Cloning and Expression of *Thermomyces lanuginosus* Phytase Gene in *Pichia pastoris*

Sada-e-Batool[1], Muhammad Hamid Rashid[1], Farooq Latif[1]

Abstract

Phytase(myo-inositol-hexakisphosphate phosphohydrolase), (EC 3.1.3.8) has been inducted in local poultry feed mills and is of high value for better utilization of the inorganic phosphates and metals released from the complex sources of plant substrates. In this regard a locally isolated fungus Thermomyces lanuginosus which was known to produce thermostable enzymes and had earlier shown substantial clearance of Phytic acid on agar plates in these laboratories, was selected. The fungal phytase gene of size 1.5 kb from genomic DNA was PCR amplified and expressed in Yeast expression system of Pichia pastoris GS115. The phytase gene was cloned into the vector pPIC9K and confirmed through PCR and restriction digestion. Recombinant vector was linearized with pme1 and transformed into P. pastoris electro-competent cells. Methanol utilization fast (Mut^{+}) selection was carried out on minimal methanol plates. Colony PCR resulted in the selection of four transformants. Recombinant resistant at 4 mg/ml of geneticin was selected as the potential phytase producing strain. It was further grown on minimal glycerol medium for inoculum and buffered methanol complex medium for the production of phytase. The crude enzyme showed 1.25 IU/mL phytase activity in shake flask. The kinetic parameters studied showed activation energy of 76.08 KJ/mol. The K_m and V_{max} were 0.091 mM and 0.074 U/mg. The recombinant P. pastoris phyS showed maximum activity at pH 4, and 40 ºC. The SDS-PAGE analysis showed a band of ~54 kDa.

Keywords: *Methanol utilization fast (Mut^{+}), Buffered methanol complex medium, Kinetic parameters, recombinant P. pastoris phyS.*

Introduction

Phytic acid acts as an anti-nutrient by binding with various proteins and divalent metal ions, reducing the bioavailability of these essential nutrients. Phytic

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acid was discovered by Pfeffer in 1872, (Cosgrove & Irving, 1980). Dvořáková, (1998) reported that the International enzyme nomenclature committee defines two types of phytases: 3-phytase and 6-phytase. Phytase 3 is for animals and phytase 6 is for plants. Difference between both of them is how they hydrolyze phytic acid and their occurrence. The 3-phytase (E.C. 3.1.3.8) firstly attacks on phytic acid at position 3, while, 6-phytase (E.C. 3.1.3.26) attacks at position 6 in phytic acid. Nielsen, Damstrup, and Hansen, (2008) categorized Phytases under a subclass of phosphatases known as histidine acid phosphatases. To fulfill the nutritional needs of animals, their feed must be supplemented with inorganic phosphate. However, the release of excessive phosphorus from animal excreta poses environmental issues, and phosphorus is a finite resource. This problem necessitates treating feed with phytase, a biotechnological solution. It was Chadha, Harmeet, Mandeep, Saini, and Singh, 2004, reported that phytases break down esters of myo-inositol and release free inositol, thereby making phosphorus available for absorption. This enzyme is naturally present in plants, animal tissues, bacteria, and fungi. To produce phytase from various strains isolated from soil samples were cultivated on a complex phosphorus-solubilizing medium (PSM). Phytase production increased on CM medium containing carbon and nitrogen source, inoculum age and level, moisture and optimizing other conditions like pH and temperature. Gulati, Chadha, and Saini, (2007) reported that phytase enzyme showed wide substrate specificity against riboflavin phosphate, sodium phytate, and ADP.

In these studies thermophilic fungus *Thermomyces lanuginosus* isolated from garden compost and was used from fungal stock of Industrial Biotechnology Division at National Institute for Biotechnology and Genetic Engineering, NIBGE, Faisalabad. The fungus was found to be a natural phytase producer. Designated vectors of *P. pastoris* GS 115, were used to clone the gene of interest into the host genome. *P. pastoris* is generally transformed by the method of electroporation or by spheroplast approach. Transformation in *Pichia* can also be performed by lithium acetate (LiAc) method. Wu & Letchworth, 2004, reported the transformation efficiency in *Pichia* can be improved 150-fold by treating the cells with DTT (Di-thio-threitol) and LiAc before electroporation. The large number of transformants will enable effective selection of random mutants and transformants showing desirable characteristics. The recombinant vectors can be transformed in the *P. pastoris* for enzyme production. *P. pastoris* strains have been chosen as a host due to the production of heterologous foreign protein extracellularly (Zhang et al., 2011). The addition of feed enzymes to pig and poultry diets has significantly enhanced nutrient utilization for over two decades. Exogenous enzymes, which are effective at degrading non-starch

