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Optimization of heterologous expression of the phytase (PPHY) of *Pichia anomala* in *P. pastoris* and its applicability in fractionating allergenic glycinin from soy protein

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Abstract The phytase (PPHY) of *Pichia anomala* has the requisite properties of thermostability and acidstability, broad substrate spectrum, and protease insensitivity, which make it a suitable candidate as a feed and food additive. The 1,389-bp *PPHY* gene was amplified from *P. anomala* genomic DNA, cloned in pPICZαA, and expressed extracellularly in *P. pastoris* X33. Three copies of *PPHY* have been detected integrated into the chromosomal DNA of the recombinant *P. pastoris*. The size exclusion chromatography followed by electrophoresis of the pure rPPHY confirmed that this is a homohexameric glycoprotein of ~420 kDa with a 24.3 % portion as N-linked glycans. The temperature and pH optima of rPPHY are 60 °C and 4.0, similar to the endogenous enzyme. The kinetic characteristics K_m, V_{max}, K_{cat}, and K_{cat}/K_m of rPPHY are 0.2 ± 0.03 mM, 78.2 ± 1.43 nmol mg^{-1} s^{-1}, 65,655 ± 10.92 s^{-1}, and 328.3 ± 3.12 μM^{-1} s^{-1}, respectively. The optimization of medium components led to a 21.8-fold improvement in rPPHY production over the endogenous yeast. The rPPHY titer attained in shake flasks could also be sustained in the laboratory fermenter. The rPPHY accounts for 57.1 % of the total secreted protein into the medium. The enzyme has been found useful in fractionating allergenic protein glycinin from soya protein besides dephytinization.

Keywords Phytase · *Pichia anomala* · Soya protein · Glycinin and β-conglycinin · Dephytinization

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Introduction

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is the major storage form of organic phosphorus in plant products, which accounts for 50–80 % of the total phosphorus [5]. In humans and other non-ruminants, bioavailability of phosphorous remains very low, as ingested phytate is largely excreted that causes both nutritional and environmental pollution problems. Phytase (myo-inositol hexakisphosphate phosphohydrolase) is an acid phosphatase capable of cleaving at least one phosphate from phytate. For improving the dietary phosphorus availability in monogastrics such as swine, poultry, and fish, mobilization of phytate phosphorus by phytate-degrading enzymes is desirable. Phytases of fungal origin are therefore added to animal feeds in order to ameliorate phosphorus availability and assimilation, and to mitigate anti-nutrient effects of phytates [9]. Phytases [myo-inositol hexakisphosphate 3-phosphohydrolase (EC 3.1.3.8) and myo-inositol hexakisphosphate 6- phosphohydrolase (EC 3.1.3.26)] belong to the family of histidine acid phosphatases (HAPs) and catalyze stepwise hydrolysis of phytic acid to inorganic phosphate and myo-inositol phosphate derivatives [15]. These enzymes are synthesized in plants, animals, and microorganisms in cell-bound or extracellular form. Since phytases from different sources vary in biochemical characteristics like temperature and pH optima, not all are suitable for industrial applications [9]. The available phytases are often unsuitable for practical use because of the constraints of thermostability and proteolytic cleavage. Phytases must sustain the heat denaturation of feed pelleting processes and proteolytic cleavage of the ingested phytase in stomach and small intestine by pepsin and trypsin. The cleavage of phytase results in the need for a relatively high level of phytase supplementation to animal feeds [24]. A versatile

phytase that overcomes these limitations would be more suitable for application as a food and feed additive.

Phytase from *P. anomala* (PPHY) is one of the well-characterized phytases. It is a cell-bound thermo-acid-stable phytase with broad substrate specificity [29] and thus is a suitable candidate for supplementation of poultry and fish feeds [30] and dephytinization of soymilk [10]. Despite extensive efforts, adequate levels of phytase production by *P. anomala* could not be attained by process optimization [11, 28]. *Pichia pastoris* has been a host of choice for heterologous expression of various proteins of eukaryotic origin [18] and therefore the *PPHY* gene has been successfully cloned and expressed extracellularly in *P. pastoris* for the first time in this investigation. A medium has been formulated that supports the production of high enzyme titers in shake flasks. The number of copies of the phytase integrated into *P. pastoris* genome has been determined and the recombinant phytase has been purified to homogeneity and characterized. The utility of phytase in the fractionation of allergenic glycinin from soy protein and dephytinization has been demonstrated.

Materials and methods

Strains, media, and oligonucleotides used

The strains, media, and culture conditions, and primer sequences used in this investigation are listed in Tables 1 and 2, respectively.

Checking the suitability of the *PPHY* gene for expression in *P. pastoris*

Before starting *PPHY* expression studies in eukaryotic host *P. pastoris*, phytase ORF (GenBank: FN641803.1) of *P. anomala* was analyzed for codon bias. The percentage usage of different codons for a particular amino acid was calculated and compared with that of a codon usage in the highly expressed proteins in *P. pastoris* [2].

