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Isolation and Characterization of Acid phosphatase from germinating Horse gram (*Macrotyloma uniflorum*) seedlings and their effect on Organophosphate pesticide degradation.

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Abstract- Acid phosphatases forms a group of enzymes catalysing hydrolysis of a variety of phosphate esters in the acidic environments. Acid phosphatases are believed to increases orthophosphate (Pi) availability under phosphorous deficient conditions. They are ubiquitous enzymes that exhibit activity against variety of substrates *in vitro*. This study reports isolation and characterization of acid phosphatase isolated from germinating Horse gram (*Macrotyloma uniflorum*) seedlings. Seeds were grown on phosphorous deficient medium. Acid phosphatase obtained from Horse gram seedling extract was purified by Ammonium sulphate precipitation, followed by dialysis of enzyme extract.

In the present study, the extents of purification of acid phosphatise was determined by SDS-polyacrylamide gel electrophoresis. Acid phosphatase was gave single band on SDS-polyacrylamide gel electrophoresis. Optimum temperature for acid phosphatase activity was 50°C and optimum pH was 5.2. Acid phosphatase activity from Horse gram seedlings was strongly inhibited by metal salts $HgCl_2$, $FeCl_3$, and strongly activated by metal salts NaCl, $CoCl_2$, and $CaCl_2$. The dialyzed enzyme was gave *Km* value 5.4×10^{-4} M and higher V_{max} when *p*NPP was used as substrate. Dialyzed enzyme extract was also used for study of degradation of organophosphate pesticide Monocrotophos ($C_7H_{14}NO_5P$), and released phosphorous was estimated by Fiske Subbarow method.

Keywords: Acid phosphatase (APase), ammonium sulphate, Horse gram seeds (*Macrotyloma uniflorum*), K_m value, SDS-PAGE etc.

INTRODUCTION

Acid phosphatase has enzyme commission number 3.1.3.2, are widely distributed in plants and animals. Acid Phosphatase have been purified and characterized from tubers (Kamenan, 1984), seeds (Gibson, 1988), root (Panara et al, 1990). Increase acid phosphatase secretion under low Phosphorus availability was seen in numbers of plants including Maize (*Zea mays*), tomatoes (Lycopersicon esculentum), Clovers (*Terifolium spp*), Lupin (*lupines spp*), Rice (*Oryza sativa*), etc (Gahan and Mclen, 1969).

Acid phosphatase catalytically breakdown a wide variety of phosphate esters and exhibit pH optima below 6 (Vincent et al, 1992). Acid phosphatase believed to increase orthophosphate availability under phosphate deficient conditions (Vance et al, 2003). Hydrolysis of phosphate esters is critical process in energy metabolism and metabolic regulation of plant cells. Secreted acid phosphatases of roots and cell cultures are relatively non-specific enzymes that appear to be important in hydrolysis and mobilization of phosphate from extracellular phosphomonoesters for plant nutrition (Stephen et al, 1994)

In plant root, acid phosphatases involved in Solubilization of macromolecular organic phosphates in soil which can then be utilized by plants. Intracellular acid phosphatases includes phytases, Phosphoglycolate phosphatases, 3-phosphoglycerate phosphatases, phosphoenolpyruvate phosphatases, etc. Acid phosphatase expression is regulated by variety of developmental and experimental conditions. Phosphate starvation induces *de novo* synthesis of extra and intra cellular APases, in cell cultures as well as in whole plant.

Acid phosphatase increases phosphate availability under low phosphorous level. Agricultural soil has 30 – 80 % organic phosphorus in comprise to total phosphorus. Free soluble

phosphate reserve plays vital role in energy transformations, metabolic regulations. It is structural constituent of many biomolecules like phytin bodies in an ungerminated seeds proteins and nucleotides (Arun Deo Sharma et al, 2011).

Phosphatases are one among them, which are believed to be important for many physiological processes, including the regulation of soluble phosphorous (Pi) (Yan et al., 2001). Phosphorus (Pi) is an essential macronutrient for plant growth and development that plays a key role in many processes, including energy metabolism and the synthesis of nucleic acids and membranes (Ehsanpour and Amini, 2003). Acid phosphatases are constitutively expressed in seeds during germination and their activities increase with germination to release the reserve materials for the growing embryo (Biswas and Cundiff, 1991). Various researchers have studied the behaviors of many enzymes during germination, using cultivars differing in salt tolerance in order to find a possible correlation between enzyme activities and the degree of salt tolerance.

