Inorganic Phosphate Solubilization by Immobilized
*Pantoea agglomerans* under *in vitro* Conditions

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It is now widely accepted that immobilized microbial cells can overcome some of the problems associated with microbial survival, stability, efficacy, storage, transportation and ease of application in agricultural environments. *Pantoea agglomerans*, a phosphate solubilizing bacterium, was immobilized in alginate, agar and gelatin carriers. All the three immobilized carriers with bacterial cells of *P. agglomerans* were compared for solubilization of tricalcium phosphate in pure liquid cultures. While alginate beads were tested for phosphate solubilization on alternate days up to five days, agar beads and gelatin cubes were subjected for one time phosphate solubilization analysis after seven days. Both alginate and agar immobilized cells of *P. agglomerans* exhibited higher efficiency in increasing the solubilization of tricalcium phosphate than gelatin immobilized cells. The culture filtrate of alginate bead inoculation treatment registered a rapid increase in soluble phosphate concentration upon incubation. A corresponding decrease in the pH of the medium was also observed in all the treatments.

**Key words**: Immobilization, *Pantoea agglomerans*, Phosphate solubilization, Tricalcium phosphate.

**Introduction**

Phosphorus is one of the major essential macronutrient for biological growth and development (Ehrlich, 1990). Compared with the other major nutrients, phosphate is by far the least mobile and available to plants in most soil conditions and always a major or even the prime limiting factor for plant growth. Though plants absorb phosphates added to soil in the form of phosphatic fertilizers, a larger portion is converted into insoluble fixed forms. Although phosphate ions can reach larger concentrations in highly fertilized soils, in many soils their concentration in the soil solution is in micro molar range that is low compared with adequate P concentrations required for optimal plant growth (Hinsinger, 2001). Moreover, problems with the fertilizer technology include energy intensive production processes with associated environmental consequences.

Considerable number of plant rhizosphere associated microbes could exert beneficial effects upon plant growth and development. Isolation and reuse of such microbes as biofertilizers for agricultural improvement would be beneficial and has been a focus of numerous researchers for more years (Rodrigues and Fraga, 1999). The most promising approach is to use the phosphate solubilizing heterotrophic microorganisms as biofertilizers (Goldstein, 1993). Biofertilizers can be taken to the fields in various forms *viz*. bulk carriers like peat, lignite, soil-sand mixtures, vermiculite, perlite or in lightweight easy to use immobilized carriers like agar, β-carragenan, alginate, gelatin, PAG (Polyacrylamide gel), etc.

Methods of immobilization by cell entrapment in gel carriers have been well accepted in the field of preparation of *Rhizobium* bearing formulations (Van Elsas and Heijnen, 1990), but only recently has the use of immobilized soil microorganisms been extended to other microbial groups including plant growth promoting rhizobacteria (Bashan, 1998; Deleereeck et al., 1996; Vassilev et al., 2001). In fermentation conditions, immobilized cell formulation systems avoid wash out of cells, ensure higher cell concentration in small volumes, and provide easy product separation (Fedirici, 1993). Advantage of immobilized cell technology applied in solubilization of insoluble inorganic phosphates and plant P acquisition has been reviewed recently (Vassilev et al., 2001).

However with easy storability and long term viability (Bashan and Gonzalez, 1999) immobilization in
lightweight easy to use carriers offers wonderful alternatives for bulk carrier materials.

In the present work, we report the results on calcium phosphate (tribasic) solubilization by \textit{P. agglomerans} immobilized in three different carriers \textit{viz.} agar, alginate and gelatin.

\textbf{Materials and Methods}

\textbf{Microorganisms} A phosphate solubilizing bacteria, \textit{P. agglomerans} described earlier (Chung, 2003) was used throughout the experiment.

\textbf{Growth Medium} Pikovskaya's broth (Pikovskaya, 1948) was used throughout the study. The pH was adjusted to 7.0 and the medium was autoclaved at 121°C for 15 min.

\textbf{Multiplication conditions} \textit{P. agglomerans} was maintained on Pikovskaya's agar slants at 4°C by subculturing monthly. For mass multiplication, the bacterium was grown in nutrient broth for 24 h in a rotary shaker (120 rpm) at 30°C. After incubation, the cells were harvested by centrifugation (10,000xg) and the pellet was washed with sterile distilled water. Later the cells were adjusted to 1 x 10^6 cells mL^{-1} concentration and used for immobilization.