Construction of rPPHY-pPICZαA

A two-step cloning strategy was used to clone the *PPHY* gene. Genomic DNA from *P. anomala* MTCC4133 was isolated according to Ledeboer et al. [14]. The phytase ORF was amplified using gene-specific primers (Phy F′ and Phy R′). The 1,389-bp PCR product flanked with *Eco*RI and *Xba*I restriction sites was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and the recombinants (*E. coli* DH5α carrying *rPPHY*-pGEMT) were selected by blue-white selection on Luria–Bertani (LB) agar supplemented with 100 μg ml^{-1} ampicillin and

Table 1 Strains, media, and culture conditions

Organism	Genotype	Growth medium	Growth temperature	Use	Medium for expression	Source
Escherichia coli DH5α	(*F-endA1 glnV44 thi-1recA1 relA1 gyrA96 deoR nupG* Φ*80dlacZΔM15* Δ (*lacZYA-argF*) *U169*, *hsdR17* (*rK− mK+*), λ−	LB[a], LSLB[b]	37 °C	Manipulation and maintenance of the–expression vector		Invitrogen, USA
Pichia anomala MTCC 4133	–	MYPG[c]	30 °C	Source of phytase ORF	CMM[d]	Lab culture collection
P. pastoris X33	Mut$^+$	YPD[e], YPDS[f]	30 °C	Mut$^+$ Expression host[h]	YMT[g]	Invitrogen, USA

[a] Luria–Bertani medium (pH 7.0)

[b] Low-salt Luria–Bertani medium (pH 7.5)

[c] Malt–yeast–peptone–dextrose medium (pH 6)

[d] Cane molasses medium (pH 5.5)

[e] Yeast extract–peptone–dextrose medium (pH 6.0)

[f] Yeast extract–peptone–dextrose–sorbitol medium (pH 7.5)

[g] Yeast extract–methano–Tween-80 medium (pH 6.0)

[h] Mut$^+$ designates methanol utilization plus phenotype and refers to the wild-type ability of strains to metabolize methanol as the sole carbon source

Table 2 Primers used in this investigation

Primers	Sequence (5′ → 3′)	Restriction site	T_m (°C)	Annealing temp (°C)
Gene amplification primer				
Phy F′	CCCGAATTCATGGTCGCTATACAAAAAGCTCTCGTTC	*Eco*RI	65	60
Phy R′	CCCTCTAGAAATCTCGTAATTCGCTCGGCGGTGGCATTGTACTG	*Xba*I	71	
Sequencing primers				
T7	TAATACGACTCACTATAGGG	–	47	36
SP6	TATTTAGGTGACACTATAG	–	41	
Primers for probe amplification				
AOX F′	GCAAATGGCATTCTGACATCC	–	62	55
AOX R′	GACTGGTTCCAATTGACAAGC	–	60	

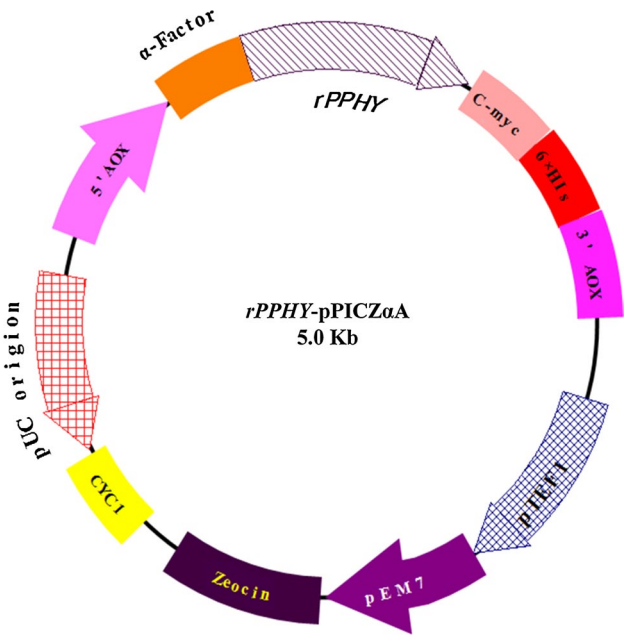

Fig. 1 Pictorial presentation of recombinant vector r*PPHY*-pPICZαA

0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 μg ml^{-1} X-gal. The insert was reconfirmed by colony PCR and double digestion. *rPPHY*-pGEMT plasmid was isolated using plasmid isolation kit (Macherey-Nagel, Düren, Germany) and double digested using *Eco*RI and *Xba*I to release the phytase fallout. The fallout was subcloned into pPICZαA (Invitrogen, Carlsbad, CA, USA), and linearized using *Eco*RI and *Xba*I. The linearized pPICZαA was dephosphorylated with CIAP (Calf Intestinal alkaline phosphatase, NEB, USA). Double-digested *PPHY* and pPICZαA were subjected to ligation using T4 DNA ligase (NEB, USA), and the ligated product was transformed into *E.coli* DH5α. Zeocin-resistant transformants were selected on low-salt Luria–Bertani (LSLB) medium supplemented with 25 μg $ml^{-1}$of Zeocin (InvivoGen, San Diego, CA, USA). Positive transformants harboring *rPPHY*-pPICZαA (Fig. 1) were screened by colony PCR and confirmed by double digestion.

