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Research article

Enhanced production of phytase from thermotolerant *Aspergillus fumigatus* isolated from rhizospheric zone of Maize fields

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Key words: Phytase, Phytic acid, Isolation, Screening, Optimization.

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Abstract

Background: Phytase (myo-inositol hexakisphosphate phosphohydrolase) catalyze the hydrolysis of phytate into inositol and phosphoric acid and finds its potential applications in the feed and food industries, environmental protection, aquaculture and agriculture. **Objective:** The objective of the present study was to locate phytase producing microbes from soil samples of maize fields of himachal Pradesh and poultry faeces and its production optimization studies. **Results:** A total of 95 microbes were isolated. They were screened for the production of phytase with calcium phytate as substrate. Out of the 79 bacterial and 16 fungal isolates obtained through primary screening, the fungal isolate *Aspergillus fumigatus* has shown maximum phytase activity and was selected for optimization to maximize phytase production. Different cultural and reaction conditions such as pH, temperature, carbon and nitrogen sources, mineral solution and inducer concentration, inoculum size, incubation time, substrate concentration, buffer system (pH and molarity), Reaction temperature were optimized by using one-variable-at-a-time (OVAT) strategy. After optimization of cultural conditions highest phytase activity (1.64U/ml) was recorded when grown for 72 hrs at pH 5.5, 40°C when inoculated with 200×10^4 spores.

Introduction

Phytase hydrolyzes phytic acid to myo-inositol and phosphoric acid and constitutes a specific group of phosphatase [1-10]. It is an enzyme of economical importance due to its applications in food and feed industry, in preparation of myo-inositol phosphate, in the paper and pulp industry and in agriculture. Although there are several sources of phytases, microbial sources offer better prospects in terms of commercial production [11].

In animal feed such as legumes and cereals, inorganic phosphorous is stored in form of phytic acid. However, it is not readily assimilated by animals [12] due to the presence of inherent phytases in insufficient quantity which is necessary to hydrolyze the phytic acid complexes in feed [13]. Consequently, this leads to the release of undigested phytate in faeces and urine causing severe phosphorous pollution of water resources [14]. Moreover phytic acid has antinutritive properties, as it forms complexes with nutritionally important metals such as calcium, zinc, magnesium and iron and proteins decreasing their bioavailability [15]. Phytic acid is also known to inhibit a number of nutritionally important enzymes *in vivo* [13]. It is to be noted that phytic acid, owing to its strong chelating ability forms cation-phytic acid complexes with multivalent cations [16,17].

Phytases were originally proposed as an animal feed additive to enhance the value of plant material in animal feed by liberating phosphate [18]. Phytase is present in about 75% of all the diets for simple-stomach animals hence its market volume exceeds US$350 million annually [19]. The current global phytase market has been estimated to account for more than 60% of the total enzyme market. The increased concern over the environmental impact of life-stock production, have paved the way for the economic success of phytases as an animal feed additive [20].

In view of its industrial importance, it is desirable to study this enzyme from as many sources as possible. There is an ongoing interest in screening new organisms that may produce efficient phytases. The ultimate objective is to produce this enzyme at a cost effective level and establish conditions for its industrial application [21].The aim of this research is to demonstrate the presence of phytic acid utilizing microorganisms in the rhizosphere of the maize fields, to isolate and identify the microbial species, to measure their phytate digesting potential *in vitro* and to isolate the potential phytase producing microorganisms which are useful for phytase production at commercial level.

Experimental

Materials and Methods

Chemicals

All the chemicals used in the experiments were of analytical grade. All the medium components used during the experiments were from Himedia, Merck and Sigma chemicals co.

Sample collection and enrichment
Soil samples for the isolation of phytase were collected from rhizospheric zone near roots of maize plants from different places of Himachal Pradesh and poultry faeces. The samples were collected during the rainy season; keeping in view that humid environment is favorable for the growth of microbes. As the samples were collected from different regions of Himachal Pradesh; temperature range was varied from 25-35°C during rainy season with approximately 60-70% humidity. For the isolation of phytase producing microbes, 1g of soil samples were added to the 50 ml of autoclaved medium containing 1.5% w/v glucose, 0.5% w/v ammonium nitrate, 0.004% w/v calcium phytate as inducer, 0.05% w/v KCl, 0.05% w/v $MgSO_4$, 0.001% w/v $FeSO_4.7H_2O$, 0.001% w/v $MnSO_4$ in distilled water. The Erlenmeyer's flasks containing above medium (both at pH 5.5) and soil samples were kept at 30°C for 48 to 72 hours on a shaker (150 rpm).

