance were chosen to sequence and the results showed high degrees of similarity to those of the GenBank reference strains (99%). There were 3/5 strains belonged to Bacilli, and 2/5 strains were Gamma-Proteobacteria. Whereas our results showed that there were some good strains for nitrogen fixation, the strain Enterobacter cloacae NDNC1e revealed as a promising candidate with multiple beneficial characteristics (good nitrogen fixation, phosphate solubilization, IAA biosynthesis and high salt tolerance). Besides, the isolated bacterial strain has the potential for application as inoculants adapted to many kinds of crops grown on poor and saline soils because it is not only famous strain, but also safe strain for sustainable agriculture in "sea level rise"condition.
Keywords: 16S rRNA Gene Sequence, Mangrove Endophytic Bacteria, Nitrogen Fixation, Phosphate Solubilization, IAA, siderophores, soil salinity

I. INTRODUCTION

Mangrove forests are among the world's most productive ecosystem that enriches coastal waters, yields commercial forest products, protect coastlines and support coastal fisheries. However, mangroves exist under condition of high salinity, extreme tides, strong winds, high temperature and muddy, anaerobic soils. There may be no other group of plants with such highly developed morphological, biological, ecological and physiological adaptations to extreme conditions [1]. Mangroves are woody plants that grow at the interface between land and sea in tropical and subtropical latitudes. These plants, and the associated microbes, fungi, plants and animals, constitute the mangrove forest community or mangal [2]. Mangroves provide nursery habitat for commercial fish, crustaceans and wildlife species that contribute to sustaining the survival of local fish and shellfish populations [3]. Mangrove root systems slow water flow, facilitating the deposition of sediment. Their adaptation to salinity condition becomes possible due to their resistance to concentration of salt, entering roots and secretion of salts from their leaves. Many mangroves have stilt root, which are aerial and acts as anchoring structure to withstand wave action [4]. Some mangroves have inverted wedge like projections on the ground from the underground root system, called pneumatophores. The plants breathe in oxygen through the pores of pneumatophores during prolonged time of submergence of the root system (Figure 1).

Figure – 1 Avicennia alba and Rhizophora mucronata in mangrove forest

Bacterial diversity from these ecosystems has been studied worldwide for their unique biochemical processes. Various groups of bacteria are typically present in the mangrove ecosystem [5] where they perform diverse activities including

photosynthesis, nitrogen fixation, and methanogenesis [6]. Bacterial communities can be found living freely in mangrove sediments [7,8,9] or as endophytes associated with the native flora [10, 11, 12, 13]. Microorganisms from mangrove ecosystems contain useful enzymes, proteins, antibiotics and salt tolerant genes, all of which have biotechnological significance [14].

The present study includes isolation, morphological characterization and identification of endophytic bacteria using biochemical and molecular biology techniques [15, 16]. Molecular biology techniques like 16S rRNA techniques are an important tool in final identification of bacteria sequencing this gene, and provide genus and species identification for isolates that do not fit any recognized biochemical profiles. It gives acceptable identification which otherwise according to conventional system of taxonomy is not possible [17].

Some studies are available for the beneficial bacteria associated with the natural mangrove habitats [18, 19, 20, 21, 22]. However, no such studies are available for artificially developed mangrove habitats. In mangrove ecosystems, high rates of nitrogen fixation have been associated with dead and decomposing leaves [23] , pneumatophores [24, 25] and the rhizosphere soil [15]. N_2 fixation in mangrove sediments is likely to be limited by insufficient energy sources. The low rates of N_2 fixation by heterotrophic bacteria detected in marine water are probably due to lack of energy sources. Phosphorous is one of the major plant nutrients, second only to nitrogen [26], so phosphate-solubilizing microorganisms (PSMs) play an important role in supplementing phosphorus to plants and allowing the sustainable use of phosphate fertilizers [27]. Fungi and inorganic phosphate-solubilizing bacteria present in the mangrove rhizosphere participate in releasing soluble phosphate into pore water [12]. Certain bacteria exhibit high phosphatase activity, capable of solubilizing phosphate [28]. However, very little information is available about beneficial bacterial diversity [9] and their activity in mangrove soil of Vietnam. Therefore, the aims of this study were (i) to isolate nitrogen-fixing bacteria and phosphate-solubilizing bacteria, (ii) to obtain their characterization as salt-tolerance, colonies, … and (iii) to identify by 16S rDNA techniques.

II. MATERIALS AND METHODS

2.1. Collect of plant samples

Plant samples were collected carefully from two species of mangroves as Rhizophora mucronata and Avicennia alba from a 5-year-old plantation site, raised along the CaMau Peninsula (Lat. 09^o 05’ 10” N; Long. 105^o 15’ 00” E), located at the end of the Mekong Delta (Vietnam) (as described in Tam and Diep, IJIET, 9(1):68-79).

The samples were collected in December, 2016. For isolation of bacterial endophytic, samples were collected during the low tide and brought to the laboratory immediately for analyses in the day.

