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Optimization and production of Extracellular alkaline phosphatase from *Bacillus megaterium*

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Abstract: 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 megaterium*** was isolated from rhizosphere soil sample of legume plant and production of alkaline phosphatase was carried out. The parameters like pH, substrates, temperature, carbon sources, nitrogen sources and additives 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, pH 8,maltose as a carbon source and peptone as a nitrogen source were suitable for higher production of alkaline phosphatase.
Keywords: Alkaline phosphatase, *Bacillus megaterium*, optimization.

Introduction

Enzymes are proteins that accelerate and mediate the rate of bio chemical reactions in the living system. The enzymes are broadly classified into two category based on their secretion as extracellular enzymes and intracellular enzymes. The intracellular enzymes which will be secreted inside the cell and governing the cell metabolism. Where as the extracellular enzymes are secreted out of the cell in order to solubilize the substrates. 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.

Alkaline phosphatase (orthophosphate monoester phosphohydrolases E. C. 3.1.3.1.) is a nonspecific metallo enzyme[1], 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 activity[2].

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 phosphatase[3-6].

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 purification[9]. Thus, the present study is proposed to conduct the Optimization of culture conditions, production, purification and characterization of Extracellular alkaline phosphatase from *Bacillus megaterium*.

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 37ºC. 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 medium[10]. The pure cultures were streaked at the centre of the sterile Hydroxyapatite plates and the plates were incubated at 37ºC 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 4ºC.

Identification of Bacteria

The positive strain that produce maximum phosphatase enzyme was selected and was given for identification in IBMS, University of Madras, Taramani.

Enzyme Production

Preparation of Inoculum

The inoculum for further production of enzyme was prepared using Luria Bertani (LB) broth[11]. The pure culture was inoculated into sterile inoculum broth and was incubated at 37ºC in a rotary shaker for over night. 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 37ºC for 48 hours. The medium was agitated at 200rpm 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 glucose[12]. 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 37ºC 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 100µl of enzyme diluted in 200Mm Tris buffer (pH-8.5), 5mM $CaCl_2$, 500µmol pNPP in a final volume of 1ml. The reaction was performed at 37ºC for 30 min and stopped by addition of 50µl of 4M NaOH. One unit of phosphatase is the amount, which hydrolysas 1µmol of substrate per min. The strandard curve obtained by adsorbance of p-nitrophenyl phosphate(0 - 500µmol) at 400nm was used for quantification of enzyme activity[13].

Protein Assay

The chemical assay for the total protein content from the sample was determined using Bradford method[14].

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

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 37ºC with shaking around 150 rpm. After incubation, around 20ml of culture was aseptically with drawned periodically at 12hours 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 ºC,32 ºC, 37 ºC, 42 ºC, 47 ºC and 52 ºC 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 ºC for 24 hours. The protein estimation and enzyme activity were estimated.

Effect of different phosphate sources

100ml of sterile production medium (pH 8.0) was prepared in different conical flasks. Each flasks were amended with different phosphate sources like Na_2HPO_4, KH_2PO_4, K_2HPO_4, $Ca_3(PO_4)_2$, NaH_2PO_4 and NH_4PO_4. The flasks were inoculated with 5% inoculum and incubated at 32ºC for 24 hours. The culture filtrate was collected and protein estimation and enzyme activity was determined.

Effect of different Carbon Sources

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

Effect of different Organic Nitrogen sources

100ml of sterile production medium (pH 8.0) was prepared in different conical flasks. Each flasks were amended with organic nitrogen sources (0.5%) such as caesin, gelatin, beef extract, yeast extract and peptone. The flasks were inoculated with 5% inoculum and incubated at 32ºC for 24 hours. The culture filtrate was collected and protein estimation and enzyme activity was determined.

Effect of different Inorganic Nitrogen sources

100ml of sterile production medium (pH 8.0) was prepared in different conical flasks. Each flasks were amended with inorganic nitrogen sources (0.5%) such as ammonium sulphate, ammonium chloride, ammonium acetate, ammonium phosphate,ammonium nitrate, sodium nitrate, pottassium nitrate and urea. The flasks were inoculated with 5% inoculum and incubated at 32ºC for 24 hours. The culture filtrate was collected and protein estimation and enzyme activity was determined.

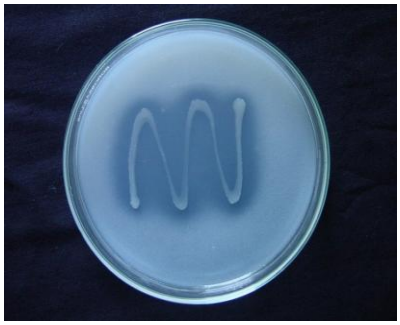
Effect of different Additives

Sterile production medium (pH 8.0) was preparedand ammended with glucose as carbon source. 0.5% of different filter sterilized additives like SDS, Triton X-100, Tween – 20, EDTA, $ZnSO_4$, $CaCl_2$, $MnSO_4$, $FeCl_2$, $MgCl_2$, $HgCl_2$ were added in separate flasks. Flasks were seeded with 5% inoculum and incubated at 32ºC for 24 hours. The total protein content and enzyme activity was estimated.

