Phosphate and potassium solubilizing bacteria from weathered materials of denatured rock mountain, Ha Tien, Kiên Giang province, Vietnam

Cao Ngoc Diep¹, ²,*, Than Ngoc Hieu¹, ², *

¹Dept. Microbiology Biotechnology, Department of Rural Development, Can Tho City, Vietnam
²Agriculture and Rural Development Dept., Biotechnology R&D Institute, Can Tho University, Can Tho City, Vietnam

Email address:
cndiep@ctu.edu.vn(C. N. Diep), thanngochieu@gmail.com(T. N. Hieu)

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Abstract: Twenty-five strains were isolated on Aleksandrov medium from sample soils/weathered rocks of Ha Tien Mountain, Kiên Giang, Vietnam. Their colonies were round or irregular, white to yellow and their shape was rod, motile. Seventeen of twenty-five bacterial strains were identified by PCR technique with specific primers fd1 and rP, they were capable of dissolving both phosphate and potassium and seven strains had high phosphate and potassium dissolution capacity (>10 mg l⁻¹ P₂O₅ and >50 mg l⁻¹ K₂O) effectively. These seven strains were chosen to sequence, DNA sequencing were compared with GenBank database of NCBI by BLAST N software. The results showed that DNV16 strain was similarity of 99% with EU977655.1 (Microbacterium hominis), TC1D strain was a 99% similarity with FN547417.1 (Flectobacillus sp.), CH9E strain was a 99% similarity of 99% with GQ861463.1 (Agrobacterium tumefaciens), TC1A strain was similarity of 99% with HM003210.1 (Bacillus cereus) and FJ976533.1 (Bacillus coagulans), CH7A strain was 99% similarity with EU048539.1 (Bacillus cereus) and GQ214131.1 (Bacillus subtilis) and TD6B strain was a 99% similarity wih AB301013.1 (Bacillus subtilis) and CH7D strain was similarity of 98% with FJ976616.1 (Bacillus megaterium). Seven strains related with Bacillus megaterium and Bacillus coagulans closely in phylogenetic tree.

Keywords: Bacillus Coagulans, Bacillus Megaterium, Denatured Rock, Identification, Phosphate and Potassium Solubilization

1. Introduction

Phosphate (P) and potassium (K) are the major essential macronutrients for biological growth and development. However, the concentration of soluble P and K in soil are usually very low, and the biggest proportion of P and K in soil are insoluble rocks, minerals and deposits[1]. The weathering of rocks plays a role in a number of important environmental processes[2] to clay, silt and sand and these minerals are important component of soil in earth. In spite of that, these sources constitute the biggest reservoirs of P and K in soil because, under appropriate conditions, they can be solubilized and become available for plants[3]. The Mekong Delta occupies 2.9 million ha (12% of the Vietnam’s total land area) and is one of the two principal areas of rice production of Vietnam. About 35% of the Mekong Delta is alluvial soil, covering 1.1 million ha along the rivers with most of the remainder acid sulfate clay soil (1.6 million ha). Both the acid and alluvial soils are deficient in phosphorus since P generally reacts with aluminium and iron under low pH conditions and forms insoluble compounds[4]. Besides that, K is also an important macronutrient for plant growth but potassium fertilizer, as potassium chloride, has been imported with big quantity every year because Vietnam has no mineral resource to produce potassium fertilizer. However, K fertilizer cost has not to stop enhance every year, this has led to increase cost of rice production and farmer’s income should reduce.

Microorganisms play a central role in the natural P and K cycle and P- or K-solubilizing bacteria in soil and in plant rhizospheres[5]; there are bacteria species having high ability to solubilize inorganic phosphate compounds[6] and silicate bacteria were found to resolve potassium, silicon and aluminum from insoluble minerals[7]. Recently
Xiufang et al.[3] found the community of microorganisms in soil of Tianmu Mountain, Zhejiang, China with herbal plants flourished and they were able to dissolve both P- and K-containing minerals. The aims of this study were (i) selection and (ii) identification the isolated P- and K-solubilizing bacteria from soil of denatured mountains of Ha Tien town, Kien Giang province, Vietnam.

2. Material and Methods

2.1. Bacteria Isolation and Growth Conditions

Bacterial stains were isolated from Ha Tien Mountain, Kien Giang Province, Vietnam (Figure 1), by virtue of their abilities to solubilize mineral P- and K. The samples were stored at $10^0$C during transit and processed immediately.

Soil samples or weathering materials were collected from the areas where herbal plants flourished (Figure 2). Each sample (2 g) was added to 25 ml of liquid Aleksandrov medium[3] with 0.2% apatite and 0.2% kaolinite and shaken for 24 h on orbital shaker at 50 rev min$^{-1}$ at temperature room.

A 10-fold dilution series were made. Dilution were plated onto the same medium and incubated at $30^0$C for 24 h. The colonies on the $10^6$ dilution plate were picked and grown in the same liquid medium, and then colonies were selected from plates and they were separate isolates, stored at medium agar tubes at 4°C and medium glycerol at –20°C.

