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Purification and characterization of acid phosphatase from monocrotophos (MCP) hydrolyzing *Aspergillus niger* ITCC 7782.10 Isolated from Local Agricultural Field

[Yerel tarım alanlarından monocrotophos'u (MCP) hidrolize eden *Aspergillus niger* ITCC 7782.10'dan izole edilen asit fosfatazın saflaştırılması ve tanımlanması]*

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ABSTRACT

Objective: The present study aimed the characterization and purification of acid phosphatases from monocrotophos (MCP) hydrolyzing *Aspergillus niger* ITCC 7782.10 isolated from local agricultural field.

Material and Methods: Intracellular phosphatases from MCP hydrolyzing *Aspergillus niger* ITCC 7782.10 were purified by four step purification procedure which were further characterized.

Results: The study demonstrated the presence of two intracellular phosphatases of varying molecular weights of 33 and 67 kDa which were purified to 126 and 76 fold respectively by four step purification strategy. Both the phosphatases showed similar pattern for pH, temperature and metal ion however, the activity of P33 was found to be higher than that of P67. The phosphatases were found to be most active at acidic pH and were found to be stable till 80°C demonstrating them to be acid phosphatases and thermostable. K_m and V_{max} values of P33 was found to be 0.28 mM and 1.21 Umg^{-1}protein respectively whereas K_m of P67 was found to be 0.72 mM and V_{max} value was 1.85 Umg^{-1}protein. This indicated that P33 had more affinity towards para nitro phenol phosphate (pNPP) as a substrate than P67.

Conclusion: This is the first report indicating the presence of two different intracellularly thermostable acid phosphatases within the same MCP hydrolyzing fungal strain *Aspergillus niger* ITCC 7782.10. Therefore, these acid phosphatases could be effectively used for the bioremediation of MCP contaminated sites.

Key words: Acid phosphatase, enzyme activity, monocrotophos, pesticide, purification

Conflict of Interest: The authors do not have any conflict of interest.

ÖZET

Amaç: Bu çalışma yerel tarım alanlarından monocrotophos'u (MCP) hidrolize eden *Aspergillus niger* ITCC 7782.10'dan izole edilen asit fosfatazın saflaştırılması ve tanımlanmasını amaçlamıştır.

Materyal ve Metot: Hücreiçi fosfatazlar, monocrotophos'u (MCP) hidrolize eden *Aspergillus niger* ITCC 7782.10'dan daha ilerde tanımlanacağı şekilde dört basamaklı bir saflaştırma yöntemi ile elde edildi.

Bulgular: Bu çalışma ile moleküler ağırlıkları 33 ve 67 kDa arasında değişen dört basamaklı saflaştırma stratejisi ile sırasıyla 126 ve 76 kata kadar saflaştırılan hücre içi iki fosfatazın varlığı gösterildi. Her iki fosfatazda pH, ısı ve metal iyonları için benzer etki gösterirken, P33 aktivitesi P67'den daha yüksek bulundu. Her iki fosfatazda asidik pH'da daha aktif ve 80°C'a kadar stabil bulundukları için termostabil ve asit fosfatazlar olarak değerlendirildi. K_m ve V_{max} değerleri P33 için sırasıyla 0.28 mM ve 1.21 Umg^{-1}protein olarak bulunurken P67 için K_m değeri 0.72 mM ve V_{max} değeri 1.85 Umg^{-1}protein olarak bulundu. Bu sonuçlar P33'ün para nitro fenol fosfata substrat olarak afinitesinin P67'den daha fazla olduğunu gösterdi.

Sonuç: Bu çalışma, aynı MCP hidrolize eden fungal hat *Aspergillus niger* ITCC 7782.10'ların içinde iki farklı hücreiçi termostabil asit fosfatazın olduğunu bildiren ilk makaledir. Böylelikle bu asit fosfatazlar MCP ile kontamine alanlarda biyolojik ajanlar kullanılarak çevre kirliliğin biyolojik olarak giderilmesinde etkin olarak kullanılabilirler.

