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# Effect of Carbon Substrates on Rock Phosphate Solubilization by Bacteria from Composts and Macrofauna

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**Abstract.** Five of the 207 isolates from different composts, farm waste compost (FWC), rice straw compost (RSC), *Gliricidia* vermicompost (GVC), and macrofauna, showed rock phosphate (RP) solubilization in buffered medium in plate culture. When tested in RP broth medium, all five strains, *Enterobacter cloacae* EB 27, *Serratia marcescens* EB 67, *Serratia* sp. EB 75, *Pseudomonas* sp. CDB 35, and *Pseudomonas* sp. BWB 21, showed gluconic acid production and solubilized RP. Based on cellulose-degrading and P-solubilizing ability, two strains were selected for further studies. In the presence of different carbon sources, both strains showed a drop in pH and solubilized RP. P released was maximum with glucose (1212 and 522 μmol) and minimum with cellobiose (455 and 306 μmol) by *S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35, respectively. Glucose dehydrogenase (GDH) activity was 63 and 77% with galactose and 35 and 46% with cellobiose when compared to glucose (100%) by EB 67 and CDB 35, respectively. Both strains solubilized RP in the presence of different crop residues. EB 67 and CDB 35 showed maximum cellulase activity (0.027 units) in the presence of rice straw and a mixture of rice straw and root. P solubilized from RP in the presence of pigeonpea root was 134 and 140 μmol with EB 67 and CDB 35. Significantly, these bacteria isolated from composts and macrofauna solubilized rock phosphate in the presence of various pure carbon substrates and crop residues and their importance in soil/rhizosphere conditions is discussed.

Intensification of agriculture and increases in population pressure have reduced the structural stability of soils. To cope with low productivity, inorganic fertilizers have been intensively used for the past two decades. This escalated the soil problems including structural degradation, reduction of organic matter, soil colloidal content, and cellulose. Agricultural residues are a rich source of cellulose, hemicellulose, and lignin in an average ratio of 4:3:3, although the exact percentage of these components may vary [1]. Cellulose is an unbranched glucose polymer, linked by 1,4-ß-D glucoside bonds, which can be hydrolyzed by cellulolytic enzymes produced by bacteria and fungi [2, 3]. Due to its polymeric nature, bioprocessing of cellulose is limited. Cellulolytic organisms are aerobic species such as *Pseudomonas* and actinomycetes, facultative anaerobes such as *Bacillus* and *Cellulomonas*, and strict anaerobes such as *Clostridium* [4, 5]. The majority of agricultural soils contain large reserves of phosphorous (P), of which a considerable part is accumulated as a consequence of regular applications of phosphatic fertilizers [6]. Soils like Indian alkaline Vertisols are rich in Ca-P complexes and have a high buffering capacity. These soils are low in organic matter and cation exchange capacity [7, 8]. Diverse groups of microorganisms in soil employ a variety of solubilization reactions to release soluble P from insoluble phosphates [9–11]. The present investigation was carried out to characterize the bacteria isolated from different composts and macrofauna for solubilizing rock phosphate and recycling crop residues, with the view of utilizing them as biofertilizer sources to boost the fertility of soils.

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## Materials and Methods

Bacteria were isolated on LB agar medium from three different composts [farm waste compost (FWC), rice straw compost (RSC), and *Gliricidia* vermicompost (GVC)] and macrofauna (body surface and excreta of earthworms, centipedes, slugs, and snails) present in FWC. A total of 207 isolates were obtained and screened for different plant growth-promoting traits including cellulose degradation on rice straw agar medium (unpublished data) and P-solubilizing ability in buffered RP agar [12]. Five of the 207 bacterial isolates (EB 27, EB 67, EB 75, CDB 35, and BWB 21) showed RP solubilization in plate culture and the quantitative studies were carried out in RP broth medium. Aliquots were taken and growth was monitored using spectrophotometer (Shimazu) $OD_{600}$ nm and a drop in pH at 24-h intervals. All the isolates were grown until the pH of the medium was reduced to 5.0. Culture supernatant was filtered using nylon-66 membrane filters of 0.22-μm pore size and subjected to HPLC with the reverse phase, ion pairing column (RP-18), and the mobile phase consisted of 0.1% phosphoric acid at a flow rate of 1 ml $min^{-1}$. Detection was performed by a UV/VIS detector at 210 nm. HPLC profile of the culture supernatants was analyzed by comparison with the elution profile of pure organic acids. P released was estimated by the ascorbate [13] method. The experiment had two replications and was repeated thrice.

