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SCREENING AND IDENTIFICATION OF MICROORGANISMS CAPABLE OF UTILIZING PHOSPHATE ADSORBED BY GOETHITE

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ABSTRACT

Information on phosphorus (P)-solubilizing microorganisms in variable charge soil is lacking. We screened soil microorganisms that can effectively utilize P adsorbed on variable charge minerals using a series of synthetic media of decreased P availability. Rhizospheric soil (Orthic plintaqualt) from a 30-y old tea plant was diluted and aseptically inoculated to a series of media containing P adsorbed on goethite at 0, 255, 50, 75, and 100% saturation. Microorganisms, which survived in the 25% P sorption saturation medium, were each by colony isolates transferred to another freshly prepared medium of the same type for growth stability test. Microbial species, which could grow and reproduce for more than 15 generations in the 25% P-saturation medium

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were considered as P-solubilizing microbes (PSMs) and used for identification tests. By this procedure, we screened one PSM population which was identified as the bacterium *Moraxella* sp by Gram staining, Gamma culture and staining, optical and electron microscopic observation, and enzyme-oxidizing reaction. The PSMs have higher maximal transport rates to P than the ubiquitous soil microorganisms and have an optimal temperature of 37°C and optimal pH of 5.5–7.5 for growth.

Key Words: Bacteria; Identification; Phosphorus solubilization; Variable-charge minerals

INTRODUCTION

Large amounts of P fertilizers are generally required for sustainable crop production on variable charge soils because of low P availability to plants (1,2). Most of the applied chemical fertilizer P accumulates in the fine soil fractions (3,4), which are readily transported to surface waters through runoff on suspended particles, especially in hilly regions (3). There have been increasing concerns over eutrophication of surface waters caused by increased P loading. Phosphorus transport from arable land has been considered a major non-point source of P (5,6). Increasing utilization efficiency of residual P in the variable-charge soils is one of the most important measures in minimizing P contamination to surface waters.

Chemical fixation of P in variable charge soils is generally attributed to the adsorption of phosphate on the surfaces of variable charge minerals such as Fe and Al oxides and kaolinite (7,8,9,10,11). The adsorbed P that is bound to either Fe or Al as bidentate or binuclear surface complexes is difficult to desorb in an aqueous solution and, therefore, is not readily available to plants (12,13,14).

The importance of microorganisms in soil nutrient cycling and their role in plant nutrition is well known. Microorganisms can increase plant availability of soil P by mediating the transformation and distribution of different P pools (15). Under pure culture conditions, microorganisms have been shown to enhance the solubility of rock phosphates (16,17) and to effectively utilize and transform specifically adsorbed P on some variable-charge minerals (18). Soil microorganisms vary in the capability of utilizing less available P (15). Some microorganisms are capable of excreting organic acids such as citric acid and oxalic acid, thereby enhancing dissolution of sparingly-soluble P sources, including Fe and Al phosphates and apatite, by means of acidification and

complexation processes (19,20). Some microorganisms in variable-charge soils also demonstrated a greater capability of utilizing and transforming adsorbed P than the others (18; Z.L. He and W. Bian, unpublished data). These microorganisms need to be identified and characterized for potential application in increasing utilization efficiency of residual P in the variable-charge soils.

Our objectives were to screen microorganisms from variable-charge soils, which are capable of utilizing and transforming adsorbed P more effectively than the average microbial community and to characterize and identify these exceptional microorganisms.

MATERIALS AND METHODS

Minerals Used for Phosphorus Adsorption

Goethite was artificially synthesized with methods proposed by Atkinson et al. (21). A natural kaolinite was collected from Yanshan, Suzhou, and a natural montmorillonite was provided by the Geology Bureau of Zhejiang Province. Each mineral was identified as the typical species by x-ray diffraction and electron microscopy. Some physico-chemical properties of the minerals were described by He et al. (10,11). Montmorillonite and kaolinite were used for the purpose of comparison with respect to bioavailability of adsorbed P.