\textbf{Immobilization procedure}

\textbf{Sodium alginate beads} Five ml of the bacterial cell suspension was mixed in 20 mL of autoclaved and cooled 2.5, 3.5 and 5% sodium alginate solutions respectively and then extruded in 200 mL of 0.5 M calcium chloride solution. The beads formed were incubated for hardening for one hour at 4°C and later were washed with sterile distilled water and stored for future studies (Woodward, 1988).

\textbf{Agar beads} Five ml of the bacterial cell suspension was mixed in 2, 3 and 4% agar (Bacto Agar, Difco) solutions respectively at 40°C and mixed well. The mixed solutions were extruded in 200 mL of vegetable oil (corn oil) and the beads formed were incubated for 1 h at 4°C and later were washed with sterile distilled water and stored for future studies (Lopez et al., 1997).

\textbf{Gelatin cubes} Five, ten and fifteen grams of gelatin was dissolved in 100 mL of water to prepare a 5, 10 and 15% (wt.) aqueous solutions and heated gently. The temperature of the gelatin solution was adjusted to 35-40°C and the bacterial suspension (1x10^8 cells mL^{-1}) was added and mixed well. To these, 2 mL of the hardening solution (20% formaldehyde, 50% ethanol and 30% water) was added. Immediately after mild vortexing the mixture was poured into a mold and kept at 4°C for 18 h to facilitate cross linking and complete gel formation. When the gels are set, they were brought to room temperature and cut into small cubes of approximately 3.5 mm and washed liberally with sterile distilled water and stored in refrigerator (de Alteriis et al., 1985).

\textbf{Phosphate solubilization studies} Pikovskaya's medium (100 mL) supplemented with 0.5 g of Ca_3(PO_4)_2, in 250 mL flasks was sterilized at 121°C after adjusting the pH to 7.0. The flasks were inoculated with 5 beads or cubes (1x10^6 cells bead^{-1} or cube^{-1}) per flask and incubated on a rotary shaker (120 rpm) at 30°C. Two set of experiments were carried out: 1) Time course analysis of alginate beads inoculation on phosphate solubilization, where samples were drawn on 0, 1, 3 and 5 d after inoculation. 2) One time analysis of tricalcium phosphate solubilization by alginate, agar and gelatin immobilized cells was performed after seven days of inoculation. Culture filtrates were obtained by centrifugation (10,000xg) and the supernatant was passed through a 0.45 μm Millipore filter and was used to determine pH and phosphate concentration. The pH of the broth was measured by directly immersing the glass electrode connected to a pH meter into the filtrate. The soluble phosphate concentration in the filtrate was estimated following the chlorostannous reduced molybdo-phosphoric acid blue method (Murphy and Riley, 1962) and the results are expressed in mg P L^{-1}.

\textbf{Results and discussion}

\textit{P. agglomerans} cells were entrapped in three different concentrations of three different carriers \textit{viz.} alginate, agar and gelatin (Fig. 1). Alginate at 2.5% concentration was found to be better than the other two concentrations \textit{viz.} 3.5 and 5%. Whereas, agar at 3% concentration was better than other concentrations studied (2 and 4%). Gelatin at 10% was better than 5 and 15% concentrations respectively. The basic characters of immobilized beads/cubes used to identify a suitable concentration were, activity of microbes in carriers, diffusion of beads
Table 1. Physical and aesthetic properties of the beads/cubes.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Bead diameter (mm)</th>
<th>Diffusion over prolonged incubation</th>
<th>Microbial cell activity</th>
<th>Appearance and stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>2.5 % 3.0</td>
<td>No</td>
<td>Active</td>
<td>Flexible and partially stable</td>
</tr>
<tr>
<td></td>
<td>3.5 % 3.0</td>
<td>No</td>
<td>Active</td>
<td>Hard and stable</td>
</tr>
<tr>
<td></td>
<td>5.0 % 3.0</td>
<td>No</td>
<td>Active</td>
<td>Hard and brittle nature</td>
</tr>
<tr>
<td>Agar</td>
<td>2.0 % 3.0</td>
<td>Yes</td>
<td>Active</td>
<td>Flexible and partially stable</td>
</tr>
<tr>
<td></td>
<td>3.0 % 3.0</td>
<td>No</td>
<td>Active</td>
<td>Hard and stable</td>
</tr>
<tr>
<td></td>
<td>4.0 % 3.0</td>
<td>No</td>
<td>Poor</td>
<td>Hard and stable</td>
</tr>
<tr>
<td>Gelatin</td>
<td>5.0 % 3.5</td>
<td>Yes</td>
<td>Active</td>
<td>Highly flexible and collapsing nature</td>
</tr>
<tr>
<td></td>
<td>10.0 % 3.5</td>
<td>No</td>
<td>Moderate</td>
<td>Flexible and partially stable</td>
</tr>
<tr>
<td></td>
<td>15.0 % 3.5</td>
<td>No</td>
<td>Poor</td>
<td>Stable; brittle nature</td>
</tr>
</tbody>
</table>

over prolonged incubation and appearance and stability of the beads (Table 1).

