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# Isolation of Alkaline Phosphatase Producing Bacteria Employing A Novel Screening Medium for Phosphatases

**P. Nalini[1]*, T. Prabhakar[1], P. Ellaiah[2] and G. Girijasankar[1]**

[1]A.U. College of Pharmaceutical Sciences, Pharmaceutical Biotechnology Division, Andhra University, Visakhapatnam - 530 003, India.
[2]Jeypore College of Pharmacy, Jeypore, Odisha - 764 002, India.

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**Phosphorus is an essential macronutrient for the crop growth. Majority of soil phosphorus is present in plant unavailable forms. Soil microorganisms enrich phosphorus to plants by producing phosphatase enzymes. This investigation was carried out for the isolation and identification of alkaline phosphatase producing bacteria using a novel defined Glucose indicator dye medium containing 5-bromo-4-chloro-3-indolyl phosphate as phosphatase screening medium. Ninety bacterial strains were isolated from phosphorus rich soils of field crops. The isolates were categorized into thirty five types based on colony morphology and microscopic studies. Ten strains gave the high ratio of hydrolyzed zone. Twelve strains showed moderate and thirteen exhibited very poor phosphatase activity. S-VI-2 isolate identified as *Streptococcus* species released maximum amount of phosphorus. The bacteria isolated from field crops were bestowed with alkaline phosphatase activity. The isolate S-V-3 recognised as new strain of *Kurthia* species produced alkaline phosphatase in highest amount. The study reveals the detection and evaluation of phosphatase activity with the designed screening medium. Growth of bacteria is directly related to phosphate solubilization and alkaline phosphatase production abilities. This is the first investigation on the isolation and identification of alkaline phosphatase producing *Kurthia* species from field crops.**

**Key words:** Novel screening medium, Phosphatases, Phosphate solubilization, Alkaline phosphatase, *Kurthia* species.

Phosphorus is a prime limiting factor of plant metabolic regulation. The optimal development of crop demands a high input of phosphate fertilizers. Most agricultural soils retain large sources of phosphorus in consequence of regular applications of phosphate fertilizers[1]. Approximately 95-99% of soil phosphorus is not available to plants[2]. Soil microorganisms are initially associated as integral component of the phosphorus cycle. Phosphorus can only be assimilated as soluble phosphate. Wide spectrum of soil microbial population can solubilize insoluble phosphates[3-8].

Phosphatases play a vital role in plant phosphorus nutrition[9,10]. Phosphorus unavailable to plants is transformed to available phosphorus by phosphatase enzymes. Microorganisms are significant in acquisition of phosphorus to plants[11]. Several soil bacteria secrete phosphatases which acts on insoluble phosphates and converts the same into soluble forms thus providing phosphorus to plants. It has been reported that there are different groups of microorganisms which increase the phosphorus availability to plants by solubilizing phosphates more accessible to them[12-14].

* To whom all correspondence should be addressed.
E-mail: nalini.p2005@gmail.com

Microorganisms producing alkaline phosphatases are ubiquitous in nature. The production of alkaline phosphatase is regulated by the phosphate concentration in the environment. Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1) is a hydrolase enzyme that functions at alkaline pH. Bacterial alkaline phosphatase provides bacteria with inorganic phosphate by reducing different phosphoester compounds available in the environment. Bacterial alkaline phosphatases have been extensively used. The wide range of applications of alkaline phosphatase in diagnostics, molecular biology, immunology and milk pasteurization have received considerable attention in scientific and commercial processing[15-20]. Bacteria are a major group of soil microflora and bacterial alkaline phosphatases play a key role in plant phosphorus dynamics.

The present study deals with the selective isolation of bacteria producing phosphatases from soils of field crops by a newly developed Glucose indicator dye medium and identification of promising isolates exhibiting high alkaline phosphatase activity as well as efficient phosphate solubilization ability.

## MATERIALS AND METHODS

### Soil collection

Phosphorus rich soil samples were collected from the field crops of brinjal, chilly, bengal gram, green gram, groundnut, maize, paddy, ragi, sorghum and turmeric. The field crops are located in Anantapur, Andhra Pradesh, India. From each plot the samples were taken at random to make a composite sample. The sampling soils were transferred to laboratory under aseptic conditions for microbial analysis.

