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Isolation and Phosphate-Solubilizing Characteristics of PSM, *Aeromonas hydrophila* DA33.

Ok-Ryul Song, Seung-Jin Lee, Mi-Wha Lee, Si-Lim Choi, Soo-Yeol Chung*, Young-Gyun Lee and Yong-Lark Choi†**

Faculty of Natural Resources and Life Science, Dong-A University
**Dept. of Food Science, Dongju College, Busan 614-103*
***Dept of Food Science, Miryang National University, Miryang, Korea*

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Abstract A bacterium having high abilities to solubilize inorganic phosphate was isolated from cultivated soils. The strain was identified as *Aeromonas hydrophila* DA33, based on the physiological and biochemical properties. The optimum temperature and initial pH to solubilize insoluble phosphate in sucrose minimal medium were 30℃ and pH 5.0, respectively. In these conditions, phosphate-solubilizing activities of the strain against two types of insoluble phosphate were quantitatively determined. When glucose was used for carborn source, the strain had a marked mineral phospahte solubilizing activity. Inorganic phospahte solubilization was directly related to the pH drop by the strain. Analysis of the culture medium confirmed the production of gluconic acid as the main organic acid released by *Aeromonas hydrophila* DA33.

Key words: insoluble phosphate solubilization, free phosphate, *Aeromonas* sp., gluconic acid

Introduction

Phosphorus is an essential nutrient for biological growth and development [1]. Bacteria and plants must often obtain their phosphorus from the external environment in a soluble ioninc form. However, phosphorus in soils is immobilized or becomes less soluble either by absorption, chemical precipitation, or both [2]. So, plants can absorb only inorganic phosphorus, and the concentration of inorganic phosphate in the soil is very low because most of the phosphorus in soils is present in insoluble form [3]. Numerous attempts have been made to improve the P-supply in soils by means of phosphate-solubilizing microorganisms (PSMs), mobilizing P from hardly-soluble inorganic phosphorus [4-6]. Illmer and Schinner have shown that some PSMs are very effetive in solubilizing calcium phosphates (Ca-Ps) [7]. Therfore, organic phosphate mineralization is an improtant soil process because it results in release of inorganic phosphorus to the soil solution for its availability to plants and soil microbes [1,8].

Taha *et al.* and Alexander reported that phosphate solubilization was due to the production of organic acid and inorganic acids and CO_2 by PSMs [1,9]. Struthers and Seiling found citiric, oxalic, butyric, malonic availability [10]. Moghimi *et al.* and Moghimi and Tate studied the release of P from calcium phospahte by rhizospere products. They reported that solubilization was associated with a fraction containing large amounts of 2-ketogluconic acid [11,12].

Microorganisms may show a preference to particular energy sources, such as farmyard manure compared with starch and molasses under these conditions. Banik reported that among four C source (glucose, sucrose, mannitol and sodim acetate) the hightest ability to solubilize apatite by microorganisms was associated with glucose in short-term experiments while sucrose was the best in longer experiments [13].

Here, we report the isolation and characterization of a new PSM from cultured soils for salts accumulation and superfluity treatment of phospahte. Secondary, we explain the effect of different C energy sources and the effect of a various concentration of C energy source.

Materials and Methods

Bacterial strains isolation and identification

Bacterial strains were isolated from the soils exposed to high salt or superfluity treatment of phosphate at Gimhae area, Korea. All soil samples were held at 4℃ for approximately 24 h before further treatment. Serial dilution of soil samples were then individually plated on sucrose minimal (SM) agar plates containing sucrose 10 g, tri-calcium phosphate 5 g, NH_4NO_3 0.27 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $MnSO_4 \cdot 6H_2O$ 1 mg, $FeSO_4 \cdot 7H_2O$ 1 mg, Yeast extract 0.1 g, agar

†Corresponding author
Phone: 82-51-200-7585, Fax: 82-51-200-6993
E-mail: ylchoi@mail.donga.ac.kr

15 g per liter of distilled water. For liquid media mineral phosphate (tri-calcium phosphate, hydroxyapatite and aluminium phosphate) was sterilized separately and then mixed with the autoclaved medium for above. Phosphate-solubilizing microorganism (PSM) could easily be identified because they developed clear zones around their colonies. Single colonies were inoculated into liquid medium containing 0.5% mineral phosphate source. The amounts of phosphate released into solution and genetic activity were criteria for choosing the most efficient phosphate solubilizing organisms.

