

Cloning of phytase gene from *Aspergillus niger* 563 and its expression in *E.coli* system

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Abstract— A *phyA* was cloned from *Aspergillus niger* by reverse transcription polymerase chain reaction. The amplified 1404 bp fragment was cloned in a T/A cloning vector and confirmed by sequencing. The isolated phytase gene showed 99.0 % sequence identity at nucleotide and protein level with *Aspergillus niger* CBS 513.88 phytase. The amino acid sequence from the *phyA* cDNA contained the consensus motifs, RHGXRRP and HD which are conserved among histidine acid phosphatases. The phytase cDNA was subcloned in pET28a(+) expression vector and expressed in *E. coli*. The expression of the gene was confirmed through SDS-PAGE analysis. A 52 kDa protein as per the calculated molecular mass of the translational product of phytase gene was observed in crude lysate of *E. coli* culture induced with IPTG. The protein was purified using nickel based His-bind resin column and checked on SDS-PAGE. The purified recombinant enzyme showed a single band of 52 kDa protein. The phytase activity of pET-*phyA* IPTG induced *E. coli* culture and purified phytase was 383.5 U/ml and 826.33 U/ml, respectively.

Keywords— *Aspergillus niger*, phytase, cloning, expression, *E. coli*

I. INTRODUCTION

Phosphorus is one of the most limiting nutrients for animals because most of the phosphorus in the plant seeds including the feeding plants is in the form of phytic acid (De Boland *et al.*, 1975), most of which cannot be digested by monogastric animals and acts as an antinutritional factor hindering the uptake of a range of minerals. Furthermore, high phytic acid content in animal manure by excretion results in elevated level phosphorus level in soil and water and accompanying environmental concerns (Lambrechts *et al.*, 1992). Phytate also chelates trace elements of iron and zinc between phosphate groups within a single phytate molecule or between two phytate molecules. The enzyme phytase is able to release the bioavailable phosphorus from phytic acid, consequently improving the phosphorus bioavailability and the uptake of minerals. On the other hand, the content of phytase in plant itself is too limited to release sufficient inorganic phosphorus (Brinch-Pederson *et al.*, 2002).

Phytases are a special class of phosphatases that catalyze the sequential hydrolysis of myo-inositol-(1,2,3,4,5,6)-hexakisphosphate or phytic acid (Ins P₆) to less phosphorylated myo-inositol derivatives and inorganic phosphate (Haros *et al.*, 2007). Phytase is the only known enzyme that can initiate the phosphate hydrolysis at carbon 1, 3 or 6 in the inositol ring of phytate. The removal of phosphate group by phytase results in releasing of calcium, iron, zinc, and other metals. Phytate degrading activity has been detected in plants, microorganisms, and in some animal tissues and phytases from several plant and microbial species (Hill *et al.*, 2007) have been purified and characterized. In plants, phytase activity is found in many plant seeds (Laboure *et al.*, 1993). Hence, although currently phytases are used mainly as animal feed additives in diets of monogastric animals, there is a great potential for the use of this class of enzymes in processing and manufacturing of food for human consumption (Jorquera *et al.*, 2008).

Phytase activity has been found most frequently in fungi, such as *Aspergillus ficuum* (Gibson, 1987), and *Aspergillus*

niger (Han et al., 2018). Phytase is also produced by gram-positive bacteria, such as *Bacillus* (Farhat-Khemakhen et al., 2012 and Saadi et al., 2021), and gram negative bacteria, like *Aerobacter aerogenes* (Greaves et al., 1967), *Pseudomonas* sp. (Singh et al., 2017), *Escherichia coli* (Greiner et al., 1993), and *Klebsiella* (Jareonkitmongkol et al., 1997). Generally, phytases from gram-negative bacteria are intracellular proteins, while phytases from gram-positive bacteria and fungi are extracellular enzymes (Kim et al., 1998; Choi et al., 2001). In this study, we cloned and sequenced the phytase gene of *Aspergillus niger* and recombinantly expressed the phytase in *E. coli*.

