Batch Culture Studies of Phosphate Solubilisation by *Micrococcus* sp PSB 7 Isolated from Rhizospheric Soil

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**Abstract:** Phosphate solubilising bacteria are among the rhizobacteria that help to improve soil fertility. Phosphate solubilisation in *Micrococcus* sp isolated from an agricultural soil was studied in batch culture. The organism had a solubilisation index of 3.6 after five days incubation. The effects of nutrient factors studied in National Botanical Research Institute’s Phosphate medium with 5% (w/v) tricalcium phosphate showed that optimal phosphate solubilisation was obtained with glucose as the carbon source at a concentration of 5% (w/v) and urea as the nitrogen source, at a concentration of 0.1% (w/v). Influence of some environmental toxicants on phosphate solubilisation estimated with the incorporation of bromophenol blue in the medium indicated that zinc at 0.2mM and phenol at 50 and 1000mg/l inhibited phosphate solubilisation by more than 20% while cadmium at 0.2 and 0.5mM stimulated the activity by more than 50%. Stimulation of phosphate solubilisation by cadmium indicates that *Micrococcus* sp has potential as bioinoculant for phytoremediation of cadmium contaminated soil.

**Key words:** Phosphate solubilisation · *Micrococcus* sp · Batch culture · Nutrient factors · Toxicants

**INTRODUCTION**

Phosphorus, an essential mineral for plant growth and development is often the limiting mineral nutrient for biomass production in natural ecosystems [1]. It is often added in form of phosphatic fertilisers in soil, however plants utilise only a few amount of it and the rest is rapidly converted to insoluble complexes in the soil which plants cannot utilise [2]. This demands that phosphate (P) fertilisers be applied regularly and frequently to soil to forestall a deficiency. This is however expensive and environmentally undesirable [3]. Soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization [4].

Phosphate solubilising ability is widespread among many bacterial genera which includes *Pseudomonas*, *Bacillus*, *Rhizobium*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter* and *Flavobacterium* [5]. *Micrococcus* is Gram positive and oxidase positive cocci, found in a wide range of environments [6]. Members of the genus can utilize a wide range of substrates, produce various useful products and are likely involved in detoxification or biodegradation of many environmental pollutants [7].

Several theories exist explaining the mechanisms of microbial P solubilization, namely: the sink theory, the organic acid theory and acidification by H+ excretion theory. In the sink theory, P solubilizing organisms are able to remove and assimilate P from the liquid medium and therefore stimulate the indirect dissolution of Ca-P compounds by continuous removal of P from broth [8]. The organic acid theory recognized and accepted by many researchers postulates that P solubilising microorganisms produce organic acids leading to acidification of microbial cells and their surroundings and, consequently, the release of P ions from the P mineral by H+ substitution for Ca+2 [9]. The acidification by H+ excretion theory explains that H+ release associated with cation assimilation causes a decrease in pH and acted as a solvent agent for P solubilization. [10] Many reported cases of microbial phosphate solubilization often has direct correlation with pH of the medium [4].

Like many other microbial processes, phosphate solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture. The solubilization activity of a microorganism is related to its organic acid production and this has been reported to be affected by...
the carbon source. Fasim et al. [12] have reported such bacterial isolates, which solubilize only in presence of glucose. Sridivya et al. [13] reported optimal phosphate solubilisation by Aspergillus sp 7 in the presence of maltose. N source present as either ammonium- or nitrate-nitrogen has been found to also affect phosphate solubilisation. Cerezine et al. [14] reported that ammoniacal sources increased the solubilization of fluorapatite by A. niger more than organic sources of nitrogen. Illmner and Schümmer [15] and Lapeyrie et al. [16] also reported a number of fungi able to solubilize phosphate only in the presence of ammonium- nitrogen as the nitrogen source. Differences in phosphate solubilization under varying nutrient conditions were attributed to the induction of different mechanisms for acid generation in the culture medium [16, 17].