### Isolation of strains

Each sample was serially diluted to tenfold dilution series with 0.85% NaCl. The diluted samples were plated on newly developed Glucose indicator dye medium containing the following ingredients in grams per liter: glucose, 8.0; NaCl, 0.5; $KH_2PO_4$, 0.1; $MgCl_2$, 0.05 and 5-bromo-4-chloro-3-indolyl phosphate as an indicator dye at a concentration of 50 µg/ml. pH was adjusted to 7.0. The indicator dye was colorless and became blue after dephosphorylation. After 48 hr of incubation at 37°C the bacterial colonies encircled with blue colored halos were picked and transferred onto nutrient agar slants. These isolates were maintained at 4°C and subcultured for every four weeks. The isolates were evaluated for their enzyme activity by measuring the hydrolyzed zone formed on Glucose indicator dye medium. Selected isolates were identified as per Bergey's Manual of Systematic Bacteriology[21,22]. Identified isolates were tested for phosphate solubilization and alkaline phosphatase production capabilities in Pikovskaya medium[23].

### Estimation of cell mass, phosphorus and alkaline phosphatase activity

Quantitative estimation of phosphorus released and alkaline phosphatase was carried out in Erlenmeyer flasks incubated on rotary shaker at 220 rpm, 30°C containing 25 ml Pikovskaya medium[23]. Uninoculated autoclaved culture medium served as control. For every 24 hr the sample quantity of 5 ml was withdrawn for bioassay. The cultures were harvested by centrifugation at 300 rpm for 20 min. The cell pellet was washed thoroughly with sterile 2% potassium chloride solution, followed by 0.85% sodium chloride solution and sterile distilled water subsequently. Washed cells were dried in oven. Cell mass was estimated by determining the dry weight of centrifuged cells. Phosphate solubilization potential of selected isolates in culture medium amended with tricalcium phosphate was determined in vitro by Molybdenum blue method[24]. The method was standardized with known concentration of phosphates. Intensely colored molybdenum blue was measured in Shimadzu (Japan) spectrophotometer at 882 nm. Phosphorus released in culture medium was expressed in µg/ml. Alkaline phosphatase activity of cell-free supernatant was assessed by Glycine assay method[25]. 0.1 ml of suitably diluted enzyme solution was added to glycine buffer reaction mixture with *p*-nitrophenyl phosphate as substrate. Alkaline phosphatase activity was observed in Shimadzu (Japan) spectrophotometer at 410 nm. One unit of enzyme activity was defined as the capability of liberating 1.0 µmol of *p*-nitrophenol per minute under the standard conditions. The recorded data is mean of three experiments.

## RESULTS

Collected samples were used for the isolation of phosphatase activity exhibiting bacteria following the serial dilution technique. After 48 hr of incubation, blue colored zone was developed around the colonies indicating phosphatase production capabilities of the bacterial strains (Fig. 1).

A total of ninety bacterial strains were isolated with designed medium. On the basis of colony characteristics, texture and microscopic observations the isolates were divided into thirty five types. The bacterial strains were coded (Table 1). Thirty five uncharacterized bacterial isolates were screened for their phosphatases activity on the prepared Glucose indicator dye medium containing 5-bromo-4-chloro-3-indolyl phosphate as an indicator dye. The principle involved in this method is that phosphatases hydrolyze 5-bromo-4-chloro-3-indolyl phosphate resulting in the formation of blue color. Phosphatase activity of the selected isolates varied from 2.5 mm to 6.3 mm.

