ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Molecular Cloning of Alkaline Phosphatase, Acid Phosphatase and Phytase Genes from *Aspergillus fumigatus* for Applications in Biotechnological Industries

Neelakantan Suresh*, Arunava Das
Department of Biotechnology, Bannari Amman Institute of technology, Erode 638401, Tamil Nadu, India.
*Corresponding author

Abstract:

The present study aims on the successful isolation and cloning of acid phosphatase, alkaline phosphatase and phytase for the genes encoding the three enzymes from *Aspergillus fumigatus* in terms of its industrial applications. 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. A typical fungal colony isolated after serial dilution, identified as A. fumigates was used in this study. After purification of the culture, culture conditions were standardized. Acid phosphatase, alkaline phosphatase and phytase cDNAs synthesized from Aspergillus fumigatus RNA by PCR reactions using primers (designed from published NCBI sequences) with reverse transcriptase resulted in amplicons corresponding to the predicted gene size of the respective enzymes. The PCR result suggested that they were amplicons of Acid phosphatase, alkaline phosphatase and phytase.

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

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 is thermophilic sine it has the ability to grow at normal body temperature of 37^0C. The conidia survive even at 70^0C, the temperatures regularly encountered in self-heating compost heaps. In immunocompromised individuals such as organ transplant recipients and people with AIDS or leukemia, the fungus is more likely to become pathogenic, over-running the host's weakened defenses and causing a range of diseases generally termed aspergillosis *Aspergillus fumigatus* is a fungus of the genus *Aspergillus*, and is one of the most common *Aspergillus* species to cause disease in individuals with an immunodeficiency. *Aspergillus fumigatus*, a saprotroph widespread in nature, is typically found in soil and decaying organic matter.

In this research work, the biopotential of *Aspergillus fumigatus* filamentous fungi has been explored for the successful isolation, production, purification, characterization, cloning of genes encoding 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 clone the acid phosphatase, alkaline phosphatase and phytase from the fungi *A. fumigatus* by amplifying the entire gene sequences of phytase-encoding gene known to secrete phytase, acid phosphatase encoding gene known to secrete acid phosphatase, alkaline phosphatase encoding gene known to secrete alkaline phosphatase in *A. fumigatus* by using designed primers for PCR reaction for further amplification of the genes and thereby performed gene cloning. 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. Use of plant-based feed in poultry and piggery is inevitable in the near future too. Phytate-rich plant ingredients restrict the bioavailability of phosphorus along with other minerals, thereby increasing discharge of phosphorus into water bodies leading to eutrophication. However, it is evident that phytase supplementation of feed improves the bioavailability of the phosphorus and nitrogen. The increased bioavailability of nitrogen and phosphorus in the diet leads to reductions in feed costs through curtailing the addition of inorganic phosphorus. Alkaline phosphatase is an enzyme with widespread use in research and industry such as protein labeling, dephosphorylation of nucleic acids, and enzyme based biosensors (Mir Mohammad et al 2008). 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 biotechnological applications of alkaline phosphatase enzymes have attracted much attention especially for recombinant DNA technology and ELISA (Guimaraes et al 2007). Due to its so many industrial uses, it is necessary to purify it on large scale for commercial and research purpose (Shah Ali ul Qader, Samina, Zaman 2009).

MATERIALS AND METHODS:

Sample collection:

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.