2.1.1 Bacterial isolation

The tree samples were washed with water to remove adherent particles and were superficially disinfected according to Araújo et al. [29]. Then, the samples were cut into fragments, and roughly 1 g was triturated in the presence of 5 mL of PBS (Phosphate Buffered Saline) buffer, transferred to a 15 mL tube and shaken for 1 hour at 180 rpm. After obtaining the suspension of microorganisms, dilutions were made in PBS buffer, and aliquots of 100 µL were inoculated onto Burk’s N free [30] and NBRIP [31] media with 1.6% agar (semi-solid], supplemented with Benomyl (50 µgmL^{-1}) to inhibit fungal growth. The plates were incubated at 28°C for 2–4 days until the pellicle was observed. Endophytic isolates were purified and inoculated into liquid 5% Tryptic Soy Broth (TSB, Merck) medium supplemented with glycerol (15% final concentration) and stored at −80°C for future experiments. For isolation of nitrogen-fixing bacteria in Burk’N free media plus 2% NaCl [30] and phosphate-solubilizing bacteria in NBRIP media plus 2% NaCl [31], cultures were streaked on media to obtain single colonies. To check for phosphate solubilization ability or nitrogen fixation ability, colonies from Burk’N free media were streaked to NBRIP media and colonies from NBRIP media were also cultivated to Burk’s N free media in order to select the colonies which developed on two media (or microbes having both N_2-fixing and phosphate-solubilizing ability).

2.2. Morphological Characterization

The morphological characterization of the bacterial colonies were carried out according to on the basis of their shape, size, colour, margin, elevation on the media and Gram staining method was also performed to decide the further determinative protocol. All isolates were tested on media (Burk’s or NBRIP) with higher NaCl concentrations (from 4.5 to 7.0% NaCl).

2.3. Screening for Biofertilizer Activities

The ability to fix N_2 was tested on Burk’N-free liquid medium incubating at 30^{o}C and the ammonium concentration in the medium was measured by Phenol Nitroprusside method after 2, 4, 6 and 8 days inoculated (DAI) and inorganic phosphate solubilizing ability was tested on NBRIP liquid medium and they were incubated at 30^{o}C and the P_2O_5 concentration was measured by ammonium molypdate method. The qualitative detection of indole-3-acetic acid (IAA) production was carried out basing on the colorimetric method [32]. Precultures were grown in Burk’s N free (100 ml) without tryptophan in 250mL-flask at 30^{o}C on a roller at 100 rpm and samples were taken from at 2, 4, 6, and 8 DAI, cell free supernatants were mixed 2:1 with Salkowki reagent (0.01 M $FeCl_3$ in 35% perchloric acid) and incubated in the dark for 20 min at RT. IAA-containing solutions were indicated by reddish color with an absorption peak at 530 nm on Genesys 10uv Thermo Scientific

spectrophotometer. Furthermore, siderophore production was assayed by the rhizopheric bacterial isolates according to Schwyn and Neilands [33] using NBRIP medium without tryptophan which was diluted five-fold. The isolates were spot inoculated onto Chrome azurol S agar plates divided into equal sectors, and the plates were incubated at 28^{o}C for 48 h. Development of a yellow, orange or violet halo around the bacterial colony was considered to be positive for siderophore production.

2.4. Molecular Analysis

2.4.1Genomic DNA Isolation

Culture was centrifuged at 10,000 rpm for 5 min. Pellet was collected and resuspended by adding 9 ml of STE buffer (0.1 mM NaCl, 10 mM Tris, 10 mM EDTA) 1 ml of SDS (10% Stock Solution). The suspension was incubated at 70˚C for 1 hr. and centrifuged at 6000 rpm for 10 min at room temperature. The supernatant was collected in fresh tube and add equal volume of Phenol:Chloroform:Isoamyl alcohol (PCI mix) (25:24:1) was added and mixed slowly. The suspension was centrifuged at 6000 rpm for 10 min. The aqueous phase in fresh tube. Equal vol. of Chloroform: Isoamyl alcohol (24:1) and mix slowly and centrifuged at 6000 rpm for 10 min.

The aqueous phase was collected and added double the vol. of absolute alcohol was added. The tube was subjected to overnight incubation in −20˚C. The solution was centrifuged at 6000 rpm 4˚C for 10 min and the pellet was resuspended in 1/10th ml of 3M sodium acetate and 10 ml of absolute alcohol and centrifuged at 6000 rpm 4˚C for 10 min. The supernatant was discarded and the pellet was air dried. The pellet was dissolved in 1 ml sterile TE buffer. The DNA quality was checked using Agarose gel electrophoresis and quantified using Nanodrop.

