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ISOLATION AND CHARACTERIZATION OF ACID PHOSPHATASE, ALKALINE PHOSPHATASE AND PHYTASE FROM *ASPERGILLUS FUMIGATUS* FOR USES IN INDUSTRIAL APPLICATIONS

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ABSTRACT

Microorganisms, especially fungi, are known for their ability to excrete phosphatases. Since soil is a reservoir for the isolation of such fungi, soil microbes were screened for phosphatases and a strain of *Aspergillus fumigatus* was isolated. For this study, protein rich soil sample was collected from Kengeri gardens of Bangalore, Karnataka, India. After purification of the culture, culture conditions were standardized. A typical fungal colony isolated after serial dilution, identified as *A. fumigatus* was used in this study. The identified phytase would be helpful for further over-expression for large scale production of cost effective phytase enzyme through fermentation and their utilization in animal feed. The identified and confirmed alkaline phosphatase would be helpful for further over-expression for large scale production of cost effective alkaline phosphatase enzyme which could find its applications in molecular biology industries especially for recombinant DNA technology and enzyme immunoassays. The enzymes obtained in this study would have a potential for producing the recombinant enzymes acid phosphatase, alkaline phosphatase and phytase in large amounts.

Keywords: *Aspergillus fumigatus*, Alkaline phosphatase, Acid phosphatase, Phytase

INTRODUCTION

Aspergillus fumigatus originally is a fungus of the genus *Aspergillus*. It is one of the most common *Aspergillus* species that occur as a saprotroph widespread in nature and plays an essential role in carbon and nitrogen recycling by decaying organic matter such as compost heaps. The fungus also causes diseases in humans suffering from immunodeficiency.

Industrial importance of Phytase, Alkaline phosphatase and Acid phosphatase enzymes

Alkaline phosphatase is an enzyme with widespread use in research and industry such as protein labeling, dephosphorylation of nucleic acids, and enzyme based. Alkaline phosphatase has become a useful tool in molecular biology laboratories, since DNA normally possesses phosphate groups on the 5' end. Removing these phosphates prevents the DNA from ligating (the 5' end attaching to the 3' end), thereby keeping DNA molecules linear until the next step of the process for which they are being prepared; also, removal of the phosphate groups allows radio labeling (replacement by radioactive phosphate groups) in order to measure the presence of the labeled DNA through further steps in the process or experiment. Another important use of alkaline phosphatase is as a label for enzyme immunoassays. Alkaline phosphatases are used in enzyme linked immunoabsorbent assays (ELISA), nonisotopic probing, blotting and sequencing systems. Phytase shows great potential for phytate bioconversion, the enzyme activities and yields need to be increased to make them possible for industrial application. Therefore, there is ongoing interest in identifying novel phytases, increasing phytase production, and improving the desired enzymatic characteristics. Industrial application of acid phosphatase is limited, but phytase, a type of acid phosphatase is used in animal feed[1].

In this research work, the bio potential of *Aspergillus fumigatus* filamentous fungi has been explored for the successful isolation, production and purification of acid phosphatase, alkaline phosphatase and phytase production from *Aspergillus fumigatus* and also to examine the possibility of employing the strains for industrial applications. Due to the very high potential uses for novel and modified acid phosphatase, alkaline phosphatase and phytase in food and biotechnology industries an attempt has been made in this study to characterize acid phosphatase, alkaline phosphatase and phytase from the fungi *Aspergillus fumigatus.*

EXPERIMENTAL

Strains and growth media

About 20 samples of about 5 gm each of the protein rich soil was collected in Kengeri gardens in Bangalore. Serial dilution was performed. On identification, *A. fumigatus* was grown on potato dextrose agar.

Isolation and identification of *Aspergillus fumigatus*

Serial Dilution

The sterile test tubes were taken and labeled as 10^{-1},10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions. One gm of saline was dissolved in 10 ml of distilled water and 9.0 ml of this solution was added in each test tube aseptically. One gm of soil sample was added into the first dilution blank of 9.0 ml of distilled water/ saline. Large particles were allowed to settle. One ml from the first dilution blank (10^{-1}) was added to the second dilution blank (10^{-2}). This kind of serial dilution was reported on till the last tube (10^{-5}) dilutions. The test tubes were shaken vigorously for 10 minutes. Lacto phenol cotton blue staining was used to identify the microorganisms in the five dilution tubes. *A. fumigatus* was identified in the 10^{-3}, 10^{-4} and 10^{-5} dilution tubes.