Preparation of Mineral–Phosphorus Complex

Mineral–P complexes were prepared by equilibrating variable amounts of P (as KH_2PO_4) with each of the goethite, kaolinite, and montmorillonite in 0.02 *M* KCl solution (with pH adjusted to 7.0) for 2 months with interval shaking at 25°C in dark. Drops of chloroform were added to stop microbial activities. The amount of P added was equal to 0, 25, 50, 75, or 100% of the adsorption capacity for each mineral as calculated from P adsorption isotherms determined with a simple Langmuir equation (Table 1). The 0, 25, 50, 75, and 100% adsorption capacities represented different bioavailability values of the adsorbed P. The mineral–P complexes were then leached with deionized water 3–5 times until no P was detectable in the leachate. This step was to ensure that only tightly bound P remained on the solid surface. Phosphorus concentration in the leachate was measured by ascorbic acid reduction blue method (22), and the amount of tightly-bound P was calculated by the difference between the total added P and P desorbed by leaching.

Table 1. Preparation of Mineral–P Complexes

Adsorption Saturation%	P Adsorbed (mg g^{-1})	Amounts of Mineral Used (g)	Amounts of P Added (mg)	Amounts of Water Added (mL)	PH Adjusted
Goethite–P complex					
0	0	18.8	0	376	7.0
25	0.871	75	65.3	1500	7.0
50	1.741	37.5	65.3	750	7.0
75	2.612	25	65.3	500	7.0
100	3.482	18.8	65.3	376	7.0
Kaolinite–P complex					
0	0	50.5	0	253	7.0
25	0.053	200	10.6	1000	7.0
50	0.105	101	10.6	505	7.0
75	0.158	67.1	10.6	336	7.0
100	0.210	50.5	10.6	253	7.0
Montmorillonite–P complex					
0	0	49.9	0	250	7.0
25	0.104	200	20.8	1000	7.0
50	0.209	99.5	20.8	498	7.0
75	0.313	66.5	20.8	333	7.0
100	0.417	49.9	20.8	250	7.0

Screening, Separation, and Identification of Phosphorus-Efficient Microbes

The mineral–P complexes were each used as the sole source of P in a culture medium with the following composition (g L^{-1}): 10 glucose, 0.5 $(NH_4)_2SO_4$, 0.3 NaCl, 0.3 KCl, 0.3 $MgSO_4 \cdot 7H_2O$, 0.03 $FeSO_4 \cdot 7H_2O$, and 0.03 $MnSO_4 \cdot 4H_2O$. Known amounts of mineral–P complexes (Table 2) and 1.2 g agar were used for preparing 100 mL of the above medium, with the C-to-P molar ratio of the medium at approximately 200 to 1. The mineral–P complex was each suspended in a proper amount of deionized water, the pH of the suspension was adjusted to 7.0, and sterilized by gamma irradiation (5 kGy) before being mixed with sterile growth medium containing agar. Soil inoculums were prepared using a freshly collected rhizosphere soil from a 30-y old tea plant. 15–20 mL of the culture medium containing each of goethite–P, kaolinite–P or montmorillonite–P complex was placed in a culture plate (2 cm height and 8 cm in diameter), inoculated aseptically with 1 mL of the soil inoculums at 10^{-4} dilution, and incubated for 3–4 d at 30°C.

The microbial species that survived in the 25% P-saturation medium were each separated and inoculated to a fresh medium of the same P saturation using the streak plate method and incubated for 1–2 d to eliminate contaminated microorganisms. The dominant microorganisms were then allowed to grow in the 25% P-saturation medium for 3–5 days and observation was recorded every 12 h. Those microbes, which continued to grow after multiplying for 15–20 generations, were considered to be the P-solubilizing microbes (PSMs) and were retained for identification.

