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A Study of the Growth Condition and Solubilization of Phosphate from Hydroxyapatite by *Pantoea agglomerans*

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Abstract The growth conditions of *Pantoea agglomerans,* a phosphate solubilizing organism, were studied in our laboratory to determine the optimal conditions. *Pantoea agglomerans* showed the highest growth rate at 30°C, pH 7.0 and 2 vvm, after 50 h cultivation. A certain relationship between pH and phosphate concentration was evident when the glucose concentration in the medium was changed. Increasing glucose concentration increased the pH buffer action of the broth. At glucose concentrations higher than the optimum concentration of 0.2 M, the cell growth was retarded. *P. agglomerans* consumed glucose as a substrate to produce organic acids which caused the pH decrease in the culture medium. The phosphate concentration in the medium was increased by the presence of the organic acids, which solubilized insoluble phosphates such as hydroxyapatite.

Keywords: *Pantoea agglomerans,* hydroxyapatite, 2-ketogluconic acid, phosphate solubilization bacteria, organic acid

INTRODUCTION

Plant growth and its phosphate nutrition are associated with soluble phosphate released from insoluble rock phosphate. When the phosphate is added into the soil in the form of phosphatic fertilizer, one part is utilized by the plants and the rest of it is converted into insoluble form.

The phosphate content in average soil is about 0.05% (w/w), but only 0.1% of this total soil phosphate can be exploited by plants [1]. As only orthophosphate ions ($H_2PO_4^-$ and HPO_4^{-2}, depending on the pH) can be assimilated in appreciable amounts [2], the free inorganic phosphate ions in soil play an important role in P-cycling and plant nutrition [3-6]. Therefore, many researchers have tried to increase the plant-available phosphate-fraction by means of phosphate-solubilizing microorganisms such as *Pseudomonas* [7], *Bacillus* [8], *Enterobacter* [9,10], *Agrobacterium* and *Aspergillus* [11]. These microorganisms are known to be involved in the solubilization of insoluble inorganic phosphate (hydroxyapatite, $Ca_{10}(PO_4)_6 \cdot (OH)_2$) [3,12]. Alexander reported that phosphate solubilization by phosphate solubilizing bacteria (PSB) was due to their production of organic and inorganic acids [13]. Sperber also identified organic acid production by PSB in culture media [14]. Citric, butyric, malonic, lactic, succinic and gluconic acids were detected in large concentration from various soil samples. Furthermore, Laheurte and Berthelin [9] have described solubilization of inorganic phosphate by the production and release of organic acid in the rhizosphere inoculated with PSB.

To understand the dissolution of insoluble phosphate by microorganisms, we examined phosphate activity by *Pantoea agglomerans* in culture medium containing hydroxyapatite. The relationships between phosphate solubilization, organic acid production and pH were investigated.

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MATERIALS AND METHODS

Microorganism and Media

Pantoea agglomerans (KCTC 2564) was obtained from the Korean Research Institute of Bioscience and Biotechnology. The media used were HY medium (glucose, 10.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; NaCl, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; NH_4NO_3, 1.5 g; hydroxyapatite, 4.0 g; yeast extract, 0.5 g; KCl, 0.2 g; peptone, 0.5 g; agar, 15.0 g per liter of distilled water at pH 7.2) and G broth ($CaCO_3$, 15.0 g; glucose, 108.0 g; peptone, 2.0 g; yeast extract 2.0 g; agar, 15.0 g per liter of distilled water at pH 7.0). All chemicals used in these experiments were of reagent grade.

Selection of Phosphate Solubilizing Bacterium (PSB)

Ten microliters of 10^2 cell/mL *P. agglomerans* suspen-

sion solution was spotted on agar medium containing hydroxyapatite. After 3-days incubation at 30°C, the phosphate solubilizing bacteria developed into colonies with clear halo [14], which were replated on the agar medium supplemented with hydroxyapatite for further isolation. One colony showing the strongest phosphate solubilizing property was selected for further characterization.