Anahtar Kelimeler: Asit fosfataz, enzim aktivitesi, monokrotofos, pestisid, saflaştırma

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Monocrotophos (MCP) is a cost effective organophosphate pesticide that is widely used to control a variety of insects on economically important crops, such as cotton, sugarcane, peanuts and tobacco [1]. MCP is a Dimethyl-(E)-1, 2-methylcarbamoylvinylphosphate that is water soluble in nature. Due to its hydrophilic nature, MCP finds its way to the surface and groundwater thereby leading to adverse effects on biological systems [2]. Exposure to MCP may cause wide range of health related problems such as irritation to eyes and skin, muscular weakness, blurred vision, and even death owing to respiratory failure [3]. Therefore, it is of great concern to remove MCP and its conversion products from soil. Microbial transformation serves as the primary biological pathway for metabolizing MCP in both soil and in water, which employs biological systems, such as bacteria, fungi, and enzymes, to degrade these harmful environmental pollutants [4]. Two different approaches including passive and active could be used for this purpose. The passive approach involves the physical attenuation of the pollutants by the existing microbes whereas; active approach employs microbial bioremediation involving the addition of specific enzymes or microbes to the contaminated area. Active remediation strategies have been proven to be more rewarding and environment friendly than the other traditional chemical or physical remediation processes [5, 6] in view of the fact that they minimizes waste disposal and heating needs [7, 8]. Therefore, numerous studies are being carried out globally to isolate and characterize the enzymes, which could potentially detoxify these harmful pesticides. In this context, an enzyme capable of hydrolyzing parathion and related organophosphorus insecticides has been reported to be isolated from a mixed bacterial culture [9]. Further, the metabolism of parathion has been shown by enzyme preparations from bacteria or by mixed-culture bacteria [10]. Another study demonstrated the isolation and purification of three unique parathion hydrolases from gram-negative bacterial isolates [11].

Organophosphate pesticides possess a characteristic phosphate–crotanamide linkage and these hydrolases cleave this bond leading to hydrolysis of pesticides. Therefore, these enzymes are referred as esterase or phosphotriesterase [12], or phosphatase [13]. Many microbes including *B. megaterium* and *A. atrocyaneus* were reported to degrade MCP by producing enzymes like phosphatase and esterase [14]. Although bacterial isolates are very well known for the production of phosphatases however, there are limited studies regarding fungal phosphatases, which can serve as a useful tool to hydrolyze MCP [15]. The background study in our laboratory revealed *Aspergillus niger* ITCC 7782.10 as an efficient degrader of MCP [16]. Since, dephosphorylation is the first step in MCP catabolic pathway, the present study aimed to purify and characterize MCP hydrolyzing phosphatases in *Aspergillus niger* ITCC 7782.10.

Materials and Methods

Chemicals

Monocrotophos of analytical grade (99. 5% purity) was purchased from Sigma and stock solution (1 $mgml^{-1}$ in ethanol) was prepared. All the other chemicals used in this study were of analytical grade and were obtained from Himedia and Rankem, India.

Medium

Modified Czapek-Dox medium was used as growth medium for the *A. niger* ITCC 7782.10 to produce phosphatase which contained sucrose, 30 g; $NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; glucose, 10 g; $FeCl_3$, 10 mg; $BaCl_2$, 0.2 g; $CaCl_2$, 0.05 g per litre and was supplemented with MCP (150 μgml^{-1}) and KH_2PO_4, 0.5 gmL^{-1} as an additional source of phosphorus.

Phosphatase Assay

Phosphatase activity of the enzyme preparation was determined by the modified method (without 0.12 mM Mg^{++}) of Tham [17]. The reaction mixture contained 100 µl of enzyme, 1 ml of 0.5 mM para nitro phenol phosphate (pNPP) and 1 ml 50 mM Tris-HCl buffer (pH-8). The reaction mixture was incubated at 60°C for 10 min and the reaction was terminated by the addition of 1 ml NaOH (1 M). Enzymatic activity was determined by measuring the released p-nitrophenol at 410 nm. Reaction mixture without enzyme was used as blank. One unit (U) of phosphatase activity was defined as µmoles of pNP released per ml per min. Enzyme activities and the total protein contents of the crude and purified fractions after each successive purification steps were determined in order to assess the specific activity and fold purification. Protein content was estimated by the Lowry method [18]. All the experiments were conducted in triplicates.