Isolation of phytase producing microorganisms
One ml of the sample from each flask was serially diluted with physiological saline (0.89% NaCl solution). The diluted inoculum (0.1 ml) was spread on nutrient agar plates and incubated at 30°C for 24 hours. Pure cultures were obtained by streaking a single bacterial colony on the nutrient agar and fungal culture on potato dextrose agar. Pure line cultures were maintained on nutrient agar slants and PDA respectively and stored in refrigerator at 4°C. The isolates were sub-cultured once a month.

Screening of phytase producing microorganisms
Fungal and bacterial strains isolated from different soil samples were assigned a separate code number and checked for phytase activity.

Production of phytase
Bacterial isolates were cultured in Nutrient broth media containing 0.004% w/v calcium phytate as inducer, 0.05% w/v KCl, 0.05% w/v $MgSO_4$, 0.001% w/v $FeSO_4.7H_2O$, 0.001% w/v $MnSO_4$ as trace elements required for growth for 24-30 hours at 30°C for mesophilic isolates. To 50 ml of enzyme production medium having same composition was added 4 ml of preculture in case of bacterial isolates and in case of fungal isolates spores were inoculated. The cultures were incubated at 30°C for 24-48 hours in an incubator shaker. Culture contents were then centrifuged at 5000 g for 15 minutes at 2-4°C. The supernatant and the cell pellet were collected and both were used to check phytase activity. For fungal isolates supernatant was assayed for three consecutive days.

Measurement of enzymatic activity
Phytase activity was determined by the modified ferrous sulfate molybdenum blue method [22, 23]. Phytase activity was estimated by measuring the amount of enzyme required to release one micro mole of inorganic phosphate per minute under assay conditions. The reaction mix consist of Buffered substrate 950µL (250 µM calcium phytate in 0.25 M Na-acetate buffer having pH 5.5) and 50µL enzyme. The reaction mixture was incubated at 55°C for 15 minutes. The reaction was stopped by 250µL of 10% TCA. For the assay of released phosphate 1 ml of reagent was added and mixed properly in reaction supernatant (7.20% w/v$FeSO_4.7H_2O$, 1.0% w/v Ammonium molybdate.$4H_2O$ and 3.2 mL H_2SO_4). The product formation was assayed by method described by Chu *et al.* [24]. The absorbance was recorded at 750 nm against a blank. The standard curve was prepared using 20–160 µg of NaH_2PO_4. One unit of phytase is defined as the amount of enzyme that liberates one µmole inorganicphosphate/ml/min under the assay conditions and expressed as units permililitre (U/ml). Activity of all the isolates was checked.

Identification of phytase producing fungus
Fungal isolate producing maximum phytase was selected and identified. Identification was done from National Fungal culture collection of India, Agharkar Research Institute Pune, India.

Optimization of production condition
In order to achieve maximum production of phytase from fungal isolate selected, attempts have been made to optimize various production parameters viz. – media, Carbon source, Nitrogen source, production pH, mineral concentration, production temperature, inoculum size, inducer concentration. The selected isolate was grown in 15 different media at 30°C (pH 5.5).

Effect of carbon source and nitrogen source
Different sugars such as starch, sucrose, glucose, maltose, fructose etc at concentration of 75 mM were used as carbon source in the production medium (pH 5.5, temperature 30°C). Medium without carbon source was used as control. Ammonium nitrate was replaced with various organic and inorganic nitrogen sources (ammonium chloride, ammonium sulphate, sodium nitrate, beef extract, malt extract, peptone and urea at concentration of 0.5% w/v).

Production pH
To investigate the effect of pH on the phytase production, the fungus was grown in pH range of 3.0–8.0. For this parameter, medium of pH 5.5 was the control. Enzyme activity was assayed to check the optimized pH leading to maximum production of phytase.