2.4.2.PCR Amplification and Phylogenetic Analysis

Amplification of 16S rDNA by PCR was carried out using the primers p515FPL and p13B [34]. The 50 µL reaction mixture consisted of 2.5 U Taq Polymerase (Fermentas), 50 µM of each desoxynecleotide triphosphate, 500 nM of each primer (Fermentas) and 20 ng DNA. The thermocycling profide was carried out with an initial denaturation at 95^{o}C (5 min) followed by 30 cycles of denaturation at 95^{o}C (30 s), annealing at 55^{o}C (30 s), extension at 72^{o}C (90 s) and a final extension at 72^{o}C (10 min) in C1000 Thermal Cycler (Bio-Rad). Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Aliquots (10 µl) of PCR pro ducts were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of selectived 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); In the best isolate(s) (high nitrogen fixation, phosphate solubilization ability, IAA biosynthesis and good salt tolerance), 5 isolates of 3 sites were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between root-associated bacterial strains [35] and phylogenetic tree were constructed by the maximum-likelihood method using the MEGA software version 7.0 based on 1000 bootstraps [35].

2.5 Data Analyses

Data from ammonium and orthophosphate concentrations in media were analysed in completely randomized design with three replicates and parameters of pot experiment also was arranged to completely randomized design with seven replications and Duncan test at P=0.01 or P=0.05 were used to differentiate between statistically different means using SPSS version 16.

III. RESULTS AND DISCUSSION

3.1 Bacteria Isolation, Colony Characteristic and Microscopic Examination

The endophytic bacteria developed to the pellicles of semi solid (in Burk's N free and NBRIP media) after 24 h inoculation (Fig. 2a and Fig 2b) as the previous results of Weber et al. [36], Thu Ha et al. [37] and our previous result [38].

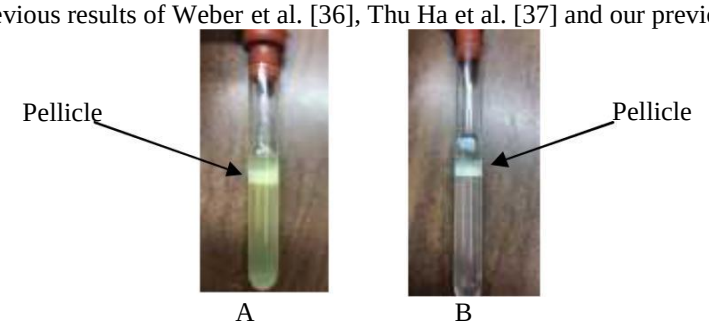

Figure 2a. Endophytic bacteric made a pellicle on the NBRIP (A) and Figure 2b. on Burk's N free media (B)

From 12 plant samples of 3 sites (villages of Nam Can district), whereas 86 isolates were isolated on two media included 52 and 34 isolates from NBRIP and Burk'N free media, respectively, 43 and 43 strains were isolated~~s~~

from roots of Avicennia alba and Rhizophora mucronata, respectively.

Almost their colonies have round-shaped; milky, white clear (on Burk's medium) and yellow, reddish yellow (on NBRIP medium); entire or loabate margin; diameter size of these colonies varied from 0.2 to 3.0 mm and all of them are Gram-positve or Gram-negative recorded by Gram stain. Especially, phosphate-solubilizing bacteria make a halo around colonies in NBRIP medium as described of Thanh and Diep [39], Nhu and Diep [40]; Tam and Diep [38] (Figure 3a and 3b). All of the isolates grew well on two media showed that they have both nitrogen fixation and phosphate solubilization abilities (Figure 3c and Figure 3d).
The cells were observed by microscopic and appearded as short rods and most of them have motility.




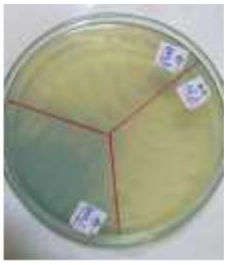

Figure 3a. Colonies of the isolate on Burk's N free medium

Figure 3c and 3d. The isolates from NBRIP were grown on Figure 3b. Colonies of the Burk's N free medium (2c) and the isolates from Burk's N isolate on NBRIP medium free were grown on NBRIP medium (2d) very well

3.2 Screening for Biofertilizer Activities
Selection of good isolates for nitrogen fixation from 2 media was presented in Table 1a and 1b

Table 1a. Nitrogen fixation of 17/34 isolates (mg NH_4/l) on Burk's N free medium

