# Effect of Physical and Chemical Parameters on the Activity of Purified Phosphatase Enzyme Produced by *Bacillus cereus*

Enas N. Danial[1,2,*] and Maha I. Alkhalf[1]

[1]*Biochemistry Department, Faculty of Science- Al Faisaliah King Abdulaziz University, Jeddah, Saudi Arabia*

[2]*Chemistry of Natural and Microbial Products, National Research Center, Cairo, Egypt*

**Abstract:** Phosphorus is one of the most important nutrients for plant growth and development. This study aimed to survey the optimum condition for phosphatase enzyme production, purified and studied its properties. Phosphatase enzyme activity was determined by end point method. Phosphatase enzyme was obtained from *Bacillus cereus* EME 66 isolate. The enzyme was purified and characterized, using a three-step purification procedure with 12.3-fold. The phosphatase enzyme was partially purified using ammonium sulfate fractionation followed by ultrafiltration. The acid phosphatase is a monomer protein purified gel filtration to 5.6 fold. Results showed that the optimum temperature for the purified enzyme activity was 60 °C and it was stable at temperatures below 60 °C. This enzyme was stable between pH 4.0-6.0, and the optimal pH activity was found to 5.0. The activity of the enzyme enhanced by heavy metals ($Fe^{3+}$, $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$ and $K^{+}$). The enzyme activity was strongly inhibited by heavy metals $Zn^{2+}$. The present article reveals on enzymatic characterization of acid phosphatase enzyme.

**Keywords:** Acid phosphatase, kinetic studies, enzyme purification, *Bacillus cereus.*

## INTRODUCTION

Acid phosphatases (EC. 3.1.3.2) are group of enzymes that catalyze the hydrolysis of many phosphate monoesters and show pH optima on acid side pH 5-6 [1]. This enzyme is able to mineralize organic phosphorus into inorganic phosphorus (Pi) that provides high (P) level for plants. Soil phosphatases play a major role in the mineralization processes (dephosphorilation) of organic phosphorus substrates. The enzymes in soils originate from animal, plant and microbial sources and the resulting soil biological activity includes the metabolic processes of all organisms [2]. The microorganisms supply most of the soil enzyme activity under favorable conditions, [3]. Activity of phosphatase enzyme is affected by some factors, that is, the amount and kind of substrate [4], pH, temperature, material of inhibitor and activator, concentration of enzyme and product, and also the kind of solvent used [5]. Besides, activity of soil phosphatase also the characteristic of physical and chemical of soil, that is, type of soil, content of organic matter, total nitrogen content, ratio of Carbon to Nitrogen (C/N) and content of total phosphorus [6]. Many microorganisms make enzymes in order to degrade complex substrates when preferred nutrients, such as glucose, ammonia, and Pi, are not available. Although all of these are essential, cells can obtain the nutrients provided by enzymes, such as proteases, lipases, and nucleases, through the metabolism of a variety of carbon or nitrogen sources [7]. Phosphate, however, cannot be synthesized by living organisms. Therefore, cells need to obtain phosphate from nucleic acids, and phosphorylated sugars, when Pi is limiting [8]. Thus, phosphatases are crucial enzymes for organism survival in nature as this enzyme can hydrolyze phosphate esters and provide Pi. Because microorganisms can face intense competition for phosphate, it would not be surprising to find that many organisms produce phosphatases and have elaborate mechanisms for regulating their synthesis and activities [9]. What is surprising, however, is the diversity of phosphatases that are being discovered. Unlike many enzyme families in which subunit size, metal ion requirements, and substrate specificities are conserved, the few phosphatases that have been studied vary greatly [3]. For example, alkaline phosphatase monomers as small as 15.5 kDa [10] and as large as 160 kDa have been reported from two different halophilic strains [11]. Many extracellular phosphatases are monomers; however, the enzyme from *Thermus aquaticus* is a 143- kDa trimer [12]. Phosphatases have high specificities in their substrate, metal ion requirements, and pH ranges. Some, such as the one from *Escherichia coli*, are periplasmic, some are membrane associated [5], and some are extracellular [13]. This picture of vast biochemical diversity is emerging from limited studies with a few microorganisms. These preliminary results suggest that the properties of new phosphatases will be particularly fruitful, because there is a high probability of discovering new types with novel properties.