Transformation and screening of rPPHY secreting *P. pastoris* integrants

Electrocompetent *P. pastoris* cells were prepared according to Cregg et al. [4]. An amount of 10 μg of *Pme*I linearized single-copy vector was used to transform *P. pastoris* electrocompetent cells. Electroporation was done according to *Pichia* expression kit manual (Invitrogen, Carlsbad, CA, USA). Yeast colony PCR was used to identify positive clones harboring phytase ORF. Spheroplasting of the *P. pastoris* was done before proceeding to colony PCR by swirling single yeast colony in 30 μl buffer containing 1 U of lyticase (Sigma). After spheroplasting, 5 μl of the suspension was used as a template in PCR mix. Yeast colonies harboring *PPHY* ORF were plated on Yeast extract-Peptone-Dextrose-Sorbitol (YPDS) agar containing 100 μg ml^{-1} Zeocin. Integrants resistant to 100 μg ml^{-1} Zeocin were grown on higher concentrations (500 and 1,000 μg ml^{-1}) of Zeocin. *P. pastoris* clones resistant to 1,000 μg ml^{-1} Zeocin concentration were screened for rPPHY production. These clones were first grown in Yeast extract-Peptone-Dextrose (YPD) broth and then in Yeast extract-Peptone (YP) medium containing 0.5 % (v/v) methanol. The production of rPPHY by these clones was checked by quantitative phytase assay.

Quantitative phytase assay

Extracellular and cell-bound phytase activity in the recombinant *P. pastoris* clones were assayed by determining the amount of phosphate liberated from hydrolysis of calcium phytate. For preparing protein extract, *P. pastoris* cells were homogenized in a French press (Constant systems, UK) at 20 Kpsi and then the filtrate containing intracellular proteins was used to assay intracellular rPPHY and the cell-free culture filtrate was used to assay extracellular rPPHY.

The reaction mixture containing 0.1 ml appropriately diluted enzyme, 0.4 ml acetate buffer (50 mM, pH 4.0), and 0.5 ml 4 mM calcium phytate was incubated at 60 °C for 15 min. For color development, 2 ml of acetone-ammonium molybdate solution (100 % acetone, 5 N H_2SO_4, 100 mM ammonium molybdate in a 2:1:1 ratio) were added to the reaction mixture, followed by 0.1 ml of 0.1 M citric acid to stop the reaction. The phosphate released was determined according to Heinonen and Lahti [6]. One unit (U) of phytase is defined as the amount of enzyme that liberates 1 nmol of inorganic phosphate ml^{-1} s^{-1} under the defined assay conditions.

Gene copy number in the recombinant *P. pastoris*

The copy number of the *PPHY* gene in recombinant *P. pastoris* was determined by Southern hybridization. Genomic DNA was isolated from *P. anomala, rPPHY* harboring *P. pastoris* clone, and host *P. pastoris* using genomic DNA isolation kit (Zymo Research). Genomic DNA was quantified in a BioSpectrophotometer (Eppendorf). DNA (2 μg) from each source was digested overnight with *Nde* I (NEB) that does not cut within the *PPHY* ORF. The fragments were separated electrophoretically on 0.8 % agarose gels (Sigma, USA) at 10 V for 18 h and transferred onto a positively charged nylon membrane (Sigma, USA) by capillary transfer using the Southern transfer protocol [20]. Hybridization was carried out with a ~500-bp probe, which was amplified using pPICZαA as template with AOX F′ and AOX R′ primers (Table 2). The *AOX* probe was biotinylated using Biotin DecaLabel DNA Labeling Kit (Fermentas). Biotinylated probes were detected with streptavidin coupled to alkaline phosphatase. The detection was performed by colorimetry using the Biotin Chromogenic Detection Kit (Fermentas) that uses BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) substrate systems. The developed blot was digitized and stored at 4 °C.

Statistical optimization of rPPHY production

The critical cultural variables for attaining maximum phytase production were first identified by Plackett–Burman (PB) design [17]. The medium composition that resulted in the secretion of the highest enzyme titer after PB screening was considered as the basal medium, and used for optimization by response surface methodology (RSM) using central composite design (CCD). The levels of three independent variables [yeast extract (A), Tween-80 (B) and methanol (C)] were selected for further optimization by RSM. The statistical software package Design-Expert 6.0 (StatEase, Inc., Minneapolis, MN, USA) was used for analyzing the experimental design and data. Each factor in the design was studied at five different levels (−α, −1, 0, +1, +α) (Table 3). A set of 20 experiments was carried out. All variables were taken at a central coded value considered as zero. Upon completion of the experiments, the average maximum phytase production was considered as the dependent variable or response (*Y*). A second-order polynomial equation was then fitted to the data by a multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

Table 3 Range of variables chosen for RSM

Variable (%)	α	−1	0	+1	−α
A: Yeast extract	0.25	0.5	1.0	1.5	1.75
B: Tween-80	0.1	0.2	0.3	0.4	0.5
C: Methanol	1.0	2.0	3.0	4.0	5.0