Heavy metals such as zinc and cadmium and synthetic organic chemicals like phenols constitute problems in the environment due to their potential adverse effects on biota. It is well documented that the presence of metals and phenolic compounds can inhibit a broad range of microbial processes through various mechanisms. Heavy metals can impose oxidative stress [18], substitute physiologically essential cations within an enzyme and bind to SH groups [19]. Phenol and its derivatives are membrane damaging microbiocides [20] whose potential toxic mechanisms of include effects on respiration, protein binding, mutagenicity and carcinogenicity [21]. However, addition of metals and phenolic compounds has also been observed to stimulate activity in some cases. Aerobic denitrification in Acinetobacter sp was stimulated by zinc, cadmium and 2, 4-dinitrophenol, [22] and in Escherichia sp by zinc [23]. In this study, nutrient conditions for optimal phosphate solubilisation by Micrococccus sp PSB 7 and the effects of zinc, cadmium and phenol on the process were investigated.

**MATERIALS AND METHODS**

**Isolation, Purification and Screening of Organisms for Phosphate Solubilisation:** Rhizospheric soil around the root of a weed growing in an agricultural farm in Nigeria, which has been left fallow for more than five years, was used for the study. One gram of the soil sample weighed into 9ml normal saline in a 15ml test tube was serially diluted. One tenth of 1ml of the 10⁻² dilution was plated on nutrient agar and incubated for 24-48h. Morphologically distinct colonies were selected, purified by repeated culturing and maintained on nutrient agar slants at 4°C. The isolates were screened for the ability to solubilise phosphate using the NBRI-BPB medium of Nautiyal et al. [24]. The medium comprised of (per liter): glucose, 10 g; Ca₃(PO₄)₂, 5 g; MgCl₂.6H₂O, 5 g; MgSO₄.7H₂O, 0.25 g; KCl, 0.2 g; (NH₄)₂SO₄, 0.1 g and bromophenol blue, 0.025g. Purified isolates inoculated onto NBRI-BPB plates were incubated at room temperature (28±2°C) for 5 days. Isolates with halo zones around them indicating production of acid that decolourised BPB were phosphate solubilizing organisms. Such isolates were identified based on the morphological and biochemical characterisation following the schemes of Holt et al. [25] and one of them was used for this study. The solubilisation index of the selected isolate was estimated on NBRI-BPB plate by the formula:

\[
\text{Solubilization index} = \frac{\text{Diameter of halo zone}}{\text{Diameter of growth zone}}
\]

**Effects of Nutrients on Phosphate Solubilisation:**

The effects of different sources and concentrations of carbon and nitrogen on phosphate solubilisation were studied sequentially in batch cultures in a mineral salt medium (NBRIY) of Nautiyal, [26]. The medium comprised of (per liter): Ca₃(PO₄)₂, 5 g; MgSO₄.7H₂O, 0.1g, KCl, 0.2 g; NaCl, 0.2g; FeSO₄.7H₂O, 0.002g; MnSO₄, H₂O, 0.002g. First, the effects of different carbon sources (glucose, maltose and starch) at 1% and nitrogen sources {NH₄Cl, SO₄, KNO₃ and urea} at 0.1% on phosphate solubilisation were compared. Thereafter, the effects of different concentrations of glucose (1-10%) and urea (0.1-1%) which were the carbon and nitrogen sources that gave optimal phosphate solubilisation were also assessed. Estimation of phosphate solubilisation was done quantitatively by the ascorbic acid- Molybdate method (Bray and Kurtz, 1945).

**Preparation and Standardization of Inoculum:** The inoculum consisted of twenty four hour cultures of the purified organism grown on nutrient broth. Cultures were harvested, suspended in normal saline and washed twice by centrifugation (3000 rpm). Washed cells were resuspended in normal saline and standardized to obtain suspensions of approximately 6 x 10⁸ cfu/ml using the McFarland standard of the nephelometry method [28]. The cell suspension served as the standardized inocula for the studies.

