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Comparison of biochemical properties of recombinant phytase expression in the favorable methylotrophic platforms of *Pichia pastoris* and *Hansenula polymorpha*

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ABSTRACT

Phytic acid is the dominant form of phosphorous in plant seeds. However, phytic acid cannot be utilized by animals and causes them serious phosphate deficiency. Utilization of phytase as an animal feed additive can affect liberation of phosphor and its mineral availability. In this study, heterologous expression of the *A. niger phyA* gene was investigated in the methylotrophic yeasts *P. pastoris* and *H.polymorpha* and its expressed active recombinant phytase biochemical and biophysical properties were studies and compared to native *A.niger* phytase. The *phyA* gene sequence of *A.niger* was designed and expression of synthetic genes was highly successfully in active form in both yeast hosts. Size of the secreted recombinant phytases, due to heavy glycosylation, varied from 50 to 65 KDa. Expressed extracellular recombinant phytase samples were purified and biochemical tests on properties demonstrated that both recombinant phytase samples had similar pH profiles with pH optima determinations of pH 2.5, pH.5.5 as acid phosphatase and optimum temperature of 60 and 50 °C respectively in *P. pastoris* and *H. polymorpha*. This study concludes that recombinant *P. pastoris* and *H. polymorpha* phytases could fulfil a series of predefined industrial quality criteria for application as animal feed supplement.

Keywords: phytase*, Pichia pastoris, Hansenula polymorpha*

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Introduction

Phosphorous is an essential mineral nutrient for animals. The major source of phosphorous is in salt form as phytate in plants and seeds such as soybean and grains that constitute the main ingredients of animal feed [1]. But some animals such as poultry, fish and swine are not able to utilize this form of phosphorus because they lack phytic acid hydrolysis in their stomach [2-4]. Furthermore phytate presence in soil has an anti nutrient effect, as chelator of divalent cations and one that decreases bioactivity of phosphorous [5, 6]. A solution to the problem of phosphorous deficiency in animals is to supply them with inorganic phosphor as animal food additive but this extra phosphorous can contribute to environmental pollution and disrupt an ecosystem [7, 8].

Phytase as an histidine acid phosphatase enzyme so it can efficiently utilize phytate and convert it to phosphorylated *myo*-inositol derivates and inorganic phosphate, however, phytase used in animal feed will increase mineral uptake and enhance utilization of phosphorous [9, 10]. Phytase is produced naturally by many different plants, animals and microorganisms but such occurrence has low expression and as such has significant effect on phytate levels in soil. Ideally, overproduction of phytase in powerful host like methylotrophic yeasts with potential high level active recombinant protein production in a cost effective medium and production technique that can be easily scaled up, could be valuable to agricultural and beneficial to environmental goals.

Accordingly, research has been done to find the most suitable source of phytate degrading enzymes and studies on a wide range phytase producing micro-organisms have revealed that *Aspergillus Niger* has a high affinity to phytic acid [11]. This determines *Aspergillus Niger* as a principle candidate for phytase production for application as feed additive but *A.niger* expresses low-level native phytase and is inactive at high pelleting temperature, so its ability for overexpression in a eukaryotic host in a cheap medium could be ideal.

The methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha (Pichia angusta)* were selected as they have the advantage of growth in a medium containing methanol as a sole source of carbon [12]. Meanwhile some academic and industrial studies have reported high heterologous eukaryotic protein expression in mentioned hosts and are easy to manipulate; *P.pastoris* and *H.polymorpha* present good candidates for recombinant phytase expression [13, 14].

To choose the most suitable methylotrophic yeast for industrial production of recombinant phytase and to compare and study biochemical properties of phytase in two main methylotrophic hosts, it was decided to investigate extracellular production of phytase in *P.pastoris* and *H.polymorpha*. Study and comparison of recombinant phytase behaviour, not only between different methylotrophic hosts but also in comparison with native *A.niger* phytase was considered as useful to make selection for an industrial compatible phytase producer for further application animal food additive.

In this study, *phyA* gene from *A.niger* (Gene Bank Accession No. p34752) was chosen and the *phyA* genes excluded pre-pro-sequence were synthesized according to *P.pastoris* and *H.polymorpha* codon performance in size of 1350bp (Figure 1). The synthetic genes were placed under the control of inducible AOX1 and FMD promoters and then transformed to *P.pastoris* and *H.polymorpha* hosts respectively and evaluations were made for biochemical properties of the active expressed phytase.