Based on cellulolytic and plant growth-promoting traits, two bacterial strains *Serratia marcescens* EB 67 and *Pseudomonas* sp. CDB 35 were selected for future studies. They were tested for RP solubilization in the presence of 100 mmol Tris-Cl $L^{-1}$, pH 8.0, and different carbon sources (glucose, xylose, galactose, mannose, maltose, cellobiose, and arabinose at 100-mmol concentrations and a mixture of all carbon source at 15 mmol each). The broth was inoculated with an overnight grown culture of EB 67 and CDB 35 in separate flasks. Each culture was washed twice with saline and subsequently diluted 1:100 times. Of this dilution, 250 μl was used to inoculate 50 mL RP broth in a 250-mL flask. The organisms were allowed to grow aerobically at 30 ± 1°C on a rotary shaker at 200 rpm. Aliquots were taken for measurement of pH and $OD_{600}$ at 24-h intervals to monitor bacterial growth and centrifuged at 5000*g* for 10 min; the supernatant was used for P estimation and organic acid produced by HPLC. For GDH activity, both EB 67 and CDB 35 were grown on glucose RP buffered medium for 30 h. After growth, cells were harvested (5000*g* for 10 min), washed with sterilized saline, and resuspended in Tris-Cl (50 mmol), pH 8.75, and the whole cell suspensions were used as a source of enzyme in GDH assay [14]. For determining the GDH activity, both the strains were grown on glucose as the sole C source and different sugars (100 mmol) were used as substrates in the GDH assay to measure the direct oxidation. Total protein was estimated using a modified Lowry's method [15]. The experiment had two replications and was repeated thrice.

In RP buffered medium, glucose was replaced with different crop residues like rice straw, rice root, mixture of rice straw and root, pigeonpea root, and grass (*Cynodoctilan* sp.) separately (at 1%), and cellulase activity [16] and RP solubilized were determined. For this, 100 mL broth with each crop residue was taken in 250-mL flasks and media were sterilized by autoclaving at 121°C for 15 min. For inoculation, an overnight grown culture of bacteria was centrifuged at 5000*g* for 10 min and the cell pellet was washed twice with saline and subsequently diluted to 100-fold. Of this dilution, 500 μl was used to inoculate 100 mL broth in a 250-mL flask. Incubation was carried out aerobically at 30 ± 1°C for a period of 12 days. The samples were collected at day 4, 6, 8, and 12, centrifuged at 15,000 rpm for 30 min, and the supernatant was used to test cellulase enzyme activity, reducing sugars and the P released. Each treatment had two replications and was repeated twice. Data are subjected to analysis of variance (ANOVA) and Duncans multiple range test (as required) using the Genstat 6.1 statistical package (Lawes Agricultural Trust, Rothamsted, UK).

## Results

Five of the 207 bacterial isolates, *Enterobacter cloacae* EB 27, *Serratia marcescens* EB 67, *Serratia* sp. EB 75, *Pseudomonas* sp. CDB 35, and *Pseudomonas* sp. BWB 21, showed mineral phosphate solubilizing (MPS) ability in plate culture conditions. When inoculated in RP broth medium, all phosphate-solubilizing bacteria (PSB) showed a drop in pH within 96 h in the presence of glucose and solubilized RP. The gluconic acid produced was highest with EB 67 (67 mmol) followed by EB 27 (35 mmol), CDB 35 (27 mmol), BWB 21 (22 mmol), and EB 75 (20 mmol). RP solubilized was highest with EB 67 (1036 μmol), followed by EB 27 (748 μmol), CDB 35 (560 μmol), EB 75 (535 μmol), and BWB 21 (430 μmol). RP solubilization was dependent on the amount of gluconic acid produced with the bacterial strains. Correlation analysis between gluconic acid and RP was significant ($r^2 = 0.95$ at $P < 0.001$).

Based on cellulolytic and other plant growth-promoting traits (unpublished data), a detailed investigation of two PSB, *S. marcescens* EB 67 (isolated from slug body surface) and *Pseudomonas* sp. CDB 35 (isolated from RSC), was done in RP broth medium using different carbon sources. Growth of EB 67 was optimum at 48 h in the presence of glucose and mixture of sugars, at 72–96 h with other carbon sources. However, there was an increase in growth of EB 67 combined with mannose even at 120 h (data not shown).

GDH activity with *S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 was highest on glucose (1323 and 679 units as 100%) followed by galactose (63 and 75%), xylose (59 and 61%), mannose (46 and 54%), maltose (44 and 48%), and cellobiose (35 and 46%) (Table 1). Drop in pH by EB 67 ranged from 5.6 with cellobiose to 3.2 with glucose. CDB 35 showed optimum growth in the presence of glucose and mannose at 48 h and with rest of the carbon sources at 72 h. Drop in pH by CDB 35 ranged from 5.0 with cellobiose to 4.1 with maltose (Table 1).