Mineral phosphate solubilizing activity of PSM

Bacterial strains were cultured in the sucrose minimal medium including 0.5% mineral phosphate. Culture conditions of 180 rev · min^{-1} and 30℃ were found to lead to the highest free phosphate yields in solution. The cultures were harvested at different growth periods in order to record the change in pH and P-concentration in the medium. Samples were centrifuged 6000 g for 10 min to receive clear solutions for analysis. The P-concentration and pH were determined from supernatant in each investigation. The P-concentration was estimated by the method of ammonum molybdate. The 50~200 $\mu\ell$ of the culture filtrate was mixed with 900 $\mu\ell$ of solution [Sigma Co. 360-3 phosphorus regent] and incubated at room temperature for 10 min. The P concentration was measured spectrophotometrically at 340 nm. In order to observe the effect of cultural conditions for mineral phosphate solubilizing, bacterial strains were cultured at different initial pH (5.0, 6.0 and 7.0), temperature (26, 30 and 37℃) and various insoluble phosphate (tri-calcium phosphate, hydroxy apatite, aluminum phospahte) conditions.

Determination of gluconic acid contents

Gluconic acid content in the culture medium was determined using gluconate dehydrogenase [14] which has a high specificity for gluconate. Gluconate dehydrogenase activity was measured spectrophotometrically at 25℃ in a reaction mixture which contained 50 mM PIPES, pH 6.5, gluconate dehydrogenase 0.1 unit, 0.4 mM pherazine metho sulfate, 0.2 mM dichlorophenol indophenol(DCIP) and the sample obtained from the medium after centrifugation [15,16]. Enzyme activity is measured as the initial reduction rate of DCIP.

Results and Discussion

Isolation of phosphate solubilizing bacteria

Some strains which showed the clear zones around their colonies for phosphate solubilizing were isolated from the salt accumulated and phosphate superfluity treatment soil. Further selection was based on the ability of strains to release phosphate in the culture medium. Altogether about several hundreds exprement were conducted to screen the strain of PSM to the most efficient strains. One bacterium, DA33, having strong phosphate solubilizing property was selected for further characterization. This bacterium was Gram-negative, rod-shaped. The strain was identified to *Aeromonas hydrophila* DA33 based on the physiological and biochemical properties (Table 1).

Characterization of phosphate solubilizing activities under culture conditions

We determined the MPS pattern of *Aeromonas hydrophila* DA33 in liquid cultures that contained known amounts of tri-calcium phophate and analytically measured the levels of P in the medium at different points of 3-day growth period at different temperature (Fig. 1). The amount of released P in the medium showed a gradually increase and reached a concentration of 120.5 $\mu g/m\ell$ in the 30℃ treatment. Concomitant with the P increase there was a drastic decrease in the pH of the medium. The pH value dropped to 4.5 from on initial value of 6.8. This observation is consistent with earlier reports which have shown the solubilization of mineral phosphate is accompanied by a decreased pH [1, 9-12]. Fig. 2 Showed the changes of P-concentration during the cultivation of DA 33 at various initial pHs with time courses. The initial pH of the medium affect the P-solubilizing ability and the optimum initial pH was pH 5.0. Phosphate solubilization of the strain *Aeromonas hydrophila* DA33 was studied using hydroxy apatite, tri-calcium phosphate and aluminum phosphate as mineral phosphate (Fig. 3). The ability of phosphate solubilization by the strain DA33 with hydroxy apatite and tri-calcium phosphate was significantly higher compared to aluminum phosphate. However, there is no significant difference in the pH of culture medium between the three mineral phosphate.