A. Native *Aspergillus Niger* PhyA gene (Gene Bank p34752)

MGVSAVLLPLYLLSGVTSGLAVPASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANESVISPEV
PAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYNYSLGADDLTPFGEQEL
VNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKFIEGFQSTKLKDPRAQPGQSSPKIDVVISEASSSNNT
LDPGTCTVFEDSELADTVEANFTATFVPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTISTSTVDTKLS
PFCDLFTHDEWINYDYLQSLKKYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDDTSSNHTLDSSPAT
FPLNSTLYADFSHDNGIISILFALGLYNGTKPLSTTTVENITQTDGFSSAWTVPFASRLYVEMMQCQAEQ
EPLVRVLVNDRVVPLHGCPVDALGRCTRDSFVRGLSFARSGGDWAECFA

B. Synthetic *Aspergillus Niger* PhyA in *P.pastoris* and *H.polymorpha*

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTT
IASIAAKEEGVSLEKRASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANESVISPEVPAGCRVTFAQ
VLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYNYSLGADDLTPFGEQELVNSGIKFYQR
YESLTRNIVPFIRSSGSSRVIASGKKFIEGFQSTKLKDPRAQPGQSSPKIDVVISEASSSNNTLDPGTCTVFE
DSELADTVEANFTATFVPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTISTSTVDTKLSPFCDLFTHDE
WINYDYLQSLKKYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDDTSSNHTLDSSPATFPLNSTLYA
DFSHDNGIISILFALGLYNGTKPLSTTTVENITQTDGFSSAWTVPFASRLYVEMMQCQAEQEPLVRVLVN
DRVVPLHGCPVDALGRCTRDSFVRGLSFARSGGDWAECFARS

Figure 1. Amino acid sequence of phytase. A. The amino acid sequence of native protein and its N-terminal native signal peptide(Underlined). B. The amino acid sequence of synthetic phytase protein of *P.pastoris* and *H.polymorpha*(the alpha mating factor signal peptide of *S.cervisiae* is underlined).

Materials and methods

Strains and Vectors

All bacteria and yeast strains, plasmid and primers used in this study are listed in (Table 1). *Escherichia coli* strain DH5α was used for cloning and transformation. *Pichia pastoris* strain GS115 (his4) and expression vector pPinkα-HC (7.9Kb) were purchased from Invitrogen, San Diego, CA). *Hansenula polymorpha* strain RB11 (*Ura3*$^{-}$) and expression vector pFPMT-MFa (7.2Kb).

Chemical and Enzymes

E.coli was cultured at 37°C in LB medium, yeasts were cultivated at 30°C in yeast extract–peptone-dextrose (YPD) medium.

For expression of recombinant protein, transformed *P.pastoris* were incubated in BMGY medium (1% yeast extract, 1.34% YNB, 2% peptone, 0.000004% biotin and 1% glycerol) with agitation of 240rpm at 30°C and to induce phytase gene expression, cells were then pelleted (2500g for 3min) and resuspended in buffered methanol complex (BMMY) medium (SD, 0.000004% biotin and 0.5%methanol) at 30°C and shaken at 240rpm.YNB-1% Glycerol (0.17% Yeast Nitrogen Base, 0.5% ammonium sulphate, 1% glycerol) was used for *H.polymorpha* phytase expression at 37°C.

Sodium phytate and phytic acid were purchased from Sigma Chemical Co., Ltd (St Louis, MO, USA). Enzyme (Taq DNA polymerase, *pfu* DNA polymerase, T4 DNA ligase and restriction enzymes were purchased from Thermo Fermentase (UAS). Endoglycosidase H (EndoHf) was purchased from new England Biolabs (Beverly, MA, USA). Protein markers were purchased from Thermo (USA), Native *A.niger* phytase purchased from BASF and used as a positive control enzyme in kinetic experiments. Oligonucleotides were synthesized at TAD Copenhagen A/S (Denmark).

Table 1. Strains, plasmids and synthetic oligonucleotide primers

Item	Description	Reference
Strains		
DH5α	*E.coli* ,α-complementation	Fermentase
P.pastoris	Protein expression host	Invitrogen
H.polymorpha	Protein expression host	
Plasmids		Invitrogen
pPinkαHC	For integration in *P.pastoris*	
pPFPMT-MFα	For integration in *H.polymorpha*	
Oligonucleotide primers		
SbuF Forward	5'-CTCGAGGCTTCTAGAAACCAATCTTCTT-3'	
SbuR Reverse	5'-GGTACCCTACTAAGATCTAGCGAAACAT- 3'	
PhyNRF Forward	5'-GTGTTTCTGCTGTTTTGTTGCCATTG-3'	
PhyNRR Reverse	5'- AACCGTGCAATGGAACAACTCTGTC-3'	

Construction of phytase expression vectors and yeast transformation

The *phyA* gene sequences encoding mature phytase with excluded pre-pro-sequence signal peptide were designed according to codon bias of *P.pastoris* and *H.polymorpha*. Designed genes were synthesized by Gene Art Company. (Gene Bank Accession Nos. JN193562.1 and JN 193563.1), respectively.

The synthetic *phyA* gene which was supposed to cloned in the pPinkα-HC vector of *P.pastoris* was amplified with *pfu* DNA polymerase by PCR using the primers listed as follows; forward primer (SbuF) and reverse primer (SbuR) with insertion restriction sites of *XhoI and KpnI* respectively at 5' and 3' ends. The PCR conditions were: 94°C for 30s, 60°C for 45s and 72°C for 1.6 min for first five cycles and then continued at 94°C for 30s, 57°C for 45s and 72°C for 1.6min for 25 cycles. Sequence of the PCR product was confirmed by DNA sequencing (Applied Biosystem, ABI, USA).