Phosphatase Purification

The experiments described below were carried out between 0 and 4°C unless otherwise specified. *A. niger* ITCC 7782.10 was incubated at 30°C for 10 days in 500 ml Erlenmeyer flasks containing 150 ml of modified Czapek-Dox medium supplemented with MCP (150 μgml^{-1}) and KH_2PO_4, 0.5 gmL^{-1}, on a rotary shaker at 100 rpm and harvested by centrifugation at 12.000× 3 *g* for 20 min. The phosphatase activity was checked in the supernatant as well as in the mycelial extract. To prepare the fungal mycelial extract, the mycelial pellet was washed twice with cold 50 mM Tris-HCl buffer (pH 8.0). Further, the mycelia were crushed in liquid nitrogen followed by addition of 5 ml of protein isolation buffer (PIB) (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2 % PVPP) per 2 g weight of mycelium and centrifuged at 608.2 *g* for 30 min. Afterwards, supernatant was collected and frozen overnight with equal amount of acetone. The mixture was again centrifuged at 608.2 *g* and the pellet was dissolved in minimum amount of 50 mM Tris-HCl buffer (pH-8). Extracted proteins were precipitated overnight with 80 % ammonium sulfate. The resulting precipitate was collected by centrifugation

at 12.000×3 *g* for 20 min, dissolved in the smallest possible volume of 50 mM Tris-HCl buffer (pH 8.0) and dialyzed twice with the 1000 fold higher volume of same buffer. The concentrated enzyme solution was loaded onto a Sephadex G-100 column (1.8 by 100 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was washed with 400 ml of the same buffer at a flow rate of 24 ml/h. Fractions (5 ml) were collected and the fractions with high specific activity were then pooled and concentrated for further purification. Sephadex G-100 active protein fractions were loaded on a DEAE-Sepharose CL-6B ion-exchange column (1.2 by 30 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was washed with same buffer (500 ml) at a flow rate of 20 ml/h and proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M. 5 ml fractions were collected and enzyme activity was calculated. Enzymes were further characterized by SDS-PAGE analysis [19]. The molecular weight of native enzymes was determined using blue dextran and molecular weight markers from Sigma as standards [20].

Optimization of Phosphatase Activity

Optimum temperature and pH of phosphatase was determined by measuring its activity at varied temperature (10°C to 37°C) and pH (1-10, at the gap of one unit). Results were expressed as % relative activity.

Phosphatase Stability

Phosphatase stability was checked by pre-incubating the enzyme at different pH (1.0-8.0) and temperature (10°C-100°C) for 18 hours. The phosphatase activity was then determined using the standard method as described above. Reaction mixture without substrate/enzyme was used as blank.

Kinetics Analysis

K_m and V_{max} of the phosphatases were calculated in 50 mM Tris- HCl buffer (pH-8) at 37°C using different concentrations of pNPP (0.06-0.84 mM) as substrate using the standard protocol. The values of kinetic constants were calculated by using Lineweaver and Burk plot [21].

Effect of Physical and Chemical Agents on Phosphatase Activity

The effects of physical agents such as UV, sunlight and chemicals (SDS, NaN_2, EDTA, potassium ferricyanide, $HgCl_2$, mercaptoethanol, $CoCl_2$, tartarate, NaF, $ZnCl_2$, $CuCl_2$, $FeCl_3$ and $MnCl_2$; 1 mM) on the phosphatase activity was determined by pre incubating the phosphatases for 30 min to the respective agents followed by carrying out phosphatase assay.

Statistical Analysis

All the experiments were carried out in triplicates and the results were expressed as means ± standard deviation. The statistical analysis was done by using SPSS 17 program (Statistical Package for the Sciences System). The variables were subjected to t-Test and One Way ANOVA (significance was set at $p \leq 0.05$).

Results

Phosphatase Extraction and Purification

Since phosphatases are considered to catalyze the initial step i.e., dephosphorylation in MCPs catabolic pathway, therefore the *A. niger* ITCC 7782.10 was analyzed for the presence of extracellular and/or intracellular phosphatase followed by their purification. For that, *A. niger* ITCC 7782.10 was grown in modified Czapek-Dox medium supplemented with MCP and KH_2PO_4 for 7 days. Phosphatase activity was checked in the supernatant as well as the fungal mycelia extract. Our results showed that phosphatases were present both extracellularly and intracellularly. However, the phosphatase activity was found to be higher in the fungal mycelial extract (11.4 U and Sp. activity 2.71 Umg^{-1}) than the supernatant (1.08 U and Sp.activity 2.35 Umg^{-1}). Therefore, the mycelial extract was used as a source of phosphatases for further purification. Purification strategy involved ammonium sulphate precipitation (80 %), followed by gel filtration chromatography (Sephadex G-100) and ion exchange chromatography (DEAE-Sepharose CL-6B). The purification data was summarized in Table 1. DEAE-Sepharose CL-6B column fractions showed two different peaks of phosphatases designating the presence of two enzymes having different molecular weights, which was further confirmed by the SDS-PAGE analysis of purified fractions (Figure 1). These two were designated as P33 and P67 based on their molecular weight. P33 phosphatase showed approx. 126 fold purification with a specific activity and yield of 294.53 Umg^{-1} and 50.35 % respectively. Similarly, P67 phosphatase exhibited 75.61 fold purification with the specific activity of 33.94 Umg^{-1} and the yield of 53.99 %.

