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# Isolation and Characterization of Bacteria of Mangrove Rhizosphere in the Mekong Delta, Vietnam

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**Abstract - Total of 125 rhizospheric bacterial isolates were isolated from 18 rhizospheric soil samples of mangrove at Ca Mau Peninsula (Ca Mau province, Mekong Delta of Vietnam). Although all of them had the abilities of ammonium synthesis and phosphate solubilization, the isolated strains had excellent phosphate solubilizing potential. The sequences from selected nitrogen-fixing and phosphate-solubilizing bacteria (22 isolates) showed high degrees of similarity to those of the GenBank reference strains (between 97% and 99%). From 22 isolates, 16 strains belonged to Bacilli, and 6 strains were Gamma-Proteobacteria. Whereas our results showed that there were some good strains for nitrogen fixation as *Bacillus subtilis* DLB4b, *Bacillus* sp. VAB2b, *Enterobacter* sp. MAB1b and *Bacillus* sp. MDB1c and strains for phosphate solubilation as *Bacillus subtilis* MLN1b, *Bacillus* sp. MLN1c, *Bacillus subtilis* VDN1f and *Bacillus subtilis* MDN1c, all of them tolerated at a concentration of 4% NaCl. However, the strain *Bacillus subtilis* MAB2b revealed as a promising candidate with multiple beneficial characteristics (both good nitrogen fixation and phosphate solubilization). Besides, the isolated bacterial strain has the potential for application as inoculants adapted to poor, soil salinity as well many kinds of crop 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 Rhizosphere Bacteria, Nitrogen Fixation, Phosphate Solubilization, soil salinity**

## I. INTRODUCTION

Mangroves are unique coastal plants which have originated due to the tectonic land shifts because of which terrestrial plants got bared to the open sea with ecological and economic importance. They not only provide socio-economic benefits to local tribes, but also provide protection to coastal areas against natural disasters and facilitate the formation of land by trapping sediments [1][2]. Around 34 major and 20 minor mangrove species belonging to about 20 genera in over 11 families have been recorded globally [3]. Mangroves constitute a significant part of tropical coastal biodiversity which occupy less than 1% of the world's surface [4] and are mainly found between the Tropic of Cancer and the Tropic of Capricorn on all continents covering an estimated 75% of the tropical coastline worldwide. Mangroves of South and Southeast Asia form the most extensive and diverse mangrove system comprising 41.4% of global mangroves in the world [5].

Bacterial diversity from these ecosystems has been studied worldwide for their unique biochemical processes. The present study includes isolation, morphological characterization and identification of rhizospheric bacteria using biochemical and molecular biology techniques [6] [7]. 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 [8].

Some studies are available for the beneficial bacteria associated with the natural mangrove habitats [9] [10] [11] [12] [13]. 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 [14] , pneumatophores [15] [16] and the rhizosphere soil [10]. $N_2$ 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 [17], so phosphate-solubilizing microorganisms (PSMs) play an important role in supplementing phosphorus to plants and allowing the sustainable use of phosphate fertilizers [18]. 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 [19]. 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

### *A. Collect of samples*

Soil samples adhering to the root system were collected carefully from four species of mangroves viz. *Rhizophora mucronata*, *Bruguiera cylindrica*, and *Avicennia marina* 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) (Figure 1).

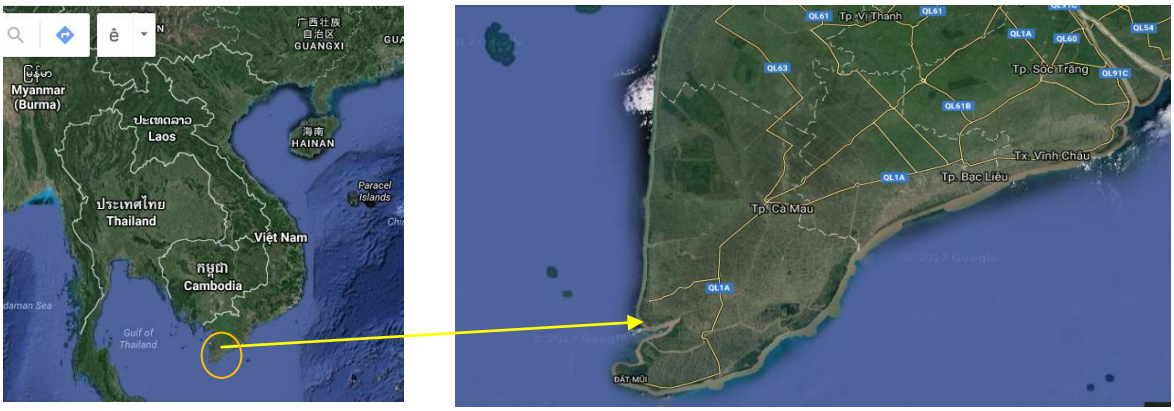


Figure 1. Sample collection sites in Ca mau Peninsula, Ca Mau province, Vietnam (the Mekong Delta, Vietnam)

The samples were collected in December, 2015. For isolation of bacterial rhizosphere samples were collected during the low tide and brought to the laboratory immediately for analyses within 3 h. Soil samples were collected by using a sterile spatula and stored in sterile polythene bags.