polysaccharides (NSP) in broiler feeds based on grains such as barley and wheat, initiated this practice (Yin et al., 2001). However, phytate-bound phosphorus (P), with limited availability, is present in all chick diets (Nelson, 1967). The broader application of phytase feed enzymes in poultry and pig diets has proven to economically increase the bioavailability of inorganic phosphorus (Pi) while also reducing the environmental impact of phosphorus. Some nutritionists highlight that phytase feed enzymes contribute to the availability of amino acids, protein, and energy, in addition to enhancing the mineralization of calcium (Ca) and phosphorus (P) (Schoevaart et al., 2004). Particularly, in human foodstuffs, the negative influence of phytate on the bioavailability of Ca and trace elements zinc has been comprehensively investigated. In certain aspects human diets containing phytate contents have potential paybacks, e.g. anti-carcinogenic properties (Selle & Ravindran, 2007).

The studies were aimed towards production of recombinant thermostable phytase from *P. pastoris* for poultry feed.

Materials and Methods

Cultures and Plasmids

Thermomyces lanuginosus a thermophilic fungus known to produce phytase was obtained from fungal culture collection of Industrial Enzyme and Biofuel, NIBGE, Faisalabad (Bokhari, Latif and Rajoka, 2009; Glocker et al., 2009). *Pichia pastoris* GS115 (Invitrogen, USA) was cultured from fresh vial available from the kit. The expression of phytase gene was analyzed with the help of vector pPIC9K as an expression vector for *P. pastoris*. The pPIC9K vector gave extracellular expression due to the presence of secretory signal. pPIC9K with *pme1* inserts gene of interest (GOI) under AOX1 promoter in GS115 (HIS^{+}, Mut^{+}).

Genomic DNA isolation, PCR amplification, Digestion and Transformation

The genomic DNA was isolated using ionic detergent cetyltrimethylammonium bromide (CTAB) to disrupt membranes and a chloroform-isoamyl alcohol mixture that separated contaminants into the organic phase and nucleic acid into the aqueous phase. The *genomic DNA* of *T. lanuginosus* was run on DNA gel electrophoresis for confirmation.

The phytase primers were designed from the sequence of phytase *phy* gene of *T. lanuginosus* (Accession # AR031151). The genomic DNA of *T. lanuginosus* was amplified with specific primers designed according to the phytase gene (NCBI). The

PCR amplified gene fragment (phytase) of *T. lanuginosus* was integrated and ligated into pPIC9K vector (gel extracted and purified, previously) in the ration of 1: 2 or 1: 3 as required. The construct (pPIC9K + *PhyS*) was confirmed by PCR and restriction digestion analysis using site specific *EcoRI* and *NotI.* This recombinant vector was then transformed into *E. coli* TOP10F′ Cells and performed mini-prep for plasmid isolation. After confirmation of recombinant through PCR and restriction digestion, it was scaled up (midi-prep) to get sufficient amount of DNA (5-20µg) for electroporation in *P. pastoris.* The DNA of insert and vector were quantified, ligated and transformed into *E. coli*. The recombinant vector was isolated and the DNA was multiplied using midi-prep and purified. It was then linearized and transformed in to *P. pastoris* electro-competent cells. Single colony PCR was performed for confirmation of recombinants.

Production of Phytase in Shake Flask

A single colony was used to inoculate 25 ml of MGY in a 1-liter baffled flask. The culture was incubated at 28–30°C in a shaking incubator at 250 rpm until it reached an OD600 of 2-6. The cells were then harvested by centrifugation at 1,500–3,000 ×g for 5 minutes at room temperature. To induce expression, the supernatant was decanted, and the cell pellet was suspended in 3-5 ml of buffered minimal methanol yeast (BMMY) medium before transferring it into 150 ml of BMMY medium in a 1-liter shake flask. Methanol (100%) was added to a final concentration of 0.5% every 24 hours to induce expression. Samples were collected every 24 hours into micro centrifuge tubes, centrifuged at high speed of 12000 rpm, in a benchtop micro centrifuge for 2–3 minutes at room temperature. The supernatant was transferred to a separate tube for extracellular expression analysis using Bradford reagent and Phytase assay. Both the supernatant and the cell pellets were stored at –80°C until they were ready to be assayed.

Phytase activity assay

Phytase activity was assessed using an assay consisting of 200 mM sodium acetate (pH 5.5), 0.5% sodium phytate, and the enzyme. In this assay, 200 µl of the enzyme was incubated with 800 µl of sodium phytate (pH 5.5) at 45°C for 30 minutes. The reaction was terminated by adding 1 ml of 5% trichloroacetic acid (Wyss et al., 1999). The released phosphate ions were quantified by mixing 1 ml of the coloring reagent. Absorbance was measured at 700 nm using a spectrophotometer (LaboMed, Inc.). Control and blank samples were also analyzed without incubation. Phytase activity was determined using 0.1 mM sodium phytate as the substrate, by measuring

the released inorganic phosphate (Pi). The intensity of the blue color increased proportionally with the phytase activity and release of phosphate.