**Medium and Incubation Conditions:** Medium used was a mineral salt medium adapted from the NBRIP medium of Nautiyal, [26] which has the same composition as the NBRI-BPB but without bromophenol blue. The carbon
and nitrogen sources and concentrations were varied. The investigation was done in a 15ml reaction volume contained in 100ml conical flasks. This consisted of 0.2ml of the standardized inoculum and 14.8ml medium constituted to obtain final concentrations of carbon (1%) and nitrogen (0.1%) sources. Flasks were incubated at room temperature (28±2°C) in the laboratory for 24h. Thereafter, the pH of the medium was measured and the flask centrifuged at 3000rpm for 10 min. The clear supernatant was used for phosphate assay.

**Quantitative Determination of Phosphate:** The amount of phosphate solubilised in the supernatant was estimated spectrophotometrically using the ascorbic acid-Molybdate method (Bray and Kurtz, 1945). In this method, orthophosphate reacts with molybdate to form phosphomolybdcic acid which is reduced by ascorbic acid to form a blue complex. To 0.05ml of the supernatant contained in a 15ml test tube was added 4.95ml of Reagent C [0.53 g L-Ascorbic Acid in deionised water + 70 mL of Reagent A made up to 500ml. Reagent A comprised of 17.14 g ammonium molybdate in 200 mL of warm deionised water + 0.392 g potassium antimonyl tartrate in 150 mL deionised water + 200 mL concentrated sulphuric acid made up to 2000ml]. The set up was left to stand for 30 min for colour development after which the absorbance was read at 885nm in a Spectronic 21 spectrophotometer. Amount of available P was estimated from a standard curve of available P earlier prepared.

**Effects of Heavy Metals and Phenol on Phosphate Solubilisation:** The effects of two heavy metals (zinc as zinc sulphate and cadmium as cadmium chloride) at concentrations of 0.2-1.0mM and phenol at 200 - 1000mg/l on phosphate solubilisation by the selected isolate were assessed qualitatively on NBRI-BPB medium of Nautiyal et al. [23]. Estimation was based on the principle that bromophenol blue decolorizes with reduction in medium pH.

**Inoculum, Media and Incubation Conditions:** The preparation and standardization of inoculum was as described earlier. Medium used was the NBRI-BPB medium of Nautiyal et al. [23]. The investigation was done in a 15ml reaction volume contained in 100ml conical flasks. This consisted of 0.2ml of the standardized inoculum and 14.8ml medium constituted to obtain final concentrations of the metals and phenol. Triplicate flasks containing each concentration of metal and phenol were incubated at room temperature (28±2°C) in the laboratory and samples were analysed for colour change immediately and after 4h and 6h. Control flasks which contained the media and the organism but without metals or phenol were also set up. At the specified time, the flasks were centrifuged at 3000rpm for 10 min and the absorbance of the supernatant measured at 600nm in a Spectronic 21 spectrophotometer.

**Estimation of the Relative Effects of Metals and Phenol on Phosphate Solubilisation:** The extents of phosphate solubilisation induced by the heavy metals and phenol were estimated by subtracting the absorbance after a specific time (4h or 6h) from the absorbance at the initial time. The relative effects of the heavy metals and phenol on phosphate solubilisation at any time were estimated using the formula (1):

\[
a = \frac{(b-c)}{b} \times 100
\]

Where

\[
a = \text{Relative effects on phosphate solubilisation} \\
b = \text{Extent of solubilisation in control} \\
c = \text{Extent of solubilisation in treatment}
\]

**Statistical Analysis:** Data generated were subjected to analyses of variance (ANOVA). Relationships between the amount of phosphate solubilized and media pH were analysed using the Pearson’s product-moment correlation coefficient.

**RESULTS AND DISCUSSION**

The organism identified as *Micrococcus* sp was a non-motile, Gram positive, oxidase positive cocci which produces only acid from mannose and sucrose and acid and gas from lactose. Members of the genera occur in a wide range of environments and have been implicated in phosphate solubilisation [29]. The organism had a solubilisation index of 3.60 after five days incubation (data not shown). This was higher than the solubilisation index of 3.29 obtained after seven days incubation recorded for the best phosphate solubilising bacterial strain PSB 107MB among the ten phosphate solubilising isolates from maize studied by Alam et al. [30]. It was also within the range of solubilisation indices of 2.16-6.23 reported for thirteen best phosphate solubilising isolates from rice rhizosphere [31].

