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Isolation and identification of phosphate-solubilizing bacterium from soybean Rhizosphere in China

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ABSTRACT

Many microorganisms in soil are able to transform insoluble form of phosphorus to an accessible soluble one, contributing to plant nutrition as plant growth-promoting microorganisms. The objective of this work was to isolate, screen and evaluate the phosphate-solubilizing activity of microorganisms in soybean rhizosphere soil, and select potential microbial inoculants. These microorganisms were selected based on the phosphate-solubilizing efficiency in a modified Pikovskaya's liquid medium culture. The isolates were identified based on the morphological characteristic and nucleotide sequence data from the 16S ribosomal DNA (rDNA) for bacteria. Strain BAⅢ, identified as Bacillus megaterium, is the most effective one for solubilization of phosphate in medium. Taken together, these results demonstrate that strain BAⅢ has the ability to convert non-available forms of phosphorus into plant-available forms, therefore, it has a great potential to develop as a bio-fertilizer to enhance soil fertility and promote plant growth.

KEYWORDS

Phosphate-solubilizing microorganisms; Rhizobacteria; Soybean; 16S rDNA; Identification.

INTRODUCTION

Phosphorus (P) is one of the major plant nutrients, and lack of phosphorus effects severely plant growth. Most agricultural soils contain large reserves of total P[1,2]. However, it is generally low in P readily available for plant growth in soil[3,4]. Currently, a large portion of soluble inorganic phosphate is fertilized into soil, but it is immobilized rapidly and becomes unavailable to plants[5]. Soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization[6]. Therefore, phosphate-solubilizing microorganisms (PSMs) have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield[7-10]. This could have a significant impact on lower yields of crops today. In the study, we isolate, screen and evaluate the phosphate-solubilizing activity of microorganisms in soybean rhizosphere soil, and select potential microbial inoculants. The isolates will be important microorganism resource to develop as a bio-fertilizer.

MATERIALS AND METHODS

Isolation of PSMs

The PSMs were isolated from the soil and roots of soybean growing in fields at Hailun and Beian, China. For the collection of the rhizosphere soil, plants were uprooted and the loosely adhering soil was obtained by mechanical shaking. The soils were then suspended in sterile saline and were shaken for 6 h on a rotary shaker. Serial dilutions of the samples were then individually inoculated on modified Pikovskaya (MPVK) agar plate supplemented with 1.5% (w/v) agar. The MPVK medium used comprised the following: 1.0% (w/v) glucose, 0.05% (w/v) $(NH_4)_2SO_4$, 0.02% (w/v) NaCl, 0.02% (w/v) KCl, 0.01% (w/v) $CaCl_2{\cdot}2H_2O$, 0.01% (w/v) $MgSO_4{\cdot}7H_2O$, 0.05% (w/v) $MnSO_4{\cdot}7H_2O$, 0.05% (w/v) $FeSO_4{\cdot}2H_2O$, 0.05% (w/v) yeast extract and 0.5% (w/v) $Ca_3(PO4)_2$ in distilled water (pH 7.5). $Ca_3(PO4)_2$ was autoclaved firstly. Then, the other sterile ingredients were aseptically mixed after autoclaving. This medium was prepared according to reference[11].

After 5 day incubation at 30 ℃, the plates were examined for the presence of colonies developing clear haloes. PSMs could easily be identified because they developed clear zones around their colonies[12]. Colonies with clear zones around them were picked up and further purified by replating on agar plate.

Screening and phosphate evaluation

Solubilization of phosphate was estimated in NBRIP liquid medium: 1.0% (w/v) glucose, 0.05% (w/v) $(NH_4)_2SO_4$, 0.025% (w/v) $MgSO_4{\cdot}7H_2O$, 0.5% (w/v) $MgCl_2{\cdot}6H_2O$, 0.02% (w/v) KCl, and 0.5% (w/v) $Ca_3(PO_4)_2$ in distilled water (pH 7.0)[13].

Two hundred fifty mililiter-erlenmeyer flasks containing 50 ml of NBRIP medium were inoculated with each of the purified bacterial strain. The flasks were incubated for 5 days at 30 ℃ with shaking (200 rpm) on a rotary shaker. The cultures were harvested by centrifugation at 17,418 g for 30 min. Phosphate in culture supernatant was estimated colorimetrically. The amount of phosphate released into culture supernatant was criteria for choosing the PSM. The bacteria with the higher phosphate-solubilizing activity were selected to further study and characterize to the species level.