The present work deals with the purification of acid phosphatase enzyme from *Bacillus cereus* EME 66 and

*Address correspondence to this author at the Biochemistry Department, Faculty of Science- Al Faisaliah King Abdulaziz University, Jeddah, Saudi Arabia; Tel: 00966509630287; E-mail: enas_mahdy@yahoo.com

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its characterization with respect to kinetic parameters, pH stability, thermal stability, enzyme concentration, and the effect of inhibitor and activator

## MATERIALS AND METHODS

### Isolation and Identification of Acid Phosphatase Bacteria

Among 30 different bacterial strains isolated from soil, a potent strain which gave a high yield of acid phosphatase was chosen for further study. The isolated strain was fully identified using morphological, biochemical and molecular biology technique as *Bacillus cereus*. This strain was routinely grown on (NA) agar medium [14] at 35 $^{o}$C for 72 hours. After this stage, the grown cells were collected in glycerol solution (50%) and stored in Cryogen vial at -80°C as master/working cells bank.

### Culture Conditions and Sample Preparation

The liquid media was used for cultivation of *Bacillus cereus* EME 66 was composed of (g/l): (% w/v): peptone 10.0; Dextrose 5.0; sodium phytate 1.0; yeast extract 5.0; $MgSO_4$ 1.0; $CaCl_2$ 1.0 (pH 7.0) Kim *et al*. [14]. The medium contents were sterilized by autoclaving at 121 °C. The broth medium (50 ml in 250 ml Erlenmeyer flasks) inoculated with 1 ml pre-culture inoculum of *Bacillus cereus* EME 66, the inoculated flasks were incubated at 30 ºC with agitation speed 180 rpm for 5 days.

### Purification of Crud Extra Cellular Acid Phosphatase Produced by *Bacillus cereus* EME 66

#### *Partial Purification of Acid Phosphatase*

The present experiment included the purification of extra cellular acid phosphatase using precipitation with ammonium sulphate, ethanol, and acetone methods according to the method of Greiner and Alminger [4].

#### *Precipitation Using Ultrafiltration*

The culture filtrate of about 2 liters was passed through Pellicon Cassette system with a membrane PLGC Cassette 10.000 NMWL. Low protein-binding.

#### *Gel Filtration of Extra Cellular Acid Phosphatase using Sephad ex G-100*

Sephadex G-100 was used as gel filtration for the enzyme purification. 7 ml of extra cellular acid phosphatase obtained from ultra-filtration contain 200 mg/ml protein was applied to a sephadex G-100 column chromatography (108 × 2.6 cm) which had been equilibrated with 0.1 M Tris buffer at pH 7.0 at 11 °C [15]. Elution was carried out with the same buffer. 5 ml fractions were collected at flow rate of 33 ml/h.

At first, the unbounded protein was removed by washing with the same buffer, and then enzyme was eluted by gradient elution using sodium chloride (0.0 to 1.0 M) in 0.1M Tris HCl buffer at pH 7.0. The flow rate was adjusted to 50 ml/h and 5 ml fractions were collected from the effluent.

#### *Acid Phosphatase Activity and Protein Assay*

The activity of acid phosphatase was measured spectrophotometrically by monitoring the release of para-nitrophenol from paranitrophenyl phosphate (*p*NPP) at 400 nm. A typical assay contained 330 mM of *p*NPP, 200 mM sodium acetate buffer at pH 5, 5 mM calcium chloride, and an appropriate amount of spent culture medium, in a final volume of 1 ml. Reactions were performed at 30 °C and stopped by addition of 5 ml of 4 M sodium hydroxide [16]. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 mM *p*NPP per min. Protein concentration was determined as described by Bradford [16].

#### *Characterization of Acid Phosphatase Activity*

The purified acid phosphatase was used for acid phosphatase activity characterization. All tests were repeated three times, each with triplicates.

#### *Effect of Temperature on the Stability of Acid Phosphatase Enzyme*

The thermal stability of the extra cellular purified enzyme was determined by storage the enzyme suspensions at different temperature in a water bath set (20-80 °C) for different times (up to 24 hours), then the enzymes activity were determined in each case.

#### *Effect of pH on the Acid Phosphatase Stability*

In this experiment, 1 ml of 0.15 M acetate buffer at pH values ranged between 3.5-6 were stored at room temperature with 1 ml of purified acid phosphatase enzyme solutions. The storage time was between 0 min. - 48 hours. The remains enzyme activity was then determined at Control conditions.