The amplified PCR product was separated by 1% agarose gel electrophoresis and a gel slice containing the expected size band (1350bp) was excised and extracted. After cloning in appropriated cloning vector, it was *XhoI/KpnI* double digested and digested fragment was sub cloned into the *XhoI/KpnI* cleaved sites of the pPinkα-HC expression vector, which contained the pro-pre-sequence of *S.cervisiae* α mating factor under control of inducible AOX1 promoter (Figure 2a). DNA manipulation was performed according to standard procedure [15].

The Synthetic *phyA* gene for *H.polymorpha* was cloned into *XhoI/KpnI* restricted sites of the pFPMT-MFa expression vector that contained pre-pro-sequence of *S.cervisiae* α mating factor for secretion under control of inducible FMD promoter (Figure 2b).

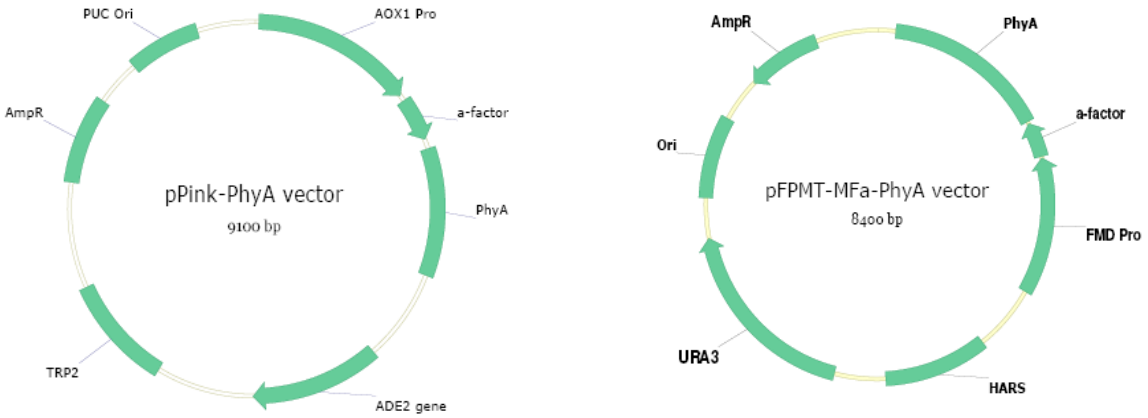

Figure 2. a) Construction of expression vectors pPinkα-PhyA and b) pFPMT-MFa-PhyA

For transformation, Adenine gene deficient *P.pastoris* competent cell was prepared and a five microgram sample of pPinkα-phyA plasmid was linearized using *Afl II* at 37°C for 60min, the digested plasmid was purified using GF1 Nucleic Acid extraction kit (Vivantis Technologies Co.) and transformed to *P.pastoris* through electroporation (BTX, ECM630, USA) at 1850 V, 200Ω and 25µFwith 2mm cuvette. Immediately after pulsing, 1 ml of cold 1M sorbitol was added to the cuvette and incubated at 30 °C for 60min, then cuvette contents were plated on PAD (*Pichia* Adenine Dropout) and incubated for 3-10 days at 30°C. As a negative control, the *Afl II* linearized pPinkα-HC was transformed to *P.pastoris*, for phytase expression assay.

H.polymorpha strain RB11 competent cell that is uracil auxotroph (Ura^-) was prepared, circular pFPMT-MFa-phyA plasmid which carry marker of URA3 expression cassette was transformed to *H.polymorpha* by electroporation (BTX, ECM630, USA) at 2100 V, 200Ω and 25µFwith 2mm cuvette. Screening of Ura^+ clones was done on YNB-Glucose medium without uracil. Transformants were grown for approximately 30-80 generations at 37°C by successive cultivation steps under selection conditions (Passage 1 to 8). During this period, plasmid copy number increased and integration occurred with onto the host genome. After final passaging transformants were cultivated under non-selective and selective conditions for stabilization and final selection, respectively. As negative control pFPMT-MFa was transformed to *H.polymorpha* for phytase enzyme assy.

Screening of genome integrated yeasts

To investigate integration of *phyA* to yeast host genomes, transformed *P.pastoris* and *H.polymorpha* were grown on YPD medium and genomic DNA were extracted from yeasts by the glass beads method as described by Hoffman and Winston [16, 17]. The genomic DNA extractions were used as templates in PCR with phyA gene specific primers of *P.pastoris* (SbuF and SbuR) and *H.polymorpha* (PhyNRF and PhyNRR), (Table1). The PCR method included 5min 95°C, followed by (30 cycles of 1min at 95°C, 30s at 57°C, 45s at 72°C and a final extension of 10min at 72°C).

Expression of recombinant phytase

The integrated *P.pastoris* clones were grown in 10 ml of BMGY medium at 30° for 24-48 hours. When OD600 reached 5-10, the cells were pelleted by centrifuge for 10 min at 8000g and resuspended in 2ml of BMMY at 30°C in a shaking incubator (300rpm).