II. MATERIALS AND METHODS

Chemicals

Sodium phytate, Isopropyl thiogalactoside (IPTG), deoxynucleoside triphosphates (dNTPs) and β -mercaptoethanol were purchased from Sigma Aldrich Chemical Company, U.S.A. All the other chemicals used were of analytical grade manufactured in India.

Strains, plasmids and chemicals

A. niger 563 obtained from National Chemical Laboratory (NCL), Pune was used to isolate a phytase gene. *E. coli* DH5 α and B121 (DE3) were used as hosts for genetic manipulation and expression, respectively. The vector pTZ52R/T (MBI Fermentas) was used for TA cloning and nucleotide sequencing, and pET28a(+) (Novagen, Madison, WI) was used for expression of phytase in *E. coli*. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium.

Total RNA isolation

The hyphae of *Aspergillus niger* was inoculated into PDA medium (20% potato extract and 2% sucrose) and shaken at 100 rpm for 2-3 days at 28°C and mycelia was collected. The total RNA was extracted by method of TRIZOL. One hundred milligram of *Aspergillus niger* mycelial mat was ground with 0.8 ml of trizol reagent followed by the addition of 0.2 ml (1/4th volume) of chloroform. After grinding, sap was transferred to a 1.5 ml microfuge tube, mixed by gentle inversion and kept as such for 3 min. The contents were centrifuged at 12,000 rpm for 20 min and an equal volume of isopropanol was added to the supernatant. After an incubation of 5 min, the mixture was centrifuged at 12,000 rpm for 10 min. Supernatant was discarded and the pellet washed with 70% ethanol. The pellet was resuspended in 100 μ l of DEPC treated water (pre-warmed to 65°C) and 13 μ l of 2.5 M sodium acetate (pH 5.5). The mixture was incubated at -70°C for 1 h after the addition of 3 volume of absolute alcohol. The contents were centrifuged at 12,000 rpm for 10 min and the pellet washed

with 75% ethanol. The pellet was air-dried and dissolved in 20 μ l of DEPC treated water. Isolated total RNA was used as template for amplification of the phytase cDNA using RT-PCR.

Cloning of the phytase gene and sequencing

RT-PCR was performed with total RNA isolated from *Aspergillus niger* with a view to obtaining the cDNA corresponding to phytase gene. First strand cDNA was synthesized according to the manufacturer's instructions (RevertAidTMH Minus First Strand cDNA Synthesis Kit # K1631, MBI Fermentas, Germany). PCR reactions were carried out in 20 μ l reaction volume containing 2.0 μ l of cDNA, 2.0 μ l of 10X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂), 0.5 μ l of 200 mM dNTPs, 0.5 μ l of 10 μ M of respective forward and reverse primers (PHYF - 5' ATGGGCGTCTCTGCTGTTCTACTTC 3' and PHYR - 5' CTAAGCAAACACTCCGCCCAATC 3'), 0.5 μ l of 1.5 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 14.0 μ l of sterile double distilled water. Amplification was performed using the temperature profile of the pre-incubation at 94°C for 5 min leading to 35 cycles of melting at 94°C for 1 min, annealing at 54°C for 1 min and synthesis at 72°C for 1 min followed by an extension of 72°C for 10 min.

The PCR product was recovered and purified from a 1.0% agarose gel using a DNA Gel Extraction kit (MBI Fermentas) according to the manufacturer's protocols, then subcloned into pTZ57R/T vector. The resulting plasmid was used for sequence analysis. The methods for *E. coli* competent cell preparation and transformation were according to the methods of Sambrook et al. (1989). The recombinants were selected on the LB agar plate containing ampicillin (50 mg/L). The presence of the gene in the recombinant clones was confirmed by sequencing using M13 primers.

Nucleotide and phylogenetic sequence analysis

The nucleotide sequencing of the phytase (*phy*) gene was done by out sourcing at Bangalore Genei (India). Nucleotide and amino acid sequence homology searches were performed on the NCBI database by BLAST search and multiple alignments were done using the CLUSTAL W program. For phylogenetic analysis, gene sequences obtained in this study and *phy* gene sequences from different *Aspergillus* sp. available in NCBI database were used.