**Table 1.** Phosphatase activity of selected isolates screened on novel Glucose indicator dye medium

| Isolate No. | Sample source | Phosphatase activity |
|---|---|---|
| S-I-1 | Brinjal fields - Sample I | 3.2 |
| S-I-5 | Brinjal fields - Sample I | 2.5 |
| S-I-7 | Brinjal fields - Sample I | 3.6 |
| S-III-2 | Bengal gram fields - Sample III | 5.1 |
| S-III-6 | Bengal gram fields - Sample III | 4.5 |
| S-III-9 | Bengal gram fields - Sample III | 4.2 |
| S-IV-7 | Green gram fields - Sample IV | 4.7 |
| S-IV-11 | Green gram fields - Sample IV | 2.7 |
| S-IV-14 | Green gram fields - Sample IV | 3.1 |
| S-IV-15 | Green gram fields - Sample IV | 4.3 |
| S-IV-16 | Green gram fields - Sample IV | 3.5 |
| S-V-3 | Groundnut fields - Sample V | 6.3 |
| S-V-4 | Groundnut fields - Sample V | 4.5 |
| S-V-6 | Groundnut fields - Sample V | 6.0 |
| S-V-7 | Groundnut fields - Sample V | 5.3 |
| S-V-8 | Groundnut fields - Sample V | 4.6 |
| S-V-9 | Groundnut fields - Sample V | 3.8 |
| S-V-10 | Groundnut fields - Sample V | 3.3 |
| S-VI-2 | Maize fields - Sample VI | 5.0 |
| S-VI-3 | Maize fields - Sample VI | 5.5 |
| S-VI-4 | Maize fields - Sample VI | 4.6 |
| S-VI-5 | Maize fields - Sample VI | 4.7 |
| S-VII-4 | Paddy fields - Sample VII | 4.3 |
| S-VII-7 | Paddy fields - Sample VII | 3.4 |
| S-VIII-1 | Ragi fields - Sample VIII | 3.7 |
| S-VIII-8 | Ragi fields - Sample VIII | 3.5 |
| S-VIII-11 | Ragi fields - Sample VIII | 4.4 |
| S-IX-1 | Sorghum fields - Sample IX | 5.1 |
| S-IX-4 | Sorghum fields - Sample IX | 5.5 |
| S-IX-5 | Sorghum fields - Sample IX | 3.6 |
| S-IX-6 | Sorghum fields - Sample IX | 4.1 |
| S-IX-9 | Sorghum fields - Sample IX | 5.7 |
| S-X-2 | Turmeric fields - Sample X | 3.2 |
| S-X-4 | Turmeric fields - Sample X | 4.2 |
| S-X-5 | Turmeric fields - Sample X | 5.8 |

Phosphatase activity is hydrolyzed zone (mm) : growth zone (mm) ratio

Of these isolates, ten isolates showed maximum hydrolyzed zone of 5.0 mm to 6.3 mm, twelve exhibited 4.1 mm to 4.7 mm and thirteen showed inconsiderable zone of <4.0 mm. This envisages the screening of phosphatases producing bacteria on the newly designed Glucose indicator dye medium. Out of all bacteria the isolate S-V-3 showed higher phosphatase activity of 6.3 mm on screening medium. Maximum number of isolates exhibiting maximum phosphatase activity

**Table 2.** Phosphorus release into the medium by the selected isolates

| Name of isolate | Isolate No. | Phosphate solubilization (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 24 hr | 48 hr | 72 hr | 96 hr |
| *Bacillus* sp. | S-III-2 | 121.9 | 250.3 | 121.6 | 120.7 |
| *Kurthia* sp. | S-V-3 | 434.2 | 618.3 | 440.5 | 134.8 |
| *Bacillus* sp. | S-V-6 | 687.2 | 709.2 | 352.6 | 257.8 |
| *Pseudomonas* sp. | S-V-7 | 210.6 | 282.6 | 164.3 | 132.5 |
| *Streptococcus* sp. | S-VI-2 | 462.1 | 859.7 | 479.3 | 320.8 |
| *Streptococcus* sp. | S-VI-3 | 271.6 | 415.3 | 342.8 | 117.2 |
| *Bacillus* sp. | S-IX-1 | 289.5 | 326.0 | 314.1 | 215.6 |
| *Streptococcus* sp. | S-IX-4 | 412.0 | 613.8 | 718.5 | 328.8 |
| *Bacillus* sp. | S-IX-9 | 330.2 | 695.5 | 554.7 | 514.9 |
| *Bacillus* sp. | S-X-5 | 367.5 | 457.8 | 802.4 | 430.5 |