The immobilized *P. agglomerans* cells (2.5% alginate, 3% agar and 10% gelatin) were incubated in 250 mL conical flasks with broth volumes of 100 mL and the solubilization of phosphates was estimated. The bacterial strain solubilized phosphates and acidified the medium in

![Fig. 1. Images showing gelatin(A), alginate(B) and agar(C) immobilized cells of *P. agglomerans*. The size of gelatin cubes, alginate beads and agar beads were 3.5, 3 and 3mm, respectively.](image)

![Fig. 2. Effect of alginate immobilized *P. agglomerans* cells on the soluble phosphate concentration(a) and pH(b) in the calcium phosphate (tribasic) amended media over a period of incubation.](image)
shake flask cultures efficiently. When the P solubilization potential of the sodium alginate immobilized cell inoculated flasks was studied on alternate day basis the results were encouraging, where the soluble P concentration was increased from 0.22 to 64.53 mg P L⁻¹ 5 d after inoculation indicating the exemplary performance of the immobilized cells to solubilize calcium phosphate (tribasic) in a rapid manner (Fig. 2a). A corresponding reduction in pH of the culture filtrate with an average acidification level of about 1.74 pH units was also noticed (Fig. 2b).

When the solubilization of calcium phosphate (tribasic) was compared using three immobilized forms of *P. agglomerans* cells, agar and alginate were found better than gelatin immobilization. The soluble P concentration observed was 72.0, 71.5 and 36.8 mg P L⁻¹ respectively (Fig. 3a). Similar results on the advantages of employing immobilized fungi and bacteria like *Aspergillus niger*, *Enterobacter* sp., (Vassilev et al., 1997a,b; Vassileva et al., 1998), *Bacillus megaterium* (Zayed, 1997) *Penicillium variabile* (Fenice et al., 2000) and yeast like *Yarrowia lipolytica* (Vassileva et al., 2000) in phosphate solubilization have been reported earlier. The pH of the three treatments stood at 5.8, 5.69 and 5.67, respectively in this study (Fig. 3b). However, the results were not comparable, as the pH in the gelatin treatment observed was less than the other two.

All the early reports indicate the advantages and superiority of this technology over free cell inoculations. According to Zayed (1997), alginate immobilization has the potential to protect bacteria from bacteriophages that may influence the activity of bacteria in the rhizosphere. Hence, immobilization could well be recommended in all phosphate solubilization systems especially in agriculture. All the studies conducted earlier address the use of agar, alginate, κ-carrageenan or polyurethane foam. Our report forms the first study to use bacteria immobilized gelatin cubes for phosphate solubilization.

The least encouraging results observed in this study on the use of gelatin could be related to the use of formaldehyde and ethanol as crosslinkers, which might have had an impact on the bacterial cells upon exposure. However, further studies are needed to evaluate the impact of crosslinkers like formaldehyde, ethanol, glutaraldehyde and gamma rays on the bacterial population inhibition and the related microbial load in gelatin cubes. Studies are underway in our laboratory to study this and use of other polymer based carriers for immobilization and phosphate solubilization.

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**References**


고정화된 \textit{Pantoea agglomerans}에 의한 난용성 인산의 가용화

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최근에는 친환경 농업을 위하여 사용되는 미생물 비료의 안정성 유지와 효율, 저장 및 운송 등에 대한 어려움 및 불편함을 극복하기 위해서 미생물을 담체에 고정화하여 생물비료로 사용하고 있다. 본 실험에서는 인산가용화 미생물인 \textit{Pantoea agglomerans}를 alginate, agar 및 gelatin에 고정화시킨 후 tricalcium phosphate가 첨가된 액체 배지에서 배양하여 인산가용화능을 비교하였으며, 또한 alginate에 고정화된 균에 의한 인산가용화능을 검
시적으로 조사하였다. 고정화된 담체에 alginate나 agar를 사용하였을 때, 고정화된 \textit{P. agglomerans}의 인산가용화능이 gelatin을 담체로 사용하였을 때보다 우수하였다. Alginate와 고정된 \textit{P. agglomerans}가 접촉된 처리구에는 배양 후 5일까지 유효인산의 농도가 크게 증가하였고 모든 처리구에서 배지 안의 pH가 감소하였다.