Construction of the expression plasmid

For the expression of mature protein, the entire ORF of the phytase gene without native signal peptide sequence was amplified with the specific primers, ECOF2 (Forward 5' TCCGAATTCCTGGCAGTCCCCGCCTCGAGA 3') and HINR2 (Reverse 5' CGCAAGCTTAGCTAAGCAAAACACTCCGCC 3') with insertion of *EcoRI* and *HindIII* sites at the 5' and 3' ends respectively. The PCR product was digested with *EcoRI* and *HindIII* and ligated into the respective sites of pET28a(+) expression vector to generate plasmid pET28a(+)-*phyA*.

The pET28a(+)-*phyA* plasmid was transformed into *E. coli* DH5a and subsequently into the expression strain *E. coli* BL21. The transformants cells were grown at 30°C in LB medium containing 50mg/L kanamycin at the mid-exponential phase and their phytase expressions induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to the cultures at a final concentration of 1mM.

Induction of phytase in *E. coli*

The *E. coli* strain BL21 harbouring the plasmid pET28a(+)-*phyA* was streaked separately on LB agar plate with kanamycin 50 mg/l and incubated at 37°C for 12-16 h so as to get individual colonies. Single colony was inoculated in 3 ml LB broth containing kanamycin 50 mg/l and grown on a rotary shaker (220 rpm) at 37°C for 16 h. From this, 500 µl of overnight grown culture was inoculated in 50 ml of LB broth and grown at 37°C and 220 rpm till the OD reached 0.3-0.5 at 600 nm. One millilitre of the aliquot was taken in an eppendorf tube and centrifuged at 10,000 rpm for 5 min and the pellet was resuspended in 50 µl of the Laemmli buffer (0.06 M Tris-HCl, pH 6.8, 10% glycerol, 5% β-Mercaptoethanol and 2% SDS) and stored at -20°C for later use as an uninduced culture. The remaining culture was induced by the addition of IPTG (isopropyl-β-D- thiogalactopyranoside) to a final concentration of 1 mM. The culture was allowed to grow at 30°C and the aliquot was collected at 1, 2, 3, 4, 5, 6 and 7 h after induction and the pellet was resuspended in Laemmli buffer. The supernatant were checked for protein expression and purity by Coomassie-stained Sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE) 12% electrophoresis.

Purification of recombinant phytase

Recombinant fusion phytase protein was purified using BugBuster® His•Bind Purification Kit (catalogue # 70793-3, Novagen, Germany). For purification, 100 ml of bacterial culture was collected by centrifugation at 10400 rpm for 10 min and protein was extracted with BugBuster™ Reagent. The cells were disrupted by sonication (5 times for 30 seconds). After centrifugation at 11400 rpm for 30 min, the supernatant containing the

target protein was purified using His•Bind Resin according to the manufacturer instruction (# TB054, Novagen, Germany).

Phytase assay

Phytase assay was carried out by taking 200 µl of crude sample of phytase into 10 ml test tubes and incubated at 37°C in water bath for 5 min. Two hundred microlitre of 1.25% (w/v) sodium phytate in 200 mM sodium acetate buffer (pH 5.0) was added for enzymatic hydrolysis of phytate, and incubated for 30 min at 37°C. The reaction was terminated by adding 400 µl of 15% trichloroacetic acid. The mixture was centrifuged at 4600 rpm for 10 min and 200 µl of supernatant was added to 1.8 ml of double distilled water. Two millilitre of fresh colour reagent (3 volume of 1 M H₂SO₄ + 1 volume of 2.5% ammonium molybdate + 1 volume of 10% ascorbic acid) was added and mixed well. The mixture was incubated at 50°C for 15 min and left at room temperature for 2-3 min. The absorbance was read at 820 nm, using water as the blank and the serially diluted potassium phosphate solutions as standards. Phytase activity was calculated per ml of culture and expressed as U/ml. One unit of phytase is defined as the amount of enzyme required to release 1 µmol of inorganic phosphate per min from sodium phytate at 37°C.