Characterization of recombinant phytase

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for extracellular expression of proteins. The resolving and stacking gels were prepared. The supernatant 20µl was added to the loading dye. The sample was heated at 95 ºC for 10 minutes and then load 30µl onto the gel. The gel was run at 70 Volts for 2-3 hours till the loading dye runs off at the specific level of the plate at bottom. After electrophoresis gel was stained with Comassie Brilliant Blue R250 staining with constant agitation for overnight to visualize the bands. The gel was de-stained by using the de-staining solution at constant agitation until the background became clear for proper visualization of blue protein band.

Optimum temperature

The optimum temperature was determined at temperature range from 35, 40, 43, 45, 47, and 50 ºC at pH 4 in sodium acetate buffer. Sodium phytate 800 µl was incubated with 200µl of enzyme at different temperature for 30 minutes. The samples were analyzed in duplicate and calculate the optimum temperature for phytase.

Optimum pH

The optimum pH was determined using phosphate/citrate buffer, sodium acetate, Sorensen's phosphate buffer, and glycine-NaOH, covering a pH range from 3.2 to 8.8. Samples were incubated at 45ºC for one hour and analyzed in duplicate to calculate the optimum pH. Amplification of *T. lanuginosus* phytase gene.

Results

The genomic DNA of was isolated from fresh culture of fungus *Thermomyces lanuginosus*. The gene of interest was successfully isolated by using these site specific primers through polymerase chain reaction (PCR). The molecular weight of forward primer (27 bp) was 8451.6, melting temperature (Tm) 69.2ºC, % (G+C) content 56 and dry mass 66.7 nmol. The molecular weight of reverse primer (28 bp) was 85925.6, Tm 71.9ºC, % (G+C) content 61 and dry mass 63.2nmol. Forward primer with *EcoR*I restriction site and reverse primer *Not*I with restriction site were designed. The PCR end product was electrophoresed on agarose gel. The band of 1.5 kb (exact size of *phy* gene was 1483bp) corresponded to the phytase gene named after the scientist as *phyS*. *The amplified PCR product of 1.5 kb was excised from the gel and eluted through gel extraction kit. The purity and integrity of eluted DNA was checked by gel*

electrophoresis (Fig.1). The PCR product of phytase gene (phyS) was digested with EcoR1 and NotI to generate the sticky ends. Similarly, the vector pPIC9K was digested with these restriction enzymes. The digested products of insert and vector were checked on the gel and then religated for transformation into E. coli TOPF'10 cells. The competent cells were grown on Luria Bertani (LB) medium containing ampicillin. The phyS gene was inserted into the multiple cloning sites (MCS) of pPIC9K.

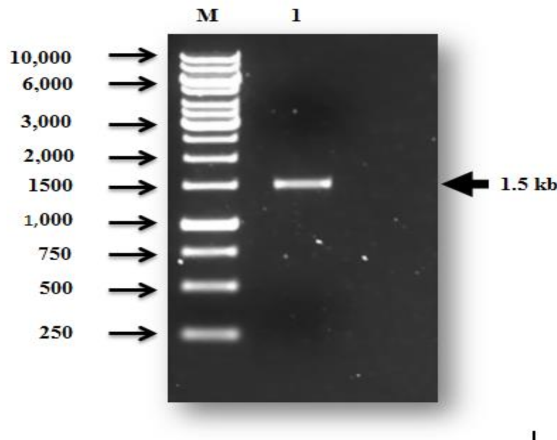

Fig. 1: Agarose gel electrophoresis of PCR amplified PhyS gene after gel extraction

Lane M: 1kb ladder, Lane 1: PhyS DNA.

*The transformants were also confirmed through restriction digestion with EcoR1 and Not1. The band of 1.5 kb was released after restriction digestion from the plasmid corresponded to the right fragment size (Fig. 2). The concentration of recombinant vector pPIC9K+phyS required for transformation was 5-20µg, so midi-prep was performed for more yields of pPIC9K+phyS. After midi-prep run the samples were run on gel and qu*antification of DNA was performed by using Nanodrop method *(Table 1).*