During 4 days of BMMY cultivation, methanol was used in the final concentration of 1% every 24 hours to induce expression of phytase. Samples were taken periodically during the 4 days of induction, lyophilized and evaluations for finally phytase activity and protein concentration were determined. In *H.polymorpha* positive phyA integrants were cultured in YNB 1% glycerol at 37°C for 48 hours with 300 rpm shaking. After 2 days of glycerol absence, derepression of FMD promoter occurred [18, 19]. Sample supernatants were collected and phytase activity beside of protein concentration was determined to select the highest *H.polymorpha* recombinant phytase producer.

Protein analysis and deglycosylaion of expressed phytase

Sample supernatants with high phytase activity were loaded on 10% SDS-PAGE gel using Mini-protein gel electrophoresis (Bio-Rad Laboratories, USA). Gels were strained after electrophoresis. Heavy glycosylaion caused expressed recombinant phytases of variable molecular size of 60 to 120 KDa.

Phytase N-glycosylaion was evaluated by assessing samples for migration shift, resulting from EndoH (Biolab) treatment. Reactions were allowed by incubating phytase supernatants with 0.2 unit of Endo H for 12 hours at 37°C according to manufactures instructions and then the phytase protein band migration shift was studied by SDS-PAGE.

Phytase assay

To determine phytase quantitative activity, phytase assay was carried out in 1.0ml final volume at 37°C, in 0.2M sodium acetate buffer pH5.5 and substrate of 20mM sodium phytate for 30 min reaction time, by following the method cited in Henonen and lahti [20, 21]. The librated inorganic phosphate was quantified spectrophotometrically using freshly prepared acetone-molybdate-acid (AMA) as a stopping reagent consisting of 2 parts anhydrous acetone, 1 part of 10mM ammonium molybdate and 1 part of 5 N sulphuric acid. After 30s, 100µlof citric acid (1.0M) was added to fix the color [22]. Absorbance was read at 380nm after blanking the spectrophotometer with appropriate negative control supernatant. One unit of phytase activity was defined as the amount of activity that released 1µmol of phosphate per minute from sodium phytate under the assay condition. Protein concentration was determined in samples by means of BSA protein assay kit (Bio-ad Laboratory, USA) using bovine serum albumin as the standard.

Phytase Purification

The culture supernatant of *P.pastoris* and *H.polymorpha phyA* transformants were centrifuged at 10.000g for 15min at 4°C, cell decried removed and supernatants were 0.2µ filtered, the filtered supernatants were concentrated by means of Amicon 30KDa centrifugal filter device ultrafiltration. Concentrated samples were loaded into Superdex size column (GE healthcare, USA) that had previously been equilibrated with 0.1M sodium acetate buffer (pH 5.5), then eluted with 0.1M sodium acetate containing 0.15M NaCl (pH 5.5) with a flow rate of 2.5ml min^{-1} using AKTA Purifier Fast Protein

Liquid Chromatography system (Amersham bioscience, USA), The flowthrough fractions were collected by automatic fraction collector. The fraction profiles of OD280 and phytase activity were check to determine desired protein peaks. The peak fractions were pooled and concentrated by Amicon centrifugal filter device and stored at -20°C for further study.

Biochemical characterization of recombinant expressed phytases.

The phytase pH profile was determined at 37°C with different buffers, 0.2M glycin-Hcl (pH1.0, 2.0 and 2.5) 0.2 sodium citrate (pH 3.0,4.0,5.0 and 5.5) and 0.2M Tris-Hcl (pH 6.0 to 8.0)[23]. The optimum temperature of phytase was tested with 200mM sodium acetate buffer at pH 5.5 at temperatures ranging from 20 to 80° adjusted at intervals of 5°C.

The kinetic parameters of Km and Vmax, Michaelis Menten constants of phytases were determined at pH5.5 with 200mM sodium acetate buffer. The phytase activity assays were made with sodium phytate salt (Sigma) at 14 different concentrations (50, 75, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900 and 1000µmol). Three parallel series of phytase assay reactions were made.

For thermostabilty evaluation purified phytase samples were incubated at temperature from 60 to 90°C for 10 min and immediately after heat treatment, samples were placed on ice for 30min, phytase residual activity was measured at 37°C and pH 5.5[23]. Phytase thermal stability was determined over a longer duration through 80°C heat treatment from 10 to 60 min and more, subsequently phytase long time stability tested for samples by determinations of remaining activity.

Results and Discussion

Construction of vector for phytase expression in methylotrophic yeasts

The *phyA* genes that contain 1350bp and open reading frame encoding 444 amino acid residue were designed by Gene Optimizer software and synthesized according to performance determined for *P.pastoris* and *H.polymorpha* codon. The artificial *phyA* genes were digested and ligated to pPinkαHC and pFPMT-MFa expression vectors and pPinkαHC–phyA and pFPMT-MFa-phyA recombinant plasmid constructs prepared. For efficient and stable expression of phytase, recombinant constructs integrated to host genomes, the pPinkαHC–phyA was linearized by *Afl II* restriction enzyme digestion at TRP2 region of plasmid and transformed to *P. pastoris* and circular pFPMT-MFa-phyA plasmid transformed to *H. polymorpha* strain RB11. For extracellular phytase secretion, mating factor alpha from *S. cervisiae* was used in both recombinant expression constructs. The *S. cervisiae* mating factor alpha pre-pro-sequence was used for secretion expression of numerous heterologous proteins in *S. cervisiae* and *Pichia pastoris* [24, 25].