Mineral solution

Effect of varying volume of mineral solution (stock solution of 600mM potassium chloride, 200mM $MgSO_4.7H_2O$, 3mM $FeSO_4.7H_2O$, 300mM $CaCl_2.2H_2O$, 6mM $MnSO_4$) on Phytase production from fungal isolate was investigated. Volume was varied from 0.00-2.5 ml.

Optimization of production temperature

The fungal isolate PPF-6 was grown at different temperatures ranging from 25-55°C (pH 5.5). Temperature 30°C was used as control.

Effect of inoculum size on phytase production

To study the effect of inoculum size on growth and enzyme production, spores were used. Varying inoculum size ranging from 50×10^4 - 225×10^4v/v was tested for phytase production (pH 5.5, temperature 40°C). 125×10^4 v/v inoculums size was used as control for experiment.

Concentration of inducer

The optimization of calcium phytate which acts as substrate inducer was tested by adding various concentrations (50µM –200µM v/v) in production medium to study the effect on phytase production. 70µM v/v calcium phytate was used as control for optimization of this parameter.

Optimization of reaction conditions for phytase assay

To get maximum yield of product, it is necessary to optimize various reaction parameters which affect enzyme activity e.g. buffer system, buffer pH, buffer molarity, incubation time, substrate concentration, temperature etc.

Effect of metal ions and inhibitors

To work out the effect of metal ions and inhibitors on enzyme activity of crude phytase different metals and inhibitors i.e. $FeCl_3$, $MgSO_4.6H_2O$, $ZnSO_4.7H_2O$, $CoCl_2$, $CuSO_4.5H_2O$, NaCl, $AgNO_3$, $BaCl_2.2H_2O$, $HgCl_2$, NaN_3, $CaCl_2.2H_2O$, $Pb(NO_3)_2$, $MnCl_2.2H_2O$, Urea, dithiothreitol (DTT), Ethylene di-amine tetra acetic acid (EDTA), phenyl methyl sulphonyl fluoride (PMSF) and polyethyleneglycol (PEG), were tested at final concentration of 1mM. Preincubation of enzyme with metal ions at 30°C for 20 min was done. Enzyme activity was assayed under previously optimized conditions.

Results and Discussion

Results

Isolation and screening of phytase producing microorganisms

Phytase producing micro organism were isolated; some of them showing significant phytase activity are shown (Table 1). Phytase activity was checked for each bacterial and fungal isolates (supernatant and cell pellet suspension) as given in material and method section. It was found that out of poultry faeces and field soil samples; soil samples showed more promising isolates producing phytase. Although number of bacterial isolated screened was large but fungal isolates produced more amount of phytase. A total of 79 bacterial and 16 fungal isolates obtained through primary screening, out of which the fungal isolate was selected; which was isolated from rhizospheric soil of maize field collected during rainy season.

Table 1. Phytase activity of isolated microorganisms

Isolated microorganism	Localization	Enzyme Activity (IU/ml)
Bacteria		
PPB – 1	IN	0.0093
PPB – 2	IN	0.026
PPB – 3	IN	0.00181
PPB – 4	IN	0.031
PPB – 5	IN	0.0010
PPB – 8	IN	0.037
PPB – 9	IN	0.002
PPB – 10	IN	0.0115
PPB – 11	IN	0.0039
PPB – 12	IN	0.00215
PPB – 13	IN	0.0158
PPB – 14	IN	0.0288
PPB - 15	IN	0.0032
	EX	0.0418
PPB - 16	IN	0.007
PPB – 17	IN	0.041
PPB – 18	IN	0.0045
PPB – 19	IN	0.097
PPB - 20	IN	0.0028
FUNGAL ISOLATES		
PPF – 1	EX	0.065
PPF – 2	EX	0.054
PPF – 3	EX	0.044
PPF – 4	EX	0.259
PPF – 5	EX	0.052
PPF – 6	**EX**	**0.664**
PPF – 7	EX	0.021
PPF – 8	EX	0.054
PPF – 9	EX	0.132

IN- Intracellular; EX-Extracellular

Identification

The fungal culture was identified as *Aspergillus fumigatus* (NFCCI Acession No. 3463). NCBI accession no. KX449335, *Aspergillus fumigatus* strain PPF-6 [Figure 1 (A), (B), (C)].