Horse Gram (*Macrotyloma uniflorum*) is one of the lesser known beans. Local name of Horse gram In Marathi is "*Kulith*". The whole seed of horse gram are generally utilized as cattle feed. However, it is consumed as a whole seed, as sprouts, or as a whole meal in India, especially in South Indian States. Medical uses of these legumes have also been discussed (Kawsar et al, 2008).Horse gram and moth beans are legumes of the tropics and subtropics, grown mostly under dry - land agriculture. The chemical composition is comparable with more commonly cultivated legumes. In local area, horse gram seeds of good quality are extensively produces by farmers. The present study report details on isolation and characterization of acid phosphatase from horse gram seeds.

Monocrotophos is an organophosphorus insecticide. It is acute toxic to birds and humans,etc. Also being a persistent organic pollutant, it has been banned in U.S. and many other countries but, it is still used in India. Modern agriculture largely relies on extensive application of agrochemicals, including inorganic fertilizers and pesticides. Even though Monocrotophos is banned due to its toxicity, it is principally used in agriculture as a relatively cheap pesticide.

MATERIALS AND METHODS

Chemicals:

- Monocrotophos 36% v/v purchases from Monodhanagrochemical company.
- All other analytical grade chemical purchases from Himedia

Collection of Horse gram seeds:

Horse gram (*Macrotyloma uniflorum*) of good variety was collected from local farmers of Ratnagiri District. After collection, the seeds were cleaned and surface sterilized by immersion in 95% ethanol for 1 min, then 4% Sodium Hypochlorite for 30 min, followed by 10 rinses of distilled water. Seeds were dried in sunlight and stored in air tight dedicator in polyethylene bag for experimental purpose.

Plant growth conditions:

Horse gram (*Macrotyloma uniflorum*) seeds were soaked in distilled water for overnight period. After hydration, germination of horse gram seeds was performed on moist cotton with phosphorus deficient condition, for 5 days in room temperature with abundant humidity. Germinating moong seeds were watered daily. After 5 days, seedlings were separated from cotton for enzyme activity assay (A. K. M. Asaduzzaman et al, 2011).

Preparation of crude enzyme extract:

The enzyme acid Phosphatase was extracted from 3 day old germinating horse gram seedlings, maintained in phosphorus deficient conditions. All operations were performed under 4 ℃. 20g horse gram seedlings were put in mortar and ground uniformly with chilled 0.1 M citrate buffer having pH 4.8. Homogenize the extract in homogenizer at chilled conditions. The homogenate was centrifuged at 10,000g for 30 minutes and supernatant was collected. All steps for enzyme purification were done at 4℃.

Clear supernatant was used as crude enzyme extract. This extract was first precipitated to 30% followed by 80% with ammonium sulphate and centrifugation at 10,000g (30 min at 4℃). Precipitate

having acid phosphatase activity was used for dialysis purpose.

Acid Phosphatase Assay:

Acid Phosphatase activity was based on conversion of para-nitro phenol phosphate- as an artificial substrate (0.02 M). The enzyme extract diluted in 0.1 M citrate buffer having pH 4.8 was incubated with 0.02 M pNPP at 37℃ for 10 minutes, then add 1 ml 2 N NaOH to stop the reaction. Finally the absorbance was measured at 420 nm spectrophotometer (Sharma et al., 2007).

Protein Estimation:

Protein concentration was estimated according to Folin - Lowry method, absorbance was measured at 660 nm. Bovine Serum Albumin was used as standard protein (Lowry et al, 1951).

SDS - PAGE Electrophoresis:

Proteins were analyzed by using Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS - PAGE).

Characterization

Effect of pH on enzyme activity:

The effect of pH on enzyme activity was determined by measuring the hydrolysis of substrate 0.02 M pNPP in series of buffers at various pH values ranging from 3.0 to 7.0. Here 0.1 M Citrate buffer was used. The optimum pH value was determined at 37℃ at 420 nm spectrophotometrically (Hoehamer et al, 2005).