For a three-factor system, the model equation is as given below:

$$Y = \beta 0 + \beta 1A + \beta 2B + \beta 3C + \beta 11A^2 + \beta 22B^2 + \beta 33C^2 + \beta 12AB + \beta 23BC + \beta 13AC$$

where *Y*, predicted response; $\beta 0$, intercept; $\beta 1$, $\beta 2$, $\beta 3$, linear coefficients, $\beta 11$, $\beta 22$, $\beta 33$, squared coefficients; $\beta 12$, $\beta 23$, $\beta 13$, interaction coefficients. To obtain the optimum concentration of the medium components, Design-Expert software was used to generate response surface curves using the above model. The model was validated by the solutions suggested by the software. The recombinant phytase production was also carried out in a 7-l laboratory fermenter (Applicon, The Netherlands) with 3-l working volume. The fermenter was operated at 30 °C and 250 rpm (rotation per minute) with 1 vvm aeration and without maintaining pH. To induce rPPHY production, methanol 3 % (v/v) was added aseptically into the fermenter via injection port every 24 h. The samples were drawn at the desired intervals aseptically and the culture filtrates were used in the phytase assays and determining soluble protein and yeast biomass.

Purification of rPPHY

The culture supernatant containing rPPHY was concentrated by lyophilization using a freeze dryer (FD-1) and freezing bath (EYELA, Japan). The lyophilized rPPHY was reconstituted in a minimum volume of sodium acetate buffer (50 mM pH 4.0). Anion exchange column chromatography was performed using DEAE Sepharose CL-6B (Sigma) matrix. A linear gradient of 0.1–1 M NaCl in 50 mM Na-acetate buffer (pH 4.0) was used for eluting the bound rPPHY. The enzyme was further purified using size exclusion chromatography

(SEC) on Sephacryl S-200 high-resolution column (16/60). Cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), sweet potato β-amylase (200 kDa), and ferritin (Mr 450 kDa) were used as molecular weight standards. Further analysis was performed by subjecting rPPHY to electrophoresis under denaturing conditions on gels containing 12 % (w/v) polyacrylamide.

Zymogram development

The pure rPPHY was run on 8 % native-PAGE and the gel was washed with 100 mM sodium acetate buffer (pH 4.0) and kept at 4 °C immersed in the same buffer for 30 min. After 2 h of immersion, the buffer was replaced with 1 % sodium phytate solution prepared in 100 mM sodium acetate buffer (pH 4.0) and kept at 60 °C for 2 h allowing rPPHY to act upon the substrate. A 10 % $CaCl_2$ solution was poured on the gel to resolve the zone of hydrolysis.

Glycoprotein staining and deglycosylation of the rPPHY

Periodic acid-Schiff (PAS) staining was carried out to assess the glycosylation level of rPPHY where oligosaccharide moieties were oxidized by periodic acid and stained with Schiff's reagent [13]. To further confirm glycosylation status of rPPHY, N-linked glycan portion was removed using 0.5 IU of endoglycosylase H (*Endo*H, NEB) for 1 h at 37 °C. Non-deglycosylated and deglycosylated phytases were run on SDS-PAGE to see the difference in running pattern on the gel.

Biochemical characterization of rPPHY

Purified rPPHY was used for its biochemical characterization. The effect of pH on enzyme activity was studied by carrying out enzyme assay in buffers of different pH. Glycine–HCl buffer (pH 2.0, 3.0), sodium acetate buffer (pH 4.0, 5.0, 6.0), Tris maleate buffer (pH 7.0, 8.0) and glycine–NaOH buffer (pH 9.0–12.8) were used in determining the optimum pH. Similarly, optimum reaction temperature was determined by assaying rPPHY activity at various temperatures (30–90 °C) at optimum pH. Kinetic constants V_{max}, K_m, and K_{cat} were determined at optimum temperature and pH by measuring rPPHY activity at different concentrations of calcium phytate and drawing Lineweaver–Burke plot.

Application of recombinant rPPHY: separation of allergenic glycinin from soy protein and its dephytinization

In six sets of reactions, 10 U of rPPHy was added to 10 mg soy protein suspended in 100 mM acetate buffer (pH 5.0) in 1 ml of reaction volume. The reaction mixture was incubated at 60 °C for 3 h in a water bath. One set was removed every 30 min for determining the released inorganic phosphate and protein analysis. The precipitated proteins were sedimented by centrifugation at 10,000×*g* for 10 min, and the supernatant and pellet fractions were analyzed by SDS-PAGE for the presence of different soy proteins. Liberated phosphorous was estimated according to Heinonen and Lahti [6].

All experiments were conducted in triplicate and the average values are presented with standard deviation.

