

Original Research Article

Optimization for the Production of Extracellular Alkaline Phosphatase from *Bacillus subtilis*

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A B S T R A C T

Keywords

Alkaline phosphatase, *Bacillus subtilis*, optimization, Total enzymatic activity, Total potent activity

Phosphatase are enzymes which hydrolyze the complex organic phosphates in to inorganic phosphates(Pi) by the process dephosphorylation which will be further used by the cell to construct the Nucleic acid, Phospholipids, ATP energy molecule etc. In this study *Bacillus subtilis* was isolated from rhizosphere soil sample of legume plant and production of alkaline phosphatase was carried out. The incubation time 24 hrs were found total potent activity 148 µg/ml and total enzymatic activity 99 U/ml. The parameters like pH, temperature, carbon sources and nitrogen sources were optimized for better production of alkaline phosphatase. The enzyme production was maximum when the medium was amended with tricalcium phosphate as substrate and it was found that temperature of 32°C results was total potent activity 158 µg/ml and total enzymatic activity 96 U/ml, pH 7 and 8 results was total potent activity 155 and 156 µg/ml and total enzymatic activity 92 and 90 U/ml, starch as a carbon source was total potent activity 157 µg/ml and total enzymatic activity 86 U/ml and Egg albumin as a nitrogen source was total potent activity 161 µg/ml and total enzymatic activity 89 U/ml were suitable for higher production of alkaline phosphatase.

Introduction

Alkaline phosphatase (orthophosphate monoester phosphohydrolases E. C. 3.1.3.1.) is a nonspecific metallo enzyme¹, which hydrolyzes many types of phosphate esters at an alkaline pH in the presence of zinc and

magnesium ions. It is present in multiple molecular isoenzymes. In animals, there are four major isoenzymes of alkaline phosphatase are intestinal (IAP), corticosteroid (CAP), bone (BAP) and liver

(LAP). Isoenzymes differ in catalytic sites and activity, immunogenecity, amino acid sequence and electrophoretic mobility. In microorganisms, alkaline phosphatase is located in the periplasmic space, external to the cell membrane and which will be released during starvation phosphate and presence of phosphate in the medium. Microbial alkaline phosphatase is comparatively resistant to inactivation, denaturation, and degradation, and also has a higher rate of activity². Mostly the bacterial strains like *E.coli.*, *Bacillus spp.*, *Pseudomonas spp.*, *Xanthomonas spp.*, *Enterobacter spp.*, *Acidovorans spp.*, *Azotobacter spp.*, *Arthrobacter spp.*, *Acetobacter spp.*, *Vibrio spp.* etc., The fungi like *Aspergillus spp.*, *Chldosporium sp.*, *Penicillium spp.*, *Mucor spp.*, *Fusarium spp.*, *Curvularia spp.*, etc, are producing extra cellular alkaline phosphatase³⁻⁶. Among this microbial population, some of the species are having symbiotic association with compatible plants and helping phosphate mobilization. Very little work has been done with respect to extracellular production of alkaline phosphatase in genus *Bacillus*. The bacterial populations which are present in the rizhosphere soil of the plant and make the availability of inorganic phosphate to the plants by solubilizing the complex phosphate. Some of the fungi are making mycorrhizal association with plant and increasing phosphate mobilization to the plants. Alkaline phosphatase is commonly used as a tool in molecular biology and clinical assays^{7, 8}. Intracellular production of Alkaline phosphatase is quite tedious and expensive process in comparison to extracellular. The extracellular Alkaline phosphatase gave higher specific activity than intracellular Alkaline phosphatase is because of short and simple steps of purification⁹. Thus, the present study is proposed to conduct the Optimization of culture conditions, production, purification

and characterization of Extracellular alkaline phosphatase from *Bacillus subtilis*.

Materials and Methods

Isolation of bacterial strain

Rhizosphere soil sample of legume plant was collected in a sterile container. The collected sample was serially diluted up to 10^7 dilutions using sterile saline as a blank and the diluted samples were plated into the sterile nutrient agar plates using spread plate method. The plates were incubated at 37°C. The isolated colonies were further purified by streak plate method using sterile nutrient agar medium. The pure cultures were inoculated into sterile nutrient agar slants and nutrient broth for further use.