Optimization of Phosphatase Activity

Purified P33 and P67 phosphatase were analyzed to determine pH (1.0-8.0) and temperature (10-100°C) optima. The pH profile of both the enzymes P33 and P67 showed maximum activity at pH-3.0 and pH 4.0 respectively indicating them as acid phosphatases (Figure 2A) and thereafter, all the experiments were carried out at pH 4.0. The temperature profile showed that P33 had maximum activity at 70°C and P67 had the highest activity at 60°C (Figure 2 B).

Phosphatase stability

The pH stability profile demonstrated that P33 was quite stable between pH 1.0 to 3.0 showing maximum activity (85 to 90%) but afterwards there was a linear decrease in the enzyme activity up to 30% at pH 8.0 (Figure 3 A). In contrast, P67 was found to be quite stable in the pH range 1.0 to 5.0, thereafter, the stability declined. On the other hand, the temperature stability profile demonstrated temperature tolerance up to 70°C for P33 and 60°C for P67. Surprisingly, both the enzymes retained their 30 and 40% activities at 100°C respectively (Figure 3 B).

Table 1. Purification profile of intracellular enzyme from *Aspergillus niger* ITCC 7782.10

		Total activity (U)	Total protein (mg)	Sp.Activity (Umg^{-1})	Purification fold	Yield %
Crude	P	812.63± 2.81	351.33±5.97	2.31±0.03	1.00	100.00
Extracted (Ppt)	P	553.73±3.52	221.90±8.45	2.49±0.11	1.07±0.03	68.14±0.67
Supernatant	P	230.18±1.31	67.27±2.14	3.42±0.12	1.48±0.07	28.32±0.07
G-100	P 33 Kd	450.02±2.64	5.26±0.35	85.69±6.29	37.04±3.07	55.37±0.16
	P 67 Kd	100.21±0.82	5.26±0.35	19.08±1.37	42.46±4.73	63.35±2.20
DEAE CL6B	P 33 Kd	408.98±1.57	1.4±0.16	294.53±35.77	126.10±14.96	50.35±0.26
	P 67 Kd	85.4±1.43	2.53±0.23	33.94±3.60	75.61±10.99	53.99±2.37