No	Bacterial Isolate	Day 2	Day 4	Day 6	Day 8
0	Control	0.00 om	0.00 op	0.00 l	0.00 m
1	BMHT1c	0.26 i	0.29 jkl	0.09 jkl	0.02 hijklm
2	BMHT2a	0.03 lm	0.49 gh	0.08 jkl	0.02 jklm
3	BĐHT1a	0.98 c	0.76 d	0.14 hij	0.05 efghi
4	BĐHT1b	0.62 gh	0.21 lmn	0.11 ijk	0.07 cdef
5	BĐHT1c	1.27 b	0.39 hij	0.09 jkl	0.07 defg
6	BĐHT1d	0.59 gh	0.26 klmn	0.19 hi	0.10 c
7	BMNC1a	0.79 de	2.46 a	2.06 a	0.06 defg
8	BMNC1b	0.26 i	0.01 p	0.05 jkl	0.06 defg
9	BMNC2a	0.02 lm	0.25 klmn	0.23 gh	0.05 efgh
10	BĐNC1a	0.86 d	1.74 c	1.74 c	0.26 a
11	BĐNC1c	0.57 h	0.77 d	0.81 d	0.09 cd
12	BĐNC2a	0.64 gh	1.88 b	1.85 b	0.04 fghijk
13	BĐNC2b	0.67 fg	1.65 c	1.76 bc	0.06 defg
14	BMĐM1b	0.73 ef	0.63 ef	0.65 e	0.05 efghi
15	BMĐM2a	0.77 e	0.69 de	0.70 e	0.02 ijklm
16	BMĐM2b	2.00 a	0.70 de	0.70 e	0.06 efgh
17	BĐĐM2b	0.97 c	0.05 p	0.42 f	0.06 efgh
	CV		6.81%		

Table 1b. Nitrogen fixation of 24/52 isolates (mg NH_4/l) on NBRIP medium

No	Bacterial Isolate	Day 2		Day 4		Day 6		Day 8	
0	Control	0.00	v	0.00	s	0.00	s	0.00	l
1	NĐNC1a	0.60	i	0.03	pqrs	0.02	pqrs	0.05	fghijkl
2	NĐNC1b	0.09	qrstu	0.11	mn	0.05	mnopqrs	0.06	efghijk
3	NĐNC1d	0.08	rstu	0.02	qrs	0.06	lmnopqrs	0.04	ghijkl
4	NĐNC1e	1.03	de	0.06	opqrs	0.02	pqrs	0.04	ghijkl
5	NĐNC1f	0.99	e	0.12	m	0.02	qrs	0.05	ghijkl
6	NĐNC2a	0.49	j	2.36	d	2.33	d	0.15	a
7	NĐNC2b	1.12	c	0.73	i	0.84	h	0.06	defghijk
8	NĐNC2c	1.27	b	1.15	gh	1.16	g	0.14	ab
9	NĐNC2d	0.76	fg	0.76	i	0.76	i	0.05	fghijkl
10	NMNC1a	0.67	hi	0.01	rs	0.11	lmn	0.06	defghijk
11	NMNC1c	0.67	hi	3.24	a	3.65	a	0.03	jkl
12	NMNC1d	0.98	e	1.18	g	1.19	g	0.12	abcd
13	NMNC1e	0.09	qrstu	0.01	rs	0.07	lmnopqrs	0.07	cdefghijk
14	NMNC2a	1.07	cd	1.76	e	1.76	e	0.05	fghijkl
15	NMNC2b	1.09	cd	1.09	h	1.16	g	0.07	cdefghijk
16	NMNC2c	1.35	a	1.25	f	1.26	f	0.08	cdefghijk
17	NMNC2d	1.27	b	2.78	b	2.76	b	0.11	abcdef
18	NMNC2f	0.83	f	0.01	rs	0.09	lmnop	0.07	cdefghijk
19	NĐĐM1a	0.34	k	0.59	j	0.56	j	0.11	abcde
20	NĐĐM1b	0.48	j	0.59	j	0.48	k	0.03	hijkl
21	NĐĐM1c	1.13	c	0.78	i	0.88	h	0.06	defghijk
22	NĐĐM1d	0.73	gh	1.18	g	1.18	g	0.09	bcdefgh
23	NĐĐM2a	0.64	i	2.66	c	2.52	c	0.06	efghijk
24	NĐĐM2d	0.67	hi	0,03	pqrs	0.12	lmn	0.05	fghijkl
	CV			5.82%					

The numbers followed by the same word not different at $p<0.01$

Good isolates for phosphate solubilization from 2 media were presented in Table 2a and 2b

Table 2a. Phosphate solubilization of 21/34 isolates (mg P_2O_5/l) on Burk's N free medium

No	Bacterial Isolate	Day 5		Day 10		Day 15		Day 20	
0	control	0.00	q	0.00	q	0.00	q	0.00	q
1	BĐHT2c	62.93	j	73.64	i	65.57	j	54.29	l
2	BĐHT2d	64.22	j	72.94	i	63.96	j	48.87	m
3	BMNC1a	66.51	j	79.79	hi	83.42	h	79.82	i
4	BMNC1b	43.40	n	82.80	h	104.75	d	77.59	i
5	BMNC1c	55.39	l	81.26	h	93.86	f	105.79	d
6	BMNC2a	57.78	jl	86.31	gh	84.51	h	88.17	g
7	BĐNC1a	61.88	k	101.13	e	101.73	e	92.78	f
8	BĐNC1b	45.01	n	97.89	ef	111.57	c	101.53	e
9	BĐNC1c	22.49	p	83.23	h	86.90	gh	82.62	h
10	BĐNC1d	53.92	l	78.63	i	101.91	e	107.87	d