A known weight of soil (1 g) was aseptically weighed and transferred to a stoppered (150 mL) sterile conical flask containing 99 mL of sterile diluent. The sediment-diluent mixture was agitated by means of mechanical shaking for about 10 min. and later subjected to bacteriological examination.

One hundred microliters from appropriate dilutions were plated on two different media, viz Burk's N free agar plus 2% NaCl [20] and NBRIP agar plus 2% NaCl [21] and they were kept to refrigerator for counting by viable drop plate count [22] (Hoben and Somasegaran, 1982) and isolation of nitrogen-fixing bacteria in Burk'N free media plus 2% NaCl [20] and phosphate-solubilizing bacteria in NBRIP media plus 2% NaCl [21]; 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 $N_2$-fixing and phosphate-solubilizing ability).

### *B. 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 were performed to decide the further determinative protocol. All isolates were tested on media (Burk's or NBRIP) with higher NaCl concentration (2.5 to 4.0% NaCl).

### *C. 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 medium was measured by Phenol Nitroprusside method after 2,4,6 and 8 days inoculated (DAI). Besides, inorganic phosphate solubilizing ability was tested on NBRIP liquid medium, incubated at 30$^{o}$C and the $P_2O_5$ concentration was measured by ammonium molypdate method.

### *D. Molecular Analysis*

*Genomic 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.

*PCR Amplification and Phylogenetic Analysis*

Amplification of 16S rDNA by PCR was carried out using the universal primers 8F and 1492R [23]. The 50 µL reactionmixture consisted of 2.5 U Taq Polymerase (Fermentas), 50 µM of each deooxynucleotide 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. 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) (especially high phosphate solubilization ability) were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between PGPR strains [24] and phylogenetic tree were constructed by the maximum-likelihood method using the MEGA software version 6.05 based on 1000 bootstraps.

*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

*A. Soil pH and bacterial population*

Soil pH of mangrove rhizosphere ranged from 5.27 to 6.68 (Table 1) and nitrogen-fixing and phosphate-solubilizing bacterial population in soil were significantly high. Particularly, population of and nitrogen-fixing and phosphate-solubilizing bacteria in rhizosphere soil of *Bruguiera cylindrica* was the highest, while that of *Rhizophora mucronata* was the least. A similar trend has been recorded with decomposing leaves of mangroves [25]. The variation recorded presently between *Rhizophora* and *Avicennia* species may be attributed to the pattern of root system.

Table 1 - Soil pH and $N_2$-fixing and phosphate-solubilizing bacterial population in mangrove rhizosphere soil

| Soil sample site | Kind of Plant | Soil pH | $N_2$-fixing bacteria population | Average | P-solubilizing bacteria population | Average |
|---|---|---|---|---|---|---|
| | | | CFU $\log_{10}$/g soil | | | |
| Phong Dien 1 | *Rhizophora mucronata* | 6.28 | 6.011 | 5.356 | 5.637 | 5.667 |
| Phong Dien 2 | | 6.24 | 5.000 | | 5.452 | |
| Phong Lac 1 | | 5.97 | 5.426 | | 3.921 | |
| Phong Lac 2 | | 6.52 | 5.784 | | 6.125 | |
| Loi An 1 | | 5.81 | 5.720 | | 5.067 | |
| Loi An 2 | | 5.27 | 6.125 | | 5.934 | |
| Phong Dien 3 | *Bruguiera cylindrica* | 6.31 | 6.861 | 6.875 | 6.831 | 6.908 |
| Phong Dien 4 | | 6.11 | 7.151 | | 7.071 | |
| Phong Lac 3 | | 6.32 | 7.102 | | 7.242 | |
| Phong Lac 4 | | 5.91 | 6.671 | | 6.882 | |
| Loi An 3 | | 5.41 | 6.871 | | 6.361 | |
| Loi An 4 | | 5.81 | 6.801 | | 6.764 | |
| Phong Dien 5 | *Avicennia marina* | 6.55 | 6.284 | 6.258 | 6.493 | 5.832 |
| Phong Dien 6 | | 6.55 | 5.962 | | 6.448 | |
| Phong Lac 5 | | 6.68 | 4.875 | | 6.875 | |
| Phong Lac 6 | | 6.60 | 6.088 | | 6.489 | |
| Loi An 5 | | 6.76 | 6.253 | | 6.004 | |
| Loi An 6 | | 6.50 | 5.530 | | 5.243 | |