Identification of *Strains of Aspergillus fumigatus*

Macroscopic appearance and Microscopic appearance of the identification of *A. fumigatus* was performed.

Culturing of pure cultures of *Aspergillus fumigatus* in PDA

The molten Potato Dextrose Agar was gently poured into the sterile test tubes and was allowed to solidify as slants. After agar gets solidified, the inoculation loop was immensely heated in the Bunsen flame. Pinch of the culture was taken from the pour plates and streaked in the solidified agar. It was incubated at 28^0 C for 4 days[2].

Screening of acid phosphatase by *A. fumigatus*

Pure cultures were maintained on basal media made of para-nitro phenyl phosphate 0.8g, KNO_3 3.5 g, KH_2PO_4 1.75 g, $MgSO_4$ 0.75 g, per liter of distilled water at pH 5.2. Glucose in the basal media was replaced with para-nitrophenyl phosphate as substrate for acid phosphatase production. 20 ml of the medium was poured into petri plates and allowed to solidify. With a sterile agar borer, 6mm bores were prepared and replaced with 5-days-old fungal culture. Plates were prepared in triplicates and incubated for 4 days at 28^0C. The development of halos and mycelium around each inoculum was observed every 12 hours[3].

Screening of alkaline phosphatase by *A. fumigatus*

Pure cultures were maintained on basal media made of para -nitro phenyl phosphate 0.8 g, KNO_3 3.5 g, KH_2PO_4 1.75 g, $MgSO_4$ 0.75 g, per liter of distilled water at pH 8.2. Glucose in the basal media was replaced with para-nitrophenyl phosphate as substrate for alkaline phosphatase production. 20 ml of the medium was poured into petri plates and allowed to solidify. With a sterile agar borer, 6mm bores were prepared and replaced with 5-days-old fungal culture. Plates were prepared in triplicates and incubated for 4 days at 28^0C. The development of halos and mycelium around each inoculum was observed every 12 hours[3].

Screening of phytase by *A. fumigatus*

A. fumigatus strain was maintained for spore propagation on malt agar at 30 ºC. It was grown in liquid starch media containing starch – 40 g, sodium phytate-10 g, $NaNO_3$ – 8.6 g, K_2HPO_4 – 0.1 g, $MgSO_4$ –0.5 g, KCl- 0.5 g, $FeSO_4$ – 0.1 g per liter of distilled water at pH 5.0. Glucose in the basal media was replaced with sodium phytate as substrate for phytase production. 0.1 cm^3 of spore suspension (2-3.10^7 spores/ cm^3 of media) was added to 30 cm^3 of nutrient medium and the cultivation was carried out for 7 days in 300 cm^3 conical flasks at 30 ºC on a rotary shaker (220 rpm). At the end of the process the biomass was removed by filtration[3].

Purification and characterization of enzymes

Crude enzyme preparations

The culture broth was filtered through Whatman Filter paper No.1. The filtrate constituted the crude enzyme.

Ammonium sulphate salt precipitation

The crude enzyme was subjected to ammonium sulphate fractionation. 70% salt cut was given to the supernatant by adding 44.2 g of ammonium sulphate in 100 ml of solution. This would precipitate out about 90% of the proteins present in the solution. Ammonium sulphate was added very slowly with continuous stirring of the solution on a magnetic stirrer in cold conditions. The solution was centrifuged at 10,000 rpm for 10 minutes at 4^0C. The pellet was collected and dissolved in 10 ml of 50 mM Tris hydrochloric acid solution and then subjected to dialysis.