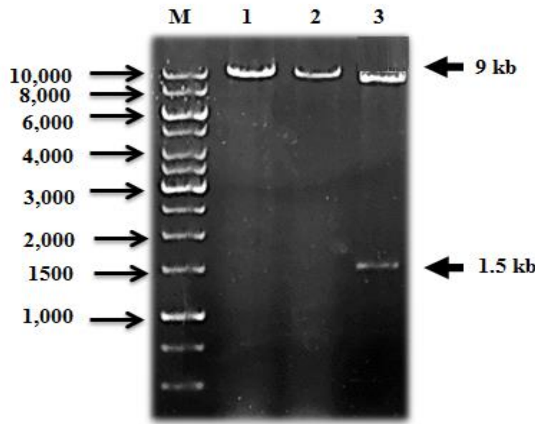

Fig. 2: Agarose gel electrophoresis of recombinant *pPIC9K+physS* after single and double restriction digestion with EcoR1 and Not1, respectively. Lane M: 1kb DNA ladder, Lane 1 and 2: linear recombinant vector of 10.8 kb with EcoR1 and Not1, respectively. Lane 3: Double digestion of recombinant vector show two fragments of size 9.3 and 1.5 kb in size respectively.

Table 1. *Quantification of pPIC9K and pPIC9K+physS*

Sample ID	Nucleic acid conc. ng/µl	Unit in µg /µl	A260	A260/280
pPIC9K	22448.5	22.44	448.97	1.39
pPIC9K-physS	22662.2	22.66	453.243	1.50

Before transformation 60µl of recombinant *pPIC9K+physS and pPIC9K were linearized using pmeI restriction enzyme. After digestion 3µl of both were loaded on the gel to check the digestion carried out properly.* In order to check the concentration of linearized recombinant vector, which should be 5-20 µg in quantity (Table 2).

Table 2. *Quantification of Linearized Vector and Recombinant Vector*

Sample ID	Nucleic acid conc. ng/µl	Unit in µg /µl	A260	A260/280
pPIC9K	18951.4	18.95	379.028	1.87
pPIC9K+*physS*	20532.5	20.5325	410.65	1.72

Pichia competent cells were prepared fresh on the day of electroporation for best results. Competent cells stored at -80℃ could be used, but the competency of the cells decreased with the passage of time. So, always competent cells were prepared freshly before electroporation. Prepared Competent cells of *P. Pastoris* were used for electroporation and kept at -80 °C. The parent vector and expression cassette were linearized with *PmeI* and quantified using nano drop. The linearized vector 20.5 µg was transformed into *P. pastoris* GS115 using electroporation according to manufacturer's protocol. The electroporated mixture was incubated at 30 °C for 1-2 h. The transformed cells 100-200 µL were spread on MD (minimal dextrose) plates for both vector *pPIC9K* and recombinant vector *pPIC9KphyS* and incubated at 30 °C until colonies appeared approximately after 2-3 days. The *P. pastoris* host strain has mutated *his4* gene that prevents it from synthesizing histidine. However, the expression vector has His4 gene and complements histidine in host for positive transformants i.e. could be selected on histidine deficient medium. Only those colonies grew that were His^+.

Table 3. ***Phytase Activity of Recombinant P. pastoris phyS at Different Time Intervals***

Time	Phytase activity U/ml
0	00
12	0.05
24	0.26
36	0.57
48	0.75
60	0.95
72	1.15
84	1.22
96	1.25

These colonies were selected for further spreading on MM (minimal methanol) plates for selection of Mut^+ (methanol utilization plus). Transformants were obtained on the MMA plates. The transformants were selected for resistance on different geneticin concentration (1mg/ml 2 mg/ml, 3 mg/ml and 4 mg/ml) on YPD plates. The bacterial kanamycin resistant gene confers geneticin resistance in *P. pastoris*. By increasing geneticin concentration the number of colonies decreased. The phytase activity was determined at different time interval. With the passage of time the activity increased after 48 hours the activity was 0.75U/ml, after 72 hours

was 1.15U/ml. The phytase activity become stationary and after 96 hours was 1.25 U/ml (Table 1. and Fig. 3). The colony was cultured in BMMY medium and phytase production was induced with 0.5% methanol after every 24 h. The supernatant was determined through phosphorus assay. The curve shows gradual increase in phytase production which becomes steady later on.

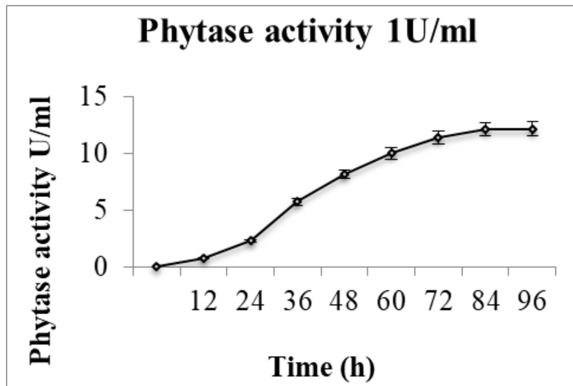

Fig. 3: Time course of recombinant phytase (phyS) by P. pastoris

Purification of phytases

The crude phytase did not show good activity of the enzyme because the presence of high phosphorous content inhibited the enzyme. Therefore, in purification table the yield at dialysis step is considered as the first slot. After fermenter study purification of enzyme was performed through ultrafiltration as the sample was passed through column with cut off value 3000 Da to remove media contents etc. the enzyme was treated against 15 liter of distilled water. The phytase was finally concentrated to about 16 fold. After ultrafiltration > 98% of phosphorus present in the media was successfully removed. In case of dialysis the yield is 100% than in ultrafiltration was 80% (Table 4). The enzyme showed better results after purification as compared to crude.