**Table 3.** Extracellular alkaline phosphatase production by the selected isolates

| Isolate | Alkaline phosphatase activity (U/ml) | | | |
|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 96 hr |
| *Bacillus* sp. S-III-2 | 16.1 | 30.7 | 15.5 | 03.5 |
| *Bacillus* sp. S-V-6 | 47.6 | 64.9 | 37.5 | 12.7 |
| *Bacillus* sp. S-IX-1 | 18.0 | 44.9 | 32.2 | 10.6 |
| *Bacillus* sp. S-IX-9 | 16.0 | 42.4 | 23.2 | 06.2 |
| *Bacillus* sp. S-X-5 | 20.4 | 48.7 | 80.2 | 37.4 |
| *Streptococcus* sp. S-VI-2 | 27.2 | 74.9 | 30.5 | 02.3 |
| *Streptococcus* sp. S-VI-3 | 03.0 | 16.1 | 06.8 | 01.4 |
| *Streptococcus* sp. S-IX-4 | 40.2 | 56.5 | 25.0 | 06.8 |
| *Kurthia* sp. S-V-3 | 70.3 | 97.5 | 55.5 | 19.7 |
| *Pseudomonas* sp. S-V-7 | 09.7 | 21.3 | 13.6 | 07.4 |

was obtained from groundnut fields. All isolates from brinjal fields exhibited negligible activity.

The ten isolates which showed significant phosphatase activity were identified as reported in Bergey's Manual of Systematic Bacteriology[21,22]. S-V-3 isolate, identified as new strain of *Kurthia* sp. exhibited highest phosphatase activity (Fig. 2). The strain has been deposited in Microbial Type Culture Collection and Gene Bank (MTCC) with accession number MTCC-9746. Identified isolates were selected for further studies.

**Phosphate solubilization efficiency**

Phosphate solubilization of an isolate is the ability to release available phosphorus into the medium. Selected isolates were tested for the efficacy to dissolve phosphate in liquid medium. Various media have been used by researchers for studying phosphate solubilization. In this study, phosphate solubilization potential of the selected isolates was carried out in Pikovskaya medium[23].

The isolates were evaluated for 96 hr at an interval of 24 hr (Table 2). Initially, within 24 hr of incubation *Bacillus* sp. S-V-6 released a greater amount of 687.2 µg/ml of phosphorus. Among all the isolates *Streptococcus* sp. S-VI-2 solubilized highest amount of phosphorus of 859.7 µg/ml from 0.5% tricalcium phosphate followed by *Bacillus* sp. S-X-5 which released 802.4 µg/ml of

**Table 4.** Growth of the selected isolates in the culture medium

| Isolate | Cell mass (mg/ml) | | | |
|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 96 hr |
| *Bacillus* sp. S-III-2 | 0.425 | 0.636 | 0.416 | 0.107 |
| *Bacillus* sp. S-V-6 | 0.361 | 0.543 | 0.281 | 0.094 |
| *Bacillus* sp. S-IX-1 | 0.417 | 0.984 | 0.686 | 0.105 |
| *Bacillus* sp. S-IX-9 | 0.284 | 0.517 | 0.351 | 0.087 |
| *Bacillus* sp. S-X-5 | 0.108 | 0.267 | 0.576 | 0.195 |
| *Streptococcus* sp. S-VI-2 | 0.516 | 0.812 | 0.639 | 0.098 |
| *Streptococcus* sp. S-VI-3 | 0.231 | 0.945 | 0.623 | 0.185 |
| *Streptococcus* sp. S-IX-4 | 0.572 | 0.613 | 0.378 | 0.127 |
| *Kurthia* sp. S-V-3 | 0.332 | 0.483 | 0.269 | 0.063 |
| *Pseudomonas* sp. S-V-7 | 0.114 | 0.359 | 0.216 | 0.096 |

phosphorus. The other isolates showed similar responses and liberated phosphorus in the range of 117.2 µg/ml to 718.5 µg/ml. Each isolate has the ability to release phosphorus in the culture medium. Only one isolate *Bacillus* sp. S-X-5 in Pikovskaya medium solubilized more amount of phosphate after 72 hr. *Streptococcus* sp. S-VI-3 liberated lower amount of phosphorus into the medium after 96 hr of fermentation. This study revealed that phosphate solubilization ability varied with each isolate. Almost all the isolates solubilized greater amount of phosphorus in the culture medium within 48 hr. The release of phosphorus content is decreased after 48 hr indicating the less growth of isolates. Phosphate solubilization ability is directly related to growth of bacteria. The result of this experiment thus demonstrates the existence of phosphate solubilizing bacteria in field crop soils.