Liquid culture of Microorganism

The G broth was used to measure the kinetic changes of glucose and phosphate concentrations. A quantity of 250 mL of G broth in a 500-mL bottle was inoculated with 200 μL of *P. agglomerans* suspension grown in LB medium overnight. Then, it was incubated either with or without hydroxyapatite. After 24 h growth in a rotary shaker at 30°C and 120 rpm, cell population, pH, and concentrations of glucose and organic acids were measured. For determination of optimal aeration rate, a 1-L (working volume 500 mL) bioreactor was used. Aeration conditions were 0, 0.5, 1.0, and 2.0 vvm.

Analytical Methods

The medium pH was measured directly by immersing a glass electrode into the culture broth, allowing the pattern of pH change to be determined. The cell population was determined by the dilution plate methods of Shan and Jelen [15].

For concentration measurement of glucose, phosphate and organic acids, a 3 mL sample was filtered through a 0.2 μm Whatman membrane filter to remove microorganisms. The glucose concentration was determined by HPLC (Shimadzu SPD-10A with LC-10AD Pumps) using an RI detector and a carbohydrate analysis column (3.9 × 300 mm, Shodex) under the following operating conditions; 60% CH_3CN as the mobile phase, a constant flow rate of 2 mL/min, column temperature 30°C, and sample injection of 5 μL. The glucose concentration was quantitatively determined by comparing the retention times and peak areas of the chromatograms with those of the standards.

The organic acid concentrations in the filtrate sample solution were measured by HPLC (Shimadzu SPD-10A with LC-10AD Pumps) using a UV-Vis detector at 210 nm and a pak KC-811 Column (Shodex) under the following operating conditions; 0.1% H_3PO_4/H_2O as the mobile phase, a constant flow rate of 0.5 mL/min, column temperature 25°C, and sample injection of 20 μL. As standards, 2-ketogluconic acid and gluconic acid were used. The organic acid concentrations were quantitatively determined by comparing the retention times and peak areas of the chromatograms with those of the standards that had undergone the same resin treatment as previously described for the unknown acids.

The phosphate concentration was measured by the Lancaster method [10]. 50 μL filtrated samples were added into 50-mL Erlenmeyer flasks containing 1.95 mL of the phosphate extraction solution. Then, 5 mL distilled water, 2 mL ammonium paramolybdate solution (12 mM) and 1 mL $SnCl_2$ (5 mM) were added and mixed well. After 5 min, the phosphate concentration was measured using a spectrophotometer (Hach DR/4000U, USA) at 660 nm.

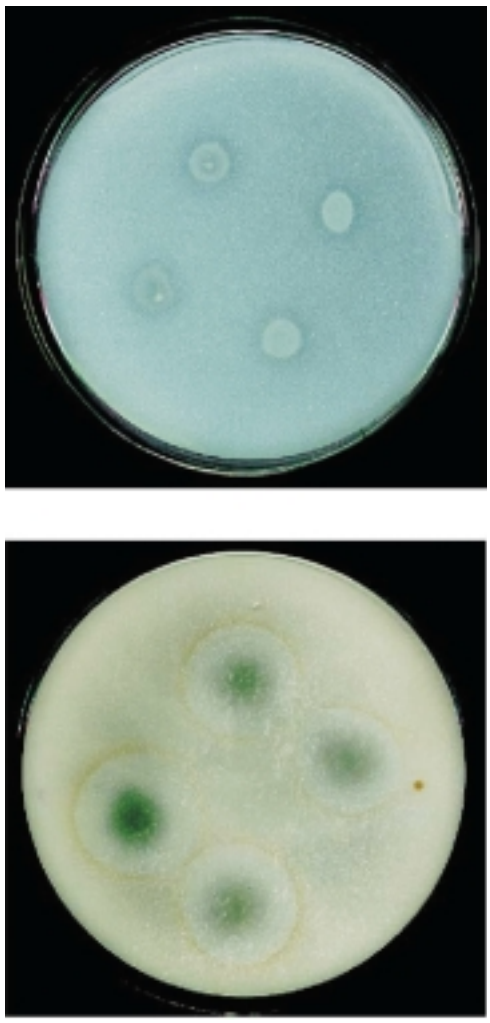

Fig. 1. Clear zones of phosphate solubilization around colonies of *P. agglomerans* on agar medium containing 0.4% hydroxyapatite (A) and G medium (B) at 30°C after 48 h incubation.