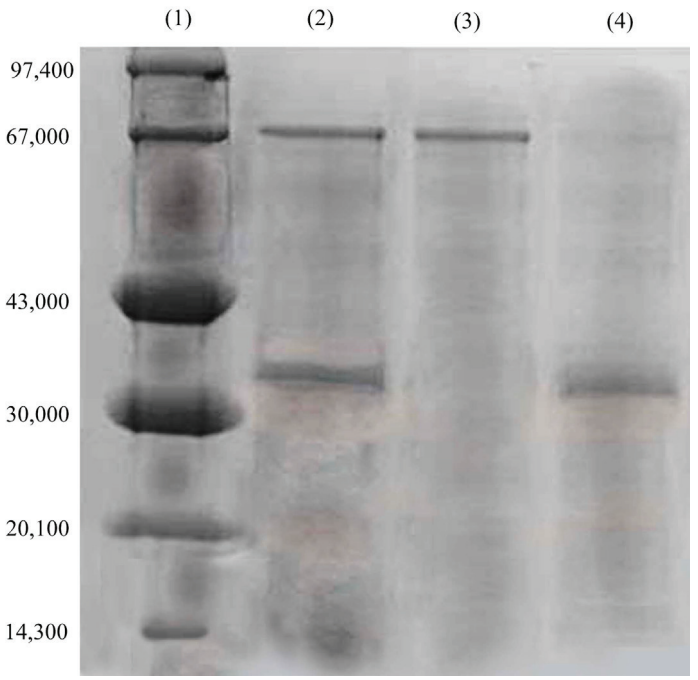

Figure 1. SDS-PAGE of the purified extracellular enzyme from A. niger ITCC 7782.10. Lane 1, marker proteins (from top to bottom) phosphorylase b (Mr, 97,400), bovine serum albumin (Mr, 67,000), ovalbumin (Mr, 43,000), carbonic anhydrase (Mr, 30,000), Soyabean Trypsin Inhibitor (Mr, 20,100) and Lysozyme (Mr, 14,300) respectively; lane 2 purified enzyme G100; lane 3 high molecular weight protein DEAE CL6B; lane 4 low molecular weight protein DEAE CL6B . The gel was stained for protein with Coomassie brilliant blue R-250 and destained in methanol-acetic acid-water (7:2:1).

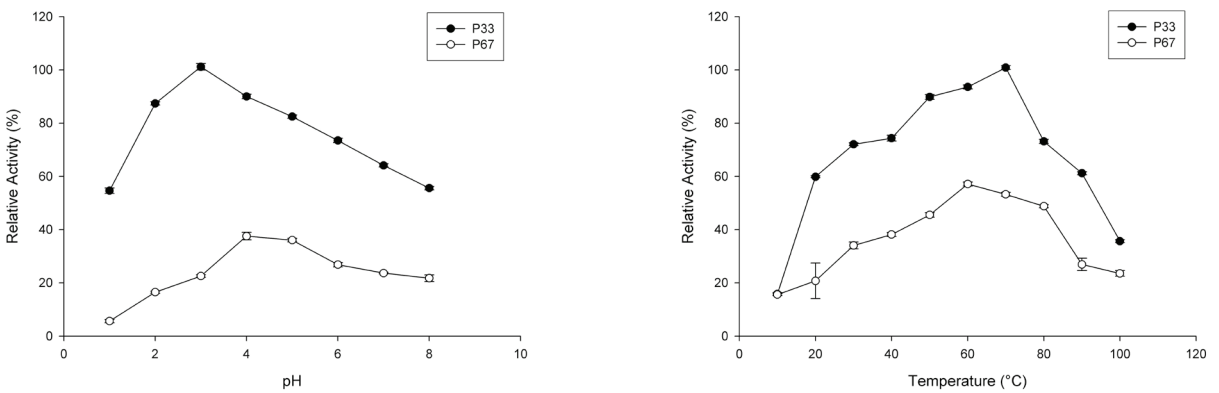

Figure 2. Effect of (A) pH and (B) temperature on the activity of P33 and P67 phosphatases.

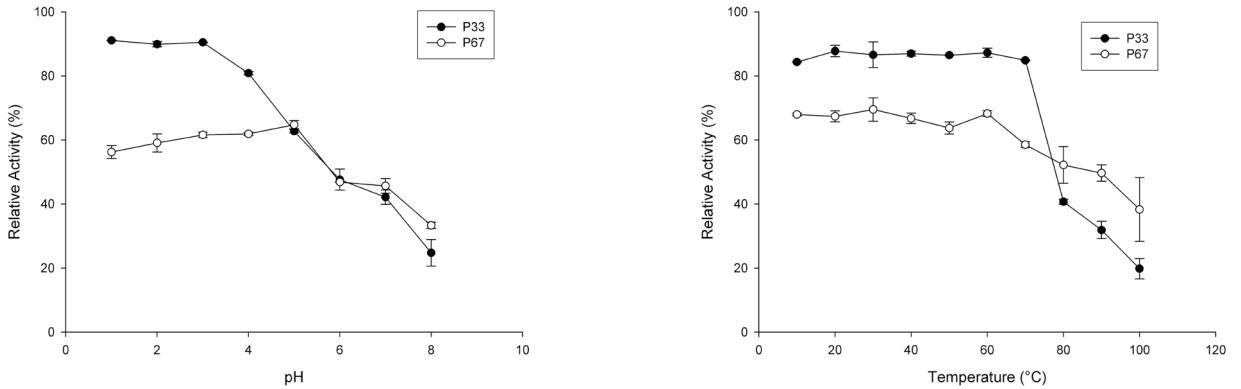

Figure 3. Effect of (A) pH and (B) Temperature on the stability of P33 and P67 phosphatases after 18 hours.