To investigate successful *phyA* genome integration, the positive transformed yeast genome was extracted and screened using specific *phyA* sequence primers by PCR, desired PCR product band showed successful genomic integration (Figure 3). In the next step, positive colons with the highest phytase activity were selected for further study.

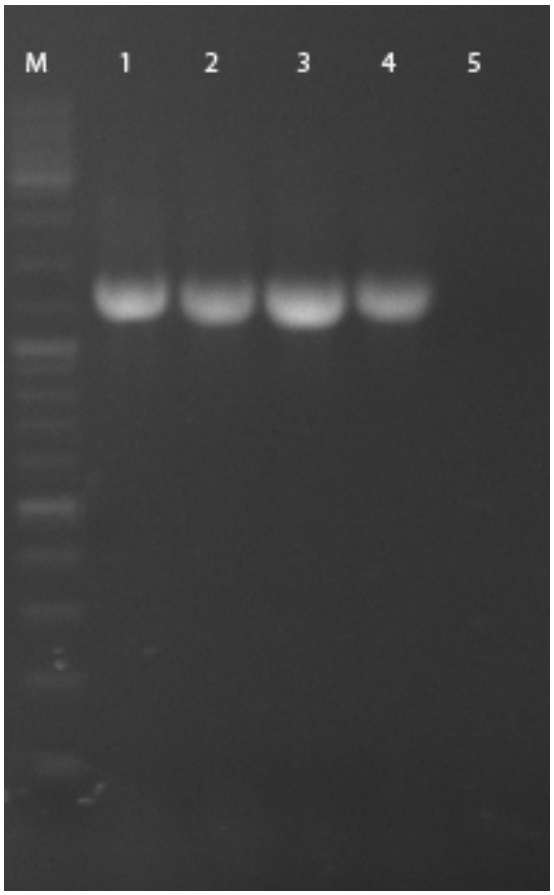

Figure 3. Yeast genome integrant PCR analysis. Lane1:PCR on transformant *P.pastoris.* Lan2: PCR on transformant *H.polymorpha* .Lan3 pPink-PhyA plasmid PCR as *P.pastoris* positive control. Lane 4.*pFMPT-PhyA* plasmid as *H.polymorpha* positive control. Lane 5 : Transformed *P.pastoris* by pPinkα-HC Plasmid PCR as negative control.

Expression of recombinant phytase in methylotrophic yeasts

To promote expression of recombinant phytase, methylotrophic yeast genome integrated colons were induced by methanol and supernatant. Collected samples were investigated by enzyme quantitative assay and SDS-PAGE. Test results indicated that *P.pastoris* and *H. polymorpha* recombinant active phytase was expressed and successfully secreted extracellularly, furthermore proteins secreted in the culture medium were determined as recombinant phytase. SDS-PAGE analysis of *P.pastoris* and *H.polymorpha* supernatants showed that due to heavy yeast glycosylation, the expressed phytase was revealed as a smear band on the SDS-PAGE with molecular size ranging from 40 to 65 KDa (Figure 4). To determine the effect of glycosylaion on molecular size of recombinant expressed phytase, samples were deglycosylated by EndoH and SDS-PAGE, evidence determined that recombinant phytase of *P. pastoris* and *H. polymorpha* had an apparent molecular weight of 49 KDa (Figure 4).The molecular weight of recombinant phytases subsequent to deglycosylaion by EndoH appeared to be similar to that of native phytase from *A.niger* [26].