Identification of the PSMs was conducted by: (1) Gram staining and morphology observation by light optical and electron microscopic (23); (2) mobility tests using solid media in tubes inoculated by a straight wire and water drop-glass micro plate method (24); (3) endospore observation by staining after culturing the bacteria in a slope surface of an endospore-production medium (23); and (4) determination of oxidase, optimal pH and temperature for growth (23). The pH test was conducted using a beef extract agar–peptone medium containing

Table 2. The Amounts of Mineral–P Complex Used for Preparing 100 mL Medium

P Sorption Saturation (%)	Goethite–P (g)	Kaolinite–P (g)	Montmorillonite–P (g)
0	0.57	9.52	4.8
25	2.3	37.74	19.23
50	1.15	19.05	9.57
75	0.77	12.66	6.39
100	0.57	9.52	4.8

beef extract agar 3 g, NaCl 5 g, peptone 10 g, agar 15–20 g, and water 100 mL. Medium (100 mL) was placed in 250 mL conical flacks with pH adjusted to 5.5, 6.0, 6.5, 7.0, and 7.5. The media were then inoculated with the PSMs at a proper dilution and incubated at 30°C. During incubation, the growth of the microorganisms and the size of colonies were recorded. There were three replications for each pH treatment. The optimal growth temperature test was conducted using the same medium adjusted to pH 6.5. After inoculated with the PSMs, the media were incubated at 25, 30, 34, 37, and 40°C, respectively. During incubation, growth of the microorganisms and the size of colonies were recorded. There were three replications for each temperature treatment.

Phosphorus Absorption Kinetics

The experiment was a split factorial design consisting of two types of inoculants and six P concentration levels in three replications. 50 mL of synthetic liquid medium that contained no P was placed in 150-mL conical flasks. Phosphorus in the form of KH_2PO_4 was added to provide P concentrations at 0, 0.1, 0.2, 0.6, 1.2, and 2.0 mg L^{-1}. All media were divided into two groups in three replications, one group inoculated with 1 mL of soil dilution at 10^4 microbes mL^{-1} and the other with the same numbers of the PSMs. The culture was conducted at 25°C on a shaker (100 per min) for 24 h. At the end of designated time, the medium was transferred into a plastic centrifuge tube and centrifuged at 20,000 × g for 15 min. Phosphorus concentration in the supernatant solution was determined, and the decrease in P concentration after microbial culture was considered to result from P absorption by the inoculated microorganisms. The experiment was repeated three times and average values of the three measurements were used for calculating P absorption kinetic parameters. The maximum absorption (Vmax) and the affinity constant (Km) were calculated based on the Michaelis–Menten equation [$v = Vmax\ C/(Km+C)$, where v is absorption rate and C is the initial P concentration]. The statistical analysis of the fitness of the data with the Michaelis–Menten equation was performed using the program of the SAS Release 6.12 (25).

RESULTS

Screening and Separation

No P was desorbed from goethite–P complex at low P adsorption saturation (0–75%) and the amount of loosely bound P accounted for only a very small proportion of total bound P (<2%) on goethite at high saturation (75%)

(Table 3). About 30–40% of sorbed P on montmorillonite was desorbed, and the desorbability of sorbed P on kaolinite was higher than goethite, but much lower than montmorillonite. Martin and Smart (14) observed by means of x-ray photoelectron spectroscopy that P is adsorbed on goethite mainly as bidentate that is a very stable six-member ring structure. The results indicate that the availability of adsorbed P on goethite is very low and tended to decrease with decreasing sorption saturation. Therefore, the medium containing goethite–P complex as sole P source of low sorption saturation was used for screening microorganisms capable of utilizing tightly bound P in variable-charge soils.