DA33 releasing gluconic acid in the culture medium

It is generally believed that the P-solubilizing activity is correlated with the production of organic acids. In order to examine whether the P-solubilizing activity is also associated with

Table 1. Biochemical, physiological characteristics of DA33.

Characteristics	*Aeromonas hydrophila* DA33
Gram strain	-
Shape	short rods
D-glucose	+
L-arabinose	+
D-ribose	+
D-sucrose	+
Maltose	+
D-sorbitol	-
2-ketogluconate	-
Histidine	+
Alanine	-
Serin	+
Citrate	-
Acetate	+
N-acetyl glucosamine	+
DL-lactate	-
3-hydroxy-benzonate	-
mannito	+

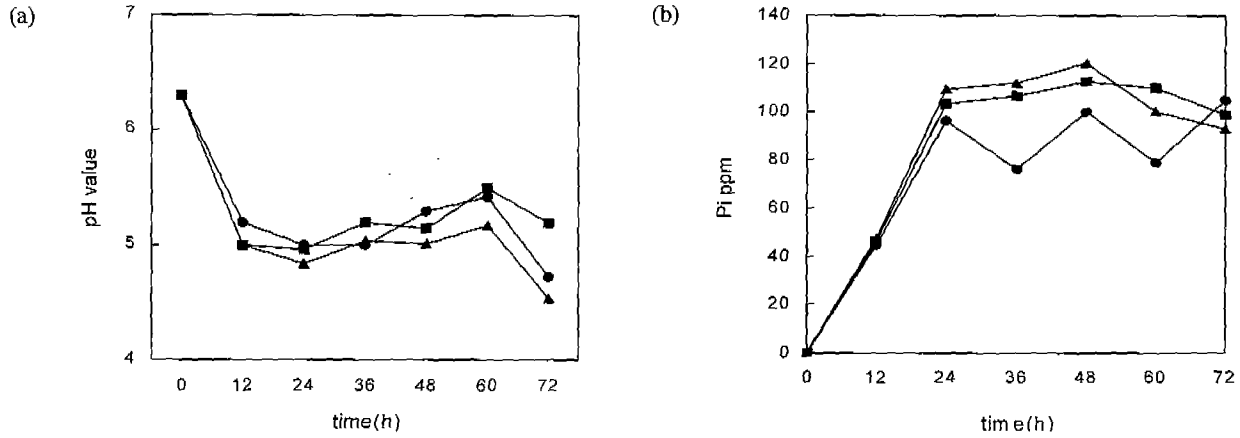

Fig. 1. Changes of free phosphate concentrations during the cultivation of *Aeromonas hydrophila* DA33 at various temperature with time courses. *Aeromonas hydrophila* DA33 was cultured in sucrose minimal medium containing 0.5% tri-calcium phosphate. ■ : 26℃, ● : 30℃, ▲ : 37℃, (a) pH value, (b) free phosphate concentration.