Effect of temperature on enzyme activity:

The effect of temperature on acid phosphatase activity was performed by using 0.1 M Citrate buffer having pH 5.6 over temperature range of 0 to 100℃. 0.02 M pNPP was used as substrate under standard conditions and absorbance was measured at 420 nm spectrophotometrically (Yoshimoto et al., 1992).

Kinetic Parameters:

Km value was determined using Michaelis-Menten Plot and Lineweaver-Burk Plot (Kundu and Banerjee, 1990).

Effect of Metal Ions and Salts on enzyme activity:

Effects of various compounds such as metal ions and its salts of different concentrations (0.05 M, 0.1 M, 0.2 M) on enzyme activity, as possible activators or inhibitors of enzyme were seen. Enzyme activity was examined by incubating various metal salts with enzyme extract prepared in 0.1 M Citrate buffer having pH 5.6 at 37℃ for 10 minutes. 0.02 M pNPP was used as substrate. The enzyme activity remained after incubation was determined under assay condition (Mizuta and Suda, 1980). Absorbance was measured at 420 nm spectrophotpmetrically.

Organophosphate Pesticide Degradation ability of Enzyme:

In this study organophosphate pesticide Monocrotophos was used. Monochrotophos in ppm concentration was incubated with enzyme prepared in 0.1 M Citrate buffer, Phosphorus released after organophosphate pesticide degradation was estimated by Fiske - Subarrow method (K. Hoehamer et al, 2005).

	Total protein (mg/ml)	Total activity (Unit/ml)	Specific activity (unit/mg)	Purification fold
Crude	0.174	117.39	674.65	-
Dialyzed	0.050.0528	286.95	5518.26	8.17

OBSERVATIONS AND RESULTS

Table 1: Acid Phosphatase purification from germinating horse gram seeds.

Ammonium Sulphate is salting out method commonly used for protein precipitation. In 30 % precipitation, enzyme comes in supernatant while after 80 % it was seen in the precipitant. As shown in table 1. Increased specific activity and purification fold reflects that this method is flexible for purification of acid phosphatase.

SDS- PAGE Electrophoresis:

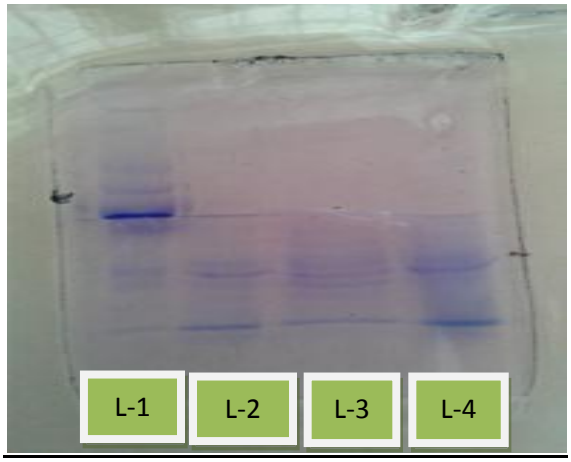


Fig 1: SDS-PAGE gel electrophoretic pattern of enzyme APase for determination of purification.

L-1: Marker

L-2: Dialyzed

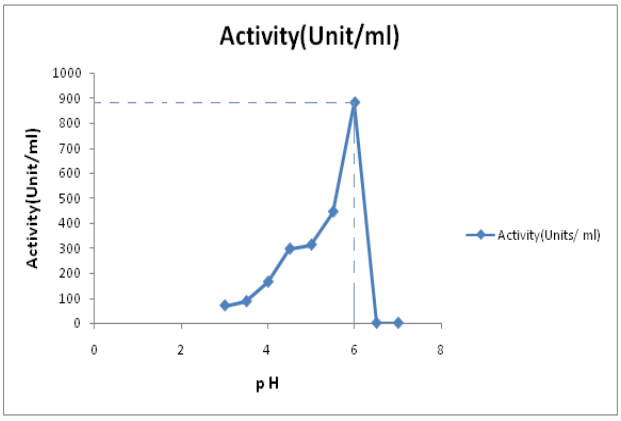
L-3: 30% precipitation

L-4: Crude

SDS-PAGE Electrophoresis reflects that purification has occurred but still required for further purification to determine exact nature of enzyme APase