#### *Effect of Reaction Period on Purified Acid Phosphatase*

The effect of reaction time at 60 °C for acid phosphatase activities was also examined. Therefore, enzyme assay mixture was incubated for 1, 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 min.

### *Effect of Enzyme Concentrations of the Purified Extracellular Acid Phosphatase on their Activities*

Different concentrations of the enzyme solutions ranging from 0.01 to 0.3 ml (1 ml of acid phosphatase enzyme contain 0.05 mg protein) were added to 1 ml of 0.2% p-Nitrophenyl Phosphate (*p*NPP) solution in 0.05 M citrate buffer at pH 6 for 60 min at 60 °C.

### *Effects of Different Ions and Cations on Acid Phosphatase Activity*

The metal ions used in the experiment were $MgCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$, $CuCl_2$, $AlCl_3$, $CaCl_2$, $CoCl_2$, $FeCl_2$, $NiCl_2$, $KCl_2$, $MgCl_2$, Na Florida, EDTA and DTT. These ions were added in concentrations 1 and 2 mM to the reaction mixture to study their effect on the acid phosphatase activity. The control was carried out without adding activators or inhibitors.

## RESULTS

### Purification of Crude Extracellular Acid Phosphatase Produced by *Bacillus cereus* EME 66

### *Partial Purification of Enzymes*

The present experiment included the purification of extra cellular acid phosphatase using precipitation with ammonium sulphate, ethanol and acetone or using ultra filtration methods. It is clear from the data presented in Table **1** that ammonium sulphate at concentration 30-60% gave a specific activity of 105 and 1.7 U/mg protein and purification fold was 0.71.

Precipitation using acetone and ethanol also done and the results is showed in Table **1**. It is clear that, the data showed that the precipitation of acid phosphatase using acetone at concentration to be 0-30% gave high specific activity than ethanol at concentration 0-30%.

### *Precipitation using Ultrafiltration*

The results showed in Table **2** two liters were concentrated to about 50 ml which contain 10000 mg protein (recovered) which represent about 64.10% of total protein while the specific activity was 2.4 U/mg proteins with purification fold 1.01 for acid phosphatase.

### *Gel Filtration of Acid Phosphatase using Sephadex G-100*

The results in Figure **1** showed the pattern of gel filtration of acid phosphatase. The data illustrated the appearance of one peak for activity fraction (31-35), which it has total activity 1080 U at 25 ml and total protein 81 mg with specific activity reached to 13 U/mg proteins.

**Table 1: Partial Purification of Acid Phosphatase Produced by *Bacillus cereus* EME 66**

| Purification steps | | volume (ml) | Total protein | Protein recovery (%) | Total units (U/min) | Units recovery (%) | Specific activity | Purification fold |
|---|---|---|---|---|---|---|---|---|
| Culture filtrate | | 50 | 390 | 100 | 925 | 100 | 2.37 | 1 |
| Acetone | 0-30% | 50 | 100 | 25.6 | 215 | 23.2 | 2.15 | 0.90 |
| | 30-60% | 50 | 190 | 48.7 | 110 | 11.8 | 0.58 | 0.24 |
| | 60-90% | 50 | 22.2 | 5.5 | 00 | 00 | 00 | 00 |
| Ethanol | 0-30% | 50 | 109 | 28 | 202 | 21.8 | 1.85 | 0.78 |
| | 30-60% | 50 | 200 | 51 | 136 | 14.7 | 0.68 | 0.29 |
| | 60-90% | 50 | 00 | 00 | 00 | 00 | 00 | 00 |
| Ammonium sulphate | 0-30% | 50 | 195 | 50 | 122 | 13 | 0.62 | 0.26 |
| | 30-60% | 50 | 150 | 38 | 254 | 27.4 | 1.7 | 0.71 |
| | 60-90% | 50 | 00 | 00 | 00 | 00 | 00 | 00 |

**Table 2: Partial Purification of Acid Phosphatase Produced by *Bacillus cereus* EME 66 using Ultrafiltration**

| Purification steps | volume (ml) | Total protein | Protein recovery (%) | Total units (U/min) | Units recovery (%) | Specific activity | Purification fold |
|---|---|---|---|---|---|---|---|
| Culture filtrate | 2000 | 15600 | 100 | 37000 | 100 | 2.37 | 1 |
| Ultra-filtration | 50 | 10000 | 64.4 | 24000 | 64.8 | 2.4 | 1.01 |