A preliminary experiment was conducted to test the ability to produce soluble phosphate in liquid cultures by the isolates. Three replicate flasks containing NBRIP liquid medium were inoculated with each isolate. Three replicates of a control treatment were included in the experiment. The initial pH was adjusted to 6.0. The cultures were centrifuged (7000 g, 10 min) after 10 days of incubation, at 27℃, with gentle shaking and 5 ml supernatant aliquots were filtered through Whatman 42 filter paper to remove thick polysaccharide-like exudates. The filtrates were assayed for soluble P, using the Murphy and Riley's (1962) colorimetric method[14]. The amount of P solubilized was obtained by subtracting the soluble P of the inoculated sample from the corresponding sample uninoculated control (i.e. P released by autoclaving of the P suspension).

Identification of isolates

The direct observation of isolated colonies was served as the first characterization comprising the color, shape, elevation, margins, diameter, surface, opacity, and texture[15]. Under light microscope all the isolates were further observed for morphology of strain. Gram-negative and positive bacterial isolates were identified using standard methods including Gram stain and general morphology, colony morphology, pigmentation[16,17].

The Characterization to the genus level of the selected PSB strain was performed by partial sequencing of the 16S ribosomal DNA gene[18]. Genomic DNA was extracted by the CTAB method[19] and amplified using PCR amplification of the 16S *rDNA*. Almost complete 16S *rDNA* genes were amplified with the forward primer 27f (5′-GAGATTTGATTCTGG CTCAG-3′) and the reverse primer 1495r (5′-CTACGGCTACCTTGTTACGA-3′). A 20 μL PCR mixture contained *Taq* DNA polymerase buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 25 pmol of each forward and reverse primers, 1 U of DNA polymerase (Promega), and 1 μL of the diluted DNA extract as template. The DNA was amplified with an thermocycler (Eppendorf, Germany) with the following program: 4 min of pre-heating at 94 ℃, 30 cycles of 30 s of denaturation at 94 ℃, 30 s of primer annealing at 52 ℃, 1 min of elongation at 72 ℃, and 10 min of extension step at 72 ℃. The amplified fragment was analyzed by running 5 μL of the PCR product on a 1.2% agarose gel[20]. The PCR products were purified, and the sequencing of 16S *rDNA* was conducted at TaKaRa Biotechnology Corporation

(Dalian, China). The 16S *rDNA* sequences were assessed by BLAST algorithm for comparison of a nucleotide query sequence against public nucleotide sequence database to find the closely related bacteria. The 16S *rDNA* sequences from the isolate were aligned and bootstrapped neighbour-joining relationships were estimated with MEGA version 4.1[21].

RESULT AND DISSCUSS

Isolation of phosphate-solubilizing bacteria

In the study, a total of 7 PSBs, showing clear and greater P-solubilizing zone on the MPVK agar medium, were isolated from the rhizosphere of soybean in Hailun and Beian of Heilongjiang province, China. They were named, HLⅠ, HLⅡ, HLⅢ, BAⅠ, BAⅡ, BAⅢ and BAⅣ, respectively. Among them, isolate BAⅢ showed the highest P-solubilizing activity as visualized by the size of the clear zone developed around the colony and computed by the ratio of zone to colony diameter after 24 h incubation.

TABLE 1 : Release of soluble-P (mg·L^{-1}) by bacterial isolates for 3 days in NBRIP liquid medium.

Isolate no.	Soluble phosphorus (mg·L^{-1})		
	Un-inoculated	Inoculated	Increase
BAⅢ	74.8 ± 4.7	264.3 ± 38.5	189.5 ± 37.2
BAⅡ	72.2 ± 5.7	194.6 ± 47.7	122.4 ± 43.1
BAⅠ	73.3 ± 8.7	246.1 ± 36.6	172.8 ± 32.2
BAⅣ	71.4 ± 5.9	210.7 ± 20.1	139.2 ± 17.5
HLⅠ	74.1 ± 7.5	219.7 ± 39.8	145.6 ± 33.2
HLⅡ	74.3 ± 9.4	254.9 ± 45.0	180.5 ± 53.7
HLⅢ	72.8 ± 5.4	236.2 ± 20.0	163.4 ± 21.8