Table 4. ***Partial Purification of Phytase from P. pastoris***

Treatment	Phytase (IU)	Protein (mg)	Specific Activity (U mg^{-1})	Purification fold	Yield %
Crude	11.5	670.4	0.017	1.00	---
Dialysis	77.76	652.0	0.119	7.00	100
Ultra-filtration	62.6	461	0.130	8.00	80

The quoted values were taken after dialysis against distilled water.

Characterization of Recombinant Phytase

Polyacrylamide gel electrophoresis of recombinant phytase

The recombinant phytase was analyzed through SDS-PAGE. Loading buffer was mixed with 20µl of protein sample and loaded on the gel. The protein bands according to the size were separated out in the gel. The phytase band was analyzed along with control (pPIC9K without insert). The phytase band was present in the recombinant *P. pastoris phyS* and absent *P. pastoris* having parent pPIC9K. The molecular mass of the recombinant phytase was also determined through SDS-PAGE. The phytase band of 54 kDa was found in recombinant *P.pastoris phyS* while absent in case of pPIC9K (Fig. 4).

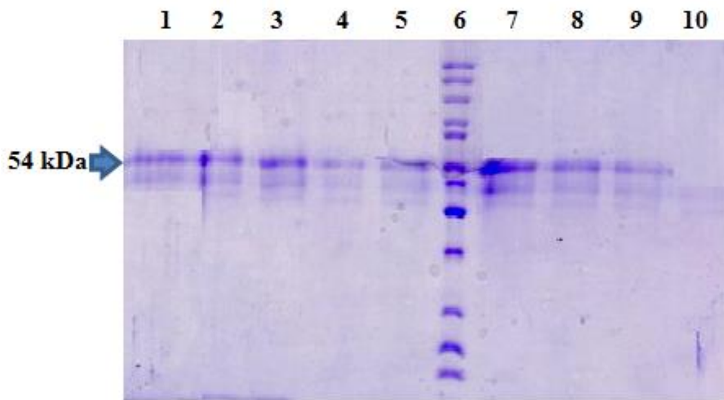

Fig. 4: Denatured polyacrylamaide gel electrophoresis of extracelluler protein of recombinant P. pastoris phyS and P. pastoris having pPIC9K

Lane 1: recombinant P. pastoris phyS after 96 hours; Lane 2: after 84 hours; Lane 3: after 72 hours; Lane 4: after 60 hours; Lane 5: after 48 hours; Lane 6: Protein ladder; Lane 7: after 36 hours; Lane 8: after 24 hours; Lane 9: after 12 hours; Lane 10: P. pastoris contains pPIC9K without phyS (control). After concentrating the 20 µl of the samples were loaded onto the gel and electrophoresed at 70 volts for 2-3 hours. Stained with Coomassie Brilliant Blue R250 and analyzed after detaining.

Effect of temperature

The recombinant *P.pastoris phyS* cultured in MGY medium, and for expression induced with 0.5 % methanol in BMMY medium. The optimum temperature recombinant *P. pastoris phyS* was found to be 45 ºC (Fig. 5).

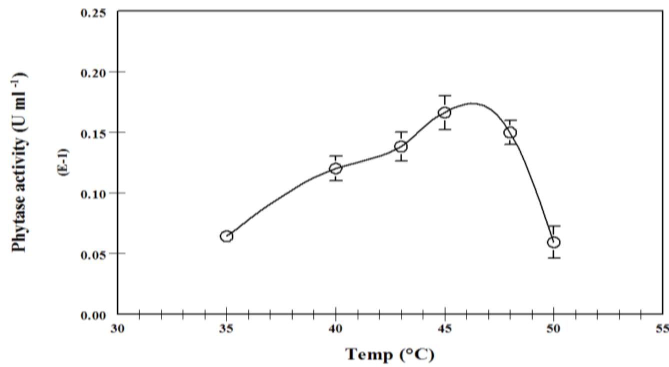

Fig. 5: Effect of temperature on the hydrolysis of sodium phytate by phytases from P. pastoris

Data presented are average SD mean of n= 3 independent experiments

The optimum temperature of recombinant *P. pastoris phyS* was determined through phosphorus assay method at temperature range from 35 to 50 ºC.