The results of the effects of carbon and nitrogen sources on phosphate solubilisation showed that diverse levels of phosphate solubilisation activities were observed in the presence of the various carbon and nitrogen sources (Table 1). For any given carbon source,
Table 1: Effects of carbon and nitrogen sources on phosphate solubilisation by Micrococcus sp PSB 7.

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Phosphate solubilised (mg/l)</th>
<th>pH</th>
<th>Phosphate solubilised (mg/l)</th>
<th>pH</th>
<th>Phosphate solubilised (mg/l)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>204.88</td>
<td>4.15</td>
<td>148.78</td>
<td>4.6</td>
<td>63.41</td>
<td>4.88</td>
</tr>
<tr>
<td>Malrose</td>
<td>134.15</td>
<td>4.76</td>
<td>102.44</td>
<td>5.17</td>
<td>68.29</td>
<td>5.83</td>
</tr>
<tr>
<td>Starch</td>
<td>239.02</td>
<td>4.11</td>
<td>92.68</td>
<td>4.75</td>
<td>41.46</td>
<td>6.71</td>
</tr>
</tbody>
</table>

Changes in nitrogen source had no significant (P > 0.05) effects on phosphate solubilisation, while for any given nitrogen source, changes in carbon source had a significant (P < 0.05) effect on phosphate solubilisation. The order of decreasing phosphate solubilisation activity in the presence of various carbon sources was glucose > maltose > starch and for nitrogen, it was urea > ammonium sulphate > potassium nitrate. Optimal amount of phosphate (239.02mg/l) was solubilised in media containing glucose as the carbon source and urea as the nitrogen source. The lowest amount of phosphate (21.46mg/l) was solubilised in media containing starch and urea as the carbon and nitrogen sources respectively. Other workers have reported solubilization in presence of a wide range of carbon sources as well. The preference of glucose for phosphate solubilisation over a wide range of carbon sources have been reported for Rhizobium sp [32] and Aspergillus sp [33]. Fasim et al. [12] also reported bacterial isolates, which solubilize only in presence of glucose, while Barcsó et al. [34] observed optimal phosphate solubilisation by Aspergillus niger with maltose as the carbon source. In the case of nitrogen sources, apart from combinations with glucose, treatments containing urea as the nitrogen source affected the least amount of phosphate solubilisation. This is contrary to the observations of Sridevi et al. [32] in which a combination of glucose with inorganic nitrogen sources yielded better phosphate solubilisation than with organic nitrogen sources. The nature of carbon source affects acid production which is a common mechanism of phosphate solubilisation [35]. Although organic acid was not measured in this study, negative correlations existed between media pH and amount of phosphate solubilised. This was strongest in the media containing glucose (-0.96) which also yielded the highest amount of phosphate solubilisation. This suggests that the major mechanism for phosphate solubilisation by Micrococcus sp PSB7 was a reduction in pH due to either the production of organic acids or extrusion of H+. H+ extrusion is associated with cation assimilation and in this study, phosphate solubilisation in the treatment containing urea was better than that in the treatment containing NH₄⁺ -N which is more easily assimilable, it seem reasonable to infer that the reduction in pH observed in this study was due to the production of organic acids. Variations in phosphate solubilisations induced by the various combinations of carbon and nitrogen sources might represent different mechanisms for the generation of acidity in the medium. Variations in the effects of different concentrations of glucose and urea were found to be statistically significant (P < 0.05). At any particular glucose concentration, increasing urea concentration above 0.1% (%) caused a significant reduction in the amount of phosphate solubilised (Fig. 1). This is attributable to the decreased media acidity induced by the high urea concentration and this resulted in low phosphate solubilisation. Optimal phosphate solubilisation (146.34mg/l) was obtained at respective concentrations of 5% (%) and 0.1% (%) for glucose and urea.