The number and size of the microbial colonies growing in the selective media decreased with decreasing P adsorption saturation. Very few microorganisms survived in the medium containing goethite–P complex at 25% saturation based on visual observation (Fig. 1). For instance, the medium containing goethite–P at 100% saturation was covered with fungal colonies of white, purplish, gray, and black color, and transparent, white or yellowish colonies of bacteria, whereas the medium containing goethite–P at the 25% sorption saturation had no fungal colony and only few small colonies of bacteria. In contrast to goethite–P, there was no difference in the growth of microorganisms in the media containing montmorillonite-P complex from 25

Table 3. Desorption and Residual P in the Mineral–P Complexes at Various P Adsorption Saturations

P Adsorption Saturation (%)	P Adsorbed ($mg\,g^{-1}$)	P Desorbed ($mg\,kg^{-1}$)	P Desorbed (%)	Remained (%)
Goethite–P complex				
25	871	0	0	100
50	1741	0	0	100
75	2612	2.6	0.1	99.9
100	3482	55.7	1.6	98.4
Kaolinite–P complex				
25	53	0.1	0.2	99.8
50	105	1.1	1.0	99
75	158	4.0	2.5	97.5
100	210	6.7	3.2	96.8
Montmorillonite–P complex				
25	104	29.2	28	72
50	209	76.5	37	63
75	313	104	33	67
100	417	138	33	67

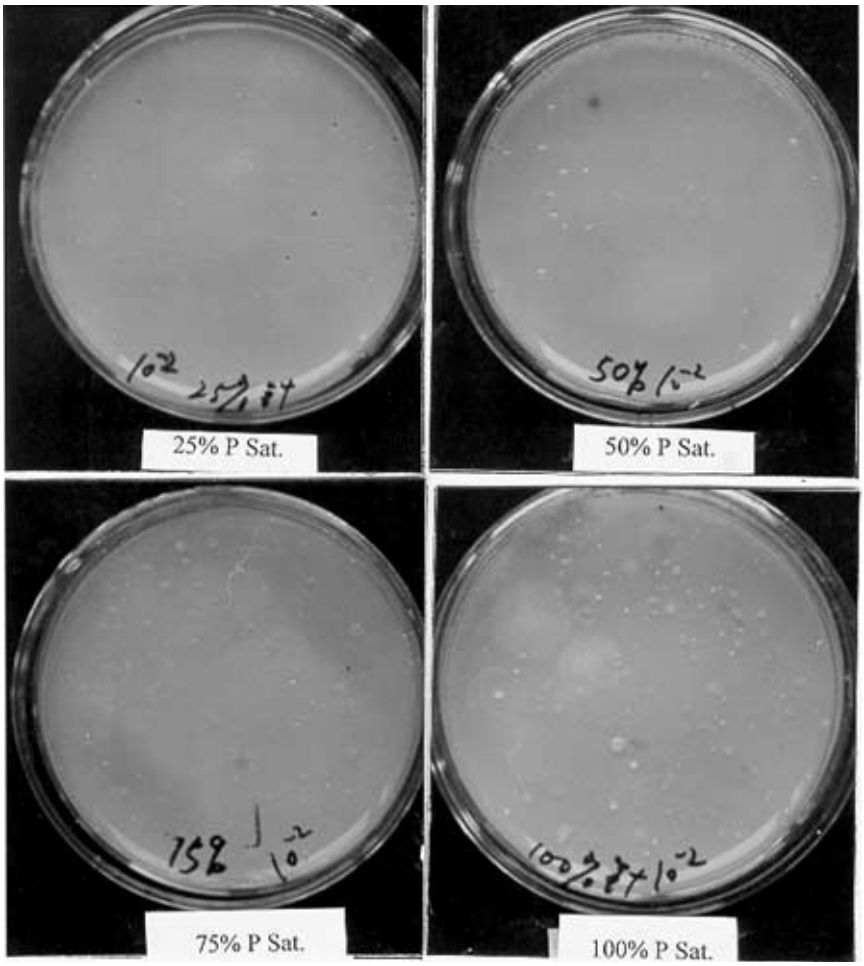

Figure 1. Microbial growth in the media containing goethite–P at different sorption saturation and inoculated with 10^{-2} soil dilution.