11	BĐNC2a	50.00	lm	64.48	jk	82.13	h	99.34 e
12	BĐNC2b	30.97	o	60.42	k	90.56	f	78.35 i
13	BMĐM1a	29.20	o	60.77	k	102.74	e	80.68 h
14	BMĐM1b	61.84	k	102.04	de	138.89	a	111.11 c

No	Bacterial Isolate	Day 5		Day 10		Day 15		Day 20	
15	BMĐM1c	71.63	i	107.66	d	126.00	b	94.21	f
16	BMĐM2a	54.15	l	69.52	j	93.58	f	92.51	fg
17	BMĐM2b	73.47	i	111.33	c	134.51	a	75.26	i
18	BMĐM2c	55.56	l	50.70	lm	100.59	d	55.04	l
19	BĐĐM1a	58.31	l	52.62	l	97.16	ef	50.24	lm
20	BĐĐM2a	56.50	l	52.62	l	94.16	f	51.44	l
21	BĐĐM2b	56.35	l	48.53	m	85.85	g	49.62	lm
	CV				6.81%				

Table 2b. Phosphate solubilization of 39/52 isolates (mg P_2O_5/l) on NBRIP medium

No	Bacterial Isolate	Day 5		Day 10		Day 15		Day 20	
0	Control	0.00	x	0.00	u	0.00	z	0.00	u
1	NMHT1a	66.51	cde	79.79	j	83.22	jklmno	79.82	1j
2	NMHT1b	43.40	nopq	82.80	j	104.75	fg	77.58	j
3	NMHT1c	55.39	gh	81.26	j	93.86	ghij	105.79	cde
4	NMHT1d1	57.78	fgh	86.31	j	84.52	jklmn	88.17	hi
5	NMHT1d2	61.88	efg	101.13	hi	101.73	fghi	92.78	gh
6	NMHT2a	45.01	lmnopq	97.89	i	111.57	f	101.52	def
7	NMHT2b	22.49	vw	83.23	j	86.90	jklm	82.62	ij
8	NMHT2c	53.93	ghijk	78.63	j	101.91	fgh	107.87	cd
9	NĐHT1a	50.00	hijklmno	64.48	klm	82.13	klmno	99.33	efg
10	NĐHT1b	30.97	tu	60.42	lmno	90.56	ijkl	78.34	j
11	NĐHT1c	29.20	uv	60.77	klmn	102.74	fgh	80.68	ij
12	NĐHT2a	61.84	efg	102.04	hi	138.89	cd	111.11	c
13	NĐHT2b	71.63	abcd	107.66	gh	126.00	e	94.21	fgh
14	NĐHT2c	54.15	ghij	69.52	k	93.58	ghij	92.51	gh
15	NĐHT2d	73.47	abc	111.33	fg	134.52	de	75.26	jk
16	NĐNC1a	52.88	hijkl	47.60	q	67.50	qrstu	67.84	klm
17	NĐNC1b	69.54	bcde	107.10	gh	104.12	fg	66.81	lmn
18	NĐNC1c	46.86	ijklmnop	47.91	q	74.90	nopqr	75.06	jk
19	NĐNC1d	68.96	bcde	100.94	hi	63.64	stuv	68.41	kl
20	NĐNC1e	36.97	qrstu	169.09	b	147.29	bc	127.69	b
21	NĐNC1f	14.65	w	64.23	klm	63.75	rstuv	102.17	def
22	NĐNC2a	57.10	fgh	139.80	c	130.66	de	58.25	opqr
23	NĐNC2b	53.01	hijkl	83.15	j	125.16	e	55.77	opqr
24	NĐNC2c	74.75	ab	117.98	ef	73.24	opqrs	60.25	mnopqr
25	NĐNC2d	43.91	nopq	140.67	c	155.24	b	81.11	ij
26	NMNC1a	67.51	bcde	53.57	nopq	66.89	qrstu	101.93	def
27	NMNC1b	69.11	bcde	52.21	nopq	62.61	stuvw	54.77	pqr
28	NMNC1c	18.55	w	20.30	t	65.68	rstuv	38.19	s

29	NMNC1d	34.20	stu	50.87	pq	101.08	fghi	37.12	s
30	NMNC1e	51.34	hijklmn	47.74	q	52.04	wxy	53.46	r
31	NMNC2a	74.39	abc	66.29	kl	77.77	mnopq	27.36	t
32	NMNC2d	77.76	a	67.15	kl	92.08	hijk	39.39	s
33	NMĐM1d	37.69	qrst	160.83	b	179.00	a	78.88	j
34	NMĐM2a	66.44	cde	97.55	i	66.80	qrstuv	135.72	a
35	NĐĐM1a	34.41	stu	48.70	q	83.27	jklmno	78.15	j