Results and discussion

The codon optimization has been reported to result in the improved co-translational folding and production of recombinant protein in *P. pastoris* [2] and therefore codons of the *PPHY* gene were checked before proceeding to the expression of PPHY in *P. pastoris*. Upon comparison of *PPHY* codons with that of highly expressed genes in *P. pastoris*, it was found that *PPHY* has a positive bias for the *P. pastoris* system. Most of the amino acids in PPHY are coded by the triplet codons, which are also preferentially used in *P. pastoris* (Suppl. Table 1) and thus the *PPHY* gene appeared to be a promising candidate for expression in *P. pastoris.*

Pichia pastoris was transformed with *rPPHY*-pPICZαA and Zeocin-resistant transformants were picked for further studies. As higher production of recombinant proteins by recombinants resistant to high concentration of Zeocin has been established earlier [1, 33], transformants were subsequently grown on increasing concentrations of Zeocin (100–1,000 μg ml^{-1}). Fifteen transformants were able to survive up to 1,000 μg ml^{-1} Zeocin. These clones resistant to high Zeocin concentration were further checked for rPPHY accumulation levels. Quantitative phytase assay and zymogram development confirmed that clone 13 produced the highest extracellular rPPHY titer (30 U ml^{-1}) and thus was selected for further studies. It was found that the clone 13 did not accumulate rPPHY in the cell-bound form. All rPPHY was present only in culture broth that suggests efficient functioning of α-secretion signal.

Southern hybridization confirmed that three copies of the *rPPHY* gene were integrated into genome of clone 13. In this investigation, the vector size plus insert size is approximately 4.9 kb. A band of ~25 kb was detected with the genomic DNA of the clone 13 and ~10 kb with the genomic DNA of the host. Thus, a ~15-kb shift in the band location was observed in comparison to the host, which is equivalent to the presence of three tandem copies of *rPPHY* at *AOXI* locus (Fig. 2). The presence of one copy of gene of interest at *AOXI* locus leads to upward shift of the band

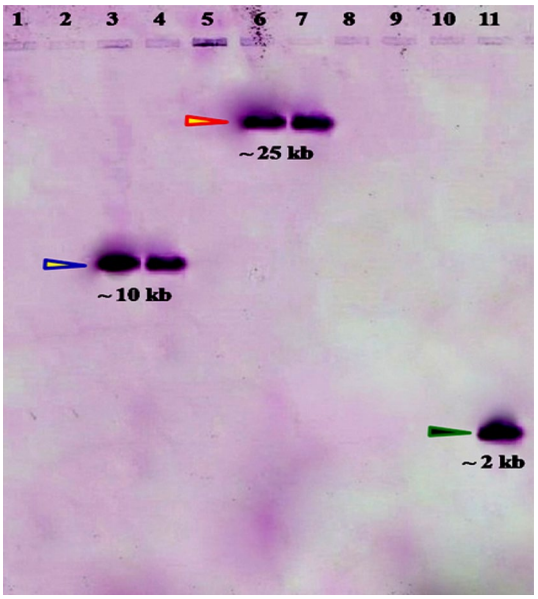

Fig. 2 Determination of *rPPHY* copy number by Southern blotting. *Lane 1* molecular weight marker. *Lanes 3 and 4 Nde*I-digested genomic DNA of *P. pastoris* X33. *Lanes 6 and 7 Nde*I-digested genomic DNA of recombinant *P. pastoris* harboring rPPHY. *Lane 9 Nde*I-digested genomic DNA of *P. anomala* (negative control). *Lane 11* AOX F′ and AOX R′-amplified PCR product (positive control)

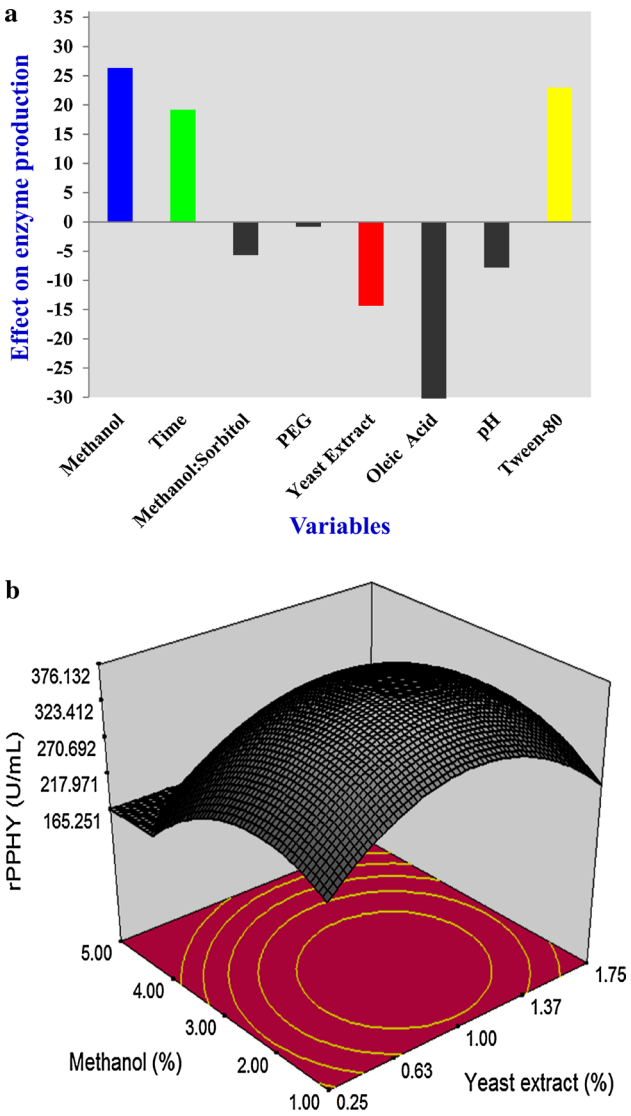

Fig. 3 Media optimization to maximize rPPHY production. **a** Pareto graph showing effect of different media components on rPPHY production (higher concentration of methanol, increased induction time intervals, and Tween-80 exerts a positive effect on rPPHY production, while the presence of co-substrate sorbitol during induction, higher concentration of yeast extract, and oleic acid exerted a negative effect. Polyethylene glycol had no observable effect on rPPHY production. **b** Response surface graph showing the effect of interaction of yeast extract with methanol on rPPHY production