Total RNA isolation:

Aspergillus fumigatus was inoculated in potato dextrose agar and incubated for 4 days at 28^{o}C. This 4-days-old *Aspergillus fumigatus* strains was inoculated into YEPD media and the cultivation was carried out for seven days in a rotary shaker incubated at 37^{o}C at 200 rpm. To construct growth curve 1 ml sample was taken at each sampling time of 24 hours and filtered through Whatman filter paper. The mycelial pellet was dried at 60^{o}C in a drying oven for several hours and weighed (Reeves et al 2004). The dry weight of the fungus was used to construct the growth curve. The fungal biomass increased at a slow rate for the initial 48 hours and thereafter showed exponential growth phase. After 5th day the rapid growth stabilized and the trend became more towards stationary phase. Ten ml of mid-exponential phase culture of *Aspergillus fumigatus* grown in YEPD liquid medium was taken for total RNA isolation. Total RNA extraction was done according to Yuping et al 2007.Ten ml of mid-exponential phase culture of *Aspergillus fumigatus* grown in YEPD liquid medium was harvested by centrifugation at 5,000 rpm for 5 minutes at 4^{o}C, washed, and resuspended in RNase-free distilled water. The cell pellet was placed into a pre-chilled autoclaved mortar and ground to powder under liquid nitrogen. This powder was transferred into a clean tube. After adding 400 µl TES solution (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 5% SDS) and 400 µl acid-phenol (water saturated), the tube was incubated at 65^{o}C for 30 minutes, with shaking every 6 minutes in a vortexer for 10 seconds. The tube was then placed on ice for 5 minutes and then centrifuged at 12,000 rpm for 10 minutes at 4^{o}C. To purify the RNA, the aqueous phase was transferred to a fresh tube and extracted with an equal volume of acid-phenol until no protein layer was observed and then extracted with an equal volume of chloroform. The RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ice-cold absolute ethanol at kept on ice for 15–20 minutes. The pellet was harvested by centrifugation at 12,000 rpm for 10 minutes at 4^{o}C. The pellet was washed with 70% ethanol and absolute ethanol. The pellet was then dried and resuspended in 100 µl of Diethylpyrocarbonate (DEPC) treated water which would inactivate the RNase enzymes for the stability and storage of the RNA. This was stored at -20 ^{o}C untill further use. Working in RNAse free conditions the half life of the RNA is longer since chemically it is quite stable. RNA was converted into cDNA as soon as possible after isolating the total RNA as complementary DNA is much more stable.

Reverse transcription (RT) reaction

Reverse transcription reaction was carried out in three separate tubes for phytase, alkaline phosphatase and acid phosphatase. In a 1.5 ml DEPC treated tube, the following reagents were added in the following order: Sterile DEPC treated water of 7 µl, RNasin (40U/ µl) 1 µl, total RNA from *Aspergillus fumigatus* sample 2 µl (3 µg), Reverse primers of phytase, alkaline phosphatase and acid phosphatase (10 µM) 2 µl individually in three separate tubes. This was heated to 70^{o}C for 10 minutes to remove the RNA secondary structure. This was quickly chilled on ice. Then added, RNasin (40U/ µl) 1 µl, 10mM dNTP mix 2 µl, 5X Reaction buffer 4 µl, Reverse transcriptase enzyme (200U/ µl) 1 µl. This was mixed gently and incubated at 42^{o}C for 1 hour. The reaction was terminated by heating at 70^{o}C for 15 minutes. The complementary DNA obtained was stored at -80^{o}C untill further use.

Primer design

The gene producing sequences for acid phosphatase, alkaline phosphatase and phytase of *Aspergillus fumigatus* without introns was taken for PCR primer designing. Primer design was performed using the National Center for Biotechnological Information (NCBI) published sequences of *Aspergillus fumigatus* acid phosphatase ***phoA*** gene Accession no. AF462065, *Aspergillus fumigatus* phytase *phyA* gene Accession no. U59804 and *Aspergillus fumigatus* Af293 alkaline phosphatase ***pho8 gene*** Accession no. XM_744353. The restriction enzyme recognition sites were included in the primers. The restriction enzyme recognition sites were obtained from literature. The forward and reverse primers of Alkaline phosphatase and Acid phosphatase had *EcoRI* and *SalI* restriction enzyme recognition sites respectively and Phytase had *Bam*HI and *EcoRI* as forward and reverse primer restriction enzyme recognition sites. Reverse transcription reaction was performed to obtain complementary DNA using Reverse transcriptase enzyme and designed reverse primers for acid phosphatase, alkaline phosphatase and phytase genes. Polymerase chain reaction (PCR) was carried out using Eppendrof Thermocycler.