Screening for phosphatase activity

The isolated pure strains were screened for the production of extracellular phosphatase production using Hydroxyapatite (Soil extract agar) as a screening medium¹⁰. The pure cultures were streaked at the centre of the sterile Hydroxyapatite plates and the plates were incubated at 37°C for 24 hours. The observation was made to see the phosphate solubilization zone around the colony. Only positive and better zone formed was taken for futher study. The CaCl_2 and KH_2PO_4 was sterilized separately and added before pouring into the plates. The pH was adjusted by sterile 1N sodium hydroxide solution before pouring into the plates. The soil extract was prepared by mixing 500g of soil in one litre of water and steaming for 20 minutes. The extract was filtered through normal filter paper.

Subculturing

The positive and better zone formed strain was subcultured on luria agar plates. The

pure cultures were retrieved every month and stored at 4°C.

Identification of bacteria

The positive strain that produce maximum phosphatase enzyme was selected and was given for identification in Kakatiya University, Warangal, Telangana, India.

Enzyme production preparation of inoculum

The inoculum for further production of enzyme was prepared using Luria Bertani (LB) broth¹¹. The pure culture was inoculated into sterile inoculum broth and was incubated at 37°C in a rotary shaker for overnight. The fresh over night cultur was used as an inoculum for the production of enzyme.

Phosphatase production medium

The enzyme production was carried out by shake flask fermentation using Bunt and Rovira broth (1955) as production media. 500ml of sterile production broth was prepared in 1litre conical flask and 5%inoculum was transferred aseptically in to the production medium. The inoculated medium was incubated at 37°C for 48 hours. The medium was agitated at 200 rpm for better aeration and growth of the organism. pH was adjusted to 6.8.

Soil extract was prepared by autoclaving 1kg soil mixed with 5litres of tap water. The medium was placed in Erlenmeyer flask each contaioning 90ml. After sterilization and just before pouring into petri dishes, 5ml of 10% KH_2PO_4 sterile solution followed by 10ml of 10% CaCl_2 sterile solution were added to each flask. The pH was readjusted to 6.8 by sterile standard NaOH solution.

Phosphatase assay plate assay

The plate assay was performed using Hydroxyapatite medium which was prepared with 1.5% of agar in distilled water excluding glucose¹². After solidification of the agar, around 10mm diameter of well was cut out aseptically with the heip of cork borer. The well was filled with the culture filtrate and incubated at 37°C for overnight in humid chamber. The observation was made to see the phosphate solubilizing zone around the well.

Chemical assay

Alkaline phosphatase activity was measured spectro photometrically by monitoring the release of p-nitrophenyl phosphate (pNPP) at 400nm. A typical reaction mixture contained 100 μl of enzyme diluted in 200Mm Tris buffer (pH-8.5), 5mM CaCl_2 , 500 μmol pNPP in a final volume of 1ml. The reaction was performed at 37°C for 30 min and stopped by addition of 50 μl of 4M NaOH. One unit of phosphatase is the amount, which hydrolysas 1 μmol of substrate per min. The standard curve obtained by adsorbance of p-nitrophenyl phosphate (0 - 500 μmol) at 400nm was used for quantification of enzyme activity¹³.

Protein assay

The chemical assay for the total protein content from the sample was determined using Bradford method¹⁴.

Preparation of Bradford reagent and Estimation (Coomassie brilliant blue)

100mg of Coomassie brilliant blue dye G-250 was dissolved in 50ml of 95% ethanol. 100ml of 85% (w/v) phosphoric acid was added and the mixture was makeup to 100ml with double distilled water. The dye was

filtered through whatman No.1 filter paper and stored in dark bottles refrigeration. 1ml of culture filtrate was taken and 5ml of Bradford reagent was added. The tube was gently tilted once for mixing and the absorbency was taken at 595nm in UV-VIS Spectrometer. The blank was prepared by mixing 1ml of distilled water with 5ml of reagent. The protein concentration was determined by comparing the value with standard graph prepared using bovine serum albumin.

