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Novel, Rapid and Sensitive Plate Assay Method for Detection and Screening of Phosphatase Producing Microorganisms

P. Nalini* and R. S. Prakasham

Medicinal Chemistry and Biotechnology Lab-Organic Synthesis and Process Chemistry Division, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad - 500007, Telangana, India.

**Corresponding author*

Abstract

Phosphatases are crucial in solubilization of phosphorus. Microorganisms producing phosphatases transform plant unavailable phosphorus into available forms. The enzymes have wide range of applications in laboratories and industries. The phosphatases producing bacteria were screened on nutrient phosphate agar whereas fungi on potato dextrose phosphate agar medium. The detection plates were flooded with Gram's iodine solution after incubation at the respective temperatures. Clear hydrolyzed zones were developed around the colonies indicating the phosphatase production. The present developed method is simple and rapid for screening of both bacteria and fungi that produce extracellular phophatases. Promising isolates producing phosphatases can be detected by measuring the hydrolyzed zones on agar media. The levels of phosphate solubilization efficiency and alkaline phosphatase production capabilities were gradually increased indicating growth associated pattern. In the present study a novel, sensitive and efficient plate assay method was developed for screening of potential microorganisms (both bacteria and fungi) producing phosphatases.

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Keywords

Microorganisms, screening, phosphatase, Gram's iodine

Introduction

Phosphorus is the essential nutrient for plant growth (Maharajan, 2018). It plays a vital role in plant physiological activities such as photosynthesis, cell division, development of reliable root system and utilization of carbohydrates. Plants acquire 0.1 % of phosphorus from soil as phosphate anion (Lambers and Plaxton, 2018) which demands for a high input of phosphate fertilizers leading to phosphorus enrichment in the soil. Phosphorous deficiency results in the turning of leaves to brown accompanied by small leaves, weak stem and slow development. Different groups of microorganisms act on insoluble phosphates and increase the availability of phosphorus to plants by solubilizing phosphates more accessible to them (Prasanna *et al.*, 2011; Tarafdar *et al.*, 2003).

Phosphorus solubilizing microbes secreting phosphatases play an important role in the nutrition of plant phosphorus (Duff *et al.*, 1994). Phosphatase enzymes transform the unavailable phosphorus to available plant phosphorus. Phosphatases are of significant headway in scientific and bio-industries. Many regulatory and industry entities identified the utility of alkaline phosphatases. Microorganisms producing alkaline phosphatases are abundant in nature. The generous availability of alkaline phosphatase in nature and its

importance in biological systems has made the enzyme activity assessments as one of the most commonly performed enzymatic tests (Miggiano *et al.*, 1983). The activity of alkaline phosphatase was shown to be sensitive to phosphate availability and specifically to the intracellular phosphate pool of microbes (Dyhrman, 2005).

The wide range of applications of phosphatases requires screening of potent isolates for extracellular enzyme production in high yields. The rapid exploitation of novel developments in this sector is substantial. The present study deals with the screening of microorganisms producing phosphatases by a newly developed, rapid, sensitive and contrasting efficient plate assay method using Gram's iodine and quantitative estimation of alkaline phosphatase activity of the selected isolates by submerged fermentation.

Materials and Methods

Chemicals

Iodine and potassium iodide were purchased from Sigma-Aldrich (India). Nessler's reagent and other chemicals used were of analytical grade from Hi-Media (India).

Serial dilution-agar plate method

Phosphorus rich soil samples were collected from paddy field crops located in Anantapur, Andhra Pradesh, India. The samples were collected at random from each plot to make a composite sample. The soil samples were collected into sterile test tubes and brought to the lab. The soil samples were serially diluted to tenfold dilution with 0.85 % sodium chloride. One ml aliquot of 10^{-6} dilution was used for each agar plating method.

Preparation of media

For extracellular phosphatases, bacteria were grown on newly developed nutrient phosphate agar (NPA) medium containing the following ingredients (gm/L): peptone, 5.0; beef extract, 3.0; sodium chloride, 5.0; dipotassium hydrogen phosphate, 5.0 and agar, 20.0. pH was adjusted to 7.0.

Fungi were grown on potato dextrose phosphate agar (PDPA) containing the following ingredients (gm/L): starch (potato), 4.0; dextrose, 20.0; dipotassium hydrogen phosphate, 5.0 and agar, 20.0. pH was adjusted to 5.6 with 1N hydrochloric acid. All the ingredients were autoclaved. Streptomycin sulphate antibiotic of 30 mg/1000 ml was added to the sterilized PDPA medium at 40 °C-50 °C.