$$\text{Phytase activity (U/ml)} = \frac{(\mu\text{moles of Phosphate released}) (\text{df})}{(30) (0.200)}$$

df = Dilution factor

30 = Time (in minutes) of assay per the Unit definition

0.200 = Volume (in milliliter) of sample used

III. RESULTS

Cloning and sequence analysis of phytase gene from *Aspergillus niger*

RT-PCR analysis with total RNA isolated from *Aspergillus niger* using phytase gene specific primers showed amplification of a 1404 bp fragment. The 1404 bp band was eluted and ligated with a T-tailed T/A cloning vector. The ligated product was transformed into *E. coli* competent cells. Colony PCR analysis of randomly selected colonies showed the amplification of 1404 bp fragment and restriction digestion of the plasmids isolated from these colonies with the restriction enzymes, *HindIII* and *EcoRI* released a fragment of the expected size (Fig.1).

Four clones (CPHY10, CPHY13, CPHY15 and CPHY20) were sequenced using automated DNA sequencer and aligned using CLUSTAL X bioinformatics tool (Fig.2).

Among the clones CPHY13 showed a maximum of 99.0% sequence identity at nucleotide and protein level with *Aspergillus niger* CBS 513.88 phytase (Accession # XM_001401676). The CPHY13 cDNA shared 99.0% sequence identity with the *Aspergillus* species (Accession # DQ192035, AB022700, AY013315) at nucleotide levels and 98.0% sequence similarity with *Aspergillus* species (Accession # AAF25481, AAR08366, ACE79229) at amino acid level. It showed 100% homology at protein level with *Aspergillus awamori* phytase (Accession # ABA29207) and followed by 99.0% with *phyA* - *Aspergillus niger* (Accession # XP_001401713, AAG40885). The cloned fragment contained a single open reading frame of 1404 bp long (from ATG start codon to TAG stop codon), which could potentially encode an approximately 52 kDa protein having 467 amino acids (Fig.3).

The amino acid sequence from the *phyA* cDNA contained the consensus motifs RHGXRX and HD which are conserved among histidine acid phosphatases. The aligned sequences formed two different clusters. Cluster dendrogram results revealed that the cloned phytase gene from the *Aspergillus niger* formed a separate sub cluster along with the phytase gene sequences of other *Aspergillus* sp. at nucleotide and protein level (Fig.4). The genbank accession number for the *Aspergillus niger phyA* sequence is JQ241266.

Expression of phytase gene in *E. coli*

For the expression of phytase in *E. coli*, the cDNA was cloned in *EcoRI* and *HindIII* site of the multiple cloning site of the *E. coli* expression vector pET28a(+). The recombinant colonies were confirmed by colony PCR analysis and restriction digestion (Fig.5). The expression of the phytase gene in *E. coli* was confirmed by SDS-PAGE analysis of crude lysate of *E. coli* BL21 cells harbouring the pET28a(+) vector alone as control and the recombinant pET28a(+) with phytase gene (both uninduced and induced with IPTG). An expected size of protein band (approximately 52 kDa) was observed in the crude lysate of induced culture, which was absent in uninduced culture and in cells with pET28a(+) vector without insert. There was an increase in the amount of induced protein with increase in time. When *E. coli strain* BL21 with pET28a(+)-*phyA* was grown at 37°C, there was no difference in the protein banding pattern of total cell extracts before and after induction with IPTG. In contrast, when the cells were grown at 30°C, an intense 52 kDa band was visible specifically in the extract of induced cells containing the recombinant plasmid (Fig.6). Phytase was purified from the 100 ml culture after 6 h induction with 50 mM IPTG using nickel based His bind resin column according to the recommendation of the manufacturer

(BugBuster® His•Bind Purification Kit, Novagen, Germany). On SDS-PAGE, the purified recombinant enzyme showed a single band of 52 kDa. The phytase activity was calculated as 826.33 U/ml for purified phytase and 383.5 U/ml for pET-*phyA* IPTG induced culture.

IV. DISCUSSION

In this study, a phytase gene (*phyA*) was isolated from the cDNA of *A. niger*. This gene contains a single open reading frame of 1404 bp long (from ATG start codon to TAG stop codon), which encodes an approximately 52 kDa protein having 467 amino acids. The phytase from *A. niger phyA* is well characterised by several earlier workers. It is encoded by a 1.4 kb DNA fragment and has a molecular mass of 80 kDa, with 10 *N*-glycosylation sites (Han and Lei, 1999). Average molecular masses of bacterial phytases are smaller than those of fungal phytases (40–55 vs. 80–120 kDa), mainly due to glycosylation differences (Choi et al., 2001; Golovan et al., 2000).