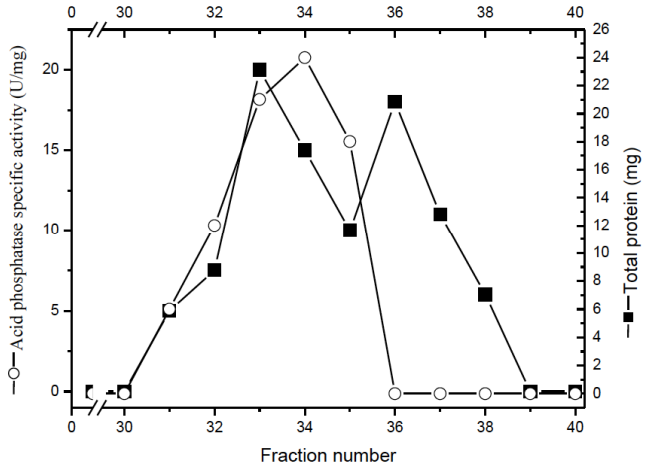


**Figure 1:** Purification of extracellular acid phosphatase by using sephadex G-100.

The active fraction of acid phosphatase was chosen for the check of their purity on polyacrylamide gel electrophoresis. Figure **2** showed that acid phosphatase was homogenous as it migrated as single protein band.

### *Summarized Purification Steps of Acid Phosphatase*

Purification steps acid phosphatase from *Bacillus cereus* EME 66 is summarized in Table **3**. The cultural filtrate obtained after centrifugation of the fermentation broth was subjected to ultrafiltration using Pellicon Cassette system with a membrane PLGC Cassette 10.000 NMWL, low protein-binding. This step yielded 1.01 fold of purification for acid phosphatase. It was chromatographed on sephadex G100 column and the enzymes were eluted with tris HCl buffer of pH 7.0.

This procedure showed that the acid phosphatase was purified approximately 5.6 fold from the culture filtrate with a specific activity about 13.3 U/mg proteins. Figure **2** showed that acid phosphatase resulted from

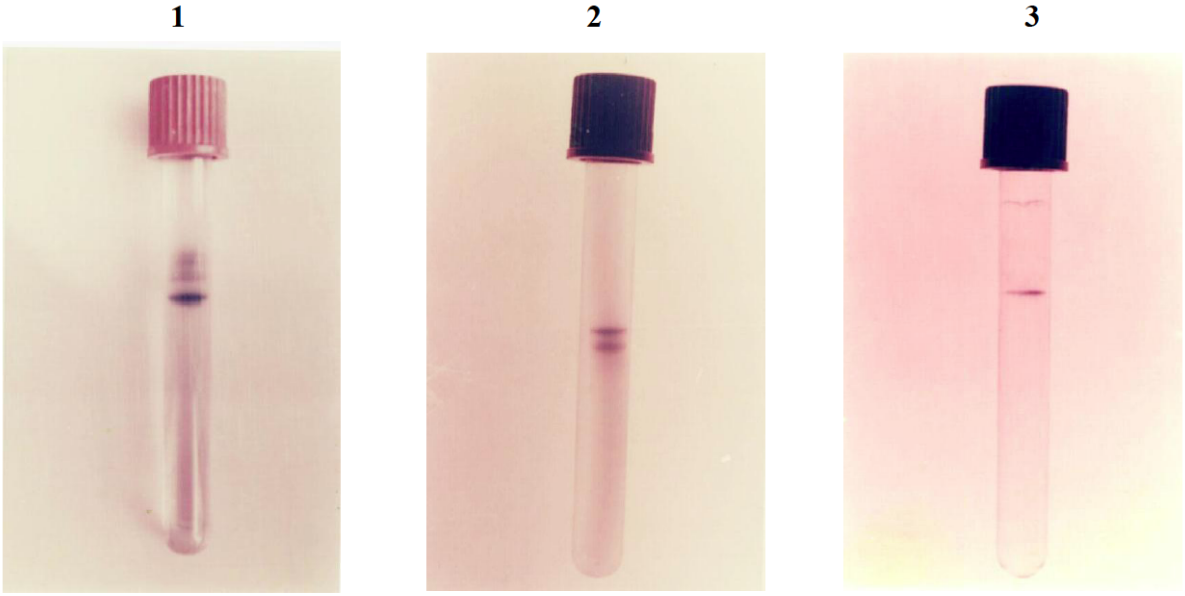


**Figure 2:** Purification steps of acid phosphatase. (1) Crude enzyme, (2) partially purified enzyme by ultra-filtration, (3) the enzyme of acid phosphatase separated on sephadex G 100.