Gram's iodine solution was prepared by dissolving the following ingredients (gm/300 ml of distilled water): potassium iodide, 2.0 and iodine, 1.0.

The NPA plates inoculated with aliquots cultures were incubated at 37 °C for 24 hr whereas the inoculated PDPA plates were incubated at 25 °C for 72 hr. Uninoculated plates served as control.

Preparation of cultures

Among 10^{-6} dilutions the plate with unselected isolates was used to check the quantitative estimation of hydrolyzed zone with Gram's iodine. A total of 110 bacterial and 80 fungal strains were isolated randomly with designed medium from the remaining plates. The selected promising isolates were identified on the basis of 16S rRNA gene and gene sequence analysis with Midilabs. The selected bacterial isolates were identified as *Bacillus licheniformis* and *Bacillus subtilis* while the fungal isolate was identified as *Aspergillus niger* and were labeled as IICT-PN-4, IICT-PN-5 and IICT-PN-6 respectively. The bacterial cultures were maintained on nutrient agar and fungal cultures on potato dextrose media. The identified *Bacillus licheniformis*, *Bacillus subtilis* and *Aspergillus niger* were subjected for further studies. These were subcultured on NPA medium and PDPA medium and preserved in refrigerator for testing. The selected isolates were subjected to streak-plate method and flooded with Gram's iodine for the detection of hydrolyzed zones. Two best isolates were selected.

Estimation of phosphorus, cell biomass and alkaline phosphatase activity

Quantitative estimation of phosphorus released and alkaline phosphatase were carried out in Erlenmeyer flasks incubated on rotary shaker at 220 rpm, 30 °C containing nutrient phosphate (NP) and potato dextrose phosphate (PDP) broth of pH 7.0. Uninoculated culture media served as controls. Five ml quantities of the samples were withdrawn for assay at 12 hr intervals. The bacterial cultures were harvested by centrifugation at 3000 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

hot air oven at 105 °C for 3 hr. The bacterial cell mass was estimated by determining the dry weight of centrifuged cells. Phosphate solubilization potential of the above isolates in culture medium amended with tricalcium phosphate was determined by Molybdenum blue method (Murphy and Riley, 1962). With known concentration of phosphates the method was standardized. Intensely colored molybdenum blue was measured in Agilent Cary 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 (Bernt, 1974). The enzyme activity was measured 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. Simillarly fungal isolate *Aspergillus niger* was also processed.

Results and Discussion

Several investigations have demonstrated the wide range applications of alkaline phosphatases (Muginova *et al.*, 2007). In recent years the microbial production of alkaline phosphatases has gained much importance. Several reports appeared in the literature on the alkaline phosphatase producing microorganisms (Bagewadi *et al.*, 2022; Yousef *et al.*, 2021; Chu *et al.*, 2019; Suresh and Das, 2014; Yadav *et al.*, 2011; Ogut *et al.*, 2010; Johri *et al.*, 1999; Kucey *et al.*, 1989). The present investigation is concerned with the isolation of prominent phosphatase producing isolates by screening the soil samples on newly designed NPA and PDPA media with Gram's iodine solution and identifying the phosphatase activity of the selected isolates.

Serial dilution technique was followed for collected soil samples to isolate phosphatase activity exhibiting microorganisms. One ml aliquots of 10^{-6} dilutions were used for pour-plate method of bacterial colonies in NPA medium (Fig. 1 c) and fungal colonies in PDPA medium (Fig. 2 c). Uninoculated plates served as controls for bacteria (Fig. 1 a) and fungi (Fig. 2 a). Among 110 isolates of phosphatase producing bacteria, two best isolates were selected and these were identified as *Bacillus licheniformis* and *Bacillus subtilis*. These were tested once again for their phosphatase production capacity by streak-plate method (Fig. 1 e and Fig. 1 g). After 24 hr of incubation the plates were flooded with Gram's iodine solution. Clear hydrolyzed zones were developed around the colonies in pour-plate (Fig. 1 d) and streak-plate of *Bacillus licheniformis* (Fig. 1 f) and *Bacillus subtilis* (Fig. 1 h). Uninoculated control plate flooded with Gram's iodine solution was completely stained (Fig. 1 b).