Dialysis

The dialysis tubing of 12 KDa cut off with pore size of 20 nM was used. About, 8 cm of the dialysis tube was cut. This was then placed in 100 ml of 2% sodium bicarbonate and 1 mM EDTA was added to chelate any metal ions. This was boiled for 10 minutes in boiling water bath and this boiling process was repeated with distilled water several times for about 10 minutes. The tube was then cooled and one of the sides was folded and tied with a rubber band. The solution containing 0.1 M Phosphate buffer saline pH 7 with the impure enzyme was placed inside the tube and the tube was sealed from the other end. This tube was now placed in distilled water in a beaker. This was subjected to magnetic stirring for 12 hours. After dialysis for 12 hours; the enzyme was further purified by Ion exchange chromatography.

Ion Exchange Chromatography

The column was washed with methanol and was dried. The chromatography column packed with DEAE cellulose was washed with distilled water one to two times. The matrix was activated using activation buffer. DEAE cellulose equilibrated with 0.1 M sodium acetate buffer pH 7.5 was packed in a 2 x 25 cm column and the protein was loaded. After washing with the buffer, bound protein was eluted stepwise using the elution buffer Tris HCl of 0.25 mM concentration and sodium chloride of concentrations 25 mM to 150 mM. The enzyme protein eluting at 150 mM was collected for enzyme assay and protein estimation[4].

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis- SDS PAGE was performed with the 40% Acrylamide stacking gel and 40% Acrylamide separating gel to determine the molecular weight and purity of protein. The protein samples were mixed with SDS-PAGE (2X) Loading Buffer Dye and loaded in the wells along with the SDS PAGE marker. The gels were stained with 0.25 % Coomasie Brilliant Blue stain for 4 hours at room temperature with shaking in a gel rocker. The gel was destained by adding 20 ml of 4% methanol, 8% acetic acid and kept at room temperature for 1 hour. The bands were then viewed under the transilluminator[5].

RESULTS AND DISCUSSION

Identification of *Aspergillus fumigatus*

Macroscopic appearance

Growth rate is rapid and the texture of colonies varies from wooly to cottony to granular. Surface colony color is smoky gray - green and the reverse is yellow, however, some isolates may show a lavender diffusible pigment and color of very mature colonies turn to slate gray while atypical colonies may remain white with slight conidiation. The colonies showed typical blue-green surface pigmentation with a suede-like surface consisting of a dense felt of conidiophores.

Microscopic appearance

Lacto phenol Cotton Blue staining was performed and the conidial head of *A. fumigatus* was observed microscopically. Conidial heads was typically columnar (up to 400 x 50 um but often much shorter and smaller) and uniseriate. Conidiophores were short, smooth-walled and have conical-shaped terminal vesicles which supported a single row of phialides on the upper two thirds of the vesicle. Conidia were produced in basipetal succession forming long chains and are globose to subglobose (2.5-3.0 um in diameter), green and rough-walled. Hyphae were septate, hyaline, acute angle branching and fan-like branching.

Screening of acid phosphatase from *Aspergillus fumigatus*

Acid phosphatase screening from *A. fumigatus* was identified by a distinct zone of clearing around fungal colony showing the appearance of transparent yellow halos around the colonies as seen in figure-1.