Media optimization

Among different media used, medium no 5* (Table 2) proved to be the best medium for the production of phytase which contained 1.5% w/v glucose, 0.5% w/v ammonium nitrate, 0.004% w/v calcium phytate as inducer, 0.05% w/v KCl, 0.05% w/v $MgSO_4$, 0.001% w/v $FeSO_4.7H_2O$, 0.001% w/v $MnSO_4$ in distilled water and was selected for the maximum production of phytase and used in subsequent experiments with modifications in the concentration of individual components such as carbon and nitrogen source.

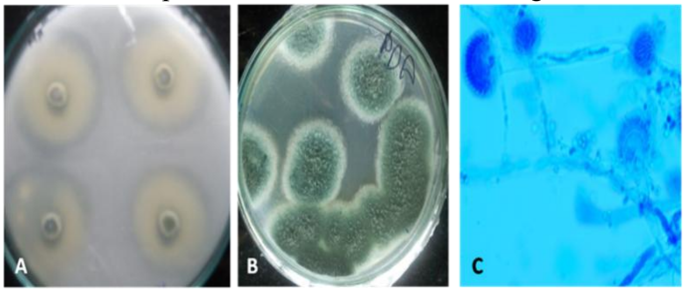

Figure 1. (A) Growth pattern of *Aspergillus fumigatus* on PSM; (B) Growth pattern of PPF-6 on PDA; (C) LCB Stained fungal isolate PPF-6.

Table 2. Screening of different media for production of phytase

Media	Enzyme Activity (IU/ml)	References
M1	0.020	[28]
M2	0.125	[42]
M3	0.000	[43]
M4	0.317	[1]
M5*	**0.677**	[24]
M6	0.027	[44]
M7	0.314	[44]
M8	0.000	[28]
M9	0.037	[45]
M10	0.030	[29]
M11	-	[46]
M12	0.011	[47]
M13	0.201	[48]
M14	-	[49]
M15	0.043	[50]

Effect of carbon sources

After incubation of fungal isolate PPF-6 with various carbon sources (75 mM) replaced by the carbon source in optimized production medium maximum activity was recorded in maltose (Figure 2).

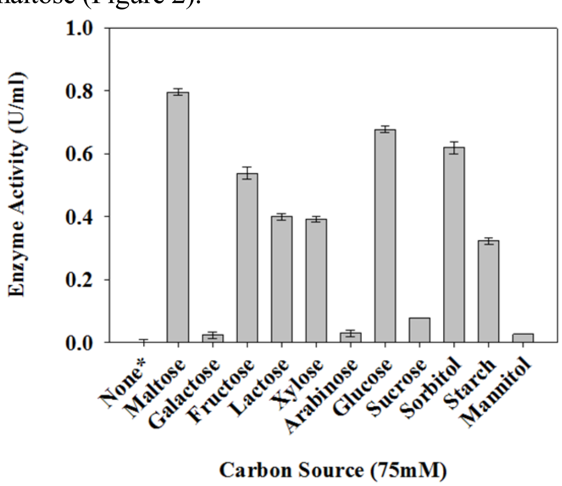

Figure 2. Effect of different carbon sources on production of phytase

Effect of different nitrogen sources

Nitrogen can act as an important limiting factor in the microbial production of various enzymes. Among various organic and inorganic nitrogen sources studied, 0.50% NH_4NO_3 has been observed to be the best source for the production of phytase (Figure 3).