*Avicennia* produces numerous, soft aerial roots (pneumatophores) that may flush the soil with oxygen, providing a congenial environment for aerobic azotobacters [26]. However our result showed that there were the highest population of nitrogen-fixing and phosphate-solubilizing bacteria in rhizosphere soil of *Bruguiera cylindrica*, this can explain the contribution of this kind of plant as leaves and roots into soils and this increased organic matter and other nutrients into soil so that there was a significant linear relationship between phosphate-solubilizing bacteria population with soil pH at $P<0.01$ in rhizosphere soil of *Bruguiera cylindrica* (Table 2). Soil pH decreased but phoshate-solubilizing bacterial population increased noticeably ($P>0.01$) perhaps these P-solubilizing bacterial strains released organic acids to dissolve non-soluble P in soils to soluble P. Bacteria solubilise phosphate in areas where the soil is oxygenated (e.g., near the mangrove roots) and may, therefore, serve an important role in P uptake by the plant [27]. It is generally accepted that the mechanism of mineral phosphate solubilization by phosphate solubilising bacteria (PSB) is associated with the release of low molecular weight organic acids [28], which through their hydroxyl and carboxyl group chelate the cations bound to phosphate, their by converting it into soluble form [29].
One hundred and twenty five bacterial isolates, included 37, 45 and 43 isolates from rhizoshere soil of *Rhizophora mucronata*, *Bruguiera cylindrica* and *Avicennia marina* respectively, were isolated from 18 soil samples in two media (Burk'N free and NBRIP medium) (Table 3) and all isolates grew well on both of media (they have nitrogen fixation and phosphate solubilizing abilities) (Figure 2a and Figure 2b).

Table 2 - The relationship between population of $N_2$-fixing and phosphate-solubilizing bacteria with soil pH in mangrove soil

| Soil pH | Population (cfu/dry soil gramme) | |
|---|---|---|
| | $N_2$-fixing bacteria | Phosphate-solubilizing bacteria |
| *Rhizophora mucronata* | r = 0.375 (n.s) | r = 0.102 (n.s) |
| | y = - 0.3488 x + 7749 | y = 0.1846 x + 4.2462 |
| *Bruguiera cylindrica* | r = 0.431 (n.s) | r = 0.922** |
| | y = 0.2319 x + 5.5241 | y = 0.8184 x + 1.989 |
| *Avicennia marina* | r = 0.031 (n.s) | r = 0.323 (n.s) |
| | y = - 0.1733 x + 6.9771 | y = 1.9032 x - 6.3152 |

n.s = not significantly

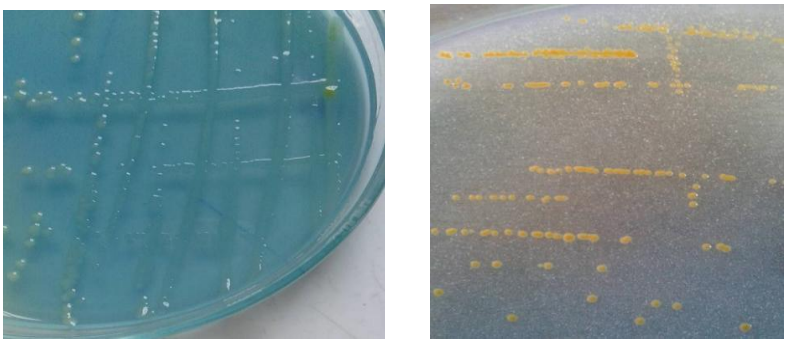

Figure 2a - The colonies of several isolates on Burk's N free media

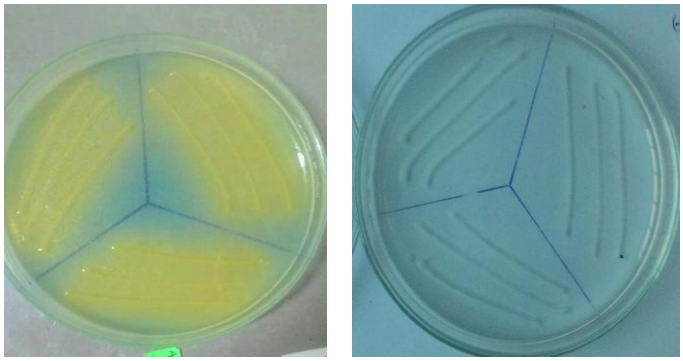

Figure 2b - The colonies of four isolates in NBRIP medium with the halos around the colonies and changed the color of medium because of organic acids

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 and Gram-negative by Gram stain. Especially phosphate-solubilizing bacteria make a halo around colonies in NBRIP medium as according to Thanh and Diep [30], Tam and Diep [31] (Figure 2b). The cells were observed by microscopic and appearded as short rods and most of them have motility.
Good isolates for nitrogen fixation (*Rhizophora mucronata, Bruguiera cylindrica* and *Avicennia marina*) were selected and presented in Table 3a, 3b, 3c.

Table 3a - Nitrogen fixation of good isolates (mg $NH_4$/l) from rhizosphere soil of *Rhizophora mucronata* (15/37)