Table 5. ***Kinetics of Sodium Phytate Hydrolysis by Phytase of P. pastoris at 45 •C, pH 4.0***

Properties	Values
V_{max} (U mg^{-1} protein min^{-1})	0.074 (0.0488 mM/min)
K_m (mg ml^{-1})	0.091 (0.0001378 mM)
V_{max}/K_m	0.813 (354.136/ min)

V_{max} and K_m were determined from Fig. 8.

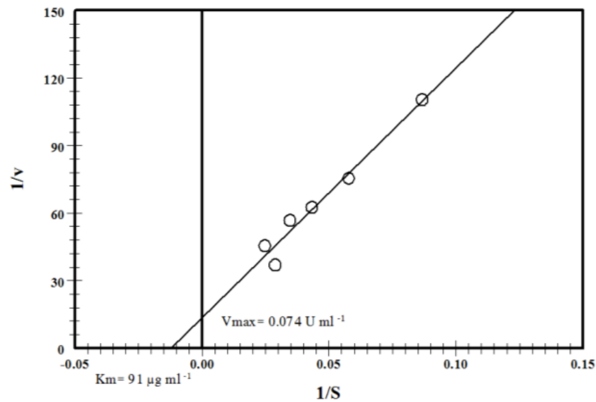

Fig. 8: Line Weaver Burk plot to determine the Michaelis Menten kinetics constants for substrate (Sodium phytate) hydrolysis.

Arrhenius plot

In Arrhenius plot the logarithm of kinetic constants (ln (K) plotted against inverse temperature 1/T. Arrhenius plots are mostly used to analyze temperature effect on a rate of chemical reaction. Arrhenius plot gave a straight line (Fig. 6). The activation energy determined for hydrolysis of sodium phytate was 76.08 KJ/mol.

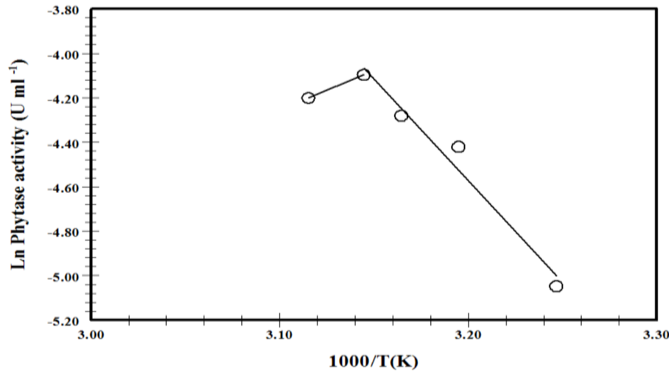

Fig. 6: Arrhenius plot to determine the Activation energy for sodium phytate hydrolysis

Table 6. *Phys*iochemical properties of *P. Pastoris* phytase at optimal conditions

Properties	Values
pH optimum	4.0
pH range	3.2–8.0
Temp optimum (°C)	45
E_a (kJ mol^{-1})	76.08

Effect of pH

The recombinant *P. pastoris phyS* was determined over a range of 3.2-8.8, as shown in (Fig. 7). The optimum pH for recombinant phytase was 4.0 after that its activity decreased in an irregular manner. The stability at this point revealed that enzyme is fully active and properly functional. Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity, for each enzyme there is also a region of pH optimal stability.

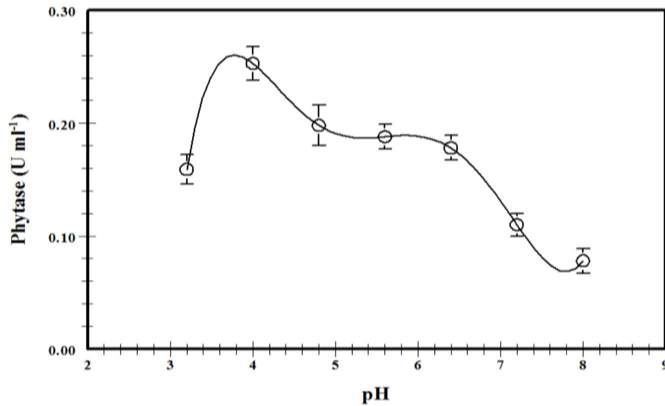

Fig. 7: Effect of pH on catalytic activity of phytase from P. pastoris at 45 °C

Data presented is average SD mean of n= 3 independent experiment.