No	Bacterial Isolate	Day 5		Day 10		Day 15		Day 20	
36	NĐĐM1b	45.89	klmnop	124.65	de	93.98	ghij	54.43	pqr
37	NĐĐM1c	46.06	jklmnop	55.60	mnopq	102.19	fgh	26.75	t
38	NĐĐM1d	34.95	rstu	51.54	opq	100.48	fghi	41.41	s
39	NĐĐM2d	69.15	bcde	129.06	d	61.26	tuvwx	54.80	pqr
	C.V			5.82%					

The numbers followed by the same word not different at $p<0.01$

From the results of Table 1a, 1b, 2a and 2b showed that the number of bacterial isolates having high biological nitrogen fixation from Burk's N free medium were higher than those of isolates from NBRIP medium. In constrast, quantity of isolates having high phosphate solubilization from NBRIP medium were much more than that from Burk's medium. Interestingly, almost the bacterial isolates had IAA biosynthesis during 8 days after
inoculation in two media. The isolates revealed generally high IAA biosynthesis in both Burk's N free medium (Fig. 3a) and NBRIP medium (Fig. 3b) without tryptophan.

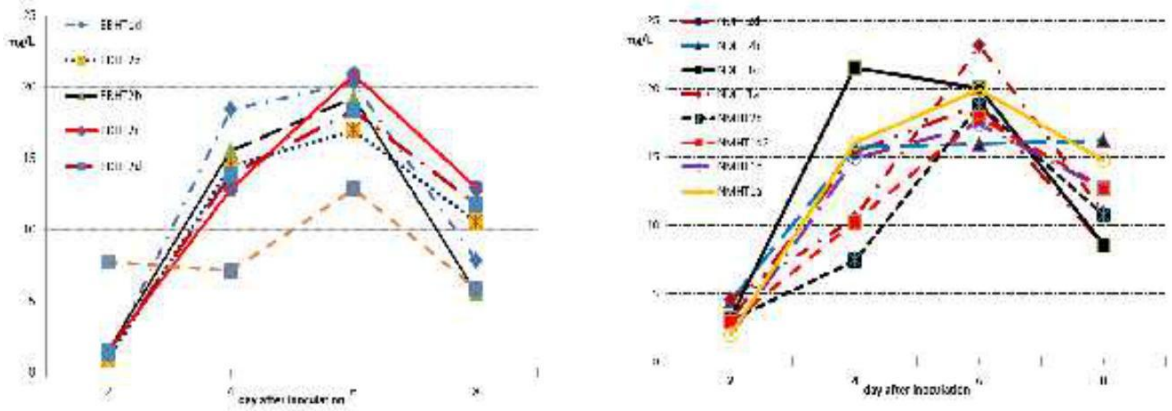

Figure - 3a. Some isolates had high IAA biosyntheis in Burk's N free medium and Figure - 3b. in NBRIP medium

There were 57/86 isolates producing siderophores (66.28%); however, the number of endophytic bacterial isolates producing siderophores with large halo were 26 strains, occupied by 30.23% (Figure 4).

3.3 Salinity tolefance

Almost the rhizospheric bacterial isolates from mangrove soil have salt tolerance capacity at 4.0% NaCl; however, the number of strains reduced significantly when saline concentrations in the media increased from 4.5 to 7.0% NaCl (Table 3). Whereas there were 17 isolates grew well at salt concentration up to 7.0% as BMHT1b, BMHT2b, BĐHT1a, BĐHT1b, BĐHT1c, BĐHT1d, BĐHT2a, BĐHT2b, BĐHT2d, BMNC2a, NMHT1a, NMHT1c, NĐHT2b, NĐNC1d, NMNC2f, NMĐM2b, NĐĐM1b (Figure. 5), there was no isolate
developed at 7.5%.

Figure – 5. Ratio (%) endophytic bacteria developed on the medium with the different NaCl concentrations

Ratio (%)	NaCl concentration in medium (%)						
Bacteria	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Developed	100	83/86 (96.51)	82/86 (95.35)	74/86 (86.05)	52/86 (60.45)	26/86 (30.23)	17/86 (19.76)
No developed	100	3/86 (3.49)	4/86 (4.65)	12/86 (13.95)	34/86 (39.55)	60/86 (69.77)	69/86 (80.24)

http://dx.doi.org/10.21172/ijiet.104.25

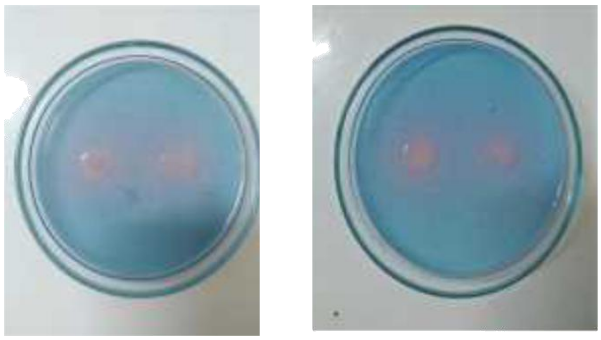

Figure - 4 Bacterial isolates made a yellow, orange halo round well contaning bacterial liquid on CAS agar after 48 h incubation