| No | Bacterial Isolates | *Rhizophora mucronata* | | | |
|---|---|---|---|---|---|
| | | Day 2 | Day 4 | Day 6 | Day 8 |
| 01 | Control | 0.000 o | 0.000 o | 0.000 o | 0.000 o |
| 02 | DDB1a | 0.308 l | 0.542 l | 0.000 o | 2.763 d |
| 03 | DDB1b | 0.024 n | 0.053 n | 2.728 d | 2.844 cd |
| 04 | DDB2b | 0.046 n | 0.289 l | 2.975 c | 2.366 e |
| 05 | DDB2a1 | 0.045 n | 0.349 l | 2.395 e | 2.445 ef |
| 06 | DDB2a2 | 0.054 n | 0.739 jk | 2.479 d | 2.388 ef |
| 07 | DAB3a1 | 0.338 l | 0.423 l | 2.395 e | 3.475 b |
| 08 | DAB3b | 0.055 n | 0.233 m | 3.409 b | 2.056 g |
| 09 | DAB3a2 | 0.085 n | 0.422 l | 2.976 c | 2.511 d |
| 10 | DLB4a | 0.017 n | 0.446 l | 2.478 e | 2.315 f |
| 11 | DLB4b | 0.085 n | 0.394 l | 2.478 e | 2.841 cd |
| 12 | DAB5a | 0.095 n | 0.355 l | 2.841 cd | 1.796 h |
| 13 | DAB5b1 | 0.083 n | 0.032 n | 1.797 i | 2.998 c |
| 14 | DAB5b2 | 0.029 n | 0.039 n | 1.998 g | 0.971 j |
| 15 | DLB6a | 0.085 n | 0.241 m | 0.971 ij | 0.782 jk |
| 16 | DAB5c | 0.584 l | 4.156 a | 0.786 jk | 0.851 jk |
| | **CV (%)** | **6.18** | | | |
| | **F calculated** | ** | ** | ** | ** |

Table 3b - Nitrogen fixation of good isolates (mg $NH_4$/l) from rhizosphere soil of *Bruguiera cylindrica* (17/45)

| No | Bacterial Isolates | *Bruguiera cylindrica* | | | |
|---|---|---|---|---|---|
| | | Day 2 | Day 4 | Day 6 | Day 8 |
| 01 | Control | 0.000 k | 0.000 k | 0.000 k | 0.000 k |
| 02 | VDB1a | 0.075 hi | 0.011 jk | 0.236 c | 0.027 j |
| 03 | VDB1b | 0.019 jk | 0.058 l | 0.185 d | 0.021 jk |
| 04 | VDB2a | 0.018 jk | 0.065 l | 0.187 d | 0.027 j |
| 05 | VDB2b | 0.028 j | 0.073 hi | 0.200 d | 0.011 jk |
| 06 | VDB2c | 0.085 h | 0.134 f | 0.084 h | 0.032 j |
| 07 | VAB1a | 0.025 jk | 0.018 jk | 0.104 gh | 0.025 j |
| 08 | VAB1b | 0.023 jk | 0.459 a | 0.032 j | 0.019 jk |
| 09 | VAB1c | 0.034 j | 0.034 j | 0.154 e | 0.022 jk |
| 10 | VAB1d | 0.032 j | 0.011 jk | 0.116 g | 0.016 jk |
| 11 | VAB2a | 0.056 i | 0.115 g | 0.105 g | 0.017 jk |
| 12 | VAB2b | 0.054 i | 0.343 b | 0.034 j | 0.044 ij |
| 13 | VAB2c | 0.011 jk | 0.016 jk | 0.121 g | 0.020 jk |
| 14 | VLB1a | 0.032 j | 0.101 g | 0.099 gh | 0.014 jk |
| 15 | VLB1b | 0.322 b | 0.026 j | 0.091 h | 0.027 j |
| 16 | VLB1c | 0.085 i | 0.066 l | 0.112 g | 0.037 j |
| 17 | VLB2a | 0.033 j | 0.019 jk | 0.153 e | 0.019 jk |
| 18 | VLB2b | 0.192 d | 0.012 jk | 0.296 b | 0.014 jk |
| | **C.V (%)** | **16.68** | | | |
| | **F calculated** | ** | ** | ** | ** |

Table 3c - Nitrogen fixation of good isolates (mg $NH_4$/l) from rhizosphere soil of *Avicennia marina* (15/43)

| No | Bacterial Isolates | *Avicennia marina* | | | |
|---|---|---|---|---|---|
| | | Day 2 | Day 4 | Day 6 | Day 8 |

| | | | | | |
|---|---|---|---|---|---|
| 01 | Control | 0.000 m | 0.000 m | 0.000 m | 0.000 m |
| 02 | MAN1a | 0.031 m | 1.993 h | 4.601 ab | 0.042 m |
| 03 | MAN1b | 0.032 m | 1.841 h | 4.322 c | 0.041 m |
| 04 | MAN2a | 0.061 m | 2.321 g | 4.393 c | 0.031 m |
| 05 | MAB2b | 0.032 m | 1.362 j | 4.151 c | 0.051 m |
| 06 | MDB1a | 0.041 m | 1.181 j | 4.433 bc | 0.023 m |
| 07 | MDN1b | 0.401 l | 1.871 h | 4.751 a | 0.053 m |
| 08 | MDB1c | 0.033 m | 2.151 gh | 4.552 b | 1.241 j |
| 09 | MDB2a | 0.071 m | 1.223 j | 4.032 c | 0.801 k |
| 10 | MDB2b | 0.082 m | 1.722 i | 3.611 e | 0.392 l |
| 11 | MLB1a | 0.041 m | 1.052 k | 3.751 de | 0.032 m |
| 12 | MLB1b | 0.061 m | 1.482 j | 4.311 c | 0.042 m |
| 13 | MLB2a | 0.062 m | 1.861 h | 4.151 c | 1.611 h |
| 14 | MLB2b | 0.151 m | 1.402 j | 3.751 de | 0.052 m |
| 15 | MLB2c | 0.031 m | 2.461 g | 3.992 d | 0.053 m |
| 16 | MLB2d | 2.831 f | 2.021 h | 3.882 d | 0.191 m |
| | **C.V (%)** | **6.56** | | | |
| | **F calculated** | ** | ** | ** | ** |