Parameter optimization studies^{16,19,27}

Effect of incubation time on phosphatase production

Around 100ml of sterile production medium was prepared and 5% inoculum was added aseptically. The inoculated medium was incubated at 37°C with shaking around 150 rpm. After incubation, around 20ml of culture was aseptically withdrawn periodically at 12-hour intervals up to 72 hours. The culture filtrate was examined for the total protein content and phosphatase activity.

Effect of temperature on phosphatase production

100ml of sterile production medium was prepared in different conical flask and inoculated with 5% inoculum. Each flask was incubated at different temperature such as 28 °C, 32 °C, 37 °C, 42 °C, 47 °C and 52 °C for 24 hours. The protein estimation and enzyme activity were estimated.

Effect of pH on phosphatase production

100ml of sterile production medium was prepared in different conical flasks and each flask was adjusted to different pH such as 4,5,6,7,8,9,10 using 0.1N NaOH and 0.1N

HCl. After sterilization, flasks were inoculated with 5% inoculum. The flasks were incubated at 32 °C for 24 hours. The protein estimation and enzyme activity were estimated.

Effect of different Carbon Sources

100ml of sterile production medium (pH 8.0) was prepared in different conical flasks. Each flask was amended with different carbon sources such as glucose, sucrose, maltose, starch and cellulose. The flasks were inoculated with 5% inoculum and incubated at 32°C for 24 hours. The culture filtrate was collected and protein estimation and enzyme activity was determined.

Effect of different Nitrogen sources

100ml of sterile production medium (pH 8.0) was prepared in different conical flasks. Each flask was amended with organic nitrogen sources (0.5%) such as casein, gelatin, tryptone Egg albumin and Bovine serum albumin. The flasks were inoculated with 5% inoculum and incubated at 32°C for 24 hours. The culture filtrate was collected and protein estimation and enzyme activity was determined.

Results and Discussion¹⁶⁻³²

Naturally occurring microorganisms are having the ability to produce various enzymes. Phosphatase is one of the important enzymes produced from several microorganisms. In this study rhizosphere soil sample of legume plant was chosen to isolate the phosphate solubilizers since the soil will have deposition of phosphate. The bacterial strain isolated from rhizosphere soil will have phosphate solubilizing capacity by producing extra cellular phosphatase. From the isolated sample, better zone by the bacterial strain was considered

based on solubilization efficiency test (Figure- 1). The phosphate solubilizing zone was found by performing plate assay in Hydroxyapatite medium. This Better zone obtained from the plate assay revealed the presence of phosphate solubilizer in isolated culture (Figure- 2).

Effect of incubation time on phosphatase production

The growth study of the organism is essential for the production of enzyme because most of the extracellular enzymes are produced during log phase of the organism. The environmental parameters show great influence in the growth of organism and production of enzymes. To optimize the incubation time, the bacterial

culture was withdrawn every twelve hours from 12 to 72 hours and the enzyme activity was investigated (Figure- 3). High amount of production was found at 24th hour (99 U/ml)

Effect of temperature on phosphatase production

To optimize the temperature for better production, productions were made in various temperature from 28°C to 52 °C. it was found that like other mesophilic bacteria, the isolate shows higher phosphatase activity (96U/ml) at 32 °C (Figure- 4). Many thermophilic bacterium like *Thermotoga neopolitana* needs 70 °C for better production of thermo stable phosphatase^{16,27}.