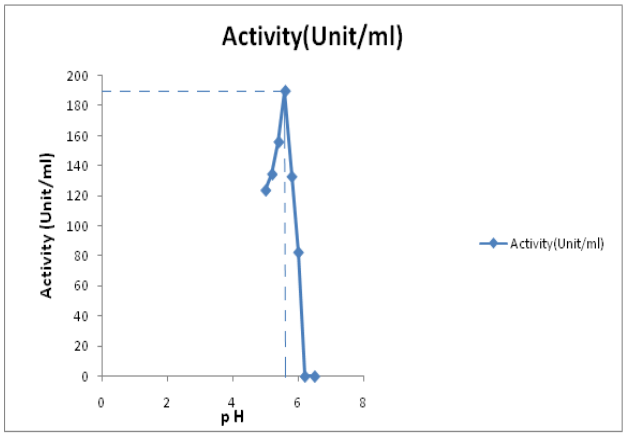
Effect of pH on enzyme activity:

Broad range



Graph 1

Narrow range

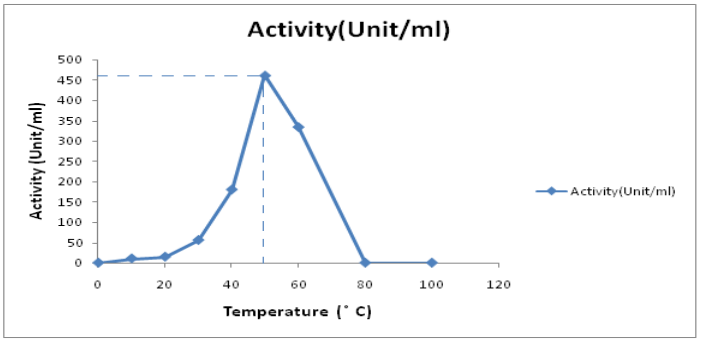


Graph 2

Graph 1, Graph 2: Effect of pH on activity of APase from germinating Horse gram seeds.

As shown in graph No. 1 and 2, at wide range of pH from 3.0 to 7.0 , Optimum activity was obtained at pH 6.0 while in narrow range of pH from 5.0 to 6.5, the enzyme was most active at pH 5.6, which was most accurate. At that pH enzyme activity was 189.68 Unit/ml. As pH increases, enzyme activity decreases.

Effect of temperature on enzyme activity:

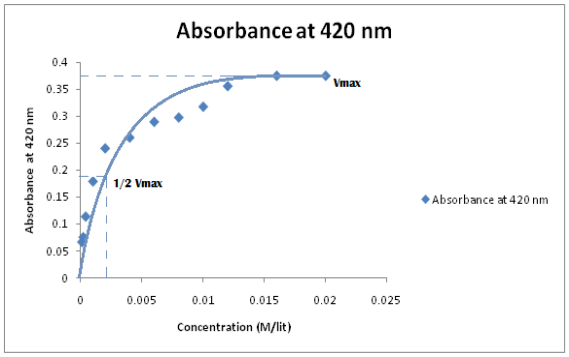


Graph 3: Effect of temperature on activity of APase from germinating Horse gram seeds.

As shown in graph No. 3 enzyme acid phosphatase was most active at temperature 50 ºC, at that temperature enzyme activity was 461.84 Unit/ml. Above 50 ºC, enzyme activity declined rapidly as temperature increases.

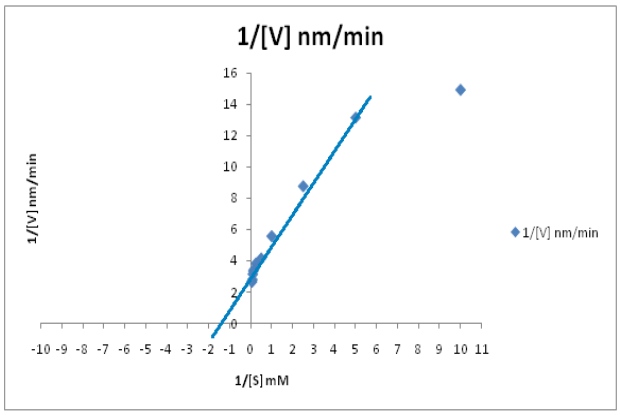
Kinetic parameters

1) Michaelis-Menten plot:



Graph 4: Determination of *Km* of APase by Michaelis-Menten plot from germinating horse gram seedlings.