Amplification of *phoA, pho8 and phyA* gene using PCR

The coding regions of *Aspergillus fumigatus* acid phosphatase ***phoA*** gene, phytase ***phyA*** gene and alkaline phosphatase ***pho8*** gene was amplified using the forward and reverse primers. Three different PCR reactions were performed for alkaline phosphatase, acid phosphatase and phytase genes using the designed primers and complementary DNA obtained from the reverse transcription reaction as template. The amplified PCR products of alkaline phosphatase, acid phosphatase and phytase genes of *Aspergillus fumigatus* were confirmed on running on 0.8% agarose gel electrophoresis. The PCR reaction amplified an amplicon size of approximately 1800 bp characteristic of alkaline phosphatase gene, 1320 bp characteristic of acid phosphatase and 1350 bp characteristic of phytase gene which covers the entire gene without introns of the fungal *Aspergillus fumigatus* genomic DNA. The cloning vector used was pET 28a (Novagen). It was expressed in *E.coli DH5 alpha.* Alkaline phosphatase and Acid phosphatase have *EcoRI* and *SalI* restriction enzyme recognition sites

respectively and Phytase have *Bam*HI and *EcoRI* as restriction enzyme recognition sites included in the forward and reverse primers for PCR reaction**.** On confirmation of the three genes after PCR reaction, alkaline phosphatase gene was digested with *EcoRI* and *SalI* restriction enzymes which would generate sticky ends, acid phosphatase was digested with *EcoRI* and *SalI* restriction enzymes which generated sticky ends and Phytase was digested with *EcoRI* and *BamHI* restriction enzymes which generated sticky ends. This restriction digested DNA was run in agarose gel electrophoresis. An incision was made in the gel ahead of desired DNA bands. The purified gel eluted insert DNA was obtained. Simultaneously, pET-28a expression vector DNA obtained from Novagen was digested with ***EcoRI* and *SalI*** restriction enzymes for the ligation of alkaline phosphatase and acid phosphatase genes. pET-28a expression vector DNA was also digested with ***EcoRI* and *BamHI*** for ligation of phytase gene. This was run in agarose gel electrophoresis.

Ligation of *phoA, pho8 and phyA* gene in cloning vector and transformation

An incision was made in the gel ahead of desired DNA bands. The purified gel eluted insert DNA was then ligated to pET-28a gel eluted purified vector DNA using T4 DNA Ligase. The ligation reaction mixture was 0.5µl of pET-28a vector, 2.5µl of insert DNA, 1µl of 10X ligase buffer, 0.5 µl of T 4 DNA Ligase and 5.5 µl of nuclease free water. The insert gene is ligated in between *EcoRI* and *SalI* restriction enzyme sites for acid phosphatase and alkaline phosphatase and between *EcoRI* and *BamH1* restriction enzyme sites for phytase in the pET28A vector individually.

Screening of recombinant clones having *phoA, pho8 and phyA* gene

Transformation was carried out by mixing 5 µl of ligation mixture with 100 µl of *E.coli* DH5 alpha competent cells. Transformation was done using heat-shock method. The transformed cells were spread on LB agar plates containing 50 µg/ml Kanamycin (antibiotic) and incubated for 16 hours at 37˚C. Transformants were screened again on LB-Kanamycin plates and the positive transformants were selected for plasmid isolation by alkali lysis method. Positive transformants were selected for plasmid isolation. The confirmation of the positive clones was carried after plasmid isolation. Alkaline phosphatase and Acid phosphatase was screened using *EcoRI* and *SalI* restriction enzymes and Phytase was screened using *Bam*HI and *EcoRI* as restriction enzymes. This would release the insert gene on restriction digestion of the sites in the pET28A vector and this was confirmed using agarose gel electrophoresis. Agarose gel electrophoresis after plasmid isolation of acid phosphatase, alkaline phosphatase and phytase genes of *Aspergillus fumigatus* confirmed acid phosphatase gene of 1340 bp after restriction digestion with *EcoRI and Sal I,* confirmed alkaline phosphatase gene of 1800 bp after restriction digestion with *EcoRI and Sal I* and also confirmed phytase gene of 1350 bp after restriction digestion with *EcoRI and BamHI.*