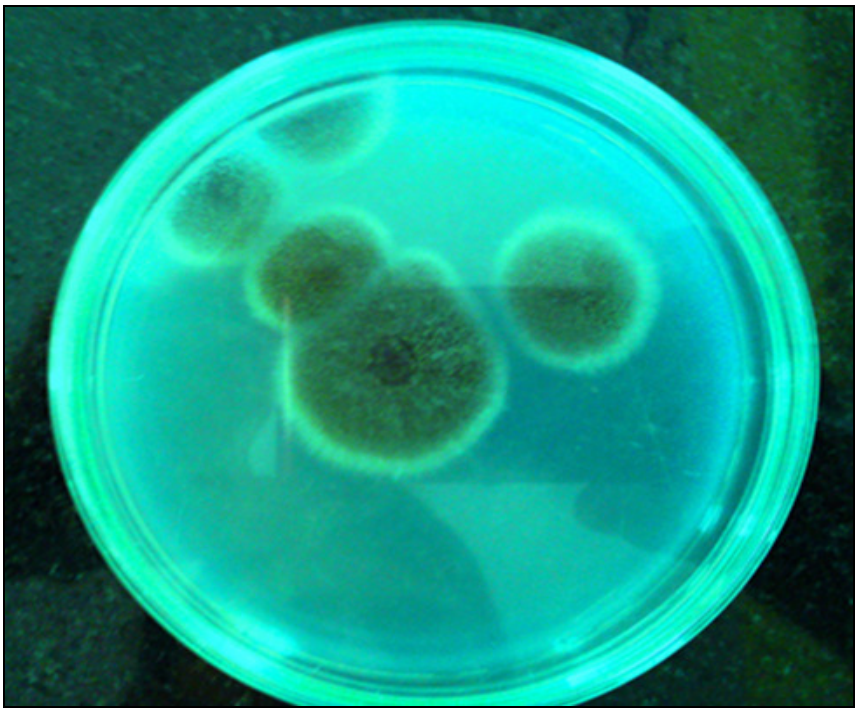

Fig.-1: Acid phosphatase screening from A. fumigatus.

Screening of alkaline phosphatase from *A. fumigatus*

Alkaline phosphatase screening from *A. fumigatus* was identified by a distinct zone of clearing around fungal colony showing the appearance of transparent yellow halos around the colonies. Effect of incubation on phosphatase activity was studied and the activity increased with the colony development as presented in figure-2.

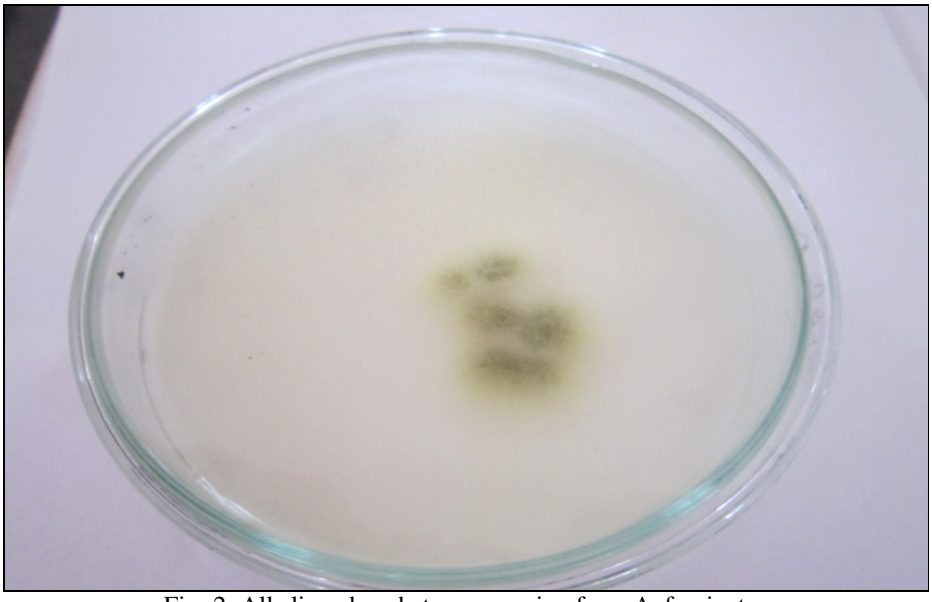

Fig.-2: Alkaline phosphatase screening from A. fumigatus

Screening of phytase from *A. fumigatus*

Phytase screening from *A. fumigatus* was identified by a distinct zone of clearing around fungal colony showing the appearance of transparent green halos around the colonies as presented in figure-3.