Means within a column followed by the same letter/s are not significantly different at p<0.01
Good isolates for phosphate solubilization (*Rhizophora mucronata, Bruguiera cylindrica* and *Avicennia marina*) were selected and presented in Table 4a, 4b, 4c

Table 4a - Phosphate solubilization (mg $P_2O_5$/l) of good isolates from rhizosphere soil of *Rhizophora mucronata* (12/37)

| No | Bacterial Isolates | *Rhizophora mucronata* | | | |
|---|---|---|---|---|---|
| | | Day 5 | Day 10 | Day 15 | Day 20 |
| 01 | Control | 0.00 s | 0.00 s | 0.00 s | 0.00 s |
| 02 | DDN1a1 | 101.99 op | 257.70 l | 632.65 c | 762.87 a |
| 03 | DDN1a2 | 141.47 no | 207.86 m | 370.53 i | 377.84 i |
| 04 | DDN1b | 346.39 j | 342.77 j | 576.54 e | 580.23 e |
| 05 | DDN1e2 | 21.04 rs | 156.29 n | 463.29 g | 422.55 h |
| 06 | DDN1e3 | 33.48 r | 341.04 j | 765.77 a | 713.17 b |
| 07 | DAN3a | 317.05 k | 405.50 h | 621.05 cd | 689.42 b |
| 08 | DAN3b1 | 272.81 l | 390.41 i | 555.21 ef | 651.69 c |
| 09 | DLN4b | 162.98 m | 265.88 l | 454.59 g | 717.56 b |
| 10 | DDN1d1 | 139.32 no | 168.39 mn | 388.13 i | 535.32 f |
| 11 | DLN4c | 83.56 o | 260.69 l | 427.88 h | 465.26 g |
| 12 | DAN5d | 51.15 p | 149.53 n | 288.13 l | 415.76 hi |
| 13 | DLN6a | 214.61 m | 266.26 l | 259.42 l | 269.09 l |
| | **C.V (%)** | **4.87** | | | |
| | **F calculated** | ** | ** | ** | ** |

Table 4b - Phosphate solubilization (mg $P_2O_5$/l) of good isolates from rhizosphere soil of *Bruguiera cylindrica* (13/45)

| No | Bacterial Isolates | *Bruguiera cylindrica* | | | |
|---|---|---|---|---|---|
| | | Day 5 | Day 10 | Day 15 | Day 20 |
| 01 | Control | 0.00 s | 0.00 s | 0.00 s | 0.00 s |
| 02 | VDN1c | 387.86 l | 340.78 m | 55.77 r | 777.21 e |
| 03 | VDN1d | 337.17 m | 341.72 m | 43.66 r | 925.05 c |
| 04 | VDN1f | 522.96 j | 605.56 h | 350.84 l | 1031.50 b |
| 05 | VDN2a | 705.99 f | 497.38 j | 178.93 p | 1003.35 b |
| 06 | VDN2b | 639.17 g | 392.04 k | 240.26 p | 1020.12 b |
| 07 | VDN2c | 507.52 j | 373.32 l | 94.96 q | 848.06 d |
| 08 | VDN2d | 382.64 l | 370.49 l | 106.65 q | 975.41 c |
| 09 | VAN2a | 416.89 k | 370.81 l | 228.29 p | 851.26 d |
| 10 | VAN2d | 34.47 r | 231.28 p | 10.66 s | 777.21 e |
| 11 | VLN1b | 313.05 m | 283.39 n | 3.72 s | 595.37 i |
| 12 | VLN1c | 655.76 g | 552.10 i | 292.76 n | 1091.18 a |

| | | | | | |
|---|---|---|---|---|---|
| 13 | VLN2c | 131.76 q | 322.69 m | 382.24 k | 445.16 k |
| 14 | VLN2e | 260.52 o | 317.04 m | 229.29 rs | 1073.41 a |
| | **C.V (%)** | **3.40** | | | |
| | **F calculated** | ** | ** | ** | ** |

Table 4c - Phosphate solubilization (mg $P_2O_5$/l) of good isolates from rhizosphere soil of *Avicennia marina* (12/43)

| No | Bacterial Isolates | *Avicennia marina* | | | |
|---|---|---|---|---|---|
| | | Day 5 | Day 10 | Day 15 | Day 20 |
| 01 | Control | 0.00 l | 0.00 l | 0.00 l | 0.00 l |
| 02 | MAB1b | 399.24 d | 134.06 ij | 206.46 h | 154.63 i |
| 03 | MAB2b | 374.01 d | 70.12 k | 132.28 j | 178.84 i |
| 04 | MLB1b | 321.16 e | 89.32 k | 128.28 j | 136.64 ij |
| 05 | MDN1a | 313.21 f | 349.06 e | 491.79 c | 624.75 b |
| 07 | MDN1b | 172.96 i | 250.79 g | 475.43 c | 703.79 a |
| 08 | MDN1d | 254.84 g | 295.76 e | 488.20 c | 600.40 b |
| 09 | MDN2b | 206.14 i | 357.24 e | 492.99 c | 745.71 a |
| 10 | MLN1b | 170.66 i | 252.68 g | 497.86 c | 715.57 a |
| 11 | MLN1c | 269.59 g | 264.78 g | 473.23 c | 710.98 a |
| 12 | MLN1f | 251.15 g | 233.34 g | 428.92 cd | 473.65 c |
| 13 | MLN2d | 183.72 i | 253.31 g | 477. 62 c | 522.75 c |
| | **C.V (%)** | **4.73** | | | |
| | **F calculated** | ** | ** | ** | ** |