RESULTS AND DISCUSSION

Selection and Growth Conditions

After 3 days solid culture at 30°C, the *P. agglomerans* showing the strongest phosphate solubilizing property was selected (Fig. 1), and further cultured at 30°C, pH 7.0, in LB medium and different aeration rates for different times. The highest cell growth shown by *P. agglomerans* was at conditions of 2 vvm aeration rate after 50 h cultivation (Fig. 2).

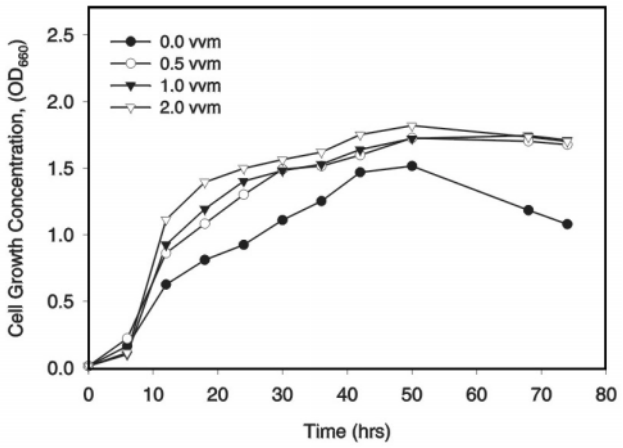

Fig. 2. Effects of several air flow rates on the cell growth of *P. agglomerans*.

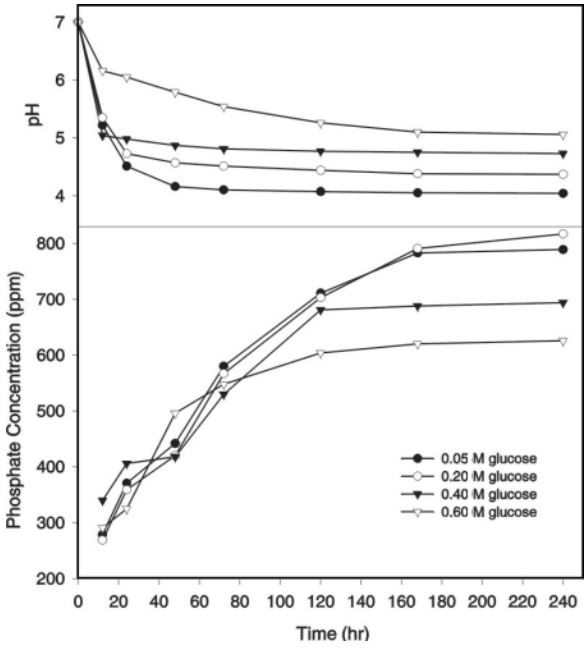

Fig. 3. Changes in pH and phosphate concentration by *P. agglomerans* over 10 days incubation in broth medium containing 0.4% hydroxyapatite at 30°C in non-areated conditions.

Properties of Inorganic Phosphate Solubilization

The relationship between pH and organic acid concentrations, and the effect on pH changes and phosphate concentration of accumulated organic acids over a 10 day incubation period were investigated. The degree of phosphate solubilization by *P. agglomerans* and associated pH changes were determined at different glucose concentrations (0.05 M, 0.2 M, 0.4 M and 0.6 M) in media containing 0.4% hydroxyapatite. The culture growth conditions were 30°C, agitation rate of 120 rpm, pH 7.0 and either the presence or absence of 2 vvm aeration. The increase of glucose concentration resulted