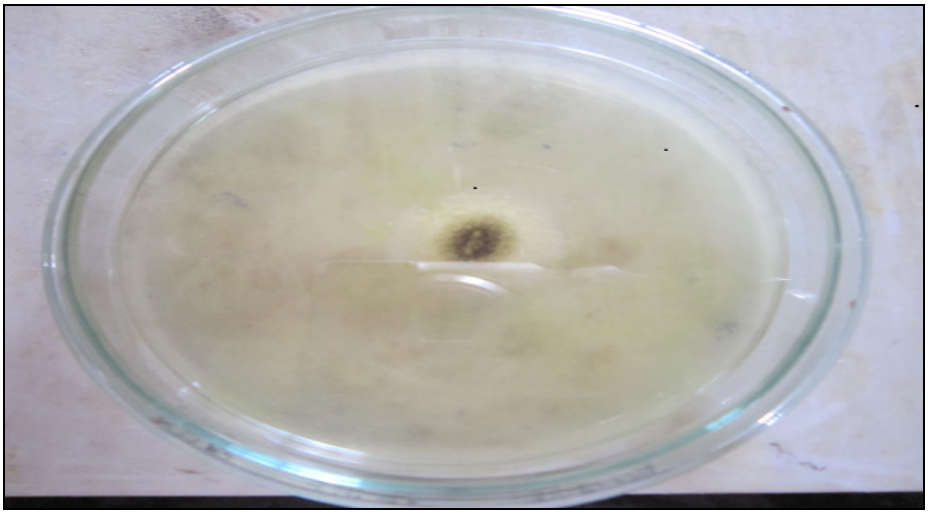

Fig.-3: Phytase screening from A. fumigatus.

Molecular weight determination of acid phosphatase by SDS PAGE

The enzymes were purified from the culture filtrate by ammonium sulphate fractionation and dialysis followed by ion exchange chromatography on DEAE-Cellulose. The molecular weights of the enzymes were calculated based on the elution profiles of the protein in relation to the standard SDS PAGE marker.SDS PAGE was performed with the 40% Acrylamide stacking gel and 40% Acrylamide separating gel to determine the molecular weight and purity of acid phosphatase. The protein samples were loaded in the wells along with the standard SDS PAGE marker. The gels were stained with 0.25 % Coomasie Brilliant Blue and destained by adding 20 ml of 4% methanol, 8% acetic acid. The bands were then viewed under the trans illuminator. Acid phosphatase showed a molecular weight of 50 KDa as given in figure 4.

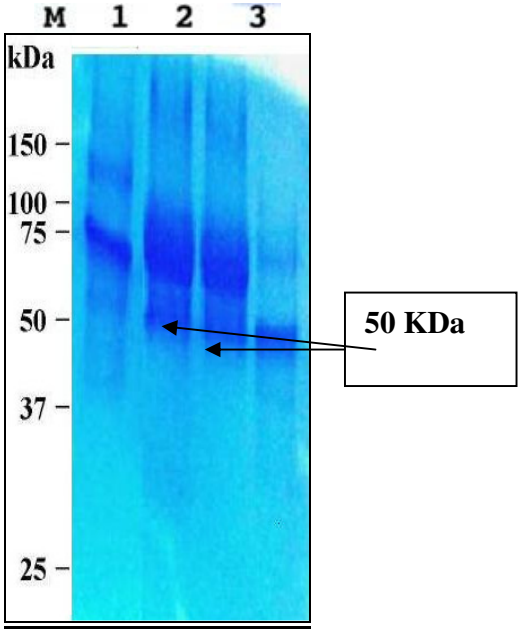

Fig.-4: SDS PAGE of purified acid phosphatase of A. fumigatus showing molecular weight of 50 KDa. [Lane M- Marker, Lane 1 – Crude Extract, Lane 2- After Ammonium sulftate precipitation and Dialysis,Lane 3- After Ion Exchange Chromatography]

Molecular weight determination of alkaline phosphatase by SDS PAGE

SDS PAGE was performed with the 40% Acrylamide stacking gel and 40% Acrylamide separating gel to determine the molecular weight and purity of alkaline phosphatase. The protein samples were loaded in the wells along with the standard SDS PAGE marker.