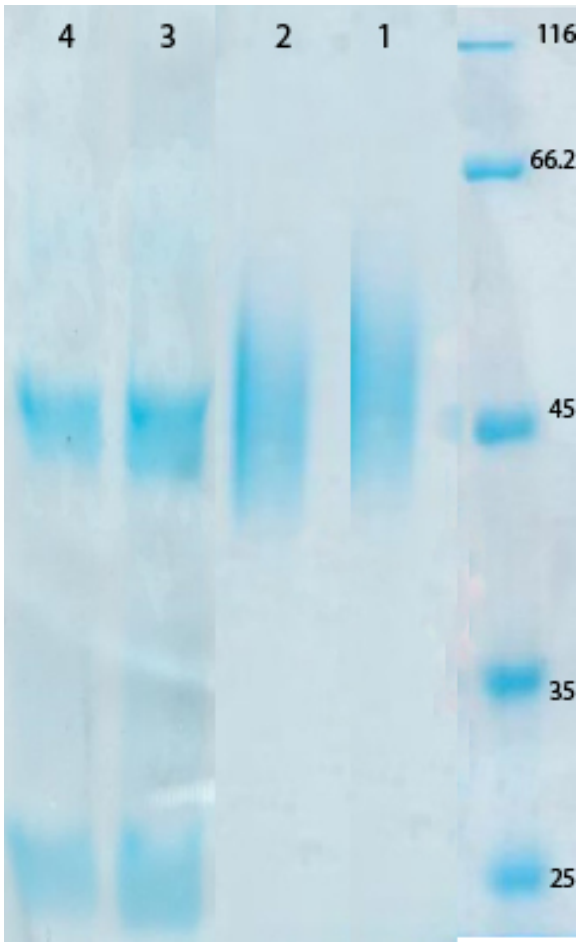

Figure 4. SDS-PAGE analysis of recombinant phytase from (1) *P.pastoris* (2) *H.polymorpha* and (3,4) protein treatment with Endo H

Phytase activity and pH profile

pH range of activity and optimum pH were determined for recombinant phytases, *P. pastoris* and *H. polymorpha* and expressed recombinant phytase activity were determined at different pH values from 1 to 8 (Figure 5). Results indicated that both recombinant phytases had two optimum pH levels of pH5.5 and 2.5, and comparison of *P.pastoris* and *H. polymorpha* activity at lower pH showed respective values of 65% and 55% of maximal phytase activity in pH of 5.5, furthermore comparison revealed that not only both heterologous expressed phytases had the same pH pattern as an acid phosphatase but they also had the potential to remain active in a wide range of pH levels; this could be considered as advantageous in terms of application in animal feed supplement [27]. Comparison of recombinant expressed phytases with commercial and native *A.niger* phytase, also showed a similar pH profile. In addition, such similar pH profiles have also been reported in *A.niger phyA* gene expression in *S. cervisiae* too [28]. It seems that despite different levels of glycosylaion between *A.niger* as filamentous fungi and *P.pastoris*, *H.polymorpha* and *S.cervisiae* yeasts, hyperglycosylaion did not have an affect on pH activity of phytase expressed in the yeast hosts [29].

Phytase temperature range of activity

In order to determine recombinant phytase behaviour at different temperatures, purified recombinant samples were assayed for phytase activity at the temperature range of 20 to 80 °C at 5-degree intervals in sodium acetate buffer, pH5.5 (Figure 6). It was discovered that recombinant phytase of *P.pastoris* and *H.polymorpha* had optimum temperatures of 50 and 60 °C respectively. Also Phytase activity assays tested at a wide range of temperature showed that recombinant phytases had maximal activity at temperatures of 50 to 65°C and loss of activity at 40% and 30% respectively at temperatures higher than 70°C. Temperature behaviour of recombinant yeasts phytases showed similar activity in comparison with study which *phyA* gene was expressed in *S.cervisiae* host with recombinant phytase optimal activity of 55 to 60°C [28]. It seems that higher optimal temperature of recombinant phytases expressed in yeasts compared with other hosts and native *A.niger* phytase, not only resulted in a higher optimum temperature range of phytase activity of 50 to 60°C, but also resulted in better thermostabilty of phytase.

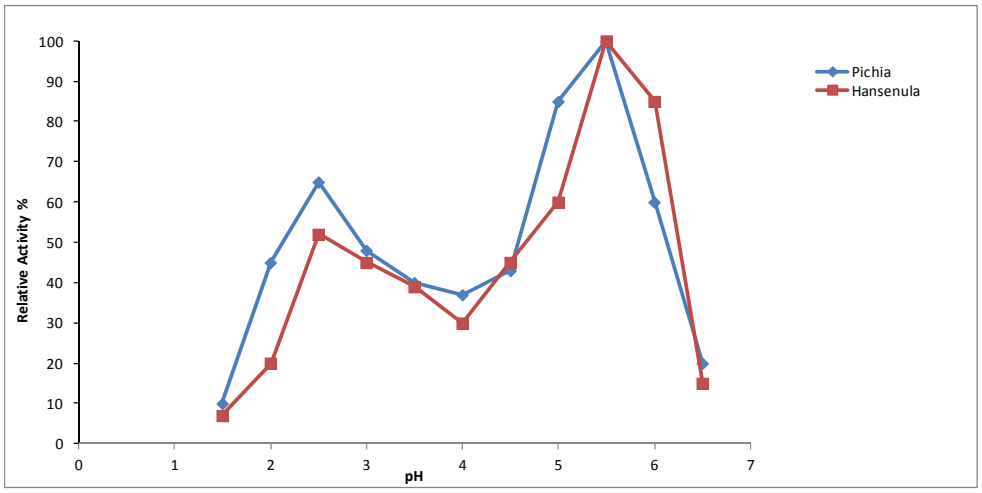

Figure 5. Characterization of recombinant phytase expressed in *P.pastoris* and *H.polymorpha*, effect of different pH on recombinant phytase assay.