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

From these results (Table 3 and Table 4), almost abilities of bacteria in rhizosphere soil of mangrove were phosphate solubilization in comparison with nitrogen fixation. This may be explained that perhaps a possible nitrogen source has been contributed by decomposition of leaves of mangrove forest while the mangrove soils mainly contain inorganic phosphate so that they need to be solubilize this phosphos for growth.
Based on the good characteristics of these isolates (Table 3 and Table 4), 22 isolates were chosen to identify.
The fragments of 1495 bp 16S rRNA were obtained from PCR with 8F and 1492R primers and sequencing. Homology searches of 16S rRNA gene sequence of selected strain in GenBank by BLAST revealved that they had similarity to sequences of Bacilli (16/22 isolates) and 6 isolates belonged to Gamma-proteobacteria (Table 5).

Table 5 - Phylogenetic affiliation of isolates on the basis of 16S rRNA genes sequences by using BLAST programmes in the GenBank database based on sequences similarity.

| **Taxonomic Group and Strain** | **Closest species relative** | **Similarity (%)** |
|---|---|---|
| | **Bacilli** | |
| MDB1c | *Bacillus* sp. YY13 (KU298561) | 97 |
| | *Bacillus subtilis* strain CR26 (KR780430) | 97 |
| MAB2b | *Bacillus subtilis* strain BS-HOT1 (HM631977) | 98 |
| | *Bacillus* sp. MN19(2014) (KM289136) | 98 |
| MAN1b | *Bacillus subtilis* strain S12 (KU206485) | 98 |
| | *Bacillus* sp. strain GY773 (KY473983) | 98 |
| MLN1b | *Bacillus subtilis* strain OTEB48 (KP225283) | 99 |
| | *Bacillus* sp. JN15 (KC121041) | 99 |
| MLN1c | *Bacillus* sp. strain AU01 (MF590123) | 99 |
| | *Bacillus subtilis* strain WJ-3 (JX673943) | 99 |
| MDN1a | *Bacillus flexus* strain ML-27 (KJ401045) | 99 |
| | *Bacillus* sp. P5'(2012) (JX083303) | 99 |
| MDN1b | *Bacillus subtilis* strain MA-40 (KX426640) | 99 |
| | *Bacillus tequilensis* strain V44.8fa (KT720325) | 99 |
| MDN2b | *Bacillus subtilis* strain CR26 (KR780430) | 99 |
| | *Bacillus* sp. strain YX48 (MF595820) | 99 |
| VLB2n | *Bacillus subtilis* strain CR26 (KR780430) | 99 |
| | *Bacillus* sp. strain YX48 (MF595820) | 99 |

| | | |
|---|---|---|
| VDB2a | *Bacillus subtilis* strain Md1-42 (MF581448) | 99 |
| | *Bacillus* sp. strain WC5 (JN975953) | 99 |
| VDN2c | *Bacillus subtilis* strain GX S-19 (KU904298) | 97 |
| | *Bacillus* sp. strain 2N-14 (KX214613) | 97 |
| VDN1d | *Bacillus subtilis* strain BJ-17 (GQ280027) | 99 |
| | *Bacillus* sp. X15 (KP262341) | 99 |
| VAB2b | *Bacillus* sp. strain Suaeda B-003 (KT981879) | 99 |
| | *Bacillus altitudinis* strain WJB15 (KU877629) | 99 |
| DLB4b | *Bacillus subtilis* strain CR26 (KR780430) | 99 |
| | *Bacillus* sp. YY-14 (JX575605) | 99 |
| VDN1f | *Bacillus subtilis* strain ZHA9 (FJ263018) | 99 |
| | *Bacillus* sp. YY-14 (JX575605) | 99 |
| VAB1c | *Bacillus circulans* strain MD1 (KT757520) | 99 |
| | *Bacillus* sp. M-B (KC853425) | 99 |
| **Gammaproteobacteria** | | |
| MAB1b | *Enterobacter* sp. WC141019 (KU245715) | 99 |
| | *Enterobacter cloacae* strain BIA145 (KU161287) | 99 |
| VDN2d | *Enterobacter* sp. strain Md1-52 (MF581458) | 99 |
| | *Enterobacter cloacae* strain LC11-B (MF498495) | 99 |
| VAN2a | *Enterobacter* sp. M3(2012) (JX081544) | 99 |
| | *Enterobacter cloacae* strain LC11-B (MF498495) | 99 |
| MLN2d | *Vibrio* sp. CR5 (KU052624) | 98 |
| | *Vibrio furnissii* strain MM5 (FJ906812) | 98 |
| VLN2e | *Vibrio* sp. QY27 (KP676706) | 98 |
| | *Vibrio fluvialis* strain LCB1 (KC210808) | 98 |
| MLN1f | *Pseudomonas stutzeri* strain W13 (KT380559) | 99 |
| | *Pseudomonas* sp. IBUN MAR3 (DQ813309) | 99 |