Results and Discussion

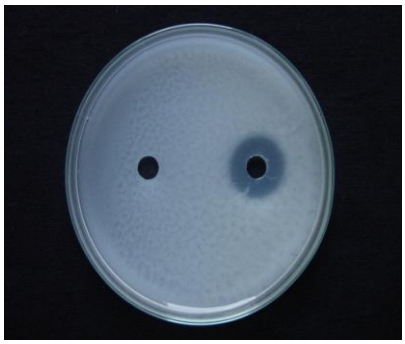
Naturally occuring microorganisms are having the ability to produce various enzymes. Phosphatase is one of the important enzyme produced from several microorganisms. In this study rhizosphere soil sample of legume plant was chosen to isolate the phosphate solubilisers since the soil will have deposition of phosphate. The bacterial strain isolated from rhizosphere soil will have phosphate solubilising 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).

Figure 1: Screening for bacterial culture



After screening for bacterial culture, The phosphate solubiling zone was found by performing plate assay in Hydroxyapatite medium. This Better zone obtained from the plate assay revealed the presence of phospate solubilizer in isolated culture (Figure- 2).

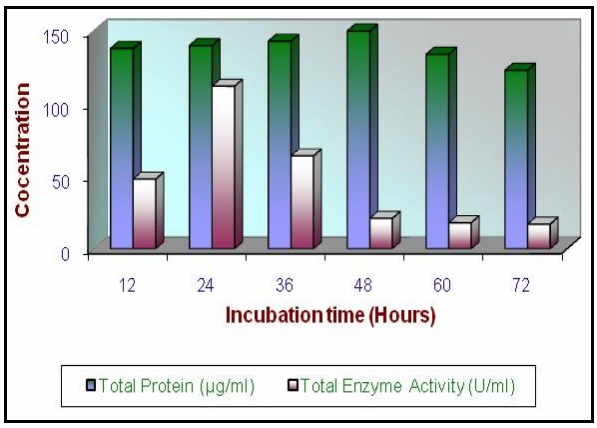
Figure 2: Phosphate solubiling zone



The identification study showed that the isolated positve strain has been assigned as *Bacillus megaterium* (Identification was done by the department of Microbiology, IBMS University of Madras, Taramani).

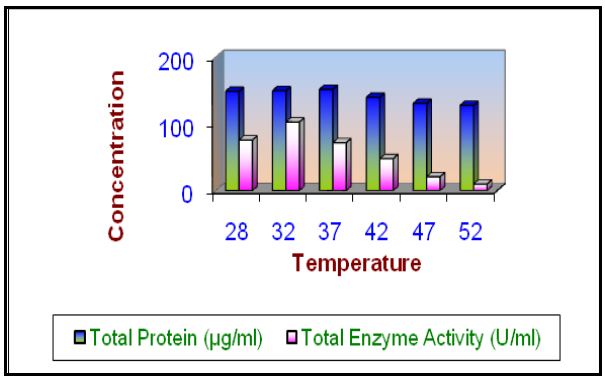
The growth study of the organism is essential for the production of enzyme because most of the extracellular enzymes are produced during log phase of the organism.The environmental parameters shows great influence in the growth of organism and production of enzymes. To optimize the incubation time, the 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 (112U/ml)

Figure 3: Effect of incubation time on phosphatase production



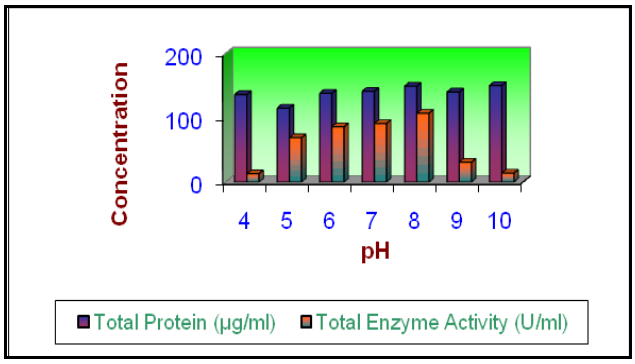
To optimize the temperature for better production, productions were made in various temperature from 28ºC to 52 ºC. it was found that like other mesophilic bacteria, the isolate shows higher phosphatase activity (103U/ml) at 32 ºC (Figure- 4). Many thermophilic bacterium like *Thermotoga neopolitana* needs 70 ºC for better production of thermo stable phosphatase[16].