to 100% adsorption saturation (Fig. 2). This may be due to the fact that P adsorbed on montmorillonite was readily desorbed and thus was highly bioavailable (Table 3). The growth of microorganisms in the media containing kaolinite–P complex was intermediate between the goethite–P and the montmorillonite–P treatment (Fig. 3). These results imply that goethite–P complex at low sorption saturation is an ideal P source for screening PSMs in variable charge soil. The microbes that survived in the media containing

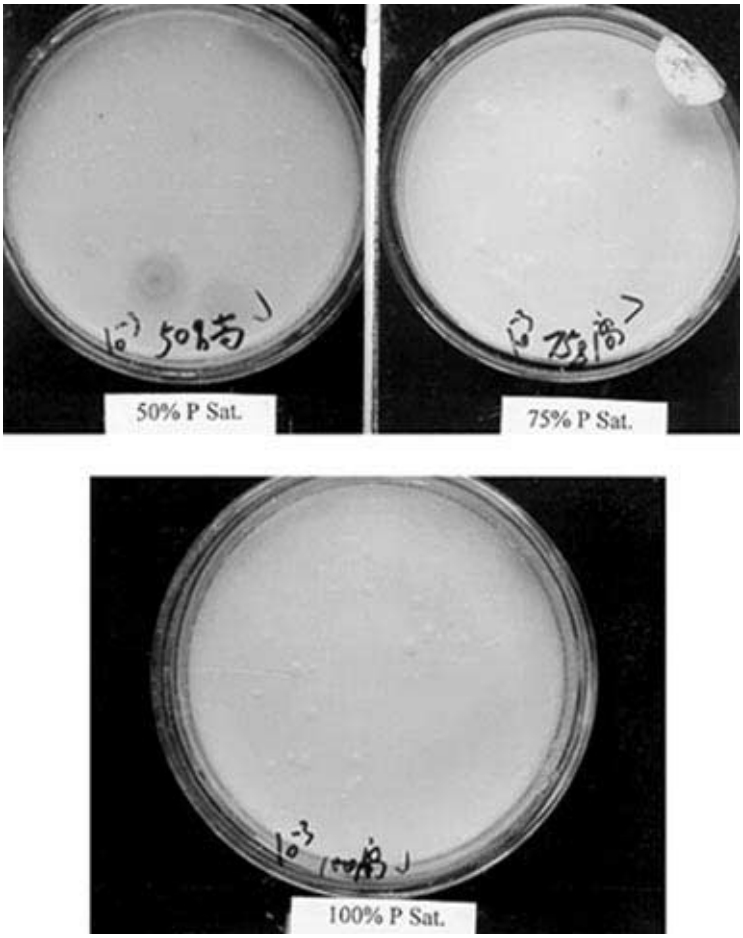

Figure 2. Microbial growth in the media containing kaolinite–P at different sorption saturation and inoculated with 10^{-3} soil dilution.

goethite–P at 25% adsorption saturation were then considered to have the ability of utilizing tightly bound P or tolerate low P stress, and selected for further tests.

Most of the bacteria that survived in the primary culture of the 25% sorption saturation goethite–P media failed to grow after 3–4 generations in a freshly prepared medium of the same media, showing that they might not have the ability of utilizing the tightly adsorbed P. These microbes were probably more tolerant to P stress than the average soil microbes. They could grow in the P-stressed medium for a short period by using their cellular P source, and died after exhausting their

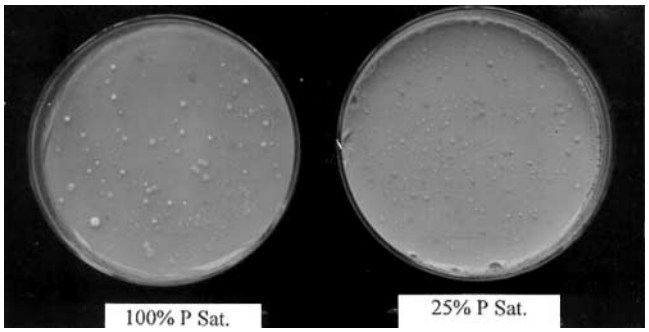

Figure 3. Microbial growth in the media containing montmorillonite-P at different sorption saturation and inoculated with 10^{-3} soil dilution.