**Table 3: Purification Steps of Acid Phosphatase**

| Purification Steps | Volume (ml) | Total protein | Protein Recovery (%) | Total activity (U/min) | Units recovery (%) | Specific activity | Purification fold |
|---|---|---|---|---|---|---|---|
| Culture filtrate | 40 | 312 | 100 | 740 | 100 | 2.37 | 1 |
| Ultra-filtration | 1 | 200 | 64.1 | 480 | 64.8 | 2.4 | 1.01 |
| Sephadex G-100 | F(1-21) | 145.8 | 46.8 | 00 | 00 | 00 | 00 |
| | F(22-30) | 267 | 85.5 | 00 | 00 | 00 | 00 |
| | F(31-35) | 81 | 26 | 1080 | 146 | 13.3 | 5.6 |
| | F(36-60) | 119.1 | 38 | 00 | 00 | 00 | 00 |

sephadex G100 column chromatography was migrated as single protein band.

## Properties of Purified Acid Phosphatase Produced by *Bacillus cereus* EME 66

### *Effect of Temperature on the Stability Acid Phosphatase*

The thermal stability of the extracellular purified acid phosphatase was determined by incubation the enzyme suspensions at different temperature in a water bath (between 20 and 80 °C) for different periods (up to 24 hours), then the enzyme activities were determined in each case.

The results in Table **4** showed that the stability of acid phosphatase was depending on the temperature and the time. In most cases, the loss in acid phosphatase activities was increased by increasing the temperature and the storage time.

The pure acid phosphatase was stable at temperature 20, 30 °C and 40 °C for 24 hours. At temperature 50 °C the enzyme lost 11% of its activity after 24 hours. The acid phosphatase completely lost its activity at 70 °C after 90 min or after 30 min at 75 °C and after 5 min at 80 °C.

### *Effect of pH on Acid Phosphatase Stability*

In this experiment, 1 ml of 0.15 M acetate buffer at pH values ranged between 3.5-6 were stored at room temperature with 1 ml of purified acid phosphatase Enzyme solutions

The remain activity of purified acid phosphatase enzyme was then determined at 60 °C and pH 6, and control was carried out using pH 6

The results recorded in Table **5** indicated that the stability pure acid phosphatase showed stability at pH 5

**Table 4: Effect of Temperature on Acid Phosphatase Stability**

| Temp.°C | Relative activity (%) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time (minutes) | | | | | | | | | Time (hours) | | | | |
| | 0 | 5 | 10 | 20 | 30 | 40 | 50 | 60 | 90 | 2 | 4 | 6 | 12 | 24 |
| 20 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 30 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 40 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 89 |
| 55 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 91 | 84 | 82 | 76 |
| 60 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 95 | 86 | 71 | 65 | 53 | 41 | 20 |
| 65 | 100 | 100 | 100 | 100 | 100 | 96 | 83 | 75 | 69 | 64 | 59 | 53 | 34 | 14 |
| 70 | 100 | 91 | 85 | 72 | 50 | 34 | 10 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 75 | 100 | 83 | 73 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 80 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 5: Effect of pH on Acid Phosphatase Stability**

| pH Value | Acid phosphatase Relative activity (%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time (minutes) | | | | | Time (hours) | | | | | |
| | 0 | 15 | 30 | 45 | 60 | 2 | 4 | 8 | 16 | 24 | 48 |
| 3 | 86 | 65 | 33 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3.5 | 91 | 82 | 68 | 42 | 30 | 18 | 6 | 0 | 0 | 0 | 0 |
| 4 | 100 | 100 | 100 | 100 | 100 | 82 | 63 | 48 | 22 | 9 | 4 |
| 4.5 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 95 | 81 | 76 |
| 5 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5.5 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 6 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 92 | 85 | 78 |
| 6.5 | 100 | 100 | 100 | 94 | 85 | 71 | 65 | 59 | 50 | 46 | 34 |

to 5.5 for 48 hours and lost some of its activity at pH 6.0 at 16 hours of incubation. At pH 3 and 3.5, acid phosphatase was completely inhibited at 60 min and 8 hours respectively. At pH 4.5 the enzyme was stable till 8 hours and partially lost its activity by increasing the time of storage.