**Alkaline phosphatase activity**

The effective phosphate solubilizers were evaluated for their efficiency to produce extracellular alkaline phosphatases. The ability of bacteria to dissolve phosphate can be determined by analyzing the alkaline phosphatase produced in the culture medium.

Bacterial cultures released greater quantities of alkaline phosphatase in the culture medium (Table 3). The isolate *Kurthia* sp. S-V-3 is the predominant bacteria involved in enhanced alkaline phosphatase production followed by *Bacillus* sp. S-X-5. A higher amount of alkaline phosphatase was detected at 97.5 U/ml. Other selected isolates exhibited alkaline phosphatase activity at the rate of 1.4 U/ml to 74.9 U/ml. Minimum alkaline phosphatase activity was recorded in *Streptococcus* sp. S-VI-3. The results of alkaline

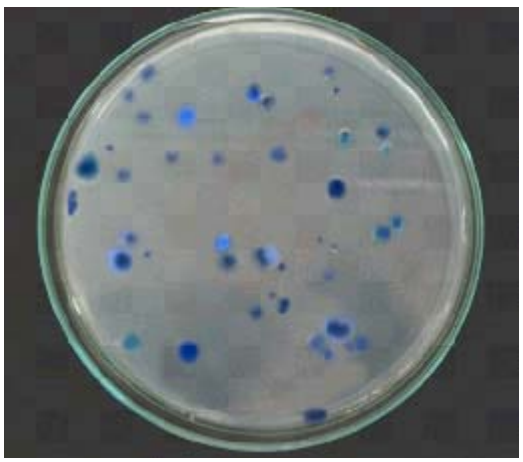

**Fig. 1.** Screening of bacteria producing phosphatases

**Fig. 2.** Phosphatase activity of *Kurthia* sp. on Glucose indicator dye medium

phosphatase production indicated that all the selected strains were able to release alkaline phosphatase under in vitro conditions. Similar to phosphate solubilization, the alkaline phosphatase production was also reduced after 48 hr with the decrease in culture growth. Likewise the phosphate solubilization abilities, more alkaline phosphatase activity in *Bacillus* sp. S-X-5 was observed after 72 hr. Compared to the activity of alkaline phosphatase with phosphate solubilization in Pikovskaya medium, the isolate *Streptococcus* sp. S-VI-3 showed greater phosphate solubilization with lower alkaline phosphatase production. The isolate *Kurthia* sp. S-V-3 produced maximum alkaline phosphatase and solubilized less content of phosphate whereas the isolate *Streptococcus* sp. S-VI-2 released higher amount of phosphorus and exhibited lower alkaline phosphatase activity. Therefore some of the isolates degraded greater amount of phosphate and produced limited alkaline phosphatase and vice versa.

**Cell mass of isolates**

Cell mass is the indication for the growth of bacteria in culture medium. To study the growth of isolates for maximum phosphorus release and alkaline phosphatase production, the fermentation samples were withdrawn periodically and cell mass was determined.

The result (Table 4) indicates that the isolates grew well in the medium and maximum cell mass of 0.267 mg/ml to 0.984 mg/ml was achieved at 48 hr. Further, increase in fermentation time exhibited a decrease in growth of bacteria. The time required to attain maximum cell mass coincided with maximum phosphate solubilization and alkaline phosphatase production. Among the identified isolates *Bacillus* sp. S-X-5 showed greater cell mass of 0.576 mg/ml after 72 hr. Maximum alkaline phosphatase and phosphorus release abilities were also observed after 72 hr of fermentation in *Bacillus* sp. S-X-5. This indicates that isolates showed maximum production abilities at good cellular growth. On comparison of cell mass to the alkaline phosphatase production and phosphate solubilization abilities in isolates, some of the isolates showed maximum phosphate solubilization and alkaline phosphatase activities with poor cellular growth while other isolates with good cellular growth exhibited lower phosphate solubilization and alkaline phosphatase production abilities. *Streptococcus* sp. S-VI-3 exhibited less phosphate solubilization, 415.3 µg/ml and alkaline phosphatase activities, 16.1 U/ml with highest cellular growth of 0.945 mg/ml. Less cellular growth of 0.483 mg/ml was observed in *Kurthia* sp. S-V-3 with greater phosphate solubilization, 618.3 µg/ml and alkaline phosphatase activities, 97.5 U/ml. However during fermentation phosphate solubilization and alkaline phosphatase production abilities of an isolate were increased with the increase in cell growth.