From the sequence analysis it was confirmed that the amino acid sequence from the *phyA* cDNA contained the consensus motifs RHGXRX and HD which are conserved among histidine acid phosphatases. The clone CPHY13 showed a maximum of 99.0% sequence identity at nucleotide and protein level with *Aspergillus niger* CBS 513.88 phytase (Accession # XM_001401676). These motifs play an important role in the phosphorylation (Kostrewa et al., 1997 and Oh et al., 2001). A highly conserved sequence motif RHGXRX (Ullah et al., 1991), involved in the catabolic reactions, is found at the active sites of phytase. Furthermore, *phyA* contains a remote C-terminal His-Asp motif (HD motif) that is also likely to take part in the catalysis. Together, it is therefore suggested that the *phyA* belongs to a member of the phytase subfamily of histidine acid phosphatases (Mitchell et al., 1997). The conserved sequence RHGXRX in the substrate-binding site interacts with the phosphate groups in the substrate to form a complex of enzyme-substrate. The HD elements in the catalyzation domain further function to release the phosphate group from the substrate (Loewus and Murthy, 2000; Mullaney et al., 2000).

Due to the enormous potential for application of phytase in the animal feed industry, several researchers have attempted to produce this enzyme cost-effectively. *A. niger phyA* phytase has been cloned and over expressed in several microbial hosts, including *S. cerevisiae* (Han et al., 1999), *P. pastoris* (Han and Lei, 1999), *A. niger* (Van Dijck, 1999) and *E. coli* (Singh et al., 2018). The pET prokaryotic expression system is one of the most effective expression systems, possesses high performance and specific

interaction between bacteriophage T7 promoter and T7 RNA polymerase to increase the expression efficacy of exogenous gene in bacteria. In this study, the recombinant pET-*phyA* construct expressed the phytase enzyme efficiently in the host *E. coli* cells. The expressed protein was purified by nickel based column affinity chromatograph conveniently because of the six histidines tag fused to the *E. coli* protein.

For the expression in *E. coli*, phytase cDNA was cloned in pET vector devoid of N-terminal signal peptide. As *E. coli* cells are not able to cleave the signal peptide of secreted proteins, the cDNA had to be modified to remove the signal peptide. An expected size of protein (approximately 52 kDa) was observed in the crude lysate of induced culture, which was absent in uninduced culture and in cells with pET28a(+) vector without insert. There was an increase in the amount of induced protein with increase in time. This demonstrated that the recombinant DNA had indeed expressed in *E. coli* cells (BL21 strain). When *E. coli* BL21 pET28a(+)-*phyA* was grown at 37°C, there was no difference in the protein electrophoretic

patterns of total cell extracts before and after induction with IPTG. In contrast, when the cells were grown at 30°C, an intense 52 kDa band was visible specifically in the induced extract from the subsequent time interval. This report was supported by Phillippy and Mullaney (1997) who reported maximum activity of 1.5 µmol/mg protein. The phytase activity of pET-*phyA* IPTG induced *E. coli* culture was 383.5 U/ml while it was 826.33 U/ml in purified phytase.

Similar results were observed in the methylotrophic yeast *P. pastoris* by the heterologous expression of *Debaryomyces castellii* CBS 2923 phytase and maximum production level obtained was 476 U/ml (Ragon et al., 2008). Chen et al. (2004) reported the successful expression of *E. coli appA* gene in *P. pastoris* with the maximum phytase activity after an induction period of 96 h, of 118-204 IU/ml at the flask scale and 1880-4946 IU/ml at high cell-density fermentation. Xiong et al. (2005) and Bei et al. (2001) also reported 865 U/ml and 165 U/ml of enzymatic activity, respectively with *A. niger* phytase in *P. pastoris*.