One of the plate of pour-plate method of 10^{-6} dilution in PDPA medium (Fig. 2 c) and plate inoculated with *Aspergillus niger* (Fig. 2 e) exhibited growth after incubation of 72 hr. Gram's iodine solution flooded fungal growth plates showed clear hydrolyzed zones around the colonies in pour-plate (Fig. 2 d) and plate inoculated with *Aspergillus niger* (Fig. 2 f). Uninoculated control plate flooded with Gram's iodine solution was completely stained (Fig. 2 b). Hydrolyzed zones of 5.3 mm and 4.9 mm were observed with *Bacillus licheniformis* and *Bacillus subtilis* and 8.9 mm for *Aspergillus niger*. The identified organisms, *Bacillus licheniformis*, *Bacillus subtilis* and *Aspergillus niger* exhibited a sharp, clear, distinct and prominent hydrolyzed zones.

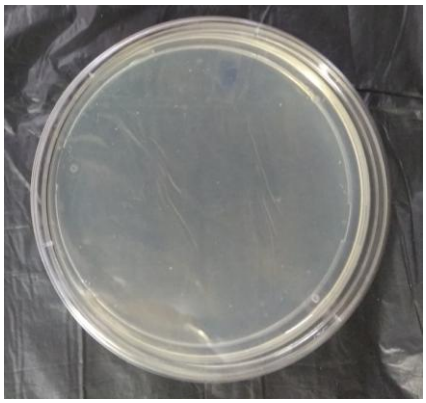
Phosphate solubilization efficiency

Soil microorganisms convert unavailable phosphorus into available phosphorus to satisfy the requirements of plants through phosphorus cycle. Phosphate solubilization is the ability of an isolate to liberate available phosphorus into the medium. In liquid medium the isolates were tested for their efficacy to dissolve phosphate. Different media have been used by researchers for studying phosphate solubilization. In the present work, phosphate solubilization potential of the selected isolates was carried out using NP and PDP broth.

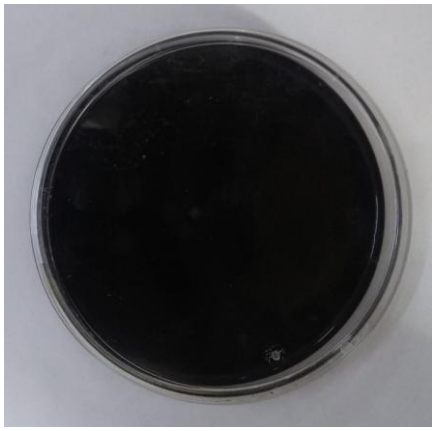
The bacterial isolates were evaluated for 96 hr taking samples at intervals of 12 hr. *Bacillus licheniformis* released a greater amount of 756.2 µg/ml of phosphorus at 24 hr, while *Bacillus subtilis* released phosphorus of 672.4 µg/ml within 24 hr of incubation. The fungal isolate *Aspergillus niger* released more amount of phosphate of 724.2 µg/ml at 72 hr. This study revealed that phosphate solubilization ability varied with each isolate. Bacterial isolates solubilized greater amount of phosphorus in the culture media within 24 hr. The liberation of phosphorus content is decreased after 24 hr with bacteria and 72 hr with fungus indicating the poor growth of bacteria and fungus respectively. The ability of phosphate solubilization is directly related to growth of microbes. The results of these experiments demonstrate the existence of phosphate solubilizing microorganisms in paddy field crop soils.

Fig. 1 Identification of phosphatase producing bacteria on NPA medium stained with Gram's iodine

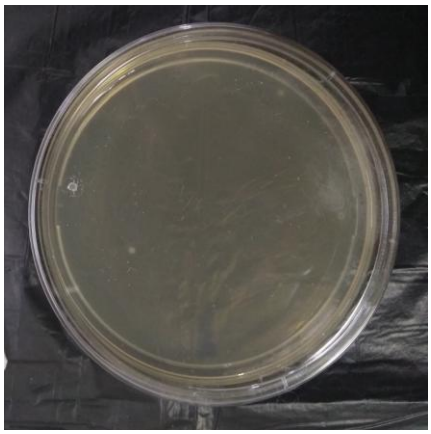
a. Control (only medium)