*S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 showed gluconic acid production in the presence of glucose whereas with other sugars there was no gluconic acid formation. It appears that the other sugars are oxidized by these strains to produce acids resulting in a drop in pH. Both the strains EB 67 and CDB 35 solubilized P in the presence of all carbon sources except arabinose. Solubilization of RP was maximum in the presence of glucose 1212 and 522 μmol followed by

Table 1. Drop in pH and P released by *S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 in RP medium in the presence of different sugars

| | *S. marcescens* EB 67 | | | *Pseudomonas* sp. CDB 35 | | |
|---|---|---|---|---|---|---|
| Carbon source | GDH activity (U $mg^{-1}$ protein) | pH | P released (μmol) | GDH activity (U $mg^{-1}$ protein) | pH | P released (μmol) |
| Glucose | 1323 (±11.4) | 3.2 (±0.04)[a] | 1212 (±10.5)[a] | 679 (±7.4)[a] | 4.2 (±0.04)[a] | 522 (±0.9)[a] |
| Galactose | 830 (±12)[b] | 3.9 (±0.37)[a] | 836 (±8)[b] | 511 (±8.7)[b] | 4.6 (±0.06)[ab] | 449 (±4.1)[b] |
| Xylose | 776 (±11.6)[b] | 4.6 (±0.28)[ab] | 775 (±6.8)[c] | 417 (±17.4) | 4.1 (±0.04)[a] | 356 (±13.1) |
| Mannose | 608 (±16.3)[c] | 4.5 (±0.37)[ab] | 573 (±3.5)[d] | 366 (±14.9)[d] | 4.5 (±0.01)[a] | 343 (±1.8)[c] |
| Maltose | 580 (±0.9)[c] | 4.7 (±0.04)[ab] | 540 (±1.5)[d] | 323 (±7.8)[eg] | 4.1 (±0.2.1)[a] | 3 66 (±4. 7)[c] |
| Cellobiose | 455 (±14.6)[d] | 5.6 (±0.33)[b] | 455 (±9.5)[e] | 305 (±0.6)[e] | 5 (±0.36)[b] | 306 (±3.0)[d] |
| Arabinose | 0[e] | 7.8 (±0.12)[c] | 0[f] | 0[f] | 7.6 (±0.27)[c] | 0[e] |
| Mixture | 536 (±5.6)[cd] | 3.9 (±0.57)[a] | 642 (±6.0)[g] | 346 (±27.6)[g] | 4.1 (±0.08)[a] | 411 (±8.1)[f] |
| Mean | 639 | 4.7 | 637 | 368 | 4.7 | 344 |
| LSD (P = 0.05) | 96.7 | 0.36 | 40.5 | 30.3 | 0.29 | 34.6 |
| *cv%* | 9 | 2 | 4 | 5 | 2 | 6 |

Drop in pH and P released after 120 h. Values in parantheses are ± standard errors. Means with the same letter(s) within each parameter are not significantly different when compared by DMRT (Duncans multiple range test).

galactose (836 and 449 μmol) and was least with cellobiose (455 and 306 μmol) (Table 1).

Both strains EB 67 and CDB 35 could utilize cellulose and rice straw as a sole carbon source and were evaluated for the cellulase activity in the presence of different crop residues. Cellulase activity was detected in submerged conditions using RP media amended with 1% crop residue, namely, rice straw (RS), rice root (RR), rice straw and root (RS + RR), pigeonpea root (PR), grass (GS), and commercially available cellulose (CL). The results of cellulase enzyme activity, RP solubilized and the sugars liberated due to cellulose (crop residues) breakdown are expressed as mean values of 4, 6, 8, and 12 days. Cellulase activity was maximum (0.027 units) in the presence of RS with EB 67 and RS + RR with CDB 35 and was least with grass (0.015 to 0.017 units) (Table 2). RP solubilized was maximum (134 μmol) in the presence of PR with *S. marcescens* EB 67 followed by RR, RS + RR, RS, GS, and CL. *Pseudomonas* sp. CDB 35 also showed maximum RP solubilization (140 μmol) in the presence of PR followed by RS + RR, RS, RR, GS, and CL (Table 2). Sugars liberated using different crop residues ranged between 103–146 μg with EB 67 and 83–145 μg with CDB 35 (Table 2).