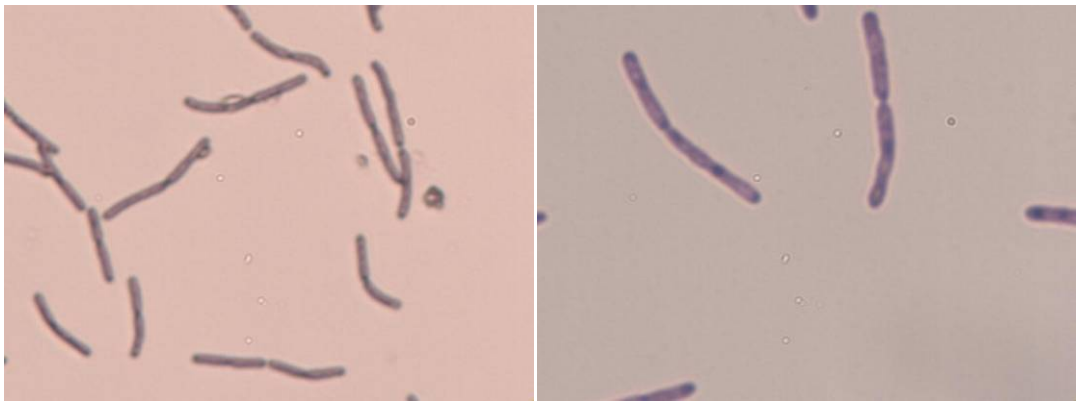

Figure 1 : Microcosmic characteristics of phosphate-solubilizing bacteria strain BAⅢ.

In the left and right Figure, the bacteria were magnified 10×40 times and 10×100 times, respectively.

Figure 2 : Phylogenetic tree based on 16S *rDNA* gene sequences, showing the relationships among selected PSB isolates and representatives of other related taxa with validly published names.

Solubilization of phosphate by bacteria from soybean soils

The solubilization of phosphate by the seven isolates was monitored up to 3 days in NBRIP medium. The phosphate-solubilizing level by BAⅠ, BAⅢ, HLⅡ, and HLⅢ increased linearly. The maximum solubilization of phosphate was achieved after 3 days of incubation. Therefore, the batch tests were analyzed after 3 days for all seven strains. Under these conditions, BAⅢ was the most efficient strain in solubilizing phosphate, followed by HLⅡ, BAⅠ, and HLⅢ in decreasing order of efficiency (TABLE 1).

Rodriguez and Fraga (1999) reported that the plate halo screening method produced contradictory results[6]. However, this method can be regarded as generally reliable for isolation and preliminary characterization of PSM. Gyaneshwar et al. (1998) found that some PSMs showing phosphate-solubilizing ability under laboratory conditions were not able to release P when they were inoculated into alkaline vertisols even other nutrients were also supplied[22]. Probably, this

was due to the high buffering capacity of the alkaline soils coupled with the inability of bacteria to secrete high concentration of organic acids[1]. Therefore, it would be better to test PSM under different conditions.

Identification of PSB

The isolates were examined for their colony morphology on nutrient agar after 24 h of incubation. The colonies of all the isolates were round, raised, smooth, pale-yellowish, and translucent with entire margins in appearance on Pikovskaya's medium but a little variation was observed in colony shape.

All 7 isolates were tested with gram staining, then the isolate HLⅢ was Gram-negative, the others were Gram-positive. The result of observed with microscope showed the isolates BAⅢ was a rod shaped bacteria (Figure 1). The selected bacterial isolate, BAⅢ, was further tested for identification. The nucleotide sequence of nearly full length (1,500 bp approximately) 16S *rDNA* gene from isolate BAⅢ was determined and compared to sequences available in data banks. Phylogenetic 16S *rDNA* sequence analysis revealed that it was 99% identical to that of *Bacillus megaterium* (Figure 2). Combining with its morphological characteristics, the BAⅢ was identified as *Bacillus megaterium.*

Application of bacterial inoculants as bio-fertilizer has been reported to improve plant growth and increase yields of plants[23]. However, Chung et al. (2005) found that there is no direct correlation among in vitro P solubilization and/or mineralization, P accumulation in plant and soil available P[24]. To test the efficiency and feasibility for the screened PSB as bio-fertilizer for soybean, further studies should be conducted under pot cultures and field conditions.

From the present study, we demonstrate that the natural soil supports a diverse group of potential phosphate-solubilizing bacteria. These bacteria could serve as candidates of an efficient bio-fertilizer for improving the P-nutrition of crop plants. The advantage of using natural isolates from soil is easier to adapt the similar soil when the isolates were inoculated into the plant rhizosphere. Meanwhile, it helps to minimize the P-fertilizer application, reduces environmental pollution and promotes sustainable agriculture via using these PSBs as bio-inoculants.

CONCLUSIONS

In the study, several bateria were selected based on the phosphate-solubilizing efficiency in a modified Pikovskaya's liquid medium culture, and the isolates were identified. Strain BAⅢ, identified as *Bacillus megaterium*, was obtained with high phosphate-solubilizing efficiency, then it has a great potential to develop as a bio-fertilizer to enhance soil fertility and promote plant growth.

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