Kinetic Analysis

Kinetic measurement of the phosphatases P33 and P67 was carried out using different concentrations of p-NPP as a substrate. As shown in the Lineweaver Burk plot, K_m and V_{max} of P33 was 0.28 mM and 1.21 Umg^{-1}protein respectively whereas P67 showed Km and V_{max} of 0.72 mM and V_{max} 1.85 Umg^{-1}protein respectively (Figure 4 and Table 2). It clearly demonstrates that P33 had more affinity towards pNPP as a substrate than P67.

Effect of Physical and Chemical Agents on Phosphatase Activity

Effect of different physical and chemical agents was determined by pre-incubating the P33 and P67 phosphatases under these conditions for 30 mins and measuring the residual activity of the enzymes. The results depicted in Figure 5 showed that both the enzymes were drastically inhibited by UV, $HgCl_2$, $CoCl_2$ and $FeCl_3$ whereas; SDS, tartarate and $CuCl_2$ significantly enhanced their activities. However, NaN_2, potassium ferricyanide, EDTA, 2-mercaptoethanol, $MnCl_2$, $ZnCl_2$ and sunlight had no or little effect on the enzyme activity of both the phosphatases.

All the results were found significant at the level of $p \leq 0.05$.

Discussion

The present study is the first report demonstrating the purification and characterization of the two acid phosphatases P33 and P67 from *Aspergillus niger* ITCC 7782.10 capable of hydrolyzing MCP [16]. Both the phosphatases were found to be thermostable and acid tolerant. The enzyme kinetic studies suggested that P33 had more affinity towards pNPP as a substrate than P67 due to its lower K_m value. However, the V_{max} of P67 was found to be higher than that of P33 indicating higher turnover number. In addition, P33 and P67 showed variable response to physical and chemical agents with respect to their phosphatase activity. UV, $HgCl_2$, $CoCl_2$ and $FeCl_3$ showed inhibitory effect on P33 and P67 activities whereas; SDS, tartarate and $CuCl_2$ significantly enhanced their activities.

In contrary to our findings regarding the presence of two intracellular acid phosphatases capable of hydrolyzing an OP pesticide, MCP; previous reports have suggested the presence of single enzyme to hydrolyze different OP pesticides. These enzymes differ in their molecular weight i.e parathion hydrolases have been reported to have a molecular mass of 35 kDa (monomer) in *Flavobacterium* sp. strain ATCC 27551, 43 kDa (monomer) in strain B-1, and 67 kDa in strain SC (four identical subunits) [11, 22].

The results of our study are concurrent with the previous studies in terms of the molecular weights of the enzymes i.e 35, 43 and 67 kDa as stated earlier, but differ in the presence of two distinct phosphatases within the same strain. In addition, it is quite evident that P33 and P67 are two distinct monomeric phosphatases since the SDS-PAGE analysis showed the presence of single band for both P33 and P67 in the DEAE-Sepharose CL-6B purified fractions (Figure 1). Alternatively, it is also possible that 33 kDa could be the proteolytic product of P67 which might have been produced during the purification process.

Both the enzymes were thermostable since they retained their full activity even after 18 hours of exposure to the temperature range 40 to 80°C. Our finding is in agreement with the previous report which showed thermostability of acid phosphatase from *A. niger* up to 70°C [23].

The effect of inhibitor and activators were almost same for both subunits. The purified enzyme was strongly inhibited by Hg^{2+}, Co^{2+} and Fe^{3+}; this may indicate that thiol groups are involved in the active catalytic site. In addition, the enzyme was completely inhibited by sulfhydryl reagent 2-mercaptoethanol, a well-known thiol group inhibitor, which supports the possibility of involvement of sulfhydryl groups in the catalytic site of the enzyme [17, 24-30]. The metal-chelating agent EDTA did not inhibit the purified enzyme activity,