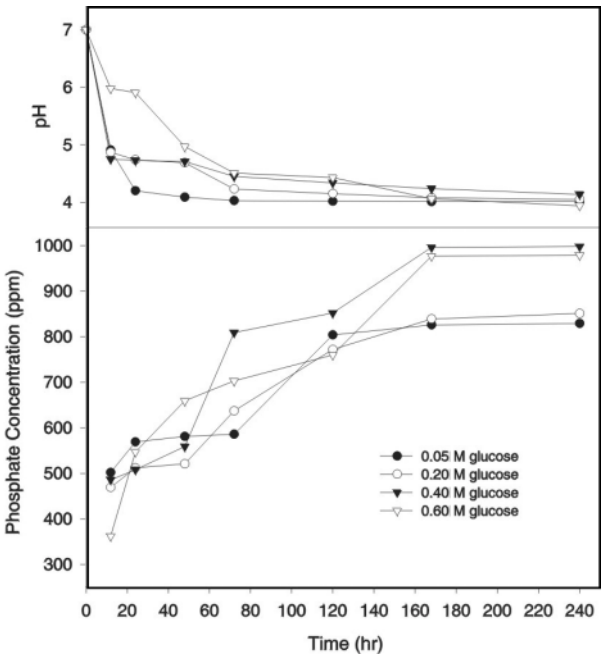

Fig. 4. Changes in pH and phosphate concentration by *P. agglomerans* over 10 days incubation in broth medium containing 0.4% hydroxyapatite at 30°C in aerated condition.

in a pH buffer action in the broth for both aerated and non-aerated conditions. Furthermore, the max growth was achieved at glucose concentrations of 0.05 M in non-aerated (Fig. 3) and 0.6 M in aerated (Fig. 4). The solubility of phosphate and decreased pH were due to the production of organic acids.

Microorganisms utilized glucose as a substrate to produce organic acids. Under the aerated condition, the glucose concentration was decreased to about 61.2 g/L after 10 days cultivation. However, the glucose concentration of the non-aerated cultivation was only reduced to about 76.5 g/L, indicating that *P. agglomerans* consumed more glucose in the aerated cultivation than in the non-aerated cultivation (Fig. 6). After 3 days cultivation with aeration, the glucose concentration decreased from about 108.7 g/L to 59.6 g/L, but in the non-aerated cultivation it was decreased from 108.7 g/L to 36.9 (Fig. 6).

The phosphate concentrations were increased independently of aeration. Under aeration, the phosphate concentration was increased from about 349 ppm to 1,675 ppm. However in the non-aerated cultivation it was increased from 242 ppm to 1,164 ppm (Fig. 5). However, more glucose was consumed under aeration.

Formation of Organic Acids by Microorganism

We observed that the retention time of each standard was about 13.2 min for 2-keto gluconic acid, about 12.6 min for gluconic acid and about 9.9 min for distilled water. A great deal of variation was initially observed in the concentrations of organic acids released by *P. ag-*

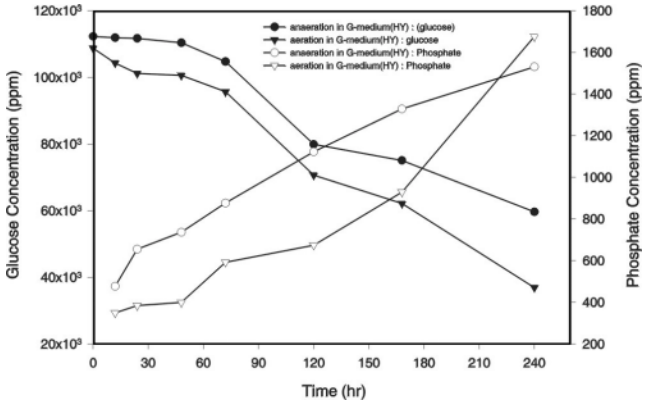

Fig. 5. Changes in glucose and phosphate concentration by *P. agglomerans* over 10 days incubation in broth G medium containing 0.8% hydroxyapatite at 30°C.