Figure 4: Effect of temperature on phosphatase production



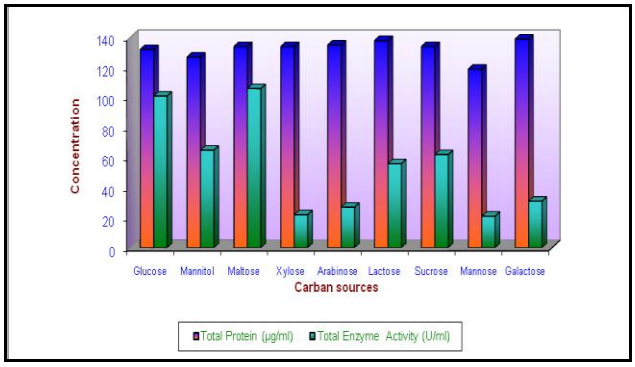
Most of the bacteria require moderate pH for its growth. The present study revealed that the higher and considerable production was recorded at the pH 8 (107U/ml), hence this bacterial strain needs alkaline pH for the maximum enzyme production. (Figure- 5) The same genus of *Bacillus subtilis* isolated from coastal region has produced the alkaline phosphatase at pH 9[17] .

Figure 5: Effect of pH on phosphatase production



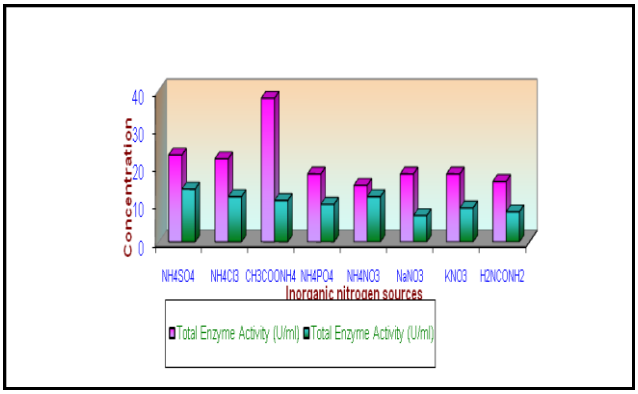
The nature of the carbon compound and the concentration may stimulate or down modulate the production of enzymes. Among the different carbon sources, maltose shown the maximum production of enzyme (106 U/ml) (Figure- 6).

Figure 6: Effect of carbon sources on phosphatase production



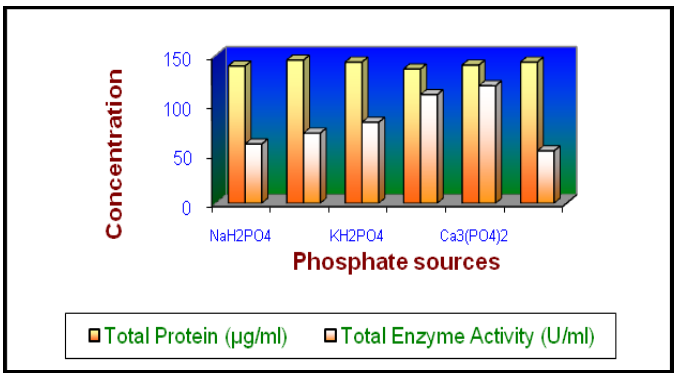
The nitrogen sources serves as the secondary energy sources for the microorganisms, which plays an important role in the growth of the microorganism and enzyme production. The organic nitrogen source peptone (107 U/ml) and inorganic nirogen source ammonium sulphate was found to be better nitrogen source for this isolate(Figure- 7).

Figure 7: Effect of inorganic nitrogen sources on phosphatase production



Although many phosphate sources are available, not all the phosphates will induce the microbes to produce the extra cellular phosphatase. Among the several phosphate sources, tricalcium phosphate (119 U/ml) was found suitable to produce phosphatase (Figure- 8). The higher concentration of phosphate induces the nutrient arrest and halted secretion of enzyme[18] .

Figure 8: Effect of phosphate sources on phosphatase production



In the case of enzyme production least quantity of additives like ionic detergents or minerals will be added to the medium to check the inhibition or enhancement of the production. In the present study, several additves like calcium chloride, zinc, manganese, magnesium ions, tween-20 etc. were used to enhance the production of phosphatase. Among which, magnesium chloride shown maximum enzyme production. These inductions may be varying fron organism to organism based on the mineral requirement and tolerance against the ionic detergents.

Conclusion

In this study *Bacillus megaterium* was isolated from rhizosphere soil sample of legume plant and production of alkaline phosphatase was carried out. The culture parameters like pH, substrates, temperatures, carbon sources, nitrogen sources and additives were optimized for better production of alkaline phosphatase.

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