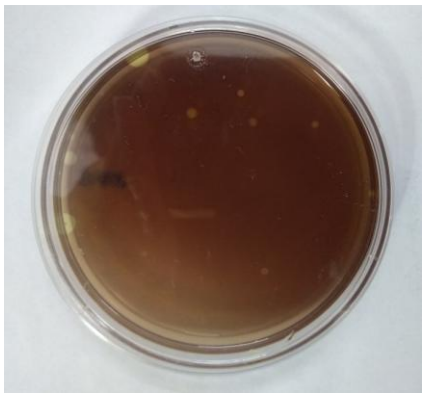
b. Control plate stained with Gram's iodine



c. Pour-plate method for isolation of bacteria in NPA medium



d. Formation of clear hydrolyzed zones of remaining isolates in NPA medium stained with Gram's iodine solution



e. Growth of identified *Bacillus licheniformis* (IICT-PN-4) on NPA medium

f. *Bacillus licheniformis* (IICT-PN-4) exhibiting phosphatase activity in NPA medium stained with Gram's iodine solution

g. Growth of identified *Bacillus subtilis* (IICT-PN-5) on NPA medium

h. *Bacillus subtilis* (IICT-PN-5) exhibiting phosphatase activity in NPA medium stained with Gram's iodine solution

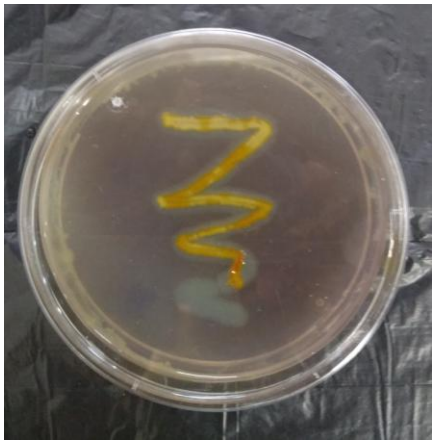
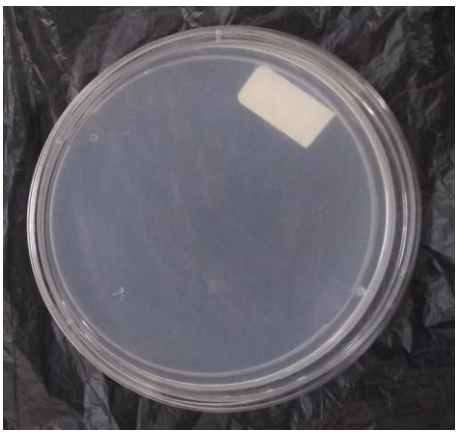
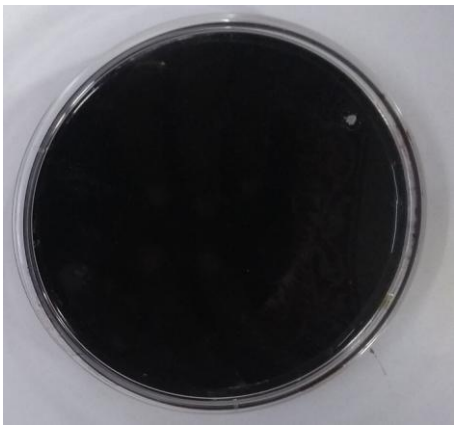


Fig. 2 Identification of phosphatase producing fungi on PDPA medium stained with Gram's iodine

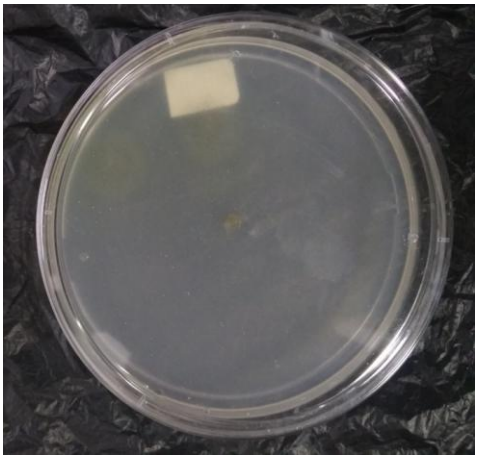
a. Control (only medium)



b. Control plate stained with Gram's iodine

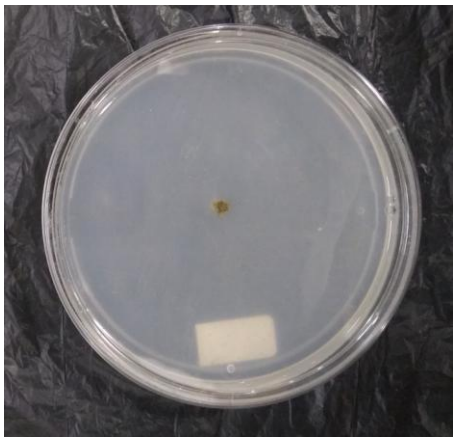


c. Pour-plate method for isolation of fungi in PDPA medium



d. Formation of clear hydrolyzed zones by fungi in PDPA medium stained with Gram's iodine solution

e. Growth of identified *Aspergillus niger* (IICT-PN-6) on PDPA medium



f. *Aspergillus niger* (IICT-PN-6) exhibiting phosphatase activity in PDPA medium stained with Gram's iodine solution