A neighbor-joining phylogenetic tree in these isolates showing the two clusters: cluster A divided into two cluster A1 and A2. Cluster A1 with cluster A11 had 5 isolates as *Bacillus subtilis* MAN1b, *B. subtilis* VDN1f, *Bacillus sp*. VAB2b, *Bacillus flexus* MDN1a and *B. subtilis* VLB2b1related very closely however they located into one cluster with strain *Vibrio* sp. VLN2e (one strain belongs to gram-negative bacteria) while cluster A12 with two strains *Bacillus subtilis* VDN1d and *Bacillus circulans* VAB1c had relationship close. Cluster A2 composed of two small clusters: cluster A21 with *Bacillus* sp. MUN1c and *Vibrio* sp. VLN2e, cluster A22 with *Enterobacter* sp. MAB1b and *Enterobacter* sp. VAN2a, both strains in cluster had relationship very close. Cluster B had cluster B1 with three strains: *Bacillus subtilis* MAB2b, *B. subtilis* VDB2a and *Pseudomonas stutzeri* MUN1f while cluster B2 composed of two small clusters: cluster B21 with *Bacillus subtilis* MDN1b, *B. subtilis* MUN1b and *B. subtilis* VDN2c related closely and cluster B22 with *Bacillus* sp. MDB1c and *Enterobacter* sp. located into one smaller cluter and *Bacillus subtisis* related *B. subtilis* DLB4b very closely.

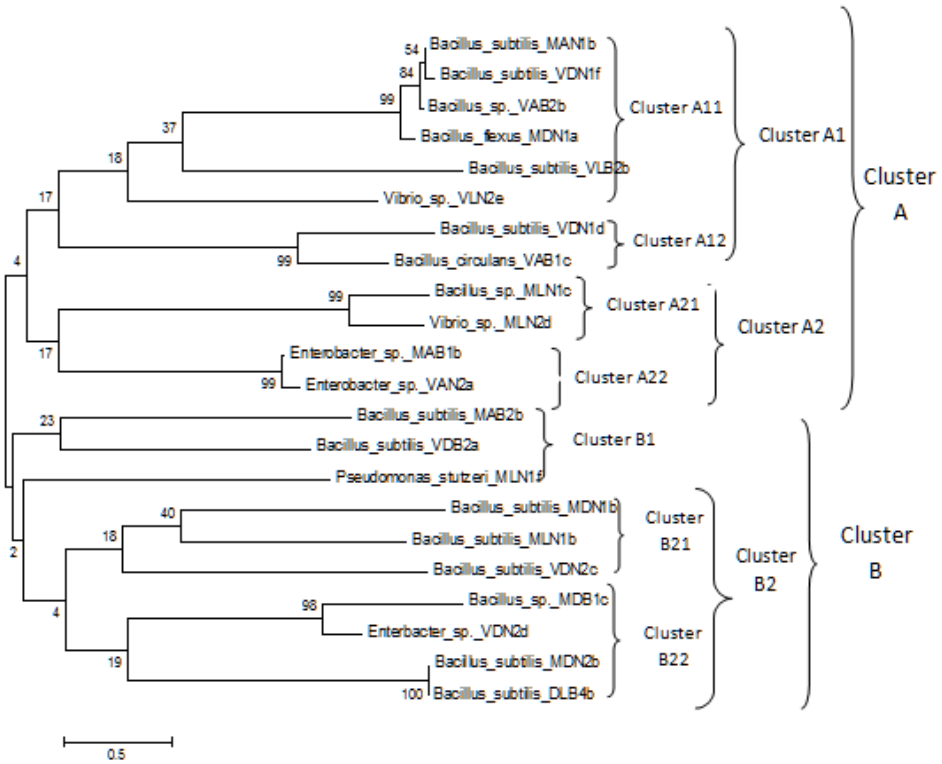


Figure 4 - Phylogenetic tree showing the relative position of rhizopheric bacteria (PGPR) by the neighbor-joining method of complete 16S rRNA sequence. Bootstrap values of 1000 replicates are shown at the nodes of the trees.

The rhizospheric bacteria has been studied and described as beneficial bacteria with Gram-positive bacteria (Bacilli) presented on Burk's N free medium and it occupied over 70% among 22 strains and 6 strains (27%) belonged to Gammaproteobacteria in our result (Figure 5).