## DISCUSSION

Several investigations have highlighted the potential uses of alkaline phosphatases. Besides the typical applications in laboratories, alkaline phosphatases are used in cosmetics for the regeneration of cells and in environmental monitoring[19]. In recent years the production of bacterial alkaline phosphatases has gained importance. This research is concerned with isolating the eminent phosphatase producing isolates by screening the samples on newly designed Glucose indicator dye medium and identifying the efficient alkaline phosphatase activity exhibiting bacteria with phosphate solubilization abilities.

Soil microorganisms solubilize nutritionally unavailable insoluble phosphates[26]. Microorganisms possess phosphatases capable of catalyzing a wide variety of phosphates to liberate plant available phosphates. Important levels of phosphatase activity in soil have been studied[27-30]. Microbial phosphatases play an indispensable role in survival of plants and microorganisms. Various groups of microorganisms have been detected to render phosphatic compounds soluble[31-34].

Isolates have been screened by an agar assay using Glucose indicator dye medium for extracellular phosphatase activity. All the isolates of soil samples were efficient in producing phosphatases. It indicates that microorganisms are a considerable source of phosphatases in soil. Based on macroscopic and microscopic observations the isolates were divided into thirty five types. From thirty five types of bacterial isolates, ten isolates showed maximum hydrolyzed zones on the solid medium (Table 1). This suggests

that detection and qualitative evaluation of phosphatase activity have been possible with novel Glucose indicator dye medium. The described screening medium can be used for plate assay of isolates producing phosphatases. Out of ten isolates, five isolates were identified as *Bacillus* sp., three isolates as *Streptococcus* sp., the isolate S-V-3 as *Kurthia* sp. and the isolate S-V-7 as *Pseudomonas* sp. according to Bergey's Manual of Systematic Bacteriology[21,22]. The isolates of bengal gram, groundnut, maize, sorghum and turmeric fields showed maximum phosphatase activity than the isolates collected from other field crops.

Microbial secretions convert the phosphorus present in the soil to plant available phosphorus[35]. Bacterial strains of *Bacillus*, *Pseudomonas*, *Rhizobium* and *Enterobacter* are effective phosphate solubilizers[26]. Based on the data obtained on phosphate solubilization (Table 2), apart from *Bacillus* sp. and *Pseudomonas* sp. the identified strains of bacterial genera *Streptococcus* and *Kurthia* also play a significant role in increasing the plant available phosphorus in soil. Phosphorus is one of the key elements for existence of life where alkaline phosphatase is crucial in biochemical processes. Among the selected isolates, *Kurthia* sp. was identified as a potential alkaline phosphatase producing strain (Table 3). The taxonomic identity of the strain S-V-3 was further ascertained by various physicochemical parameters with MTCC, Chandigarh, India. The selected isolates of soil samples secreted alkaline phosphatase in the culture medium. This revealed alkaline phosphatase activity is involved in the mechanism of phosphate solubilization. Isolates collected from groundnut fields showed maximum alkaline phosphatase production and phosphate solubilization capabilities. The levels of alkaline phosphatase production and phosphate solubilization abilities increased during the culture growth (Table 4).

Activity of alkaline phosphatases by soil amendments increases the phosphorus availability to plants. The enzyme activity compared between the days was significantly different. Difference in the isolates of the same sample as well as from different samples was observed. Alkaline phosphatase production and phosphate solubilization efficiency can be variable with strains and even within the same bacterial species. The overall results indicate that alkaline phosphatases are also responsible for the release of insoluble phosphates. Phosphate solubilization and alkaline phosphatase production have a positive correlation with growth of bacteria. This is the first investigation of isolation and identification of alkaline phosphatase producing *Kurthia* sp. from field crop soils.

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