On confirmation of the insert gene, the remaining plasmid DNA which was not subjected to restriction enzymes was sequenced using automatic DNA sequencer using T7 promoter primer and T7 terminator primer. DNA sequencing was performed using automatic DNA sequencer using the T7 promoter primer and T7 terminator primer which anneals to the region in the vector. DNA sequencing revealed an Open Reading Frame of 1824 base pairs downstream from a potential ribosome-binding site that encoded alkaline phosphatase gene beginning with an ATG (start codon) and ending with TAG (stop codon) was identified as given in figure 1,2 and 3.

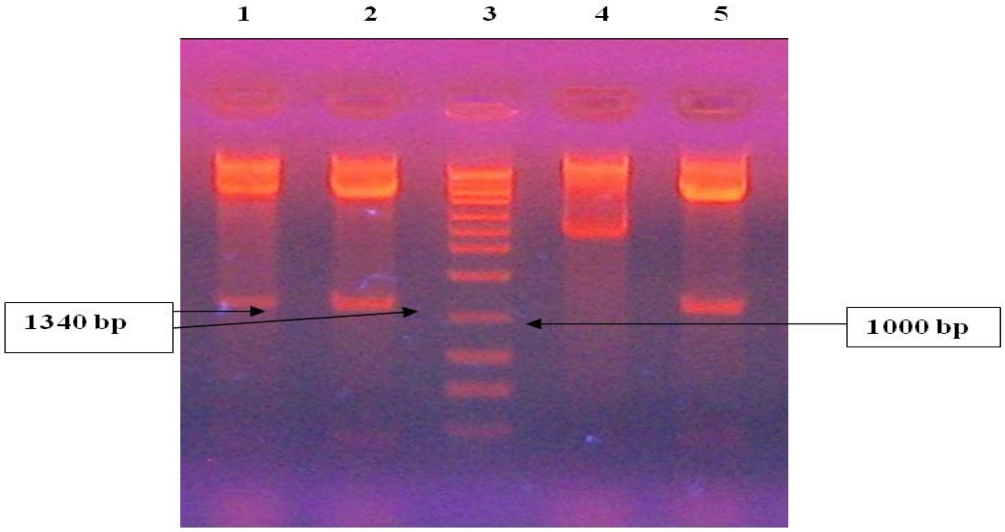

Figure 1

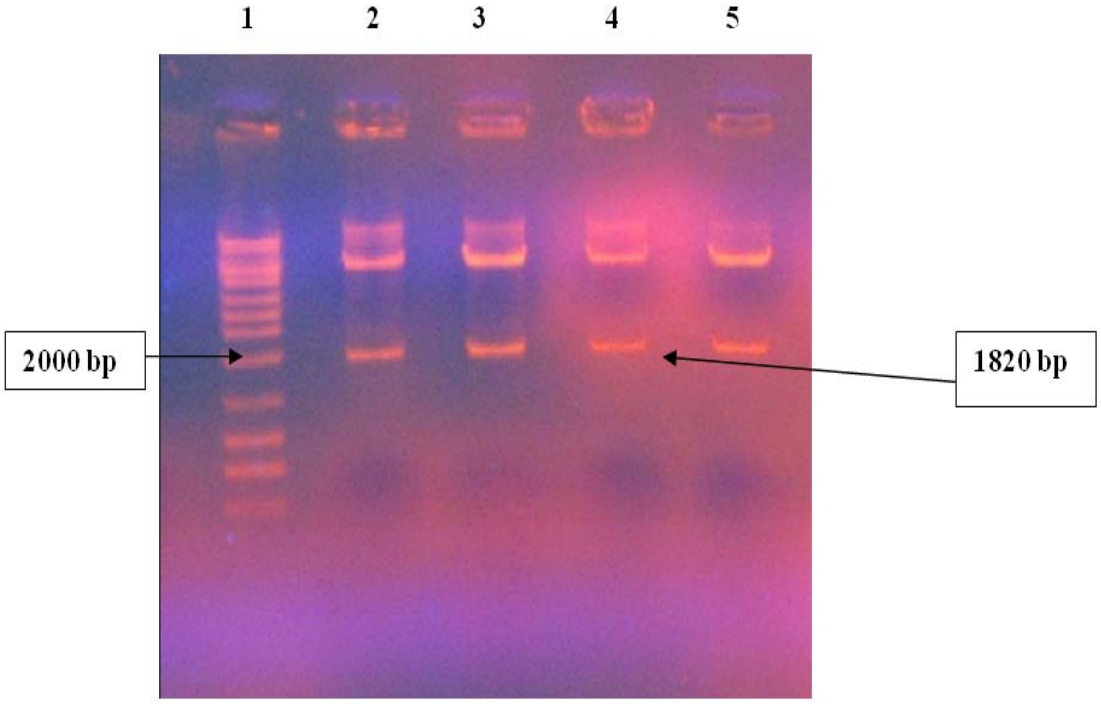

Figure 2

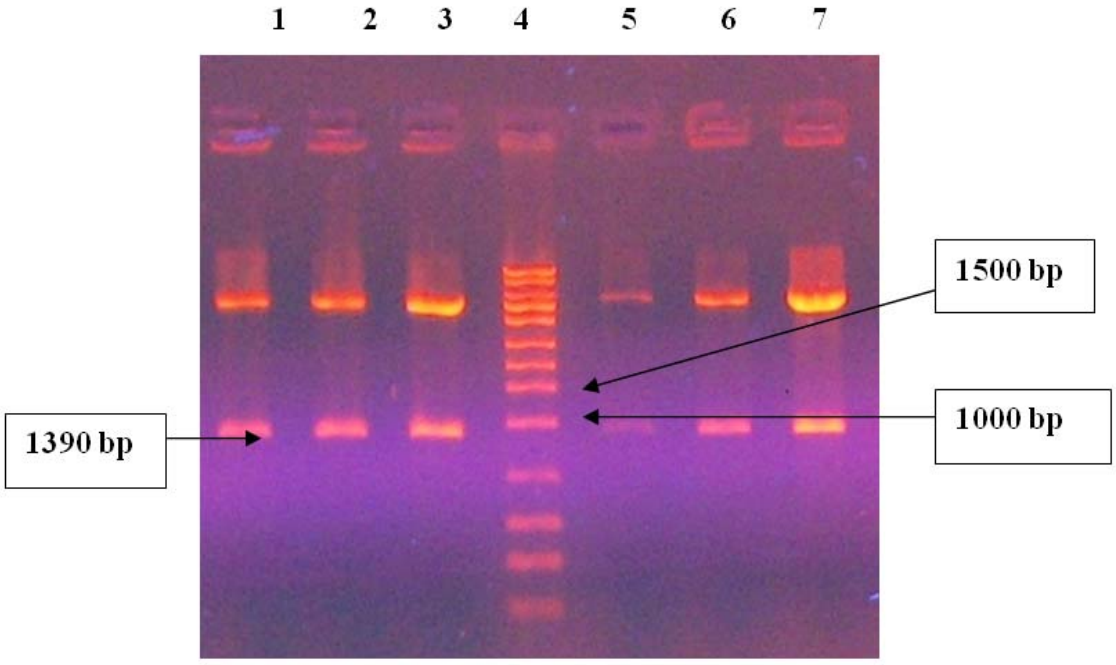

Figure 3

Acid phosphatase gene after restriction digestion

Agarose gel electrophoresis confirmed acid phosphatase gene after double restriction digestion with *EcoRI and Sal I*. This was obtained after viewing in UV transilluminator after ethidium bromide staining. Lane 3 indicates 1 kb DNA ladder, Lane 1, 2 and 5 indicates acid phosphatase gene of 1340 bp. **(Figure 1)**