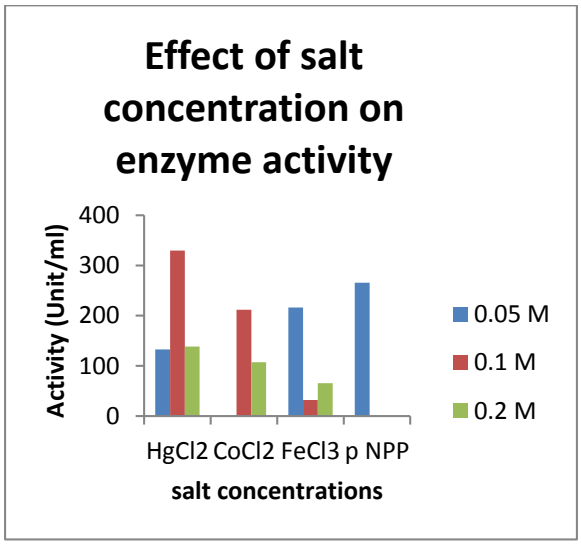
2) Lineweaver-Burk plot:



Graph 5: Determination of *Km* of Acid phosphatase by Lineweaver – Burk Plot from germinating horse gram seeds.

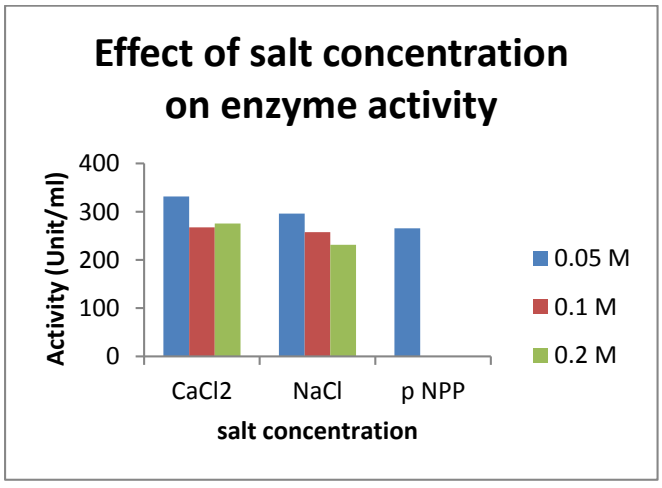
Enzyme activity of purified acid phosphatase was analyzed using different concentrations of pNPP as Substrate. As shown in graph No. 4 and 5 *Km* value of APase and Lineweaver–Burk plot was 0.5 Mm.

Effect of metal ions and salts on activity of APase from germinating Horse gram seedlings:



Graph 6: Effect of salts (0.05 M, 0.1 M, 0.2 M) on activity of APase from germinating Horse gram seedlings.

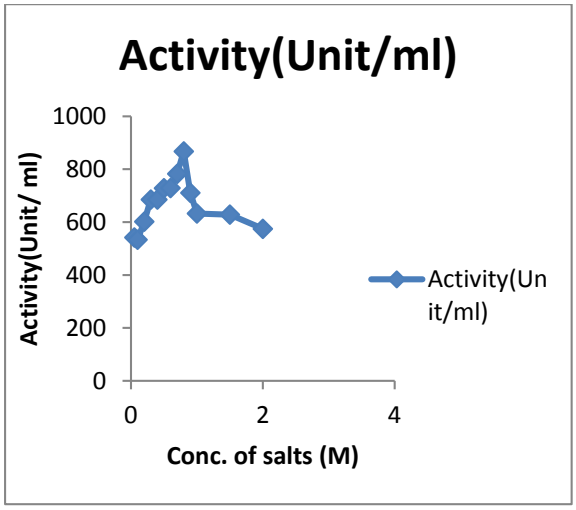
AS shown in graph 6 CoCl2 showed total inhibition of enzyme activity at 0.05 M conc. while reduced activity was seen at 0.1 M and 0.2 M conc (2.4 times decrease). HgCl2 showed decreased enzyme activity at 0.05 M and 0.2 M conc. while increased activity was seen in 0.1 M (1.2 times) conc. FeCl3 showed decreased enzyme activity. Decreased activity i.e. 8.3 times, 4.5 times was observed in presence of $FeCl_3$ 0.1 M conc. and 0.2 M conc. respectively.