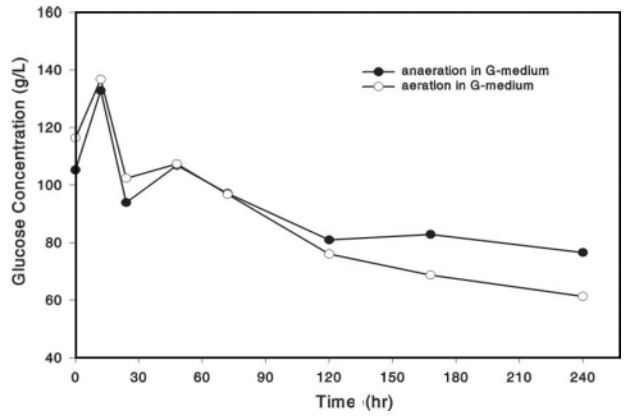

Fig. 6. Changes in glucose concentration by *P. agglomerans* over 10 days incubation in broth G medium containing no hydroxyapatite at 30°C.

glomerans. The retention times based on the range of the standards were 13,990 and 23,402 after 3 days, 14,960 and 42,508 after 5 days, and 28,519 and 70,611 after 7 days, for 2-keto gluconic acid and gluconic acid, respectively. The amount of organic acid was increased with time. El-Gibaly *et al*. reported that the pH values during the cultivation of the selected microbial isolates showed an inverse correlation with the quantity of soluble phosphate [16]. The inverse correlation between the pH value and the released amount of phosphate indicated that the phosphate solubility was directly correlated with the concentration of produced acids. According to Louw and Webley [12], PSB could release many kinds of organic acids, such as oxalic, citric, lactic, gluconic, acetic and 2-ketogluconic acids.

Relationship Between pH and Bacterial Population

Under aeration condition, pH was decreased from 7.33 to 3.62, whereas in non-aerated cultivation pH was decreased only from 7.24 to 5.49 (Fig. 7). For both aerated and non-aerated conditions, the CFU (colony

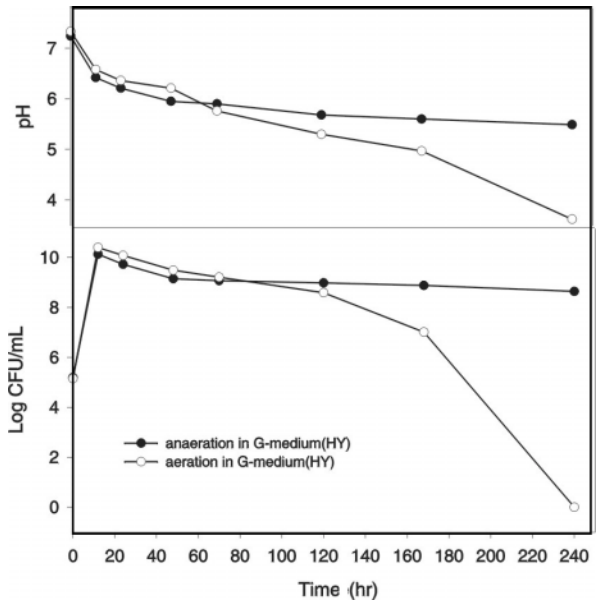

Fig. 7. Changes in pH and population of *P. agglomerans* over 10 days incubation in broth G medium containing 0.8% hydroxyapatite at 30°C.

forming unit) was nearly the same after 10 h cultivation; aerated being 10.40 log CFU/mL and non-aerated 10.13 log CFU/mL. After 120 h cultivation, *P. agglomerans* maintained its CFU under the non-aerated condition but decayed under the aerated condition (Fig. 7). A pH buffer action was observed in the non-aerated condition but not in the aerated condition. The decrease of pH due to the organic acid production and nutrients exhaustion might have reduced the *P. agglomerans* population. Therefore, these results regarding the changes in pH and population of *P. agglomerans* (Fig. 7) suggest that the extinction of *P. agglomerans* due to increasing organic acid production indicates the absence, or at least delay, of phosphate solubilization (Figs. 3, 4).

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