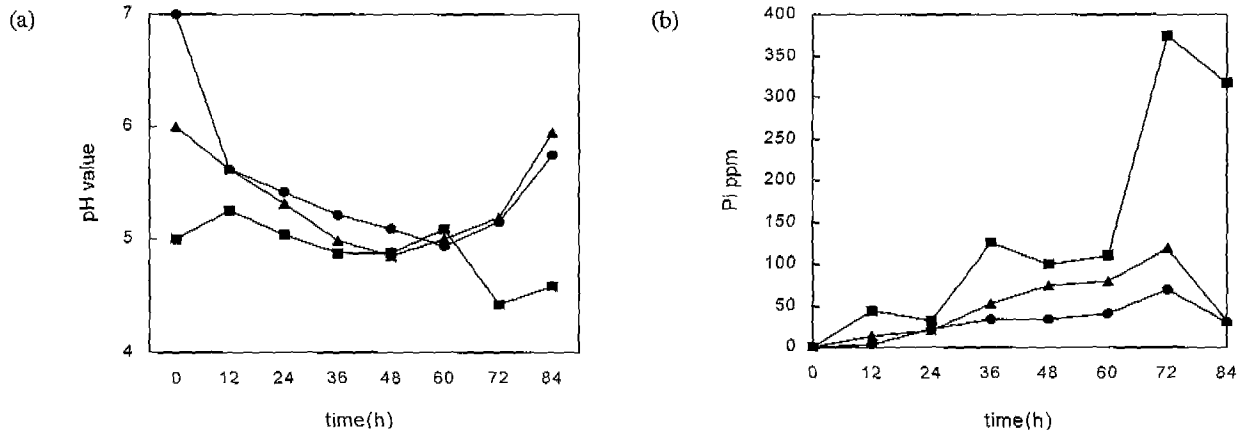

Fig 2. Changes of free phosphate concentrations and pH values during the cultivation of *Aeromonas hydrophila* DA33 at various initial pHs with time courses. *Aeromonas hydrophila* DA33 was cultured in sucrose minimal medium containing 0.5% tri-calcium phosphate at 26℃.
■ : initial, pH 7.0, ● : initial pH 6.0, ▲ : initial pH 5.0, (a) : pH value, (b) : free phosphate concentration

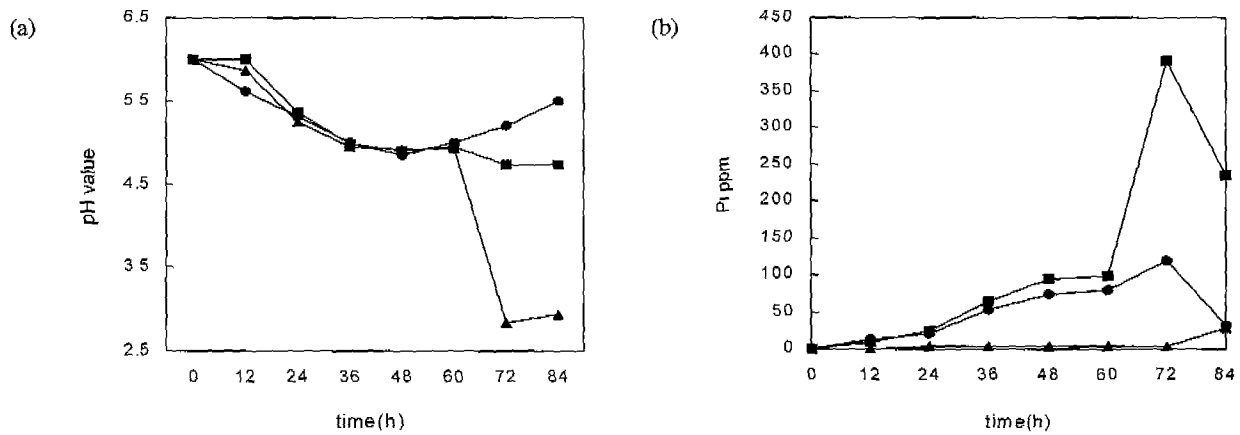

Fig. 3. Changes of free phosphate concentrations during the cultivation of *Aeromonas hydrophila* DA33 at various insoluble phosphate with time courses. *Aeromonas hydrophila* DA33 was cultured in sucrose minimal medium containing 0.5% insoluble phosphate at 26℃. ■ : tri-calcium phosphate, ● : aluminium phosphate, ▲ : hydroxyapatite, (a) pH value, (b) free phosphate concentration.

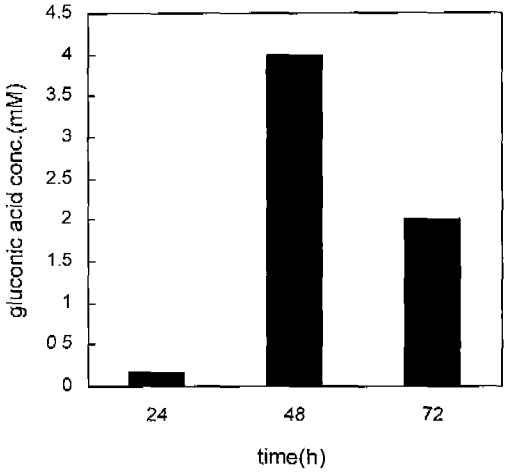

Fig. 4. Gluconic acid production by *Aeromonas hydrophila* DA33. *Aeromonas hydrophila* DA33 was grown in glucose minimal medium containing 21 mM potassium phosphate buffer, pH 6.8