equivalent to vector size plus insert size [8] and thus the insertion of three copies of the *rPPHY* at the *AOX I* locus in *P. pastoris* was confirmed.

An optimized combination of fermentation variables is known to improve recombinant protein production in *P. pastoris* [31]. The effect of methanol concentration, time of induction, presence of co-substrate sorbitol, PEG-8000, yeast extract, oleic acid, and pH of the medium on rPPHY production was assessed using Plackett–Burman (PB) design. An *F* value of 24.08 and the values of 'Prob > *F*' less than 0.05 indicated that the model terms are acceptable (Suppl. Table 2a). The dummy variables included had no impact on rPPHY production, implying that the model is significant. The factors that affect rPPHY production (yeast extract, Tween-80, time of induction, and concentration of methanol) have been identified. A lower level of yeast extract and a high level of methanol supported rPPHY production (Fig. 3a). Both lower and higher concentrations of methanol have been reported to affect recombinant protein production due to the limitation of carbon source for growth of the yeast and toxicity, respectively [4], and therefore it is necessary to determine optimal levels for maximum product formation. The production of rPPHY was supported by high levels of Tween-80 and longer induction interval. The inclusion of oleic acid in the medium has been shown to increase protein expression under alcohol oxidase promoter [12, 16]. In contrast, oleic acid exerted a negative effect on rPPHY production, while other factors had no observable effect (Fig. 3a). The concentrations of methanol, Tween-80, and yeast extract were optimized by response surface methodology (RSM), where interactions between these parameters were analyzed. The *F* value of the model was 59.70 and the 'Prob > *F*' value was <0.0001, indicating that the model is highly significant. The coefficients of all linear model terms *A*, *B*, and *C* and squared model terms A^2, B^2, and C^2 are significant. The interaction of *A* with *C* is also a significant model term (Suppl. Table 2b). The coefficient of