2.2. Mineral Dissolution

Apatite from Lao Cai factory, North Vietnam and Kaolinite mineral (commercial) were added to liquid Aleksandrov medium as the sole P and K source to test the ability of the isolates to solubilize these minerals and the isolates with the highest solubilization capacity were kept on Aleksandrov medium for further study.

Quantitative estimation of P and K solubilization was carried out in Falcon tubes (50-mL) containing 30 ml of Aleksandrov medium, and inoculated in triplicate with tested isolates (1 ml inoculum with approximately $3 \times 10^7$ cfu ml$^{-1}$). Autoclaved, uninoculated medium served as controls. The falcon tubes were incubated for 10 days on orbital shaker with 10 rev min$^{-1}$ at temperature room. Samples were taken at two times: 5 and 10 day after incubation, pH value was measured with a pH meter, after that samples were centifuged at 8000 g for 10 min. The supernatants were used to assay the solubilized P (5 and 10 day after incubation) and K (only at 10 day after incubation). P was estimated using the molybdo-vanado-method (Oniani method) and K was examined using atomic absorption spectrometry[8]. Values are recorded and presented with average value for each sample, and differences were considered to be significant at the P<0.05 level with LSD or Duncan test.

2.3. Isolation of Bacteria

Cultivation-based techniques were used to gain insight into the abundance and species composition of bacterial communities, and to reveal the poly-P accumulation of bacteria. Serial dilutions ($10^2$ to $10^5$) of composite samples were prepared. Dilutions (0.05 ml) were aseptically plated on the agar-based culture medium. Plates were incubated at $30^0$C for 5 days. Bacterial colonies were differentiated on the basis of colony morphology and pigmentation. Colonies were subculture on the agar-based subculture medium plates by striking technique and re-incubated at $30^0$C for 5 days. This isolation process carries out in shifts of the agar-based culture medium to the agar-based subculture medium until monocultures were obtained. Monocultures were culture on the agar-based culture medium slant in the test-tube (12 ml) and incubated at $30^0$C for 4 days following by stored $10^0$C in refrigerator.

2.4. Colony Characteristic and Microscopic Examination

The characteristics of colony such as size, color, shape….were presented in each group, cell morphologies of the isolates were observed using an optical microscope and they were also observed on scanning electron microscope.
2.5. Phylogenetic Analysis of 16S rRNA Gene Sequence

Bacteria universal primers, the forward primer fD1 and the reverse primer rP[3] were used to amplified partial length of 16S rRNA gene sequence.

Genomic DNA was extracted from the cultures grown in Aleksandrov medium 30°C for 24 h[9]. The 16S rRNA gene sequence was amplified in a PCR mixture, composed with 1 µmol l-1 of each primer. 200 µmol l-1 of each dNTP, 50 mmol l-1 KCl and 1.5 mol l-1 MgCl2 in 10 mmol l-1 Tris/HCl (pH 8.3) buffer. DNA (0.1 µg) and 2.5 U Taq DNA polymerase[Fermentas] were added in 100 µl PCR mixture. PCR amplifications were performed at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, anneling at 55°C for 50 s and extension at 72°C for 105 s; and a final extension at 72°C for 10 min. Partial 16S rRNA genes of some good bacterial strains was sequenced by MACROGEN, Republic of Korea (dna.macrogen.com) and they were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequence to show relationships between other P&K-solubilizing bacterial strains[10] and the phylogenetic analysis was constructed by the neighbour-joining method based on 1.000 bootstraps.

3. Results and Discussion

3.1. Bacteria Isolation and Colony Characteristic

From 10 soil samples/weathering materials, 25 isolates were isolated on Aleksandrov medium. They developed very well on this medium from 36-48 h at 30°C, this showed that these isolates had P and K-solubilizing capacity. Their colonies had round-shape, cliny, smooth, colourless or milk-color, yellow and some colonies appeared to have much larger size (Figure 3). This result was the same Xuufang’s experiment (2006) which was done at Tianmu Mountain, China however these colonies was the same Xuufang’s experiment (2006) which was done at Tianmu Mountain, China however these colonies was the same Xiufang Xiufang’s experiment at 30°C. The colonies appeared to have much larger size (Figure 3). This result was the same Xuufang’s experiment (2006) which was done at Tianmu Mountain, China however these colonies was the same Xiufang Xiufang’s experiment at 30°C. However K-solubilizing ability of all strains was higher than P-solubilizing and pH values were measured at 5 and 10 day of incubation and the results showed that pH value were always higher than 6.5 perhaps they originate from denatured mountain at seaside (Thailand Bay).

![Figure 3. Characteristics of colonies of bacterial isolates after grown on Aleksandrov medium.](image)

In the modified medium with apatite (Lao cai, Vietnam) and potassium mineral (kaolinite), the ability of the isolates to solubilized different P and K minerals was also investigated (Table 1).

All of 11 strains showed significantly higher solubilization of potassium mineral (kaolinite) than control and strain TC1A was the most efficient strain in solubilizing mineral K and this strain also had P-solubilizing ability after 10 days of incubation (Table 1). However K-solubilizing ability of all strains was higher than P-solubilizing and pH values were measured at 5 and 10 day of incubation and the results showed that pH value were always higher than 6.5 perhaps they originate from denatured mountain at seaside (Thailand Bay).