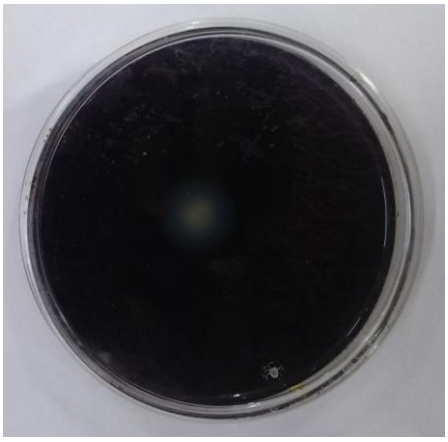
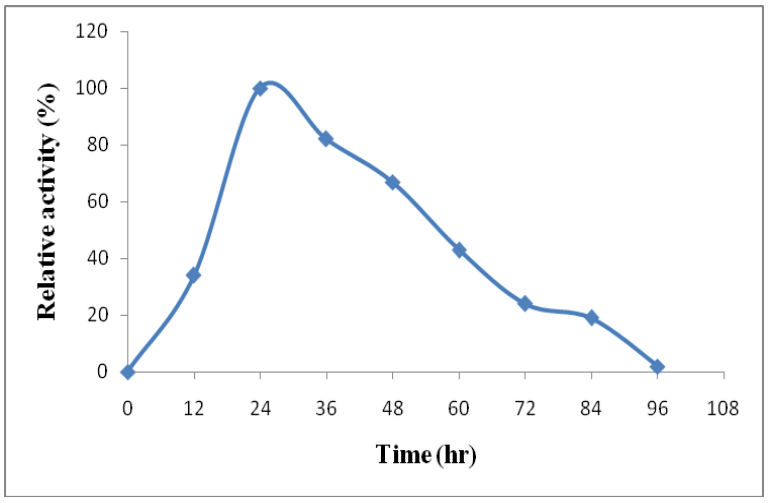
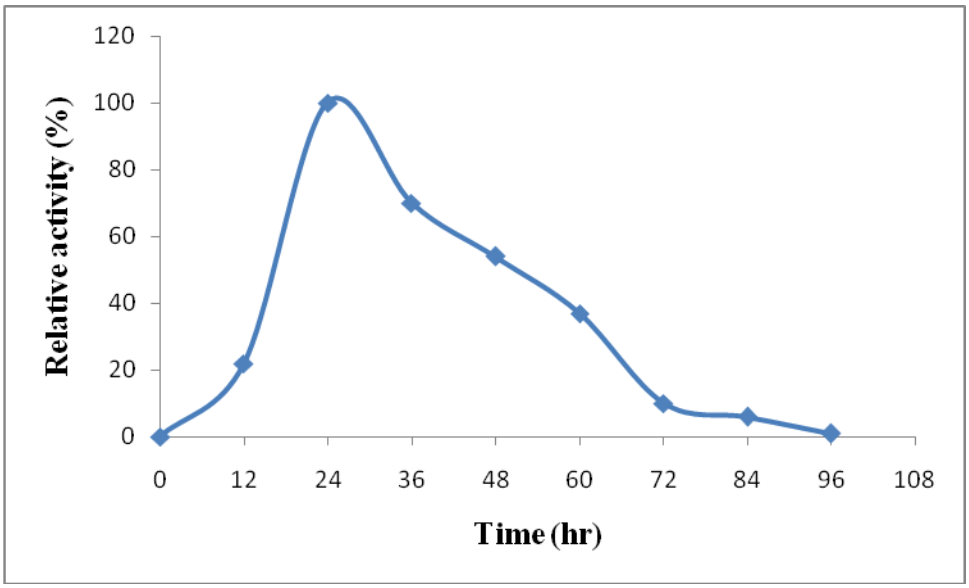