internal P. The microbial species that grew well in the 25% sorption saturation goethite–P medium for more than 15–20 generations is assumed to have the ability of utilizing the tightly bound P, and was therefore considered to be the PSMs. We observed that the PSMs lost forever their ability in utilizing the tightly bound P after they were grown in a P-enriched medium for a few days. Therefore, the PSMs should be maintained in a low available P medium and incubated every other week during the storage In addition, the PSMs need to be incubated for 18–24 h after being inoculated into a fresh medium and then re-inoculated from this medium to the slope surface of a solid medium prior to storage at 2–10°C.

Identification of the PSMs

Colony Characteristics

Colonies of the PSMs grown on either selective (containing goethite–P at 25% adsorption saturation) or complete media were roundly shaped, distinctly raised with a smooth and sticky surface. The colonies grown on the selective media were non-pigmented and small, like small drops of water, whereas those grown on the complete media were pigmented with a light yellow color (Fig. 4).

Morphology of Cells

Cell of the PSMs was short rod-shaped, in pair or short chains. The PSMs had no flagella or endospore, but had a capsule and tiny pilli that can only be observed under an electronic microscopy (Fig. 5).

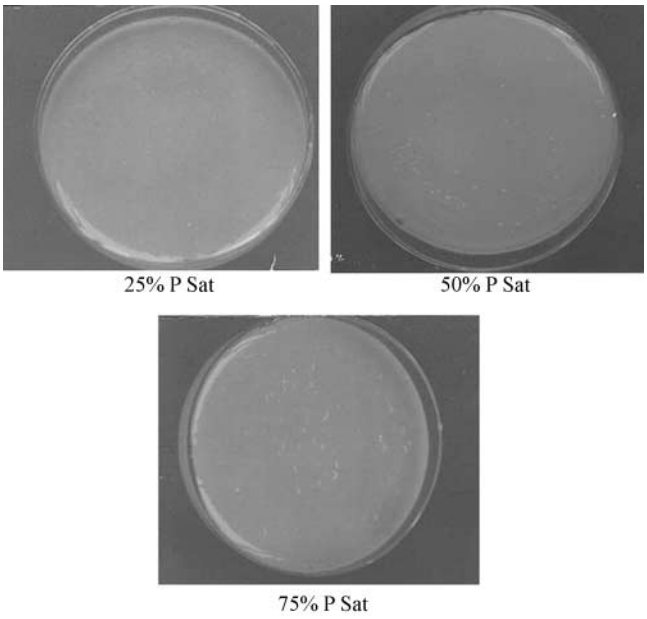

Figure 4. Growth of P-solubilizing microbes in the media containing goethite–P at different sorption saturation.

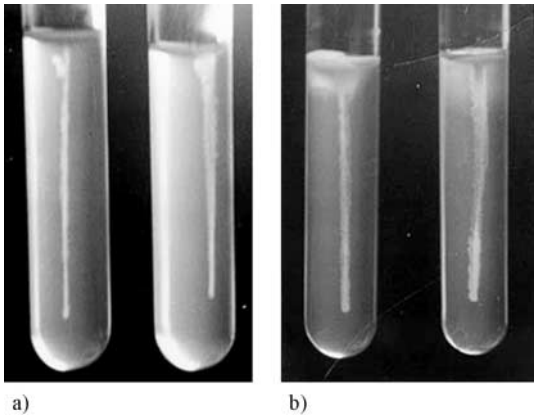

Figure 5. Liquidization of semi-solid agar media by the P-solubilizing microbes (PSMs): (a) without PSMs and (b) with PSMS.