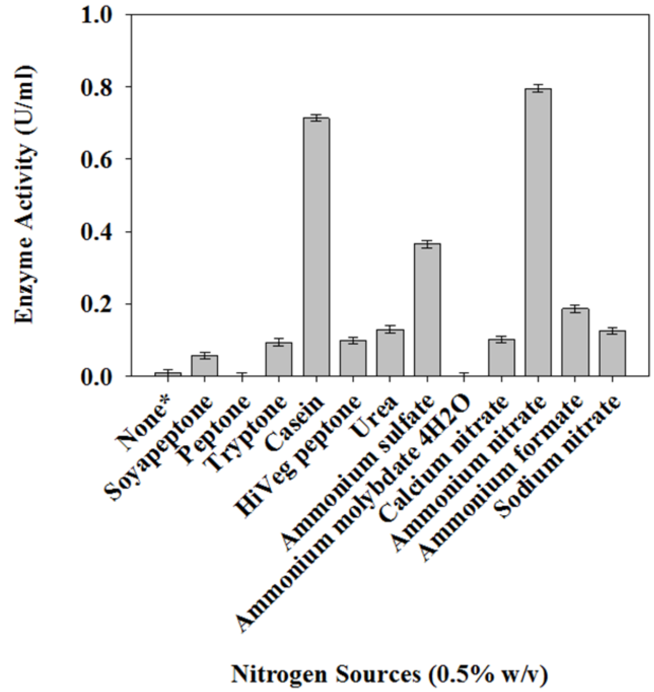

Figure 3. Effect of different nitrogen sources

Effect of pH on phytase production

The fungal isolate PPF-6 was grown in medium from pH range 3.0 to 8.0. The maximum phytase production was recorded at pH 5.5.

Temperature

Phytase production was studied at various temperatures varying from 25°C to 50°C. The fungal isolate produced higher amount of phytase at 40°C as compared to other temperatures tested (Figure 4).

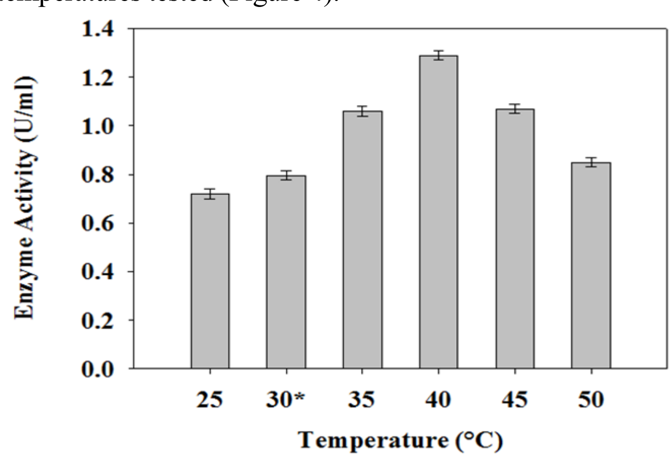

Figure 4. Effect of incubation temperature on phytase production from *Aspergillus fumigatus*

Mineral solution

Effect of varying volume of mineral solution (stock solution of 600mM potassium chloride, 200mM $MgSO_4.7H_2O$, 3mM $FeSO_4.\ 7H_2O$, 300mM $CaCl_2\ .2H_2O$, 6mM $MnSO_4$) on Phytase production from PPF-6 was investigated. Volume was varied from 0.00-2.5 ml (Figure 5).

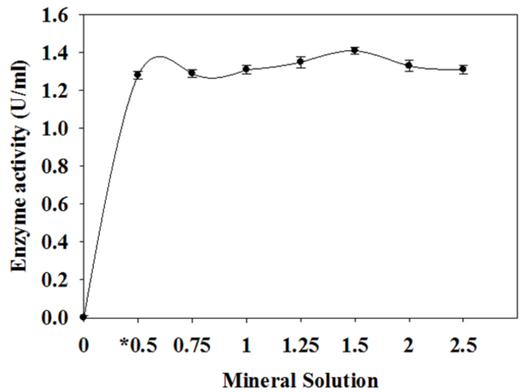

Figure 5. Optimization of Mineral solution for phytase production

Inoculum size

Production media was inoculated with varying number of fungal spores as inoculum (100×10^4-225×10^4 % v/v) and maximum activity was observed at inoculum size 125×10^4 v/v (this inoculum size was the control for optimization of this parameter (Figure 6).