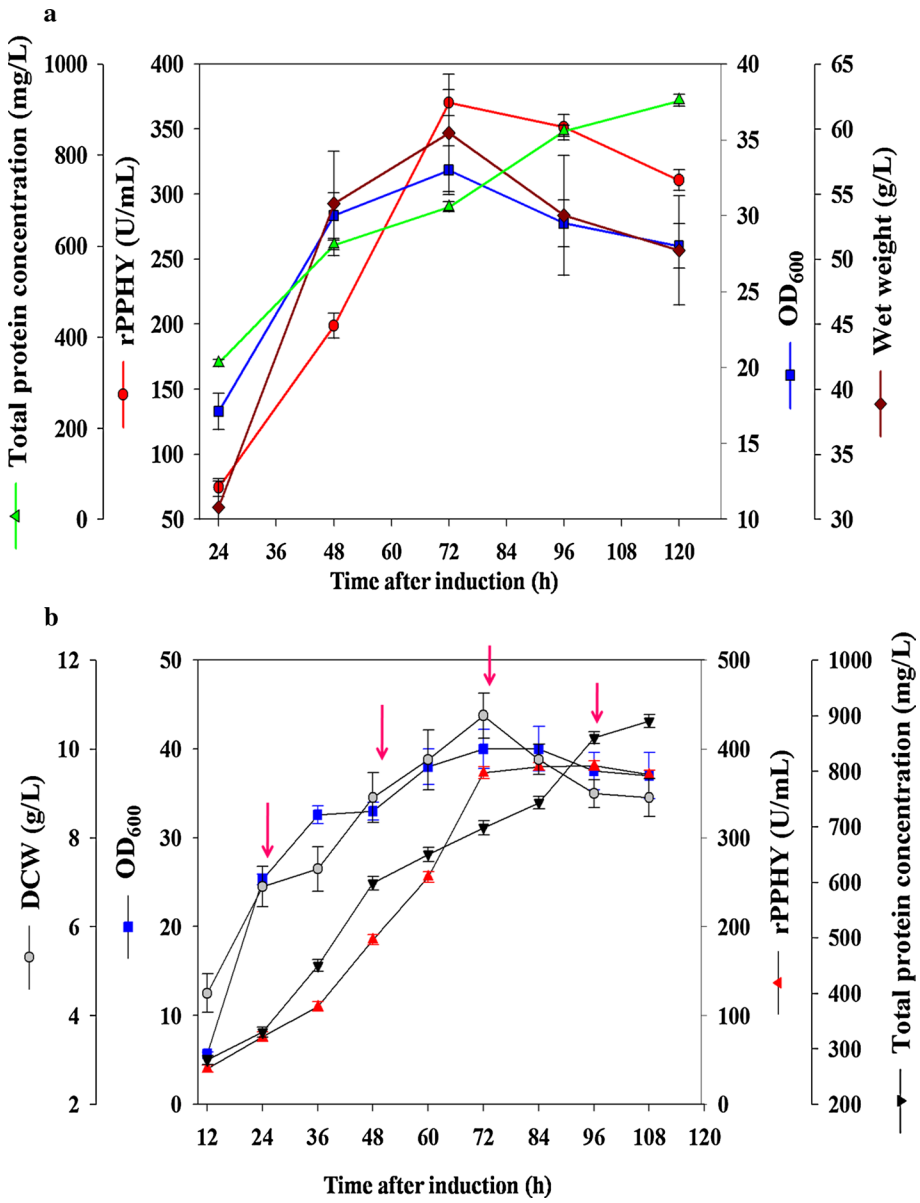

Fig. 4 Production of rPPHY. **a** The production of rPPHY in a 7-l laboratory fermenter: *open circles* denote dry cell weight (g l^{-1}), *filled circles* rPPHY production (U ml^{-1}), *filled squares* OD_{600}, *filled triangles* total protein (mg l^{-1}). *Down pointing arrow* indicates time of addition of methanol. **b** Production profile of rPPHY in 0.25-l shake flasks under optimized conditions. *Filled circles* rPPHY (U ml^{-1}), *filled squares* OD_{600}, *filled up pointing triangles* total protein (mg l^{-1}) and *filled down pointing triangles* dry cell weight (DCW) (g l^{-1})

Table 4 Improvement in rPPHY production due to optimization

Organism	Phytase production (U ml^{-1})	Fold improvement
P. anomala	17.0	1.0
P. pastoris Clone 13 (unoptimized)	30.0	1.8
P. pastoris Clone 13 (optimized)	371.0	21.8

determination (R^2) was 0.9817 for rPPHY production. The predicted R^2 (0.8536) and adjusted R^2 (0.9653) are in reasonable agreement with each other (Suppl. Table 3a). The adjusted R^2 corrects the R^2 value for the sample size and for the number of terms in the model. Adequate precision is a measure of the signal-to-noise ratio and a value greater than 4.0 is desirable. The adequate precision value of 21.34 indicated an adequate signal and suggested that the model can be used to navigate the design space. The following model equation explains the model:

$$(Y) = +368.97 + 23.91 * A + 28.92 * B - 59.59 * C - 101.28 * A^2 - 31.99 * B^2 - 98.81 * C^2 - 17.62 * A * B + 22.13 * A * C - 9.38 * B * C$$

where Y represents the response, i.e., phytase production, and A, B, C, A^2, B^2, C^2, AB, AC, and BC are the variables.

Maximum rPPHY production was attained at 3 % (v/v) methanol when added at every 24-h interval, while keeping Tween-80 and yeast extract at 0.3 % (v/v) and 1 %

Table 5 Summary of purification of rPPHY

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Fold purification
Culture filtrate	32,396.34	68.34	474.33	100.00	1.00
Lyophilized concentrate	30,256.73	41.30	732.61	93.40	1.55
Anion exchange chromatography	9,586.00	12.40	773.10	29.60	1.63
Gel-filtration chromatography	8,721.00	10.50	830.60	27.00	1.80