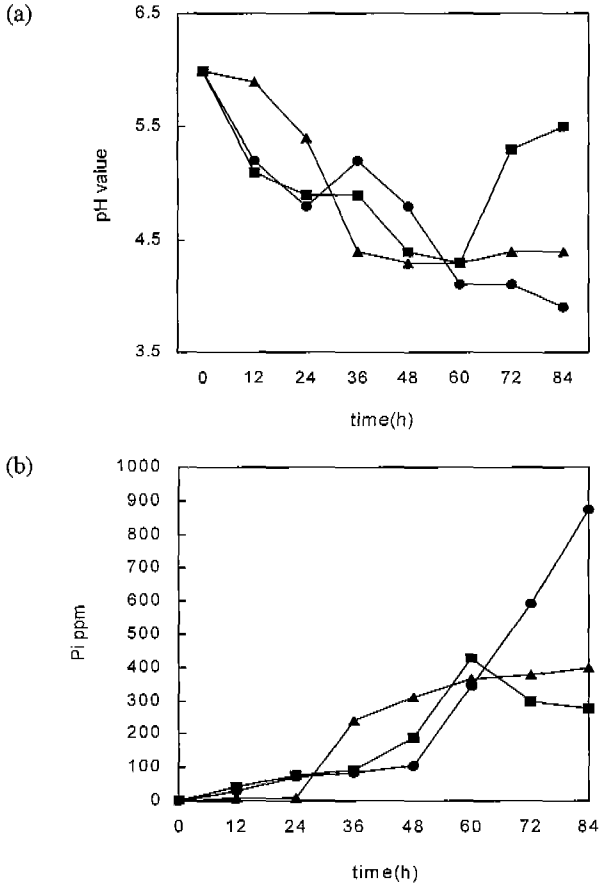

Fig. 5. Changes of free phosphate concentrations during the cultivation of *Aeromonas hydrophila* DA33 at various concentration of glucose in medium with time courses. *Aeromonas hydrophila* DA33 was cultured in glucose minimal medium containing 0.5% tri-calcium phosphate at 26℃.
■ : glucose 1%, ● : glucose 3%, ▲ : glucose 5%, (a) pH value, (b) free phosphate concentration.

organic acid production, we determined the concentration of gluconic acid present in the culture medium. As shown in Fig. 4, *Aeromonas hydrophila* DA33 produced 4 mM gluconic acid when grown with 0.4% glucose medium in the 2-day culture. This result indicates that the phosphate solubilizing ability of this bacteria was caused by the modification of pH brought about by the release of organic acids. It is reported that *Penicillium billaji* secretes 10 mM each of citric acid and oxalic acids [17] and *Citrobacter koseri* and *Bacillus coagulans* secretes various organic acid in the range 1~5 mM [18]. However, the concentration of these acids required to reduce the pH of the soil was 20~50 times more.

Effect of C-source on phosphate solubilizing

To elucidate the influence of C-source of the medium, the phosphate solubilization in *Aeromonas hydrophila* DA33 was estimated in the glucose or sucrose minimal medium. The effect of glucose on the phosphate solubilization was illustrated in Fig. 5. The free phosphates solubilized from sucrose medium were 10 times less than that of glucose medium. It was observed that phosphate solubilization ability increased by about 3% in the glucose medium. P-solubilization was more effective when the glucose concentration in the medium was raised from 1% to 3%. This results is consistent with the earlier report which P-solubilizing ability was increased with increasing concentration of glucose in *Pseudomonas* sp [13]. The ability of the strain to solubilize mineral phosphate in 3% glucose medium was increased at a high level up to 5 days (data not shown).

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