## Discussion

The results of screening bacteria from composts and macrofauna for P solubilization in buffered medium showed that only five of the 207 isolates solubilized RP and this corroborates with the fact that buffering capacity reduces the phosphate-solubilizing activity of the microorganisms [17]. The release of soluble phosphate by microrganisms from RP usually involves production of organic acids and a decrease in pH of the medium [18]. Mineral phosphate-solubilizing (MPS) bacteria utilize the direct oxidation pathway to produce gluconic acid and 2 keto-gluconic acid. As a result, the activity of periplasmic or membrane-bound GDH is one of the best-studied mechanisms by which MPS bacteria liberate P from poorly soluble mineral phosphates. GDH activity of two strains, *S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35, varied depending on the nature of sugars used as a carbon source and was highest with glucose followed by galactose, xylose, mannose, maltose, mixture, and cellobiose (Table 1, after 30 h), whereas the reported GDH activity of *Enterobacter asburiae* PSI3 was highest with galactose followed by glucose and other sugars and least with cellobiose [19]. P released by *E. asburiae* PSI3 was maximum with glucose followed by cellobiose and least with a mixture of sugars [19]. Plants secrete various root exudates such as carbohydrates, organic acids, amino acids, and so on, to varying extents [20]. EB 67 and CDB 35 can use root exudates or a broad range of carbon substrates in soil and supply P to plants in the rhizosphere. *Pseudomonas* and *Serratia* sp. show MPS activity by the non-phosphorylating oxidation pathway for aldose sugars. HPLC analysis of the culture supernatant revealed that acidification of the medium was possibly due to conversion of different sugars to their respective aldonic acids by GDH-mediated direct oxidation [21, 22]. GDH has been characterized from bacteria such as *Acetobacter calcoaceticus*, *Escherichia coli*, and *Glucanobacter oxydans* for understanding its role in basic carbohydrate metabolism [23].

*S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 showed cellulolytic activity and solublized RP in the

Table 2. Cellulase activity, RP solubilized and sugars liberated with *S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 in the presence of different crop residues in submerged growth conditions

| | Cellulase (U $ml^{-1}$ $min^{-1}$) | | P release (μmol) | | Reducing sugars (μg $mL^{-1}$) | |
|---|---|---|---|---|---|---|
| Carbon source (1%) | EB 67 | CDB 35 | EB 67 | CDB 35 | EB 67 | CDB 35 |
| Cellulose (CL) | 0.018 (±0 0009) | 0.019 (±0.0017) | 71 (±1.3) | 61 (±1.3) | 103 (±17.5) | 100 (±7.9) |
| Rice straw (RS) | 0.027 (±0.0013) | 0.024 (±0.0023) | 97 (±21.1) | 116 (12.0) | 126 (±1.9) | 145 (±7.3) |
| Rice root (RR) | 0.021 (±0.0023) | 0.023 (±0.0038) | 126 (±45.2) | 105 (±9.4) | 109 (±10.8) | 123 (±22.1) |
| RS + RR | 0.026 (±0.0030) | 0.027 (±0.0034) | 123 (±21.2) | 123 (±10.6) | 146 (±9.8) | 127 (±8.5) |
| Pigeonpea root (PR) | 0.022 (±0.0036) | 0.024 (±0.0039) | 134 (±16.1) | 140 (±21.8) | 120 (±21.4) | 125 (±28.5) |
| Grass (GS) | 0.017 (±0.0039) | 0.015 (±0.0036) | 73 (±16.9) | 67 (±15) | 106 (±14.3) | 83 (±15.4) |
| Mean | 0.022 | 0.002 | 104 | 102 | 118 | 117 |
| LSD ($P = 0.05$) | 0.029 | | 13.3 | | 13.6 | |
| CV% | 6.1 | | 7 | | 5.3 | |

One unit of cellulase activity is equivalent to 1 μmol of reducing sugar (glucose) equivalents per minute. data given are the mean of different days. Values in parantheses are ± standard errors.

presence of crop residues. Both the strains presented a similar behavior towards reducing sugar liberation and consumption, as a peak of sugar concentration was observed on day 6 after inoculation (data not shown), followed by a sharp decline, which indicated carbon source consumption. There was not much difference in the pH drop in the presence of crop residues with EB 67 and CDB 35 and it ranged from 5.5 to 6 (data not shown). Solubilization of RP in the presence of crop residues could be due to their ability to break down cellulose into glucose. In the absence of organic acids, the release of protons accompanying respiration and/or ammonium assimilation were also related to P solubilization [24]. Crop residues (grass, straw, etc.) applied as cellulosic biomass consisting cellulose and hemicellulose contain 55–75% carbohydrates. Cellulose is a polymer of glucose and hemicellulose consists of xylose, arabinose, glucose, galactose, and mannose [25]. In this study, both the strains solubilized RP in the presence of crop residues that could be due to the release and utilization of the above-mentioned sugars (Table 2).

When biomass is added as surface mulch, microbial activity of the soil surface might not be sufficient for its decomposition. To enhance mineralization and decomposition of crop residues, phosphate-solubilizing bacteria with cellulolytic activity may be preferred. The action of cellulases is synergistic over substrate, especially for microorganisms isolated from environments where agro-residues are biodegraded [26, 27]. In our study, both the strains were from composts, confirming their ability to use crop residues as a carbon source. Such strains with a dual trait of cellulase and RP-solubilizing ability can be a better source of bioinoculants/biofertilizers and can enhance plant growth when applied to soil.

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