3. MATERIALS AND METHODS
3.1 Soils

Black and red soils from agricultural fields of Kandukur and Akuthotapally villages of Anantapur district, Andhra Pradesh were collected respectively. The soils were air-dried and after breaking the clods were sieved through a 2 mm sieve. The physico-chemical properties of the soil samples were determined and are presented in Table 1.

3.2 Incubation

Soil samples (5 Kg) of both the soils were placed in earthen pots. Moisture content of the soil was maintained at 60% water holding capacity (WHC) throughout the incubation period. Triplicate samples (pots) were maintained for each sampling.

3.3 Sampling

Soil samples were collected after 30, 45, 60 and 75 days of incubation. A composite soil sample (10 g) was collected from triplicate samples for each sampling. The experiment was conducted twice. The first experiment was conducted between January and March 2001 while the second experiment was conducted between July and September 2001.

3.4 Isolation of phosphate-solubilizing bacteria from the soil samples

Pikovskaya’s medium (modified by Sundara Rao and Sinha, 1963) with the following composition was used for enumeration (g/l):
Table 1. Characteristics of the soils used in the experiments

<table>
<thead>
<tr>
<th>Location</th>
<th>Texture</th>
<th>pH&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Sand&lt;sup&gt;2&lt;/sup&gt; (%)</th>
<th>Silt&lt;sup&gt;2&lt;/sup&gt; (%)</th>
<th>Clay&lt;sup&gt;2&lt;/sup&gt; (%)</th>
<th>Organic matter&lt;sup&gt;3&lt;/sup&gt; (%)</th>
<th>Total Nitrogen&lt;sup&gt;4&lt;/sup&gt; (%)</th>
<th>Total Phosphorus&lt;sup&gt;5&lt;/sup&gt; mg P/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kandukur Black</td>
<td>Black</td>
<td>7.3</td>
<td>72.1</td>
<td>16.2</td>
<td>11.7</td>
<td>1.12</td>
<td>0.29</td>
<td>92</td>
</tr>
<tr>
<td>Akuthotapally</td>
<td>Red</td>
<td>6.4</td>
<td>70.2</td>
<td>18.6</td>
<td>11.2</td>
<td>0.47</td>
<td>0.25</td>
<td>69</td>
</tr>
</tbody>
</table>

1 Measured by taking 1:1.25 soil water slurry

2 Bouyoucos hydrometer method

3 Estimated by Walkley-Black Method (Jackson, 1973)

4 Estimated by Kjeldahl method (Jackson, 1973)

5 Estimated by sodium bicarbonate method (Olsen et al., 1954)
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Tricalcium phosphate</td>
<td>5.0</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.1</td>
</tr>
<tr>
<td>Manganous sulphate</td>
<td>Traces</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>Traces</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5</td>
</tr>
<tr>
<td>Agar agar</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Ten-fold serial dilutions of the soil sample (10 g) were prepared. 0.1 ml suspension of soil dilutions from $10^{-3}$ to $10^{-6}$ was inoculated to Pikovskaya’s agar plates (5 plates for each dilution) and spread uniformly under aseptic conditions in a laminar flow chamber. The plates were incubated at $30 \pm 2^\circ C$ temperature for 4 days.

Total number of bacterial colonies that developed on Pikovskaya’s agar plates were counted using a colony counter. Similarly, the number of phosphate-solubilizing bacterial (PSB) colonies with clear transparent zones around the colonies were also counted. The populations of both total and phosphate-solubilizing bacteria were estimated by the Most Probable Number (MPN) method.
3.5 Purification of phosphate-solubilizing bacterial isolates obtained from the soil samples

All the individual and discrete colonies, with transparent zones of clearing around them, were picked up. The colonies were purified following standard procedures. The selected single colonies were streaked repeatedly on Pikovskaya’s agar plates until isolated single colonies are obtained. Further purification of the single colonies was carried out on Pikovskaya’s agar slants. The single colonies thus purified were maintained on Pikovskaya’s agar slants for further studies. Before use, they were checked for purity by streaking on Pikovskaya’s agar slants. After confirmation of their purity, the isolates were used in subsequent experiments.

3.6 Morphological characteristics of phosphate-solubilizing bacteria

The morphological features of the phosphate-solubilizing bacterial colonies such as shape, margin, colour and size were recorded.

3.7 Biochemical tests

Gram staining was performed for all the pure cultures. Further, the isolates were subjected to the following biochemical tests:

a) Carbohydrate fermentation
b) Starch hydrolysis
c) Casein hydrolysis
d) Indole test
e) Methyl red test
f) Voges-Proskauer test

g) Citrate utilization test

h) Oxidase test

i) Catalase test

Based on the results obtained from the biochemical tests, an effort was made to identify the isolates.

3.8 Solubilization of phosphorus by pure cultures of bacteria

The pure cultures of phosphate-solubilizing bacteria were grown in 50 ml aliquots (sterilized) of Pikovskaya's liquid medium (broth) at 30 ± 2°C for 10 days. The bacterial cultures were filtered through Whatman No. 1 filter paper and centrifuged at 10,000 rpm for 10-15 min. Filtration and centrifugation was repeated until a clear solution is obtained which was finally made up to 50 ml.

To 10 ml aliquot of the clear filtrate, 2.5 ml of Barton's reagent was added and the volume was made up to 50 ml. After 10 minutes, the resultant yellow colour was read in a colorimeter at 430 nm.

Preparation of Barton's reagent

The reagent was prepared as per the following procedure (Sundara Rao and Sinha, 1963):

Solution A

25 g of ammonium molybdate was dissolved in 400 ml of distilled water.
Solution B

1.25 g of ammonium metavanadate was dissolved in 300 ml of boiling water, cooled and then 250 ml of concentrated nitric acid was added.

The solutions A and B were mixed and the volume was made up to 1 litre with distilled water.

A standard curve was prepared by dissolving 0.2195 g of potassium dihydrogen orthophosphate in distilled water. The solution was made up to one litre (1 ml = 59 ppm P). Further dilution of 10 ml into 250 ml was made so that 1 ml is equivalent to 2 ppm of P. Aliquots of 2, 3, 4, 5, 6, 8, 10, 15 and 20 ml of the 2 ppm stock solution were taken in 50 ml volumetric flasks to which 2.5 ml of Barton's reagent was added and the volume was made up to 50 ml with distilled water. After 10 minutes, the yellow colour developed was read in a colorimeter at 430 nm. A standard graph was then prepared from which P values for experimental samples were calculated (Koenig and Johnson, 1942).

3.9 Statistical analyses

In all the cases, analyses of significant differences of \((P \leq 0.05)\) between values of each sampling and treatment were performed using Duncan’s New Multiple Range (DMR) Test (Duncan, 1955).