Graph 7: Effect of salts (0.05 M, 0.1 M, 0.2 M) on activity of APase from germinating Horse gram seedlings.

As shown in graph 7 increased activity (1.2 times) increase was observed in presence of $CaCl_2$ at 0.05 M conc. Slight increase in activity of enzyme was seen in presence of 0.1 M and 0.2 M concentration of $CaCl_2$. NaCl showed slight increased enzyme activity at 0.05 M conc. while slight decrease in enzyme activity was seen in presence of 0.1 M and 0.2 M concentration of salts.

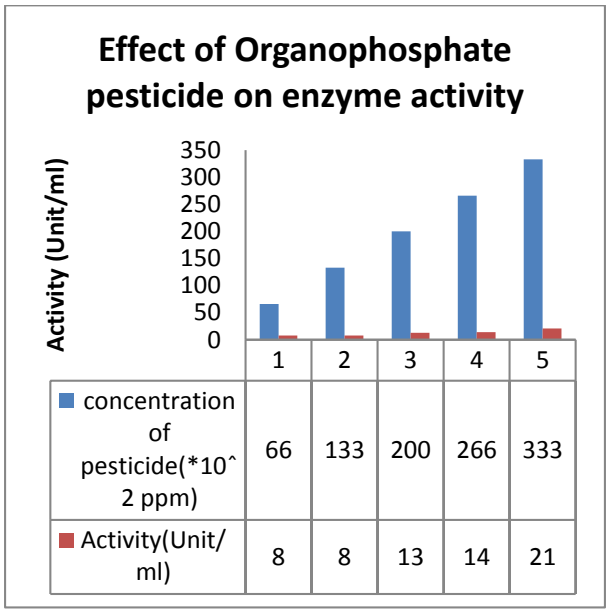
Effect of various concentrations of NaCl:



Graph 8: Effect of various concentrations of NaCl on enzyme activity

As shown in graph No. 8 maximum activity of APase i.e. 1.1 times increase was seen in presence of 0.8 M concentration of NaCl. As shown in graph 8 activity at 0.8 M conc. is 867.76 Unit/ml. Slight decrease was observed in rest of the concentrations but the enzyme activity was not lost. Hence, APase is resistant to increased salinity. As salt concentration goes on increasing, enzyme activity goes on increasing. But after 0.8 M conc. of NaCl, decrease in activity was observed.

Organophosphate pesticide (Monocrotophos) degradation ability of enzyme:



Graph 9: Effect of Organophosphate pesticide on activity of APase from germinating horse gram seedlings

Organophosphate pesticide Monocrotophos was actively degraded by APase. As shown in graph No. 9 increased concentration of pesticide, increased activity of enzyme APase was observed.

DISCUSSION

Several studies have been devoted to the purification of acid phosphatase from germinating seeds. Protocol from the former studies with little modification is used in this study to understand the role played by acid phosphatase during horse gram seed germination. Acid phosphatase from the crude extract of horse gram seedling germination was purified by Ammonium Sulphate precipitation and then by Dialysis (Gibson,1988; Granjerio, 1998). Maximum purification of enzyme was seen after 2nd precipitation (80% Ammonium Sulphate precipitation) followed by Dialysis using Citrate Buffer (pH 4.8)

The APase showed maximum activity at pH 5.6 and temp. 50℃. The Optimum pH determined for the horse gram seedlings acid phosphatase was largely consistent with other acid phosphatase having

pH optima between 5.0 to 6.0 (Ferreira et al, 1998; Duff et al, 1991; Gonnety et al,2006). However, APase from peanut seedlings and soyabean seeds exhibited maximum catalytic activity at 55℃ (Gonnety et al,2006). Horse gram unable to germinate in winter or rainy season but, germinate during temperature more than 35℃ reflects that this enzyme is very much important for germination.