![Figure 4. Electron micrographs (bars, 2 µm) of cells of bacterial isolates grown on Aleksandrov medium](image)

<table>
<thead>
<tr>
<th>Name of isolate</th>
<th>Solubilization of potassium (mg K l⁻¹) Day 5*</th>
<th>Solubilization of phosphate (mg PO₄³⁻ l⁻¹) Day 10*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC1D</td>
<td>68.53 def</td>
<td>17.46</td>
</tr>
<tr>
<td>TC1B</td>
<td>70.21 d</td>
<td>5.11</td>
</tr>
<tr>
<td>TD6A</td>
<td>68.78 de</td>
<td>5.13</td>
</tr>
<tr>
<td>TD6B</td>
<td>65.33 g</td>
<td>5.27</td>
</tr>
<tr>
<td>CH7D</td>
<td>65.04 g</td>
<td>8.52</td>
</tr>
<tr>
<td>CH7A</td>
<td>70.33c</td>
<td>9.82</td>
</tr>
<tr>
<td>CH9E</td>
<td>72.56 bc</td>
<td>3.32</td>
</tr>
<tr>
<td>DVNV16</td>
<td>67.27 efg</td>
<td>16.24</td>
</tr>
<tr>
<td>TC1A</td>
<td>76.78 a</td>
<td>4.33</td>
</tr>
<tr>
<td>TD13A</td>
<td>62.57 h</td>
<td>12.18</td>
</tr>
<tr>
<td>TD13B</td>
<td>61.02 h</td>
<td>4.19</td>
</tr>
<tr>
<td>Control</td>
<td>0.69j</td>
<td>1.77</td>
</tr>
</tbody>
</table>

* days of incubation
** numbers following the same word not diffrence at 1% level

P-solubilizing bacteria and silicate bacteria play an important role in plant nutrition through the increase in P and K uptake by the plant[3]. Application of Phosphate solubilizing microorganisms have been used as P-biofertilizer for crop cultivation[11][12]. Silicate bacteria provided less polluting and less energy-consuming approaches[13] widely used in improvement of available K for assimilation by plants, and removal of impurities from minerals[14]. As a strain effective in both P- and K-solubilization, strain TC1A should have to potential to be applied as a P and K biofertilizer to increase P and K assimilation and crop yield simultaneously.

3.2. Microscopic Examination

Microscopic observations showed that the cells of bacterial isolates were motile, rod, Gram-positive and Gram-negative (Figure 4), short-rods (0.8x1.3 µm) and long-rods (0.8x1.7 µm).
3.3. Phylogenetic Analysis of 16S rRNA Gene Sequence

Bands of approximately 1500 bp were obtained from the amplification of 16S rRNA gene sequence from 17 isolates among 25 isolates. With this result showed that 17 isolates were identified as P- and K-solubilizing bacterial strains (CH7D, TD13C, DNV16, TD6A, CH7A, TC1B, TD6C, TC1C, BS8B, CH9E, TD12B, TD6B, TC1F, BS8C, TC1A, TD13A and TD13B) as Xiufang et al.’s experiment[3]. Simultaneous the similarities of seven isolates (CH7A, TC1A, CH9E, CH7D, TC1D, DNV16 and TD6B) were 98-99% with Microbacterium hominis, Flectobacillus sp., Bacillus cereus, Bacillus subtilis and Bacillus megaterium.

A neighbor-joining phylogenetic tree in P- and K-solubilizing bacteria showing 2 clusters: cluster A with two cluster A1 and A2 among cluster A1 Flectobacillus sp. with CH9E isolate and cluster A2 Bacillus megaterium with TC1D, TD6B and TC1A isolates. Cluster B Microbacterium hominis with three isolates: CH7A, CH7D and DNV16. The results showed that P- and K-solubilizing bacteria mainly are genus Bacillus.

About of 75% of the world’s coastline is rock cliff[15]; denatured rock at Ha Tien mountain, Kien Giang province, Vietnam originates from calcite-sedimentary rock but it had been formed from seism with low level. The denatured rock has been formed by mud-rocks and shales and the weathering of mud-rocks and shales, fine-grained sedimentary rocks made from clay minerals of important environmental processes, especially shales contain clays that are the products of silicate weathering prior to rock formation.

Microorganisms are known to mediate rock weathering[16][17][18] and Cockell et al.[2] demonstrated that the physical and chemical micro-environment of weathered shales of coastline cliffs in the northwest of England was Proteobacteria, with phylotypes closely associating with Methylocella and other members of the γ-subdivision. Our results showed that the bacterial strains closely related with Bacillus perhaps these bacterial strains developed with the presence of plants, the results of this study also determined the our experiment which was done on weathered materials of calcaceous mountain, Kien Giang, Vietnam[19].

4. Conclusions

From soils or weathering materials of denatured mountain, Ha Tien, Kien Giang, Vietnam appeared many bacterial strains which solubilized phosphate and potassium minerals. Strain TC1A exhibited higher solubilization of Phosphate and Potassium in comparison to all strains and it will be used to produce biofertilizer in the future.

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References