Fig. 3 Alkaline phosphatase production

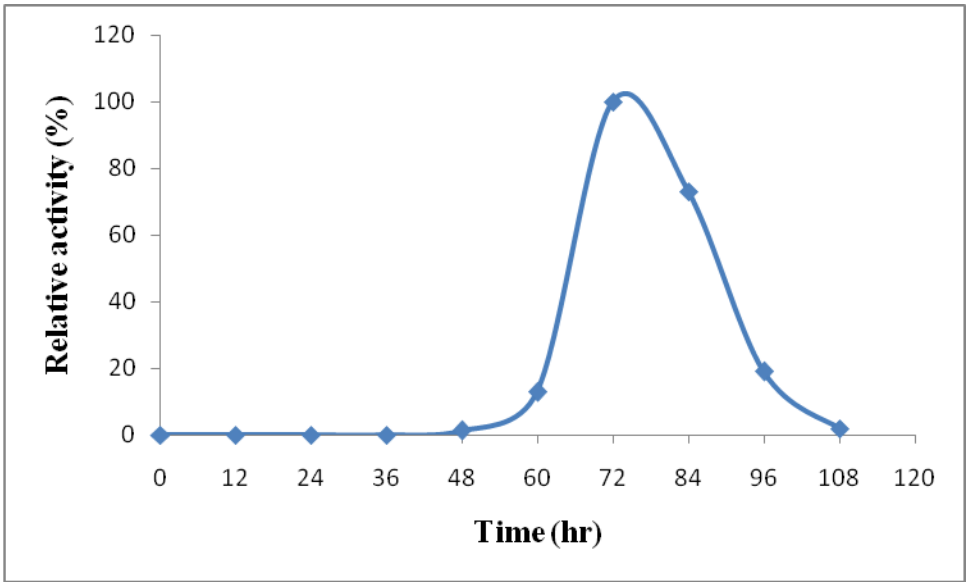
a. *Bacillus licheniformis* (IICT-PN-4)



b. *Bacillus subtilis* (IICT-PN-5)



c. *Aspergillus niger* (IICT-PN-6)



Alkaline phosphatase activity of bacterial isolates and their cell mass

The bacterial isolates were evaluated for their efficacy to produce extracellular alkaline phosphatase. The ability of microbes to dissolve phosphate can be evaluated by analyzing the alkaline phosphatase produced in the culture medium. All the isolates released good quantities of alkaline phosphatases in the culture media. The yield of alkaline phosphatase was found to be 86.5 U/ml by *Bacillus licheniformis* while *Bacillus subtilis* showed 67.5 U/ml at 24 hr incubation. The yield of the enzyme was gradually decreased after 24 hr with both bacteria (Fig. 3 a and Fig. 3 b). The fungal isolate, *Aspergillus niger* exhibited maximum enzyme production at 72 hr incubation (Fig. 3 c). The results of alkaline phosphatase activity indicated that all the selected isolates were able to produce alkaline phosphatase under submerged conditions (Fig. 3).

The fermentation samples were withdrawn periodically to determine the growth of bacterial cell mass. The

isolates grew well in NP medium and maximum cell mass of 0.897 mg/ml was achieved for *Bacillus licheniformis* and 0.678 mg/ml for *Bacillus subtilis* at 24 hr. An increase in fermentation time showed a decrease in bacterial biomass. Maximum enzyme production abilities were exhibited at optimum cellular growth by all the three selected isolates. It is also observed that an increase of fermentation time gradually reduced microbial biomass. The results indicate that during fermentation the phosphate solubilization and alkaline phosphatase production abilities of each isolate were increased with the increase in cell biomass.

This suggests that detection and qualitative evaluation of phosphatase activity was possible with Gram's iodine solution using NPA and PDPA media. The described screening method can be used for isolation of potent isolates producing phosphatases. Based on results of phosphate solubilization efficiency, microorganisms play a vital role in phosphorus liberation. The identified isolates secreted alkaline phosphatase in the culture media indicating the involvement of alkaline phosphatase in phosphate solubilization. The relative activity of phosphatase has been suggested as a potential measure of microbial biomass. Phosphate solubilization and alkaline phosphatase production abilities of the isolates were increased with culture growth.

The newly developed method envisages the screening of phosphatase producing bacteria on NPA medium and fungal phosphatase on PDPA medium with Gram's iodine solution. The developed Gram's iodine method gives a clear and sharp colour contrast between the hydrolyzed and unhydrolyzed phosphorus. Clear zone of hydrolysis around each isolate can be observed which is useful for screening of potent isolates. This suggests the detection and qualitative evaluation of phosphatase activity with the newly developed method. The described method can be used for plate assay of isolates producing phosphatases. The developed method is easy, rapid and efficient for the screening of potent microorganisms producing phosphatases.

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