Effect of substrate

It had been clear from the experiment that if enzyme concentration was kept constant and the substrate concentration was increased gradually, reaction velocity had increased until it reached to maximum. After achieving V_{max}, increase in substrate concentration will not increase the velocity. At maximum velocity, all concentration of available enzyme had been converted to enzyme substrate (ES) complex. This was known as V_{max} in (Fig. 8) and value was 0.074 U/ml. Michaelis gave a mathematical equation to calculate enzyme activity in terms of reaction velocity. The Michaelis (K_m) constant is defined as the substrate concentration at 1/2 the maximum velocity. The value of K_m was 91µg/ml or 0.091mg/ml.

Discussion and Conclusion

Thermomyces lanuginosus is a common and widespread thermophilic fungus. Numerous strains belongs to this have been described for high level production of extracellular enzymes, including phytase (Singh, Madlala, & Prior, 2003). The recombinant product was biochemically characterized (Berka, Rey, Brown, Byun, & Klotz, 1998).

The phytase gene *(phyS)* of *Thermomyces lanuginosus* was expressed in *P. pastoris* GS115. The thermophilic fungi secrete enzymes with distinctive properties like high temperature, tolerance against organic solvent, longer shelf-life and broad range of pH, were most desirable characteristics for commercial purposes (Maheshwari, Bharadwaj, & Bhat, 2000; Reilly, 1999). The thermophilic

fungi secrete phytases extracellularly, and have simple nutritional requirement than bacteria and yeast (Maheshwari et al., 2000; Vohra & Satyanarayana, 2003; Wodzinski & Ullah, 1995). Phytases from thermophilic fungi like *T. lanuginosus* are highly thermostable and have broad range of pH 4-10 (Berka et al., 1998; Chadha et al., 2004; Gulati et al., 2007; Singh & Satyanarayana, 2009).Thermophilic fungal phytase are reported to be protease resistant (Singh & Satyanarayana, 2006; Wang, Gao, Su, Wu, & An, 2007). This property is helpful in commercial application of phytase in feed and food industries to tolerate acidic environment of intestine during digestion (Greiner & Konietzny, 2006; Suhairin et al., 2010). Thermophilic fungi have further advantage to grow in solid-state fermentation than yeast and bacteria (Vohra & Satyanarayana, 2003).

Fungal phytases are more stable in organic solvents (Gulati et al., 2007; Singh & Satyanarayana, 2009) as compared to other phytases (Vats & Banerjee, 2005). The one major limitation of thermophilic fungal phytase is low production of enzyme compared to mesophilic microbes (Singh & Satyanarayana, 2008). In recent approaches, problem could be overcome by using cloning and expression of the phytase genes from thermophilic molds (Mitchell, Vogel, Weimann, Pasamontes, & van Loon, 1997; Pasamontes, Haiker, Henriquez-Huecas, Mitchell, & van Loon, 1997) In this research project, phytase gene was isolated from fungus *T. lanuginosus* due to high temperature optimum, broad pH range, organic solvent tolerance and dephytinization and expression in eukaryotic host system. The maximum production of recombinant *P. pastoris phyS* was found to be 11.5 U/ml of growth in 10 L fermenter after 96 h. However, this was possible only, after 16 fold concentration of the enzyme through ultrafiltration. This could be due to many reasons such as low copy number, presence of high concentration of phosphate in the BMMY medium, problems in secretion of the enzyme, post transcriptional and translational modification. The copy number of a gene is a critical factor in *P. pastoris* for heterologous expression. In some cases, high or multiple copy clones exhibit a significant increase in expression, up to 200-fold, compared to low copy number clones. This suggests that low transcript levels could be a limiting factor in the expression of foreign genes, whereas higher transcript levels may overcome other limiting factors, such as mRNA secondary structure or protein instability (Fu et al., 2008). While transgenes with low copy numbers allow host cells to grow normally, clones with high copy numbers may experience metabolic stress, ultimately leading to a reduction in the expression of foreign proteins (Yang, Johnson, & Murthy, 2012). The other reason is the use of K_2HPO_4/KH_2PO_4 buffering system as recommended in

the *Pichia* manual provided by the supplier of Invitrogen as described above. In order to get better expression this problem could be tackled by moving toward another buffer system.