Physiological and Biological Characteristics

The PSMs were gram negative, had limited mobility when growing into the semi-solid agar media. However, they could liquidize agar (Fig. 6) and showed positive reaction to oxidizing enzyme tests. Colony sizes of the PSMs varied little in the pH range of 6.5–7.5, but decreased at pH 5.5 or below (Fig. 7). The optimal temperature for PSMs growth is 37°C. The colony sizes of the PSMs were larger at 30 or 35°C than at either 25 or 40°C (Fig. 8). According to morphological, physical, physiological, and biological characteristics of colonies and cells, the PSMs were identified as *Moraxella* sp.

Phosphorus Absorption Kinetics of the PSMs

There was no difference in the absorption rate of P between the PSMs and the average soil microorganisms at very low P concentration range (0–0.2 mg L^{-1}). However, at higher P concentrations (>0.2 mg L^{-1}), the PSMs had a greater uptake rate of P than the average soil microorganisms (Fig. 1). The PSMs had a Vmax value of 88.5 (10^{-4} mg d^{-1} $number^{-1}$), as compared to 28.3 (10^{-4} mg d^{-1} $number^{-1}$) for the average soil microorganisms (Fig. 9). These results indicate that the PSMs have a greater capacity for P uptake than the average soil microorganisms.

DISCUSSION

Microorganisms play an important role in the turnover of organic P, and in the cycling of P in soil (26,27). About 20% of microorganisms in soil were

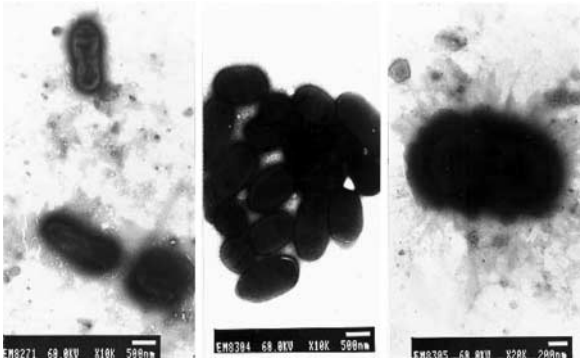

Figure 6. Morphological observation of the P-solubilizing microbes under electronic microscopy.

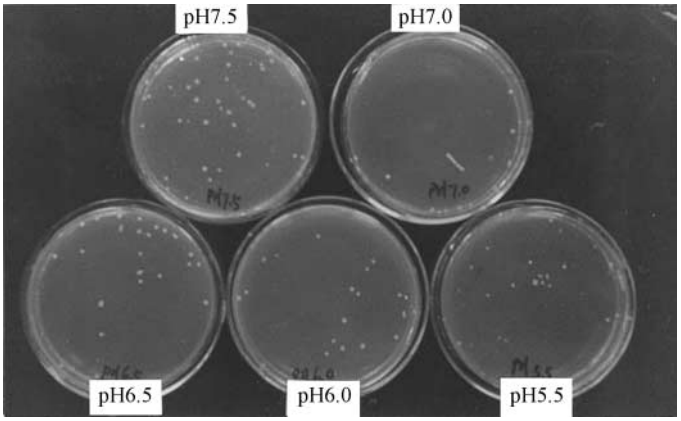

Figure 7. Optimal pH for the growth of P-solubilizing microbes.

reported to have the ability to solubilize insoluble inorganic phosphate, such as apatite and $AlPO_4$ (15,17). The mechanisms involved in microbial solubilization of inorganic phosphate include acidification and chelation by organic acids produced by the microorganisms (15,19).

From our study, it appears that a small percent of microorganisms have also the ability of utilizing P tightly bound on the surfaces of variable-charge minerals. The optimal temperature for growth of the PSMs appeared to be 35–37°C, higher than the average soil microorganisms (25–35°C) (28), which

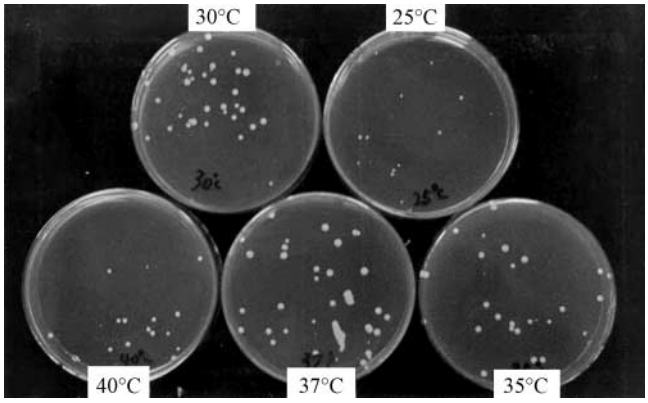

Figure 8. Optimal temperature for the growth of P-solubilizing microbes.