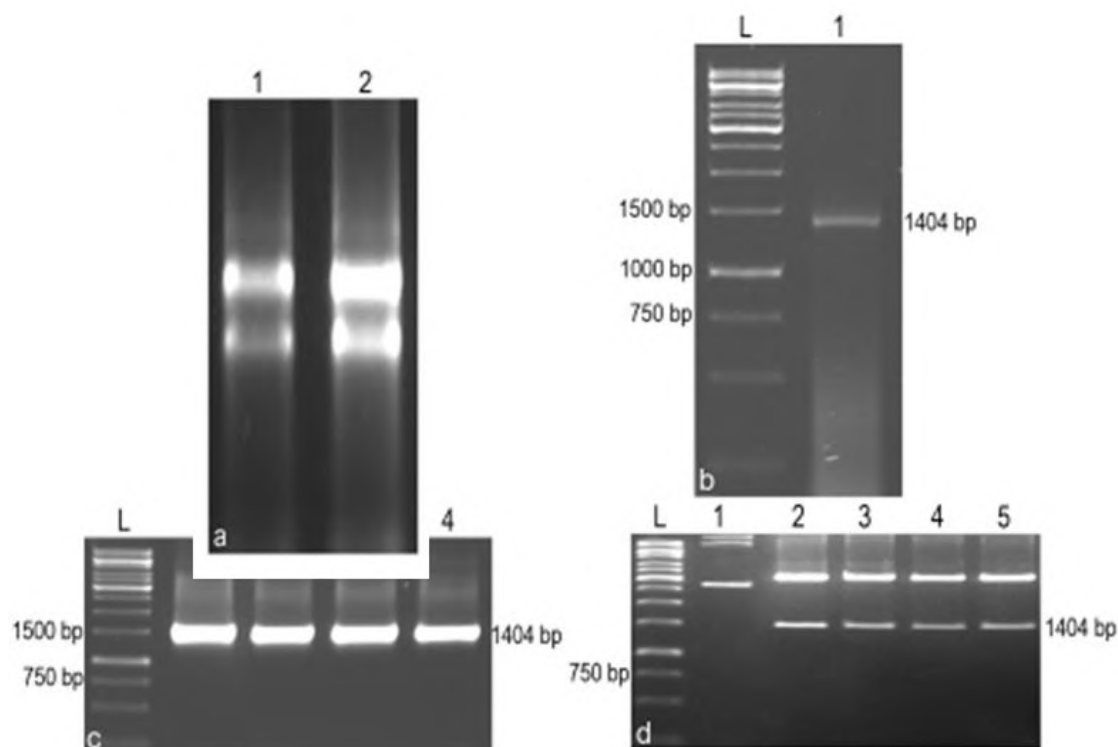


Fig.1. PCR amplification and cloning of phytase gene in T/A cloning vector. a. Total RNA isolated from *Aspergillus niger*, 1 to 2: Total RNA of *Aspergillus niger*. b. PCR amplification of phytase gene, L: 1 kbp ladder, 1: Amplification of phytase gene from cDNA of *A. niger*. c. Colony PCR analysis, L: 1 kbp ladder, 1 to 4: Phytase gene amplification from selected colonies. d. Restriction analysis, L: 1 kbp ladder, 1: Undigested pTA-*phyA* plasmid, 2 to 5: Plasmid (from positive clones) digested with *Hind*III and *Eco*RI



Fig.2. Multiple sequence alignment of nucleotide sequence of CPHY13 phytase gene from the *Aspergillus niger* with other related sequences



Consensus motifs of histidine acid phosphatase

. Indicates stop codon

Fig.3. Nucleotide and amino acid sequence of phytase gene (*phyA*)