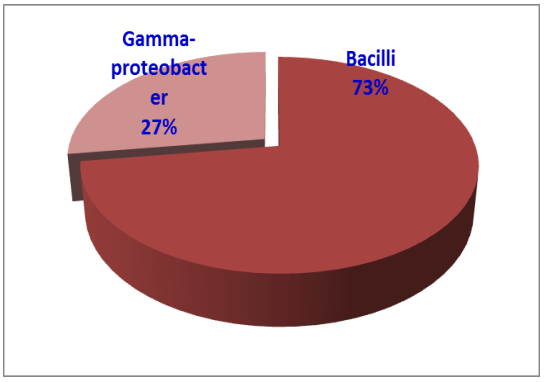


Figure 5 - The proportion of group and they distributed in two clusters

Almost rhizospheric bacteria from mangrove soil have ability of saline tolerant with 2.0% NaCl however when increasing NaCl concentration from 2.5 to 4.0% NaCl in the media, amount of bacteria reduced especially bacteria from rhizosphere of *Rhizophora mucronata* (Table 6) perhaps their roots developed from the air for a short time before they fell to the ground, and as a result, the strains from rhizosphere soil of *Rhizophora mucronata* were less salt tolerance than *Bruguiera cylindrica* and *Avicennia marina* which roots of two these kinds grew from saline soil.

Table 6 - Ratio (%) rhizospheric bacteria of mangrove soil with three kind of plant

| Ratio (%) of Rhizospheric Bacteria in three kinds of plant | NaCl concentration in medium (%) | | | | |
|---|---|---|---|---|---|
| | **2.0** | **2.5** | **3.0** | **3.5** | **4.0** |
| *Rhizophora mucronata* | 100 | 97.22 | 88.89 | 66.67 | 25.25 |
| *Bruguiera cylindrica* | 100 | 100 | 100 | 86.67 | 86.17 |
| *Avicennia marina* | 100 | 100 | 92.90 | 92.90 | 89.30 |

Mangroves provide a unique ecological environment for diverse bacterial communities. Heterotrophic bacteria are very important in mangrove habitats as the bacteria decompose the mangrove litter, recycle the nutrients and produce the detritus food for many fishes [32][33]. Abundance and activities of the bacteria are controlled by various physicochemical parameters in the mangrove environment [34] [35] [36] [37] [38 [39 ]. Among heterotrophic bacteria, $N_2$-fixing bacteria are efficient in using a variety of mangrove substrates [40].

Phosphorus (P) is one of the essential elements for the growth and reproduction of bacteria and plays a very significant role in many aspects of cell metabolism. It is the second most important plant nutrient after nitrogen [41]. Phosphorous usually precipitate because of the abundance of cations in the interstitial water of mangrove sediments making phosphorus largely unavailable to plants, thus organisms that solubilise P can have important implications for plant growth, especially in nutrient-limited environments. This may be due to the fact that the bacteria are active in converting the insoluble forms of phosphorus compounds in mangrove soils to soluble forms that are readily transferred from soil to underlying water and or utilized by plants and microbes [42] [43] [44] [11] [12]. Muniyandi [45] has also observed higher level of phosphorus in the core mangrove areas than in the back mangrove areas. Venkateswaran [46] has reported that phosphate is efficiently absorbed by fine sediments of muddy areas than coarse areas. The changes in the levels of phosphorus can be linked with the influx of phosphorus from upstream regions and with regeneration into the overlying water column. Walsh [47] has reported that mud releases phosphates and nitrates during low saline conditions and it absorbs them from overlying water when it becomes more saline. In general, mangroves in low nutrient carbonate soils are limited by phosphorus, what phosphorus is present may be bound with calcium, efficiently holding it within the sediments [48].

Promod and Dhevendran [49] reported phosphate solubilisation by IPSB *Vibrio* sp. and *Pseudomonas* sp. of 0.5-0.55 mg/l from Cochin, India. Similarly seven bacterial sp such as, two *Bacillus subtilis*, three *Pseudomonas* sp. and two *Azotobacter* sp. reported from mangrove soil of Chollangi, East Godavari exhibited solubilising ability of 80-100 ug/ml of phosphate [50]. Genera of phosphate-solubilizing bacteria, like *Pseudomonas, Bacillus, Corynebacterium, Vibrio, Micrococcus* and *Alcaligenes,* were studied by Venkateswaran and Natarajan [51] in mangrove biotopes in Porto Novo, Chennai water and sediment. Our result found that Bacilli occupied over 70% of the total rhizospheric bacteria of mangrove soil, some good strain for nitrogen fixation as *Bacillus subtilis* DLB4b, *Bacillus* sp. VAB2b, *Enterobacter* sp. MAB1b and *Bacillus* sp. MDB1c and strains for phosphate solubilation as *Bacillus subtilis* MLN1b, *Bacillus* sp. MLN1c, *Bacillus subtilis* VDN1f and *Bacillus subtilis* MDN1c. All of them tolerated at a concentration of 4% NaCl but strain *Bacillus subtilis* MAB2b is not only good nitrogen fixation but also high phosphate solubilization, so that it becomes a promising strain to produce biofertilizer for the crops which cultivated in soil salinity.

## IV. CONCLUSION

From 18 rhizosphere soil samples of three kinds of plants in mangrove forest as *Rhizophora mucronata, Bruguiera cylindrica,* and *Avicennia marina,* 125 isolates were isolated in two media (Burk's N free and NBRIP). They were identified as rhizospheric bacteria and 22 isolates having good plant growth promotion were chosen to analyse their relationship. These isolates were identified as Bacilli (more than 70%) and Gammaproteobacteria on mangrove soil. Among them, one strain will be suggested to produce for crop cultivation on soil salinity in the future.

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