### *Effect of Reaction Period on Purified Acid Phosphatase*

The effect of incubation time on enzyme activity was monitored by incubating the enzyme reaction mixture in different time period (10 to 90 min). The results in Figure **3** showed that the rate of *p*NNP hydrolysis of reached its maximum at 60 min, then decreased with the incubation time. The activity of acid phosphatase was measured at different periods of incubation (10 to 90 min). The enzyme activity slowly increased from 10 to 70 min. The maximum of enzyme activity was obtained at 70 min and after that, the activity gradually decreased.

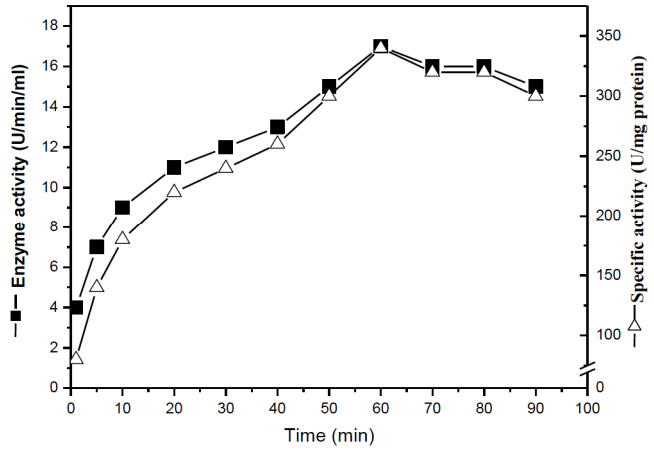


**Figure 3:** Effect of the incubation period on the purified acid phosphatase.

### *Effect of Enzyme Concentrations of the Purified Extracellular Acid Phosphatase on their Activities*

The data recorded in Figure **4** showed that the acid phosphatase activity was increased gradually with the increase of enzyme concentrations up to 0.1 ml enzyme, at which the acid phosphatase showed a maximum activity (500 U/mg proteins). Above this concentration the specific activity decreased.

### *Effect of Different Concentrations of Activators and Inhibitors on the Acid Phosphatase Activities*

In this experiment, the metal ions used in the experiment were $MgCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$, $CuCl_2$, $AlCl_3$, $CaCl_2$, $CoCl_2$, $FeCl_2$, $NiCl_2$, $KCl_2$, $MgCl_2$, NaF, EDTA and DTT. They were added in concentrations 1 and 2 mM to the reaction mixture to study their effect on the acid phosphatase activities and their substrates. The results recorded in Table **6** showed that the enzyme activity was greatly inhibited by $FeCl_2$, Na Florida, EDTA, $AlCl_3$, $ZnCl_2$ and DTT. On the other hand the addition of $MnCl_2$, $BaCl_2$, $CuCl_2$ and $NiCl_2$ showed partial inhibition of acid phosphatase while, $MgCl_2$, $CoCl_2$, and $KCl_2$ had no effect on the both enzymes**.** Moreover, $CaCl_2$ made a slight increase in the activity of phytase when added at concentration in 1 mM, the activity was decreased with decreasing the concentration, but there is no effect on acid phosphatase activity.