The results of the effects of Zn²⁺, Cd²⁺ and phenol on phosphate solubilisation by Micrococcus sp PSB7 are shown in Table 2. Extent of solubilisation was determined by measuring absorbance of filtrate at 600nm based on decolourisation of BPB in broth medium due to production of acid which strongly correlated with phosphate solubilisation. Relative to the control which induced a reduction in absorbance amounting to 0.230 units after 6h incubation, addition of Cd²⁺ at 1, 0.5 and 0.2mM reduced absorbance by 0.250, 0.350 and 0.385 units respectively while addition of Zn²⁺ at 1mM reduced absorbance by 0.265 units. On the contrary, reductions in absorbance after 6h due to the effect of phenol at all the concentrations studied and Zn²⁺ at 0.5 and 0.2mM were lower than reductions in the control (<0.230 units). This implies that phosphate solubilisation by Micrococcus sp PSB7 was stimulated by Cd²⁺ at 1, 0.5 and 0.2mM and Zn²⁺ at 1mM but inhibited by Zn²⁺ at 0.5 and 0.2mM and phenol at 200-1000mg/l. Rate of solubilisation in the control was uniform throughout the period of incubation but varied with the addition of different concentrations of the metals and phenol. Variations in the response of Micrococcus sp PSB7 to chemical exposure.
might be related to the synthesis of biochemical constituents which might play a role in the responses of cells to toxic chemicals. Ravikumar et al. [29] reported increases in the content of total sugars and protein in phosphobacterial isolates in cadmium treated medium and Wu et al. [36] also reported the synthesis of some common proteins upon exposure of cyanobacteria to heavy metal stress. The degrees of inhibition or stimulation of phosphate solubilisation upon exposure to the different chemicals are shown in Fig. 2. Stimulation by Cd" at 0.2mM was greater than 50% of the control after 6h exposure of the organism and this decreased with increasing concentration. Ravikumar et al. [29] also reported higher phosphate solubilising activity in halophytic strains of Micrococcus roseus.
and other bacteria when Cd\(^{2+}\) was added to the culture media. Stimulatory effects of Cd\(^{2+}\) on phosphate solubilising activity of Micrococcus sp PSB7 is not necessarily indicative of a lack of negative effect of the metal on other microbial processes but it is suggestive of the potential for the inclusion of the organism or its gene responsible for phosphate solubilisation in the development of biofertilisers for use in cadmium contaminated soils.

Zn\(^{2+}\) at 1mM induced greater than 10% stimulation of phosphate solubilisation and at 0.5 mM and 0.2mM induced 12% and 22% inhibition respectively (Fig. 2). Phenol at 200mg/l caused 30% inhibition of phosphate solubilisation and this decreased with increasing concentration. Thus for zinc and phenol, lower concentrations were more toxic to phosphate solubilisation than higher concentrations. A similar pattern of toxicity was reported for cadmium on NAPH biodegradation by a Burkholderia sp. [37] and for 4-bromophenol on the dehydrogenase activity of Acinetobacter sp [38]. It is possible that high concentrations of Zn\(^{2+}\) and phenol may have induced a resistance mechanism important in alleviating the toxic effects of these substances. The inhibitory effects of Zn\(^{2+}\) and phenols on microbial organisms have been reported. Zinc is a potent inhibitor of the respiratory electron transport system [39] and was reported by Nweke et al. [40] to inhibit dehydrogenase activities in four planktonic bacteria at concentrations greater than 0.2mM. Phenol and its derivatives are membrane damaging microbiocides and their overall toxic effects is caused by distinct and complex mechanisms such as narcosis, the inhibition of growth and the uncoupling of adenosine triphosphate synthesis [20].

**CONCLUSION**

Phosphate solubilisation in Micrococcus sp PSB7 was influenced by both nature and level of carbon and nitrogen nutrients. Optimal phosphate
solubilisation was in the presence of glucose (5\% (\text{v/v})) and urea (1\% (\text{v/v})), and the major mechanism was the reduction of pH. The varying degrees of stimulation and inhibition of phosphate solubilisation observed upon chemical exposure provides useful information for effective use of the organism in the formulation of microbial inoculants. Stimulation of phosphate solubilisation by Cd$^{2+}$ is suggestive of the potential for the use of the organism as a biofertiliser in Cd$^{2+}$ contaminated soils.

REFERENCES

1. **MISSING**


5. **MISSING**