Alkaline phosphatase gene after restriction digestion

Agarose gel electrophoresis confirmed alkaline phosphatase gene after double restriction digestion with *EcoRI and Sal I*. This was obtained after viewing in UV transilluminator after ethidium bromide staining. Lane 1 indicates 1 kb DNA ladder, Lane 2, 3, 4 and 5 indicates alkaline phosphatase gene of 1820 bp. **(Figure 2)**

Phytase gene after restriction digestion

Agarose gel electrophoresis confirmed phytase gene after double restriction digestion with *EcoRI and BamHI*. This was obtained after viewing in UV transilluminator after ethidium bromide staining. Lane 4 indicates 1 kb DNA ladder, Lane 1, 2, 3, 5, 6 and 7 indicates phytase gene of 1390 bp. **(Figure 3)**

RESULTS AND DISCUSSION:

In the present study, *A. fumigatus* was isolated, identified and grown in PDA medium. p-nitrophenyl phosphate was used as a substrate for both acid phosphatase and alkaline phosphatase enzymes production at pH 5.2 and pH 8.2 in the production media respectively. Phosphatase activity was identified by distinct zone of clearing around fungal colony giving the appearance of transparent yellow halos around colonies. Sodium phytate was used as substrate for phytase enzyme production. Phytase production activity was identified by a light green halos around the colonies. The PCR reaction amplified an amplicon size of approximately 1800 bp characteristic of alkaline phosphatase gene, 1320 bp characteristic of acid phosphatase and 1350 bp characteristic of phytase gene which covers the entire gene without introns of the fungal *A. fumigatus* genomic DNA. Agarose gel electrophoresis after plasmid isolation of acid phosphatase, alkaline phosphatase and phytase genes of *A. fumigatus* confirmed acid phosphatase gene of 1340 bp after restriction digestion with *EcoRI and Sal I,* confirmed alkaline phosphatase gene of 1800 bp after restriction digestion with *EcoRI and Sal I* and also confirmed phytase gene of 1350 bp after restriction digestion with *EcoRI and BamHI*. After the confirmation of the insert, the plasmid was sequenced by automated DNA sequencing method. Similar observations and identification criteria were followed in the previous studied (Luis Pasamontes, Monika Haiker, Markus Wyss, Michel Tessier, 1997) for phytase. Similar observations and identification criteria were followed in the previous studied (Muriel Bernard, Isabelle Mouyna, Guy Dubreucq, Jean-Paul Debeaupuis, Thierry Fontaine, Constantinos Vorgias, Claus Fuglsang and Jean-Paul Latge, 2002) for phytase.

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 and confirmed *pET28A-phyA* clone would be helpful for further over-expression of this gene 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 *pET28A-pho8* clone would be helpful for further over-expression of this gene 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. Homology modeling and phylogenetic analysis revealed high identify for acid phosphatase *phoA* gene, phytase *phyA* gene and alkaline phosphatase *pho8* gene with other genes of the same *Aspergillus* species. This would be helpful in investigation of the structure, evolution and mechanisms of the enzyme action. In the light of these findings the cloned genes obtained in this study will have a very high potential for producing the enzymes alkaline phosphatase, acid phosphatase and phytase of *A. fumigatus* in large quantities.