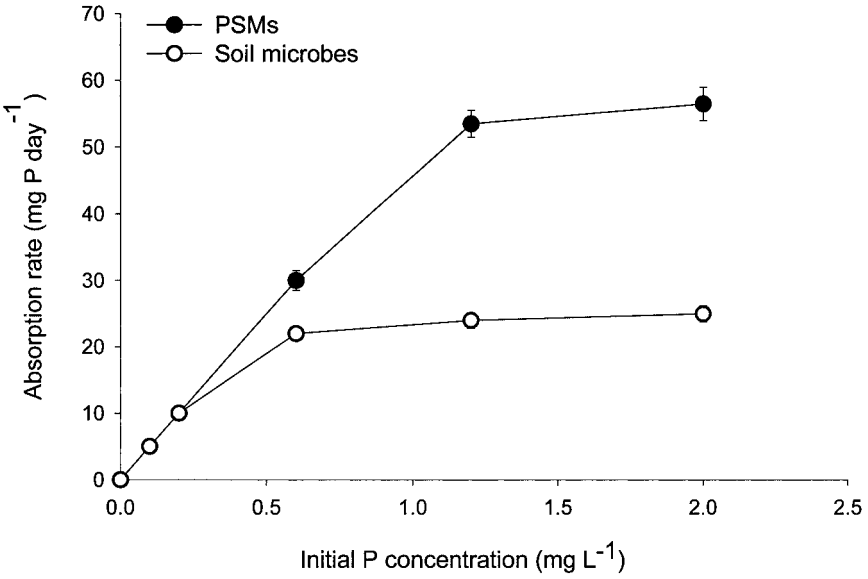

Figure 9. Kinetics of P uptake by the screened P-solubilizing microbes (PSMs) as compared with soil microbes (concentration of microbes: $10^4\,L^{-1}$) (The error bar to each data point indicates standard deviation of the mean value).

renders the PSMs able to actively participate in the transformation of P in variable charge soils in the tropical and subtropical regions.

With regards to mechanisms of microbial utilization of adsorbed P, microorganisms can produce low molecular weight organic acids. A number of organic acids such as citric acid, oxalic acid, lactic acid, 2-ketogluconic acids, glycolic, fumaric, and succinic acids have been identified in culture (15,29,30). Therefore, mineral phosphate solubilization is often accompanied by a decrease in medium pH (18,30). These organic acids enhanced the dissolution of minerals by chelating with Fe and Al, thus releasing the adsorbed P. Moreover, a series of organic anions strongly compete with phosphate for adsorbing sites on the surfaces of iron (Fe) and aluminum (Al) oxides (20,31,32), and release adsorbed P through ligand exchange reactions (33). In addition to organic acid release (our unpublished data), the PSMs have a greater capacity for P transport from external sources into the cells and therefore, can more efficiently absorb and incorporate P into the cells. As a result, more P can be desorbed from the tightly bound sites by the PSMs than the average soil microorganisms (Z.L. He and W. Bian, unpublished data).

The trait of mineral phosphate solubilization has been displayed by a wide range of bacteria (34,35) and is regulated by external levels of available inorganic P (36), indicating that bacteria have mineral phosphate solubilizing genes (34). These genes have been successfully cloned in *E. Coli* and showed great ability of solubilizing phosphate in culture (35). The PSMs obtained from our study demonstrated similar traits in responding to external available P level, implying that they may have functional genes for utilizing P adsorbed by variable-charge minerals.

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