$65^{0}/_{00}$ 0

70 $/_{00}$

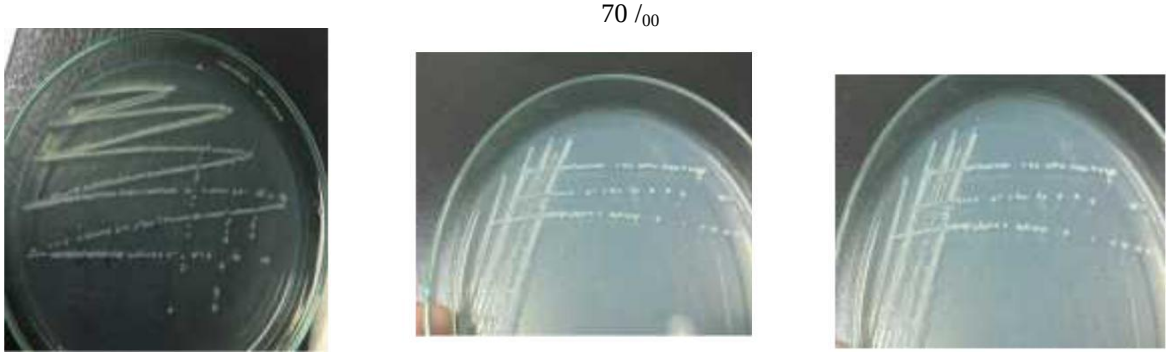

Figure – 5. The isolates developed well on medium with salt concentration up to 7%

Based on the characteristics as high nitrogen fixation, phosphate solubilization, IAA production, siderophores and well growth on media supplemented high salt concentrations, 5 good isolates were chosen to identify with universal primers p515FPL and p13B and sequence as NĐNC1d; NDNC1b; BMNC2a; NDNC1f,; NMNC1e.

3.4 DNA analysis and sequencing

The fragments of 900 bp 16S rRNA were obtained from PCR with p515FPL and p13B primers and sequencing. Homology searches of 16S rRNA gene sequence of selected strains in GenBank by BLAST revealved that 3/5 isolates had similarity to sequences of Bacilli and 2/5 isolates belonged to Gamma-Proteobacteria (Table 3) (Figure 6)

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

Taxonomic Group and Strain	nucleotide	Closest species relative	Similarity (%)
NĐNC1d	838	HQ844260 Bacillus sp. AS6	98
		KF417548 Bacillus flexus strain PHCDB20	99
		JQ833743 Bacillus megaterium strain p50-A06	99
NĐNC1b	838	KX470412 Bacillus sp. strain CA4	99
		KX881940 Bacillus subtilis strain K-18	99
		LN827663 Bacillus tequilensis, strain CEES, isolate CEES#2	99

		KJ195697 Bacillus amyloliquefaciens strain PJ-5	99
BMNC2a	858	KC212004 Bacillus subtilis strain Z214	99
		KX161425 Bacillus flexus strain WSH3	99
NĐNC1f	861	HM755542 Pseudomonas oryzihabitans strain C-G-NA8	99
		LT986201 Pseudomonas psychrotolerans, isolate VrL4	99
		KY653040 Pseudomonas sp. strain Po-C2-3	99
NMNC1e	859	KT260944 Enterobacter cloacae strain RCB732	99
		CP028538 Enterobacter hormaechei strain SCEH020042	99
		LT992502 Enterobacter bugandensis isolate EB-247	99

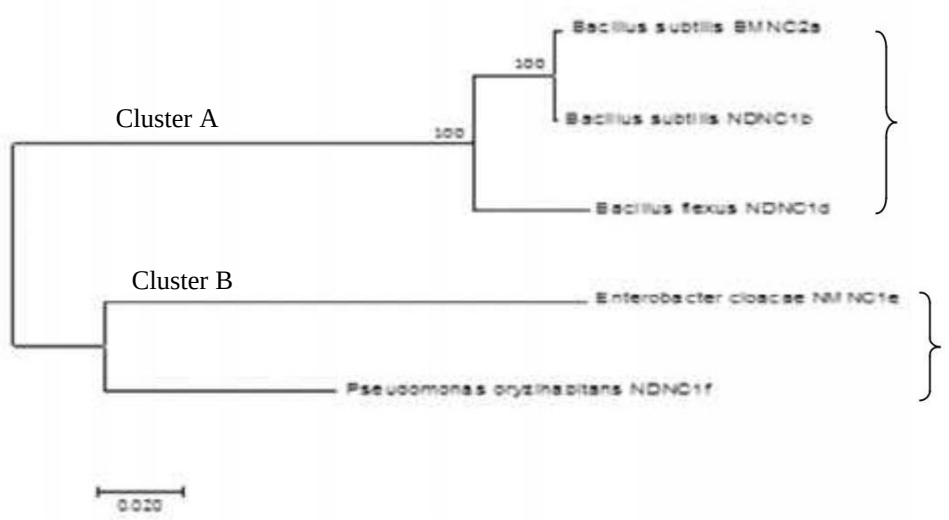

Figure 6 - Phylogenetic tree showing the relative position of endophytic bacteria by the maximum-likelihood method of complete 16S rRNA sequence. Bootstrap values of 1000 replicates are shown at the nodes of the trees.