ACKNOWLEDGEMENT:

The experimental work was performed in Azyme Biosciences Pvt Ltd, Bangalore and Bhat Biotech India Private ltd, Bangalore

REFERENCES:

1. K. Lamrani, H. Lakhtar, M. Ismaili-alaoui, M. Ettalibi, P. Boiron, C.augur, I. Gaime-perraud and S. Roussos, Production of fumagillin by *Aspergillus fumigatus* isolated from traditional trituration units, "maasra", in morocco, Micologia Aplicada International, 20(1), 2008, pp. 35-41.
2. C., L. Quan, Zhang., Y. Wang and Y. Ohta (2001); Journal of Bioscience and Bioenginering., 9 (2): 154-160. Production of Phytase in Low Phosphate Medium by a Novel Yeast Candida krusei.
3. Mahesh.M, Guleria Neha, Rajesh.T.S, Somashekhar.R, Puttaiah.E.T, Isolation And Characterization Of Extracellular Thermostable Alkaline Phosphatase Enzyme From *Bacillus* Spp; Azyme Biosciences Private Limited, Jayanagar, Bangalore, Karnataka-560069.
4. Muriel Bernard, Isabelle Mouyna, Guy Dubreucq, Jean-Paul Debeaupuis, Thierry Fontaine, Constantinos Vorgias, Claus Fuglsang and Jean-Paul Latge; Characterization of a cell-wall acid phosphatase (PhoAp) in *Aspergillus fumigatus.*
5. M.V. Sariyska, S.A. Gargova, L.A. Koleva, A.I. Angelov; *Aspergillus Niger Phytase*: Purification And Characterization; University of Food Technologies, Department of Biotechnology, Plovdiv, Bulgaria; University of Food Technologies, Department of Biochemistry and Molecular Biology, Plovdiv, Bulgaria.
6. Jaya Prakash Goud, M; Vinaya Sagar Goud, J; Singara Charya, M. Replica Plate Screening Method for detecting Phosphatase activity using 1-Napthyl phosphate as a chromogenic substrate, Department Of Microbiology, Kakatiya University, Warangal, 506009-A.P, India.
7. Luis Pasamontes, Monika Haiker, Markus Wyss, Michel Tessier, and Adolphus P. G. M. Van loon; Gene Cloning, Purification, and Characterization of a Heat-Stable Phytase from the Fungus *Aspergillus fumigatus*; Vitamins & Fine Chemicals Division, F. Hoffmann-La Roche Ltd., 4070 Basel, Switzerland.

8. Yan Wang, Xiaorong Gao, Qiao Su, Wei Wu, Lijia; Cloning, Expression, and Enzyme Characterization of an Acid Heat-Stable Phytase from *Aspergillus fumigatus* WY-2 ;Department of Bioscience and Biotechnology, School of Environment and Life, Dalian University of Technology, Dalian, 116024, P.R. China.
9. Xueying Wang, SuchartUpatham, Watanalai Panbangre, Duangnate Isarangkul, Pijug Summpunn, Suthep Wiyakrutta and Vithaya Meevootisomd; Purification, Characterization, Gene Cloning and Sequence Analysis of a Phytase from *Klebsiella pneumoniae* subsp. pneumoniae XY-5.
10. Luis Henrique (2006); Screening of filamentous fungi for production of enzymes of biotechnological interest; Brazilian Journal of Microbiology 37:474-480.
11. S. J, Howson and R. J. Davis; J. Enzyme Microbiol. Technol. 1983, 5: 377-382, Production of Phytate-Hydrolysing Enzyme by Fungi.
12. J. Anim. Sci. 83: 1062-1067, T. W Kim, and X. G. Lei. (2005); An Improved Method for a Rapid Determination of Phytase activity in animal feed.
13. Yetti Marlida , Rina Delfita , Neni Gusmanizar, and Gita Ciptaan (2010); Identification Characterization and Production of Phytase from Endophytic Fungi; World Academy of Science, Engineering and Technology 65.
14. N. McCLENNY; Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach San Francisco State University, San Francisco, CA, USA.