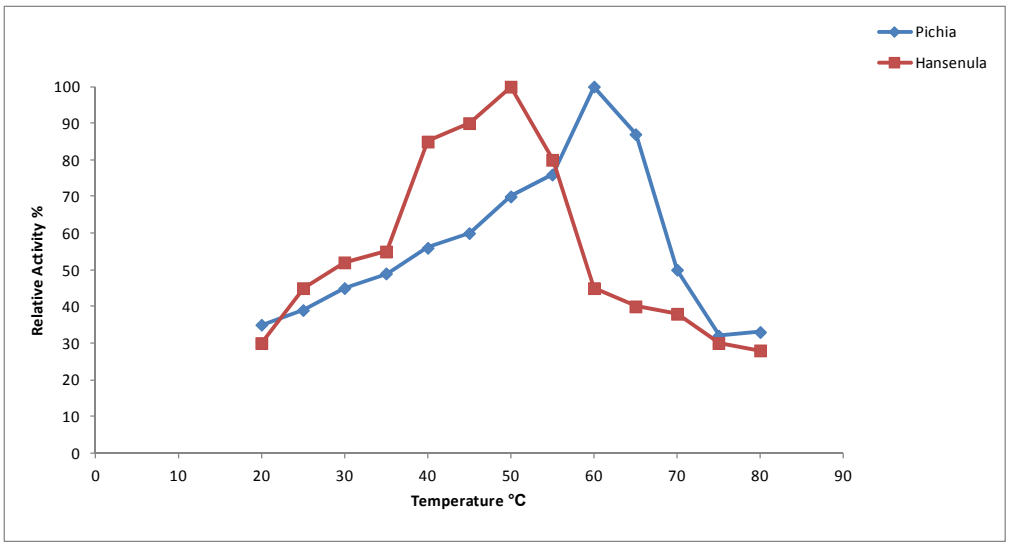

Figure 6. Effect of different temperature on recombinant *P.pastoris* and *H.polymorpha* phytase assay

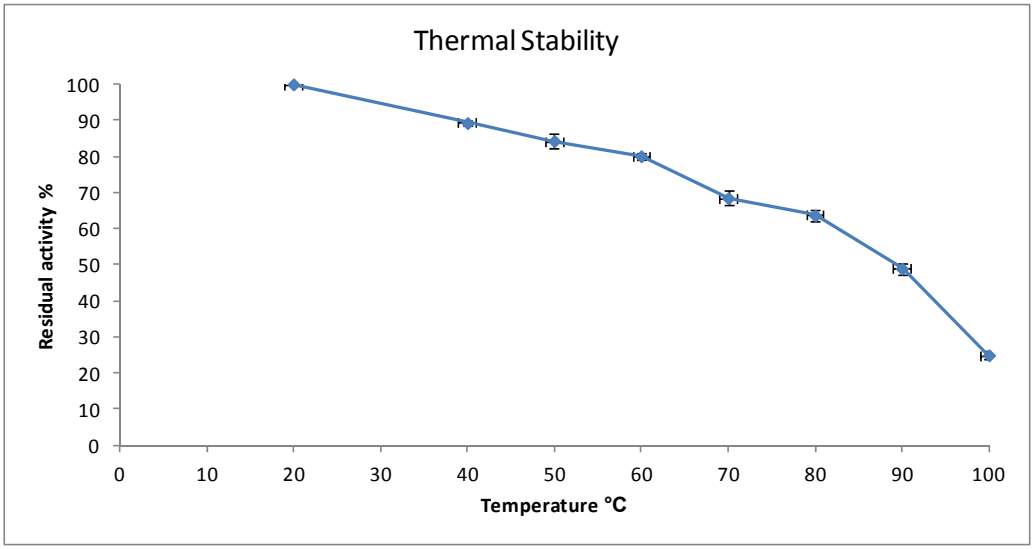

Figure 7. Recombinant *P.pastoris* thermostabilty test . Residual enzyme activity after being heated at various temperatures for 10 min.

Thermal stability of recombinant phytase

Thermostabilty of purified recombinant expressed phytases determined after heating at 20 to 90°C for 10min with 10°C interval, then samples were cooled on ice and residual phytase activity of the treated samples was determined at 37°C in sodium acetate buffer (pH 5.5). Study of phytase thermostabilty revealed that the phytase expressed in yeast had higher thermostabilty compared to native *A.niger* phytase, group of native phytase producers and phytases that were expressed in different hosts other than yeast. Thermal stability study showed that when recombinant expressed phytases were heated at 70, 80 and 90°C for 10 min, *P.pastoris* recombinant phytase retained its phytase activity 68, 60 and 50% (Figure7) and recombinant phytase expressed by *H.polymorpha* retained its corresponding phytase activity at 70, 60 and 46% whereas native phytase of *A.niger* only retained 40% of its activity at 68°C heat treatment [30]. Also phyA gene expressed in

soybean cells declined rapidly at temperatures above 63°C [31]. Results of thermal satiability tests revealed that higher thermostabilty of phytase expressed in *P. pastoris* and *H.polymorpha* could be attributed to the impact of yeast glycosylaion pattern that impact on stability of expressed phytases [29, 32, 33], also SDS-PAGE results indicate high glycosylation pattern in yeasts (Figure 4).