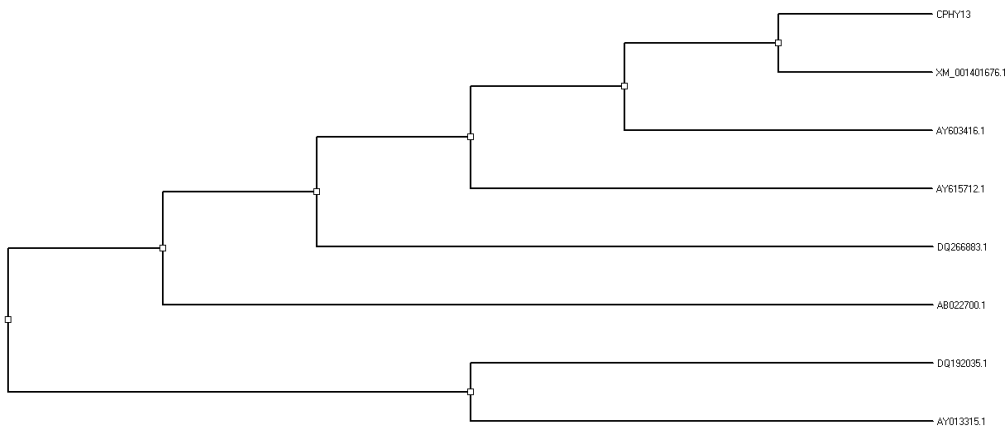


Fig.4.Cluster dendrogram illustrating the phylogenetic relationship based on the multiple sequence alignment of the nucleotide sequences of known phytase gene and the phytase gene from *Aspergillus niger*.

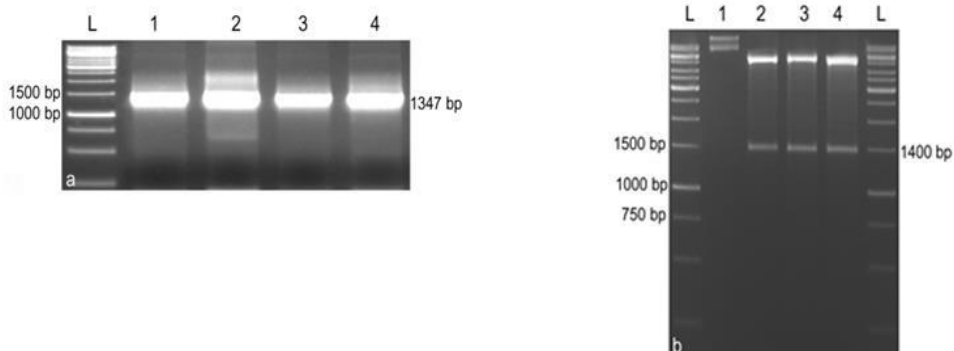


Fig.5. Cloning of phytase gene in *E. coli* expression vector *pET28a(+)*.

a. Colony PCR analysis, L: 1 kbp ladder, 1 to 4: Amplification of phytase gene from *E. coli* colonies. b. Restriction analysis, L: 1 kbp ladder, 1: Undigested *pET28a(+)-phyA* plasmid, 2 to 4: Plasmids (from selected colonies) digested with *HindIII* and *EcoRI*

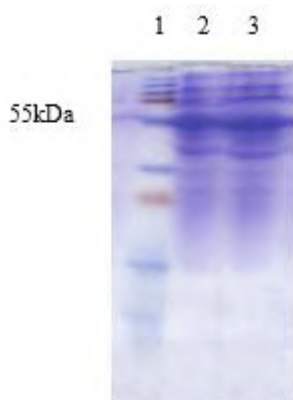


Fig.6. SDS-PAGE analysis of crude lysate of *E. coli* cells harbouring pET28a(+)-phyA 1: Protein marker, 2&3: Phytase expression after IPTG induction of 6 and 7 h.

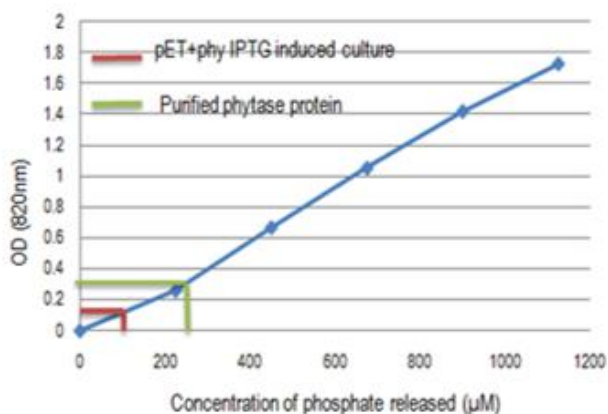


Fig.8. Standard curve for the phosphorus concentration measurement

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