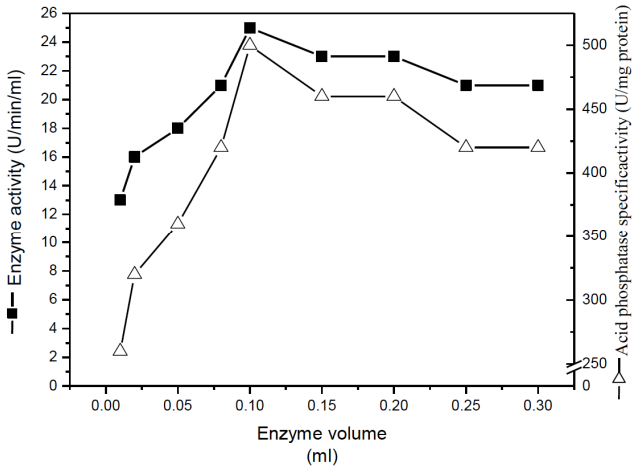


**Figure 4:** Effect of enzyme concentrations of the purified extracellular acid phosphatase on their activities.

**Table 6: The Effect of Different Concentrations of Activators and Inhibitors on the Acid Phosphatase Activities**

| Additive | Acid phosphatase Relative activity % | |
|---|---|---|
| | 1 mM | 2 mM |
| Control | 100 | 100 |
| $MgCl_2$ | 98 | 91 |
| $MnCl_2$ | 93 | 87 |
| $ZnCl_2$ | 78 | 70 |
| $BaCl_2$ | 86 | 83 |
| $CuCl_2$ | 89 | 80 |
| $AlCl_3$ | 78 | 69 |
| $CaCl_2$ | 100 | 99 |
| $CoCl_2$ | 100 | 95 |
| $FeCl_2$ | 60 | 43 |
| $NiCl_2$ | 91 | 89 |
| $KCl_2$ | 96 | 91 |
| $MgCl_2$ | 84 | 68 |
| EDTA | 78 | 65 |
| NaF | 69 | 52 |
| DTT | 74 | 56 |

## DISCUSSION

Bacilli have been widely used in many fermentation industries since many years [16]. This based on their high capacity for the production of bioactive metabolites. In addition, they also considered as one of the best enzyme biofactories based not only their ability to produce wide range of enzymes in high concentration but also due to their high secretion capacity and export of protein to the fermentation medium [17-21].

The present study is demonstrated the purification and characterization of acid phosphatases from *Bacillus cereus* EME 66. First attempts to separate acid phosphatase activity by the one-step procedure described by Tham [22], for acid phosphatase purification was unsuccessful. This procedure was based on the difference of isoelectric points of enzyme. When chromatographed on a strong cationic-exchanger (SP-Trisacryl) column at pH 4.5, soybean phosphatase was retained on the column, whereas acid phosphatase was excluded. Under the same conditions, enzyme activities were retained and eluted at the same saline concentration.

Purification of enzyme phosphatase was by ammonium sulfate precipitation followed by dialysis. The optimum temperature for enzyme purified activity was 40 °C and the enzyme was stable at temperatures below 60 °C when preincubated at various temperatures for 30 min. Cheng and Yang, reported Thermo-tolerant phosphate-solubilizing microbes with six types of enzyme activities and three types of inorganic phosphate-solubilizing activities at 25 and 50°C were isolated from the composts and biofertilizers [1]. Acid phosphatase of *Penicillium citrinum* (cold-tolerant fungus) had an optimum temperature of 60°C. The dried enzyme extract is stable at a temperature of up to 50°C for at least 1 h [4]. In addition, pH had a statistical effect on the activity of acid phosphatase where, in more acid Acid phosphatase isolated from *Burkholderia gladioli* was stable after 6 h of incubation at 45 °C in 100 mM acetate buffer at pH 6.0. The rate of hydrolysis enzyme reached a maximum at pH 6.0 [23].

The phosphatases were found to be thermostable and acid tolerant the enzyme was thermostable since the retained their full activity even after 2 hours of exposure to the temperature range 40 to 65°C. Our finding is in Agreement with the previous report which showed thermostability of acid phosphatase from *A. niger* up to 70°C [24,25].

In the reaction medium the incubation time might be due to stability of enzyme. The enzyme was also found to be thermostable with 60 °C as its optimum temperature. Enzyme activity was decreased after 60 °C might be due to irreversible denaturation. Acid phosphatase (from Red kidney bean purple) had optimal enzyme activity at 60 °C [26]. The acid phosphatase from soybean seeds was shown to be heat stable [12]. However, the obtained temperature 70 °C for acid phosphatase in germinated mung bean seed was higher than the value obtained for the enzyme in cotton seedling [27] and in *Vigna aconifolia* [28].

Large number of metal ions was tested for their effects on the acid phosphatase activity (Table **4**). The enzyme was not affected by $Ca^{+2}$, $Mg^{+2}$, $Ba^{+2}$, $Mn^{+2}$ and $Co^{+2}$ in the reaction mixture, indicating that divalent cations are not required for the catalytic activity of this enzyme. A similar neutral effect of these ions was also reported for acid phosphatases isolated from axes and cotyledons of germinating soybeans [22].

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