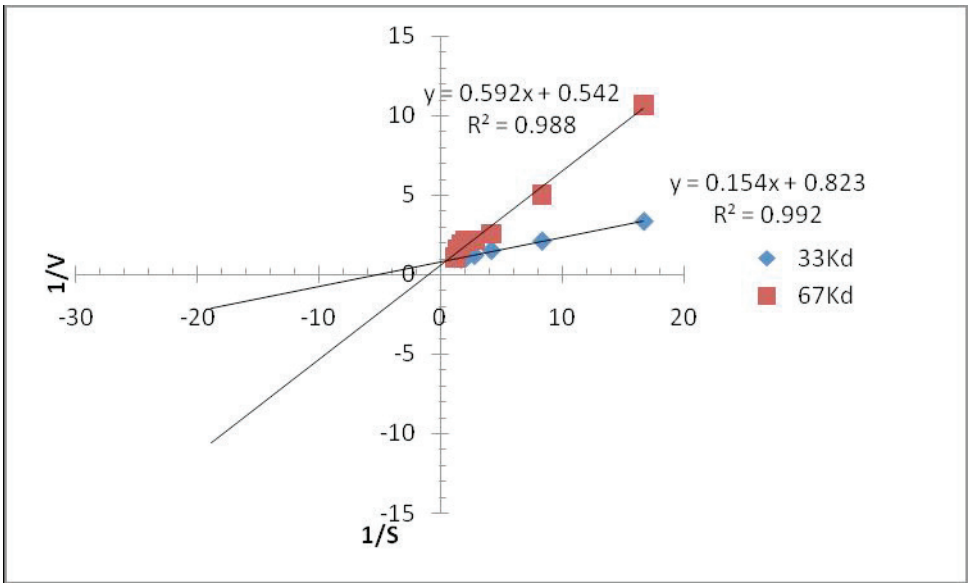

Figure 4. Lineweaver Burk plots of P33 and P67 Phosphatases

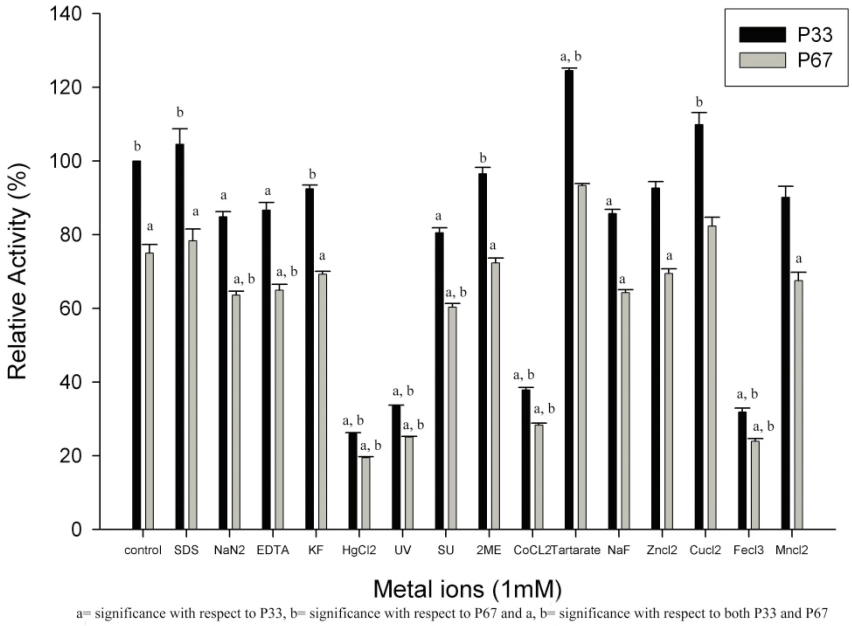

Figure 5. Comparative effect of metal ions on the activity of P33 and P67 subunits. SDS- Sodium Dodecyl Sulphate, KF- Potassium Ferricyanide, 2ME- 2-Mercaptoethanol, SU- Sunlight, Tartarate- Sodium Potassium Tartarate.

Table 2. K_m and V_{max} of P33 and P67 Phosphatases

Kinetic Parameters	P33	P67
K_m mM	0.28	0.72
V_{max} Umg^{-1}protein	1.21	1.85

suggesting the absence of any role of divalent cations for the activation of the enzyme. However, Cu^{2+} exhibited activation of the enzyme activity. Similar finding has been shown for parathion hydrolase of strain SC [11]. The anionic detergent SDS significantly enhanced the phosphatase activity that might be due to increased substrate accessibility to the enzyme.

Therefore, it could be concluded that the P33 and P67 from *Aspergillus niger* ITCC 7782.10 are intracellular phosphatases that are thermostable and pH tolerant having high affinity for the substrate along with higher turnover number. These properties make them excellent candidates for the bioremediation of OP pesticide contaminated sites.

Acknowledgement

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