Methylotrophic recombinant phytase half-life

To evaluate long time recombinant phytase stability and half-life, purified samples were heat treated at 80 °C for 60 min and more. Residual enzyme activity revealed that recombinant phytase expressed by *P. pastoris* and *H.polymorpha* loss of phytase activity of 35% after 60 min heat treatment, whereas *A.niger* native phytase when heat treated at 63.3°C had 60-70% reduced activity [34]. Furthermore thermostabilty study determined that *P. pastoris* and *H.polymorpha* recombinant phytases had respective half-life evaluations of 19 and 17 minutes at 80 °C. So, according to test results both recombinant phytases were determined as thermopile acid phytase.

Kinetic Characteristic

Kinetic constant Km and Vmax values of recombinant phytases, calculated at different concentrations of sodium phytate substrate and by the Michaelis–Menten equation are shown on Table 2. Assessment of kinetic parameters showed that Km value of *P. pastoris* recombinant phytase was considerably lower than that of *H.polymorpha*. The lower Km value is an advantage of *P.pastoris* recombinant phytase, so it has higher affinity for phytate higher and has sufficient degradation rate even at lower substrate concentration. Comparison of *P. pastoris* and *H.polymorpha* catalytic efficiency indicates that *P.pastoris* had higher K_{cat} / K_m rates, which might be an effect of variant extent of glycosylation pattern which could have a main role on phytase substrate binding site conformation and substrate affinity.

Conclusion

Although the phyA gene has been expressed as an active enzyme in *A.niger* [35], *A.terreus* [36], tobacco seeds [37] or leaves and soybean cells [38], the low expression level of phytase in these hosts is unsuitable for production of heterologous phytase for application as animal diet [39]. Application of microbial phytate degrading enzymes as a feed supplement requires a highly efficient and cost-effective production process by recombinant microorganisms. Among micro-organisms, methylotrophic *P.pastoris* [19] and *H.polymorpha* [40] expression hosts have advantages such as strong inducible promoter, post–translational modification and high expression in a cost effective medium; these are all strong reasons that both systems were selected as hosts for high phytase expression to make comparisons of relevant recombinant expressed phytases and biochemical properties. Since *A.niger* phytase has high affinity to phytate, the *phyA* gene from *A.niger* was selected, proper *P.pastoris* and *H.polymorpha* codon performance were optimized and genes were synthesized and successfully expressed extracellularly.

Table 2. Kinetic parameters of the purified Recombinant phytase of *P.pastoris* and *H.polymorpha*

Values are mean ±SE (n=3).

	K_m(μM)	V_{max}(μmol $min^{-1}mg^{-1}$)	K_{cat}(s^{-1})	(Kinetic efficiency) ($s^{-1}mol^{-1}$)
P.pastoris	148	135	168.75	1.14×10^6
H.polymorpha	170	113	116.16	0.685×10^6

Characterization of the biochemical properties of recombinant phytases in these tests was the first report of optimized synthetic *phyA* expression, biochemical and kinetic parameters comparison of two yeasts hosts as main candidates for highly industrial phytase production.

Biochemical characterization of purified recombinant phytate degrading enzymes, indicated that both recombinant enzymes had the same optimum pH of 5,5 and acidophilic character with broad pH range activity, which makes both recombinant phytases useful for application as feed additive. Comparison of temperature activity patterns determined that both recombinant phytases performed at slightly wide range of temperatures, which was probably due to yeast heavy glycosylaion potential. Recombinant phytase expressed by *P.pastoris* had optimum activity at 60°C in comparison with 50°C optimum temperature of *H.polymorpha* recombinant phytase, high optimum temperature activity potential could be an advantage of recombinant phytases that adopted them during the feed pelleting procedure. Comparison of thermostabilty in different heating treatments at 60 to 90°C by assessment of enzyme residual activity determined that both recombinant expressed phytases had similar behaviour and were active in the range of 60 to 90°C. Also, in comparison with native *A. niger* phytase, both recombinant phytases had significantly higher thermal stability which was probably related to higher level of yeast glycosylaion ability. Thermostabilty is an important issue in feed pelleting which is performed at temperature between 60 to 90°C [41], so both recombinant expressed phytases could be candidates for application in feed pelleting. Kinetic experiments demonstrated that phytase expressed in *H.polymorpha* in comparison with that of *P.pastoris*, showed a considerable decrease in catalytic efficiency and recombinant *P.pastoris* phytase have higher phytate affinity. In general, such different biochemical properties for behaviour of two recombinant expressed phytases could be the role of variant extent of carbohydrate moiety glycosylation pattern that is variable between yeasts. In conclusion, characterization of biochemical behaviour and kinetic parameters of recombinant phytases which were expressed in *P. pastoris* and *H. polymorpha* revealed that *P. pastoris* recombinant phytase successfully fulfilled a series of predefined industrial application criteria such as a broad range of optimum pH, good stability during storage, feed pelleting and substrate specificity, so it has good potential for application to a wide range of industries [29].

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