For the enzymes, Kinetic study correlates well with substrate specificity and showed Lineweaver – Burk Plot. Here purified acid phosphatase showed highest catalytic efficiency towards pNPP. During study, Km value of acid phosphatase from horse gram seedling was found to be 0.5 mM. The value obtained was substaintially lower than 0.7 mM of acid phosphatase obtained from *Vigna radiate* (Kundu and Banerjee, 1990) reflects that the enzyme has higher affinity for the substrate.

The purified acid phosphatase from germinating horse gram seedlings was sensitive to the ions at various degrees depending on nature of ions and their concentrations. The requirements of metallic ions for acid phosphatse activity has also varied according to Plant species, development stage (Scandalois, 1974; Panara et al.,1990; Tso and Chen, 1997). However, Ca^{2+} was potent activator, while Co^{2+}, Hg^{2+}, Fe^{3+} were potent inhibitors. Na^{+} showed resistance with increased salt concentration.

Monocrotophos is a widely used organophosphate pesticide by all farmers to control the attack of pest, insects etc. Monocrotophos known for their toxic effects on all organisms from soil microorganism to human. Monocrotophos is weakly sorbed by soil particles because of its hydrophilic nature. Leaching of Monocrotophos may pollute the surface and groundwater, ultimately resulting in adverse effects on biological systems (Subhas and Singh, 2003). Moreover the persistence of monocrotophos depends upon the microbial composition, pH, temperature and availability of sunlight. Hence, the half life of monocrotophos varies. Farmers use these pesticides in solution form but, the rate of hydrolysis in solution form is low, hence very less degradation. The range of degradation of monocrotophos after cooking or other processing varies from 35% to 95%. Further, the monocrotophos used in this study is Dimethyl (E)-1-methyl-2-(methylcarbamoyl)vinyl phosphate which on hydrolysis forms N-methylacetoacetamide. N-methylacetoacetamide can cause breathing problems, as an irritant to skin, eye: Proper data is still not available properly (TCI AMERICA SAFETY DATA SHEET,Revision no.1, Revised date: 19/11/2013). To avoid these harmful effects, the persistent pesticide should be degraded. According to current study, acid phosphatase can free phosphate from organophosphate pesticide by attacking terminal phosphate forming hydroxide form of used monocrotophos in this study. Hence, in present study, purified enzyme acid phosphatase from germinating horse gram seeds were tested for degradation of Organophosphate pesticide monocrotphos and it may work as an important tool in degradation of organophosphate pesticide monocrotophos. This enzyme has ability to degrade pesticide Monocrotophos even at higher concentrations. There is no marked decrease in pesticide degradation capacity of enzyme implies that after further purification and proper immobilization, we can use this enzyme in field which are contaminated with pesticide monocrotophos. Microbial degradation by bacteria such as *Pseudomonas fluorescens*, *Bacillus subtilis*, *Klebsiella sp*. And fungi- *Aspergillusniger* are also studied (KaviKarunya S. et al, 2012; Jain Rachna et al, 2012).

CONCLUSION

Germination of Horse gram seedlings which accompanied by the synthesis or activation of enzyme acid phosphatase, which is responsible for degradation of reserve materials present in horse gram seeds. APase are involved in the metabolic process of germination and maturation of plants. They are constitutively expressed in seeds during germination, to release reserved materials for the growth of embryo (Biwas and Cundiff, 1991; Thomas, 1993). Various salts of different concentrations showed activation or inhibition of acid phosphatase. Ca^{2+} and Na^{+} showed increased activity while Hg^{2+}, Co^{2+}, Fe^{3+} reduced the APase activity. No enzyme inhibition even in presence of high NaCl concentration reflects that this enzyme sustained in salty land.

Enzyme produced by roots of plant degrades pesticides (Christopher F. Hoehamer et al., 2004). Acid phosphatase from horse gram seedlings degrades Monocrotophos. APase reduces the load of pesticide in soil, which makes enzyme acid

phosphatase from horse gram seedlings as potential tool to reduce Monocrotophos in agricultural lands.

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