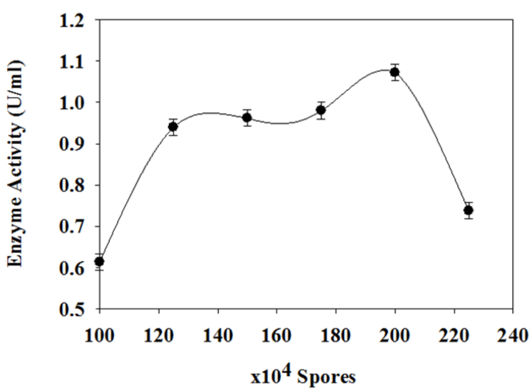

Figure 6. Optimization of inoculum size

Concentration of inducer

Calcium phytate act as substrate inducer for phytase production. So, concentration of calcium phytate was varied from 50µm–200µm (v/v). Maximum activity was observed at 70 µm concentration.

Effect of buffer systems and buffer pH on crude phytase activity

Enzyme activity is much dependent on pH of buffer system. In order to select an appropriate buffer system and pH for enzyme reaction, different buffers at 0.25 M concentration were used. The extracellular phytase activity from *Aspergillus fumigatus* was determined in each buffer with 250 µM Ca-phytate at 55°C for 15 min (Figure 7).

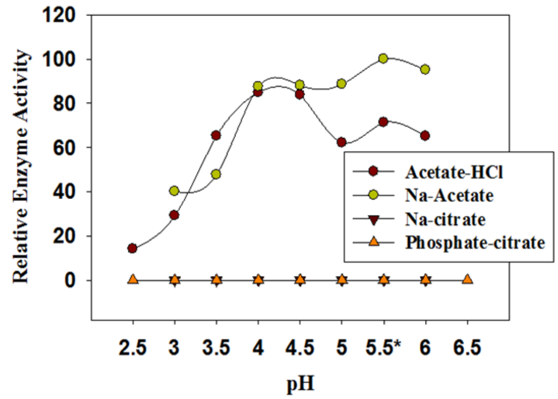

Figure 7. Effect of buffer systems and buffer pH

Effect of the buffer molarity on Phytase activity

The molarity of the selected Na-acetate buffer (pH 5.5) was varied (100-500 mM) and the activity of crude and purified phytase was measured. The reaction was performed with 250 µM Ca-phytate at 55ºC for 15 min.

Optimization of reaction temperature

The phytase activity was assayed in Na-acetate buffer of pH 5.5 (450 mM) at different incubation temperatures (35-70ºC) to find out the optimum reaction temperature with Ca-phytate as substrate. The maximum phytase activity was found at 55ºC.

Effect of substrate concentration

Varying concentration (50-500 µM) of Ca-phytate was used to find out the suitable concentration of substrate for assay of phytase from *Aspergillus fumigatus*. The maximum phytase activity was found with 250 µM (Figure 8).

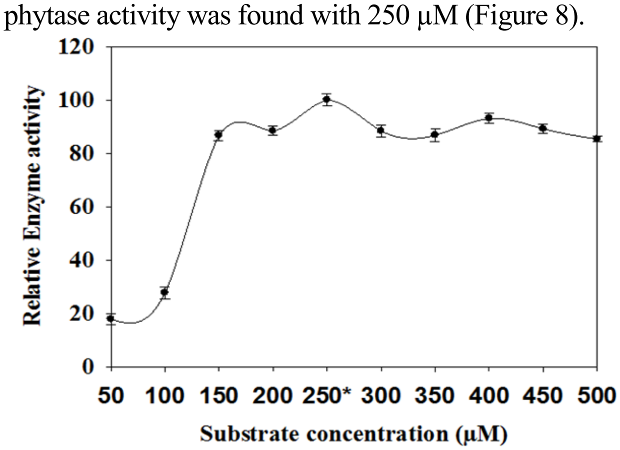

Figure 8. Effect of substrate concentration

Effect of metal ions, inhibitors and additives on crude phytase activity

Metal ions were added to the reaction mixture at 1mM concentration to determine their effect on phytase activity. Various inhibitors and additives such as DTT, EDTA, PMSF, sodium azide, PEG, urea and 2-mercaptoethanol were also used in the concentration of 1mM to determine their effect on the phytase (9).