Other studies reported that the high concentrations of inorganic phosphorous (P_i) in the medium favor the growth of microbes but inhibit the expression of the phytase gene (Van Hartingsveldt et al., 1993). The buffering system (K_2HPO_4/KH_2PO_4) used for growing *Pichia* recombinants was not favourable for phytase production as claimed by who used *P. griseoroseum* for phytase production. Phytase requires phosphate free media. The other buffering systems e.g HEPES, MOPS and SCM were selected for higher phytase activity in the culture supernatant and no inhibition of recombinant strain growth (Teixeira Filho et al., 2013). An increase of 5.1 times was observed in phytase activity for recombinant *P. griseoroseum* T73 as compared with the host strain *P. griseoroseum* PG63 using SCM buffer system after 72 h. In other research experiments *phy*B gene from *A. niger* var. *awamori*, was cloned into the pFF1 plasmid transformed into *A. niger* var. *awamori* ALK02268 cells to get a super-producing phytase mutant. Phytase activity was increased up to seven-fold IU/mL (Piddington et al., 1993). The *E. coli* phytase, AppA2 gene has been transformed into three inducible yeasts: *Schizosaccharomyces pombe, S. cerevisiae* and *P. pastoris.* The later showed highest activity of enzyme (272 IU/mL) than other yeast systems (Lee, Kim, Stahl, & Lei, 2005). The modified *Aspergillus niger* yielded 865 U/mL phytase after cloning in *P. pastoris* and produced 6.1 g/L protein after purification (Xiong et al., 2005). Phytase gene from *Eupenicillium parvum* (BCC17694) expressed in *P. pastoris using* expression vector pPICZαA, produced 2.7 IU/mL. After purification through gel filtration the expression level was 21.8 U/ml (Fugthong et al., 2010). The yield of phytase by jar fermenter cultivation was reported as 23 g/l (Watanabe, Ikeda, Masaki, Fujii, & Iefuji, 2009). Recombinant *P. pastoris* GS115 pPIC9CT*phyA* showed 9 U/mL phytase activity after 48 h and 75 U/mL measured after 72 h.

Phytase protein band on SDS–PAGE of 75 kDa was obtained by mutant GS115/pPIC9CT*phyA* (Zhao, Wang, Mu, Sun, & Wang, 2007). Data presentation showed that the enzyme activity was low due to the interference of native signal peptides with the extracellular secretion. Due to high concentration of phosphate in the medium the host cells suffer metabolic stress and leads to low yield of proteins. By using constitutive promoter than strongly regulated promoter P_{AOX1} the yield can be increased up to eight to ten fold. Previous study has specified that by changing the pH from 6.0 to 4.0 of the medium the stability of secreted proteins could be enhanced

(Clare et al., 1991; Koganesawa et al., 2002). Total activity was increased in rLlALP2 case, in BMGY media and not dependent on the pH of the medium. The supplementation of media with Triton X-100 significantly enhanced the extracellular production and extraction (Baldi, Scott, Everard, & Loewus, 1988; Scott, 1991), Addition of Triton (X-100) into BMGY, three fold increased the enzyme activity (Vogl & Glieder, 2013).

The heterologous expression system of *P. pastoris* has certain limitations. During fermentation for cell density all controlled conditions are provided and induction through methanol, are all stress factors. All these conditions are different from natural environment and limiting the expression of foreign proteins. To replace methanol in industrial scale fermentations, new effective alternatives for induction have to be found ((Delic, Mattanovich, & Gasser, 2013; Guerfal et al., 2010; Prielhofer et al., 2013). Glucose is an attractive carbon source at industrial scale applications, due to its low price and easy availability. Literature history reported that recombinant phytase by *P. pastoris* YY113 gives production of 2200 FTU phytase ml^{-1} using glucose as carbon source than and yield 0.25 FTU ml^{-1} using methanol. Study revealed that glucose can used as a sole carbon source for large scale production of the recombinant proteins in *P. pastoris* (Hang et al., 2009).

The recombinant *P. pastoris phyS* showed maximum activity at pH 4, so it has potential chance of commercialization in food and feed industry after optimization because monogastric animals require phytase that is stable at acidic pH of stomach and broad range of pH. The temperature of recombinant *P. pastoris phyS* was 40°C and for feed and food industry the required enzymes carry temperature range from 37-40°C, so the optimum temperature of this enzyme is ideal. The low temperature and lower growth rate results in reduced protein synthesis ultimately leads to proper protein folding (Çalık et al., 2015). Recombinant phytase (BCC17694) showed highest activity at pH 5.5 and 50 °C. Optimum pH and temperature of recombinant phytase differed from the crude native enzyme which was 60 °C and pH 4.0 (Promdonkoy et al., 2009). Crude and purified recombinant phytase have a clear difference between their properties (Singh & Satyanarayana, 2009). Many fungi and microbes produces phytase to moderate level, however, the focus is on commercialization so we need best expression system that give enzyme production in less time and is also cost effective.

In future the enzyme activity could be improved by changing the media recipe or using alternate substance for phosphate buffer to maintain pH of the media. The expression can be further improved through codon optimization, optimizing

fermentation conditions and by inserting gene of interest in high copy number plasmid. By constructing P_{GAP} constitutive promoter, modifying new signal peptides for proper protein folding and efficient secretion. The metabolic stress could be overcome by providing low temperature for growth.

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