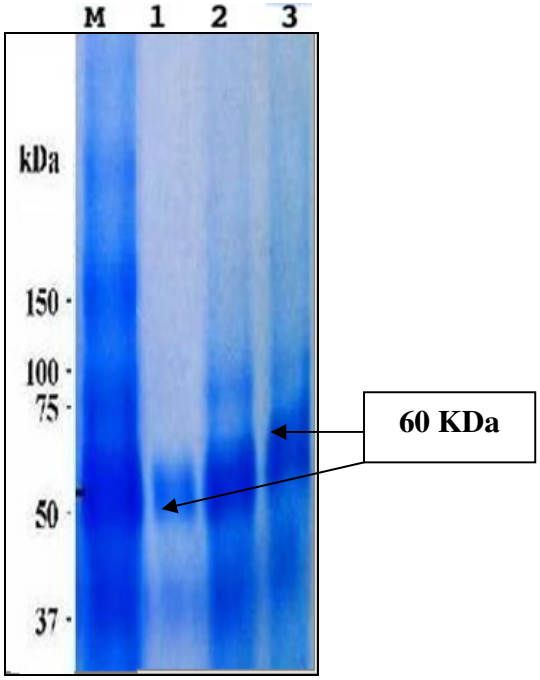

Fig.-5-SDS PAGE of purified alkaline phosphatase of A. fumigatus showing molecular weight of 60 KDa. [Lane M- Marker, Lane 1 – After Ion Exchange Chromatography, Lane 2- After Ammonium sulftate precipitation and Dialysis, Lane 3- Crude Extract]

The gels were stained with 0.25 % Coomasie Brilliant Blue and destained by adding 20 ml of 4% methanol, 8% acetic acid. The bands were then viewed under the trans illuminator. Alkaline phosphatase showed a molecular weight of 60 KDa as given in figure 5.

Molecular weight determination of phytase by SDS PAGE

SDS PAGE was performed with the 40% Acrylamide stacking gel and 40% Acrylamide separating gel to determine the molecular weight and purity of phytase. The protein samples were loaded in the wells along with the standard SDS PAGE marker. The gels were stained with 0.25 % Coomasie Brilliant Blue and destained by adding 20 ml of 4% methanol, 8% acetic acid. The bands were then viewed under the trans illuminator. Phytase showed a molecular weight of 47 KDa as given in figure 6.

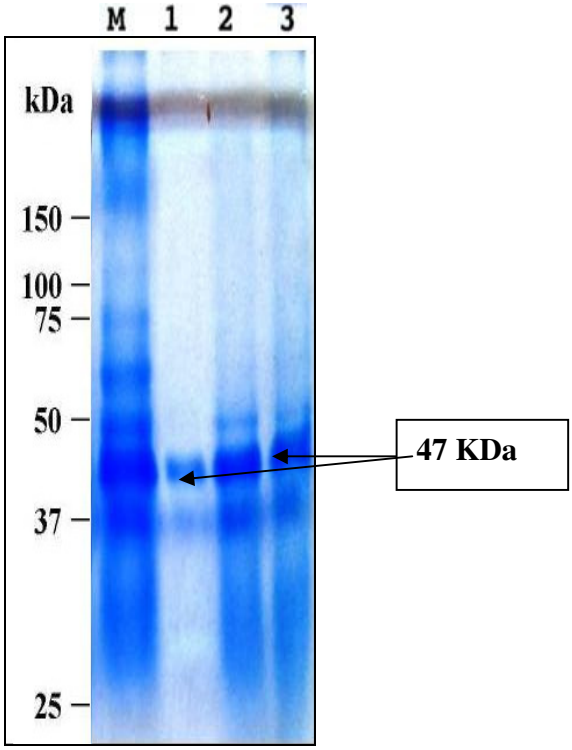

Fig.-6: SDS PAGE of purified Phytase of A. fumigatus showing molecular weight of 47 KDa. [Lane M- Marker, Lane 1- After Ion Exchange Chromatography, Lane 2- After Ammonium sulftate precipitation and Dialysis, Lane 3 – Crude Extract]

CONCLUSION

Phytase is a promising candidate for applications in the feed industry to enhance the quality for poultry and piggery by supplementing it in their diets. The identified phytase would be helpful for further over-expression for large scale production of cost effective phytase enzyme through fermentation and their utilization in animal feed. Alkaline phosphatase has become an important tool in molecular cloning and DNA sequencing. It also used as an important part of diagnostic kits component of different ELISA base kits. The identified and confirmed alkaline phosphatase would be helpful for further over-expression for large scale production of cost effective alkaline phosphatase enzyme which could find its applications in molecular biology industries especially for recombinant DNA technology and enzyme immunoassays. The enzymes obtained in this study would have a potential for producing the recombinant enzymes acid phosphatase, alkaline phosphatase and phytase in large amounts for industrial applications.

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