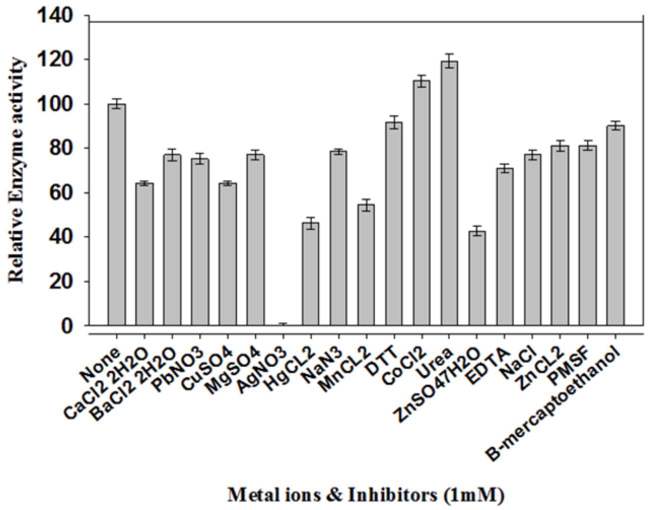

Figure 9. Effect of metal ions, inhibitors and additives on phytase activity

Discussion

The ability of soil microorganism to solubilize various forms of precipitated phosphorus is well documented [25]. Several types of phosphatase, such as phytase, are able to increase the rate of the dephosphorylation (hydrolysis) of organic compound. These enzymes are normally present in soils, where they originate from microorganisms [1]. In the present study 95 micro organisms were isolated for the production of phytase amoung which maximum phytase production was observed from *Aspergillus fumigatus* in submerged media. Similarly 203 fungal strains belonging to the genera *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* and Seven most efficient phytase and phosphatases producing fungi were reported [26,27]. Different cultural conditions such as pH, temperature, carbon sources, nitrogen sources, inoculums sizeand inducer concentration were tested to maximize phytase production from fungal isolate. The best carbon source selected was maltose whereas in some reports [28,1,29] glucose was optimized as carbon source for phytase production from *Bacillus subtilis, Pseudomonas* sp., *Aspergillus niger*113 respectively. Along with the maltose as carbon source, ammonium nitrate suited best as nitrogen source for phytase production which is also reported previously [28].

The effect of environmental factors on the growth and production of phytase by *A. fumigatus* was studied in submerged media. There are reports regarding the production of phytase from *Aspergillus sp.* using cornstarch and semi-synthetic media by submerged fermentation [30,21].Temperature is one of the most critical parameters to be controlled in any bioprocess and the optimum temperature for production of phytases by many microorganisms was 25-37°C [31]. In the present study when growth was carried out at different temperatures (25, 30, 37, 45, 50 °C), the isolate was found to grow best at 40 °C with a considerable production of phytase at 45 °C and above suggesting the fungal isolate to be thermo-tolerant [32,33]. *Aspergillus fumigates* has been reported as thermo-tolerant fungus and has the ability to grow over a range of 12-57°C [34].

The maximum phytase production was recorded at pH 5.5, Similar production pH was reported by for phytase produced from *Aspergillus niger* [35] whereas pH 5.0 and 7.0 was reported from phytase production from *Klebsiella pneumoniae* [36] and from *Bacillus subtilis* [28], *Mucor indicus* [37] respectively. It is well known that the phytase production is inducible, and the presence of phytate, wheat bran or some other inducer in the medium is necessary for enzyme formation [38,39]. Induction for phytase production from *Aspergillus fumigatus* was checked using ca-phytate as an inducer. Different reaction conditions for phytase assay were optimized. Sodium acetate buffer having pH 5.5 was best for phytase assay. Similarly sodium acetate buffer was reported earlier for phytase assay [40,37,22]. Effect of different metal ions on phytase activity was also checked. It was found that mostly metal ions inhibit the enzyme reaction. But exceptionally, enzyme was activated by Al^{3+} and Co^{2+}, but was strongly inhibited by Hg^{2+}in enzyme reaction performed by Wang [36]. Similarly the crude phytase from *Enterobacter* sp. 4 was inhibited by each addition of 1 mM Zn^{+2}, Ba^{+2}, Cu^{+2}, Al^{+3}, and ethylenediamine tetraacetic acid (EDTA) [41].

Conclusion

In conclusion we screened phytase producing microbes acting actively around rhizospheric zone in maize fields specifically in Himachal Pradesh. Phytase production by thermotolerant fungi *Aspergillus fumigatus* was optimized**.**

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