A maximum-likelihood analysis of phylogenetic tree in these isolates showed in the two clusters: cluster A composed of three species of genus Bacillus as strains Bacillus subtilis BMNC2a, Bacillus subtilis NDNC1b and Bacillus flexus NDNC1d related closely even though they were isolated at other sites in Nam Can district. Cluster B including only 2 strains belong to Gamma-proteobacteria as Enterobacter cloacae NMNC1e and Pseudomonas oryzinabitans NDNC1f (Figure 6).

Mangroves are unique intertidal ecosystems of the tropical and sub-tropical regions of the world that support genetically diverse groups of aquatic and terrestrial organisms [41]. Nearly 60–70% of the world's tropical and subtropical coastlines are covered with mangroves, which are known to be highly productive ecosystems of immense ecological value. Despite being fragile and sparsely distributed, these ecosystems are highly productive all over the world [42].

Endophytes are microorganisms that live inside of plants without causing any harm to their hosts [43]. Endophytic bacteria have been isolated from root nodules and the stems, leaves and fruits of a wide variety of plant species including citrus [44], sugarcane [45], maize [46], eucalyptus [47,48], soybean [49,50], and strawberry [8], among others. However, some endophytic communities remain unexplored in studies describing the bacterial communities from tropical native plants. Consequently, studies on the endophytic bacteria of plants from different ecosystems (mangroves, for example) offer a great opportunity to discover new compounds and resources with biotechnological potential that can be exploited [51]. Microorganisms from mangrove ecosystems contain useful enzymes, proteins, antibiotics and salt tolerant genes, all of which have biotechnological significance [14].

In a recent study, Khianngam et al. [52] isolated and screened endophytic bacteria from mangrove plants in Thailand for the presence of hydrolytic enzymes. Twenty isolates showed activities associated with proteases, lipases, amylases or cellulases. The Rhf-2 strain, which was isolated from the fruit of Rhizophora mucronata, produced all of these enzymes; the strain was later identified as Bacillus safensis. Castro et al., [53] isolated endophytic microorganisms from two mangrove species, Rhizophora mangle and Avicennia nitida, that are found in streams in two mangrove systems in Bertioga and Cananéia, Brazil. Bacillus was the most frequently isolated genus, comprising 42% of the species isolated from Cananéia and 28% of the species from Bertioga. However, other common endophytic genera such as Pantoea, Curtobacterium and Enterobacter were also found. In our experiment, 3/5 strains were identified as Bacillus and 2/5 strains were Gamma-proteobacteria and our result was the same with described results previously as Liu et al. [12] dertemined Bacillus was the most abundant genus isolated from all samples. Various Bacillus sp. have also been isolated from several fish, mollusks, sediments and marine waters in Canada [54]. Ravikumar et al. [55] isolated many endophytic bacteria from mangrove halophytic plants collected from the Pichavaram mangrove forest in India. Among the isolates, the authors identified two endophytes, Bacillus thuringiensis (MB4) and Bacillus pumilus (MB8), which were able to control many bacterial and fungal pathogens. Similarly, the endophytic strain Bacillus amyloliquefaciens (RS261) is a biological agent isolated from the leaf of R. stylosa [13]. In our experiment, all of them tolerated at a concentration of 4% NaCl, but strain Enterobacter cloacae NDNC1e is not only good nitrogen fixation, high phosphate solubilization but also good IAA biosynthesis and salt tolenace, so that it may become a promising strain to produce biofertilizer for the crops which cultivated in soil salinity.

VI. CONCLUSION

From 12 plant samples (Rhizophora mucronata and Avicennia alba) from mangrove forest of Nam Can district, Ca Mau province, Mekong delta, Vietnam, 86 isolates were isolated and identified as endophytes, with 5 isolates having good plant growth promotion from 3 different sites were chosen to analyse their relationship. The results showed that bacterial diversity was very high; 3/5 strains belonged to Bacillus and 2/5 strains were Gamma-proteobacteria. Among them, one strain will be suggested to apply to crop cultivation on soil salinity in the future.

V. ACKNOWLEDGEMENTS

The authors thank the helpfulness of Microbiology BSc. Students and technicians in the Environment Microbiology; Ms. DAO THI MINH CHAU, Environment Microbiology Laboratory, Biotechnology R&D Institute, Can Tho University, Vietnam for grammartical english.

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