Figure.1 After screening for bacterial culture



Figure.2 Phosphate solubiling zone

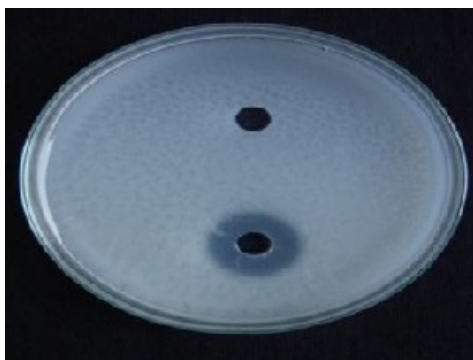


Figure.3 Effect of incubation time on phosphatase production

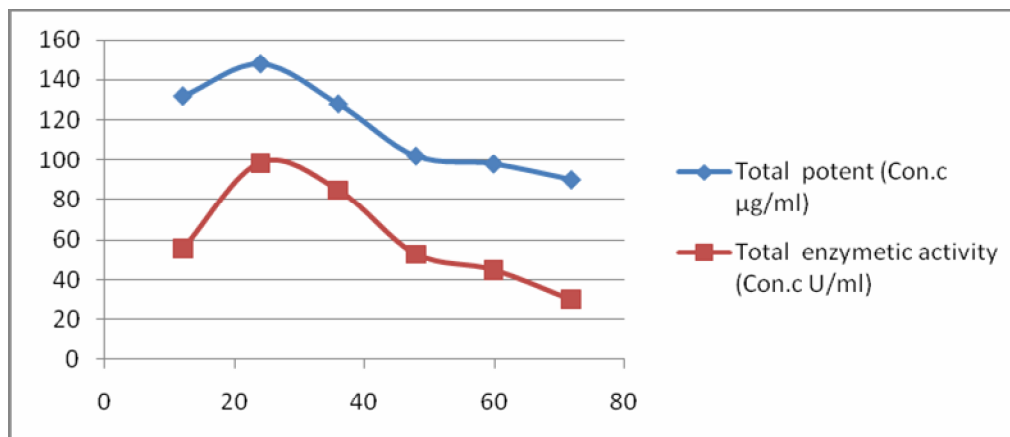


Figure.4 Effect of temperature on phosphatase production

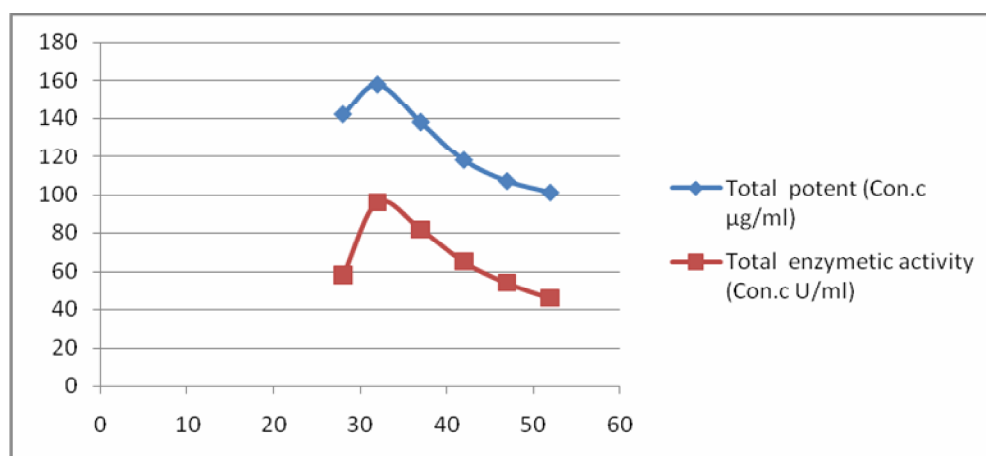


Figure.5 Effect of pH on phosphatase production

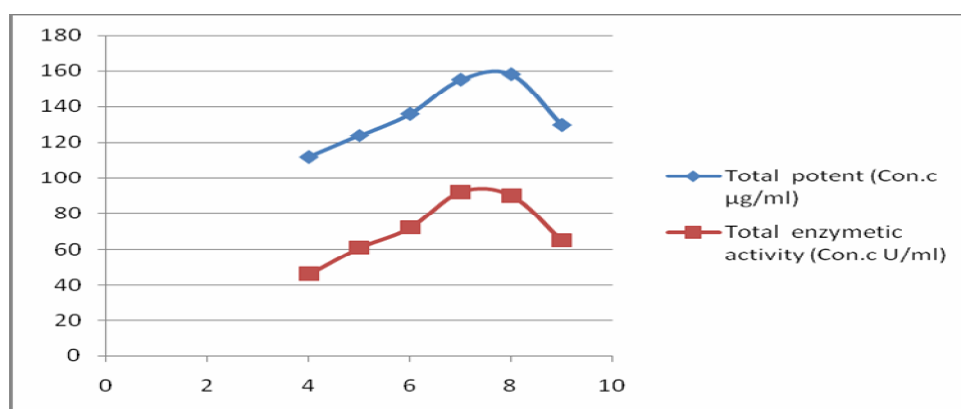


Figure.6 Effect of carbon sources on phosphatase production

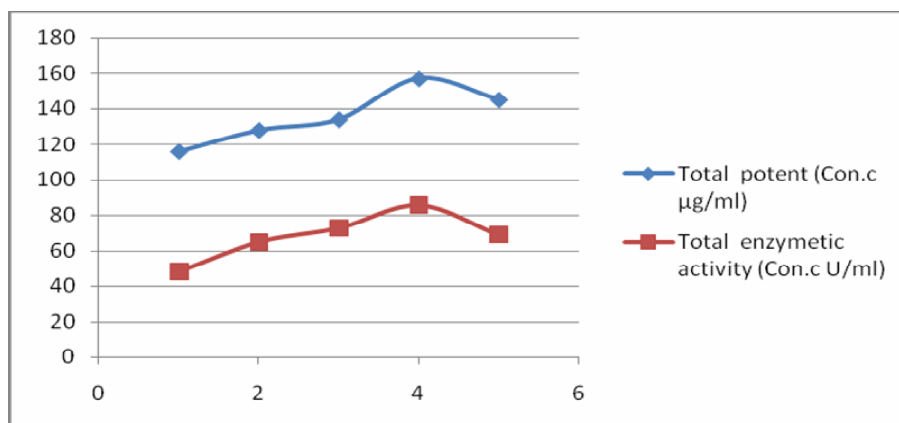
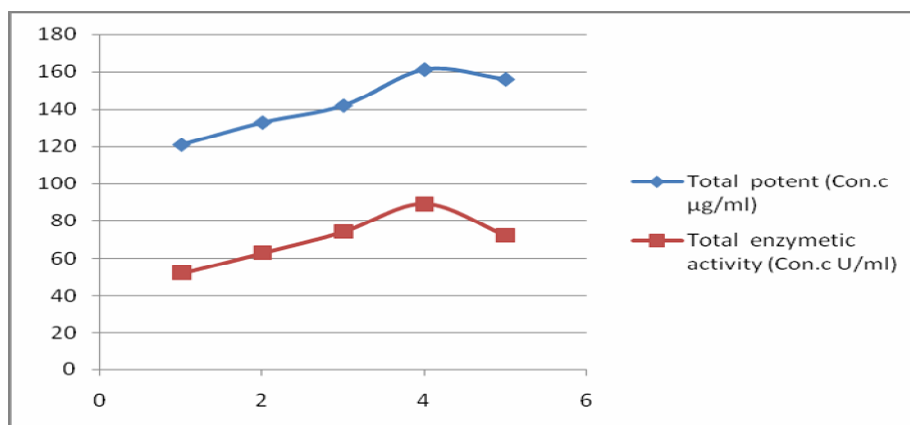


Figure.7 Effect of inorganic nitrogen sources on phosphatase production



Effect of pH on phosphatase production

Most of the bacteria require moderate pH for its growth. The present study revealed that the higher and considerable production was recorded at the pH 7 and 8 (92 and 90 U/ml), hence this bacterial strain needs alkaline pH for the maximum enzyme production. (Figure- 5) The same genus of *Bacillus subtilis* isolated from coastal region has produced the alkaline phosphatase at pH 9^{17,27}.

Effect of different carbon sources

The nature of the carbon compound and the concentration may stimulate or down modulate the production of enzymes.

Among the different carbon sources, starch shown the maximum production of enzyme (86 U/ml) (Figure- 6).

Effect of different nitrogen sources

The nitrogen sources serves as the secondary energy sources for the microorganisms, which plays an important role in the growth of the microorganism and enzyme production. The organic nitrogen source Egg albumin (89 U/ml) (Figure- 7).

In this study *Bacillus subtilis* was isolated from rhizosphere soil sample of legume plant and production of alkaline phosphatase was carried out. The culture parameters like pH, temperatures, carbon sources and

nitrogen sources were optimized for better production of alkaline phosphatase.

Acknowledgements

Authors are thankful to University of Kakatiya University and Pingle College of Science Warangal, Telangana, India for providing Technical support in the form of instruments, encouragement and guidance during research work.

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