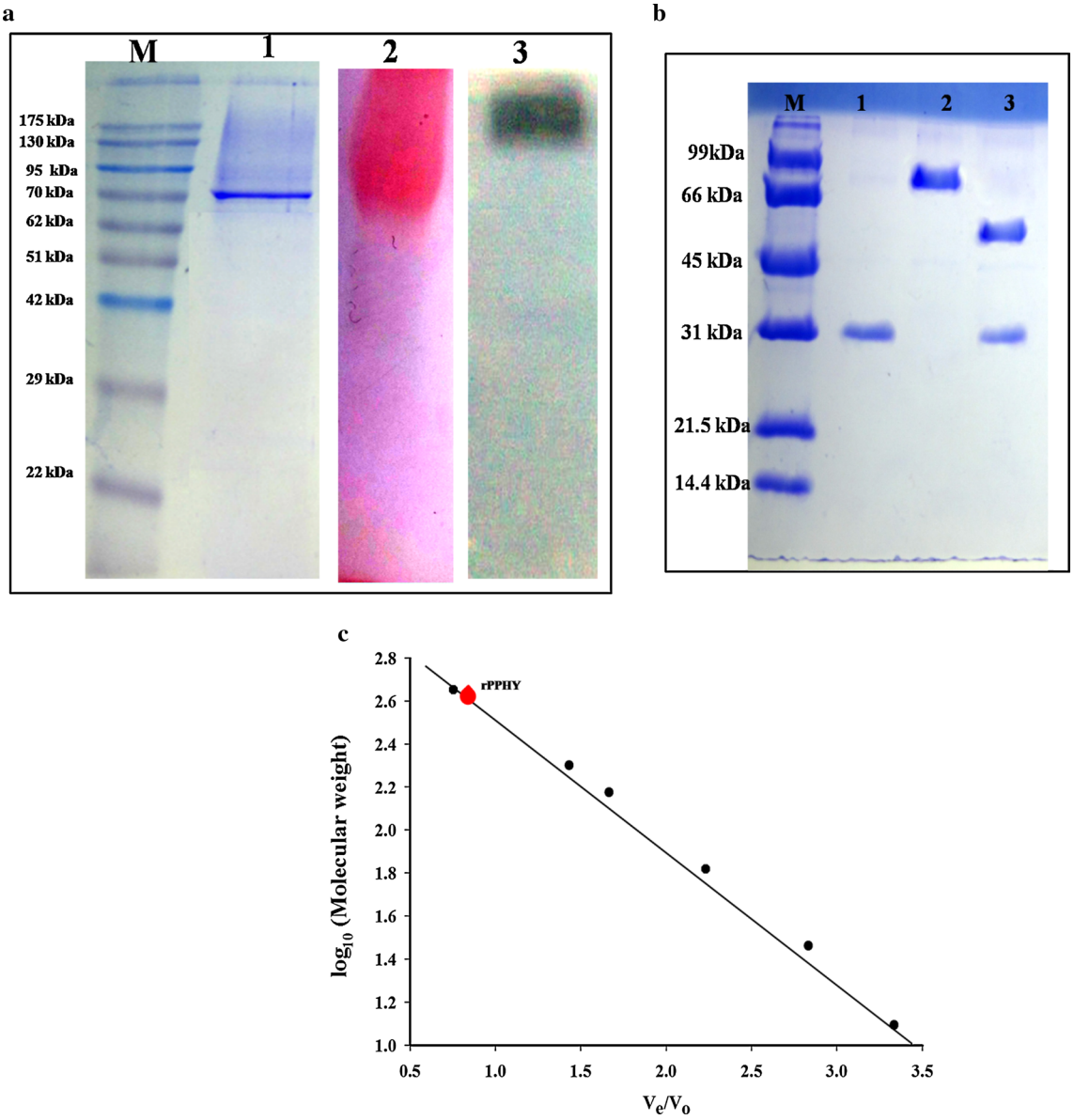

Fig. 5 Purification of rPPHY to homogeneity. **a** PAGE analysis of rPPHY. *Lane M* molecular weight marker. *Lane 1* purified rPPHY. *Lane 2* PAS stained rPPHY. *Lane 3* zone of hydrolysis due to hydrolytic action of rPPHY. **b** Removal of N-linked glycan portion from rPPHY. SDS-PAGE analysis of glycosylated and deglycosylated rPPHY. *Lane M* molecular weight markers. *Lane 1 Endo*H. *Lane 2* rPPHY after deglycosylation with *Endo*H. *Lane 3* rPPHY before treatment with *Endo*H. **c** Plot of V_e/V_o against semi-log of molecular weight of proteins on Sephacryl S-200 high-resolution column (16/60) for α-Lactoalbumi (12.4 kDa), carbonic anhydrase (30 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), sweet potato β-amylase (200 kDa), rPPHY (~420 kDa), and ferritin (Mr 450 kDa)

(w/v), respectively. A positive interaction was observed only between yeast extract and methanol (Fig. 3). When the model was validated in shake flasks, the experimental and predicted values were very close and thus proved the validity of the model (Suppl. Table 3b). Final enzyme production profile in shake flasks is presented in Fig. 4a. The enzyme production is sustainable in 7 l of laboratory fermenter (Fig. 4b). The medium containing 1 % yeast extract (w/v), 0.3 % Tween-80 (v/v), and 3 % methanol (v/v) supported highest rPPHY production. Optimization of fermentation parameters led to a 21.8-fold improvement in rPPHY production as compared to that of the endogenous yeast (Table 4). Enhancement in the production of recombinant proteins had been achieved by optimizing variables like methanol concentration, addition of co-substrates such as sorbitol during induction phase, Tweens, and oleic acid [32, 34].

The purification of rPPHY from cell-free culture supernatant led to a 1.8-fold purification with 27 % yield (Table 5). The purity of the rPPHY was confirmed by SDS-PAGE analysis and the identity of this protein was confirmed by zymogram analysis on native PAGE as indicated by clear zone of phytate hydrolysis (Fig. 5a).

Table 6 Comparison of properties of rPPHY with endogenous cell bound phytase

Property	rPPHY	Endogenous phytase [29]
pH optimum	4.0	4.0
Temperature optimum (°C)	60	60
Native mw (homohexamer)	~420 kDa	~384 kDa
Subunit mw	~70 kDa	~55 kDa
Calculated mw	~50.82 kDa	~51.74 kDa
V_{max} (n mol mg^{-1} s^{-1})	78.13 ± 1.43	105.67 ± 2.84
K_m (mM)	0.2 ± 0.03	0.2 ± 0.02
K_{cat} (s^{-1})	65,655 ± 10.92	ND
K_{cat}/K_m (μM^{-1} s^{-1})	328.28 ± 3.12	ND

mw molecular weight, *ND* not determined

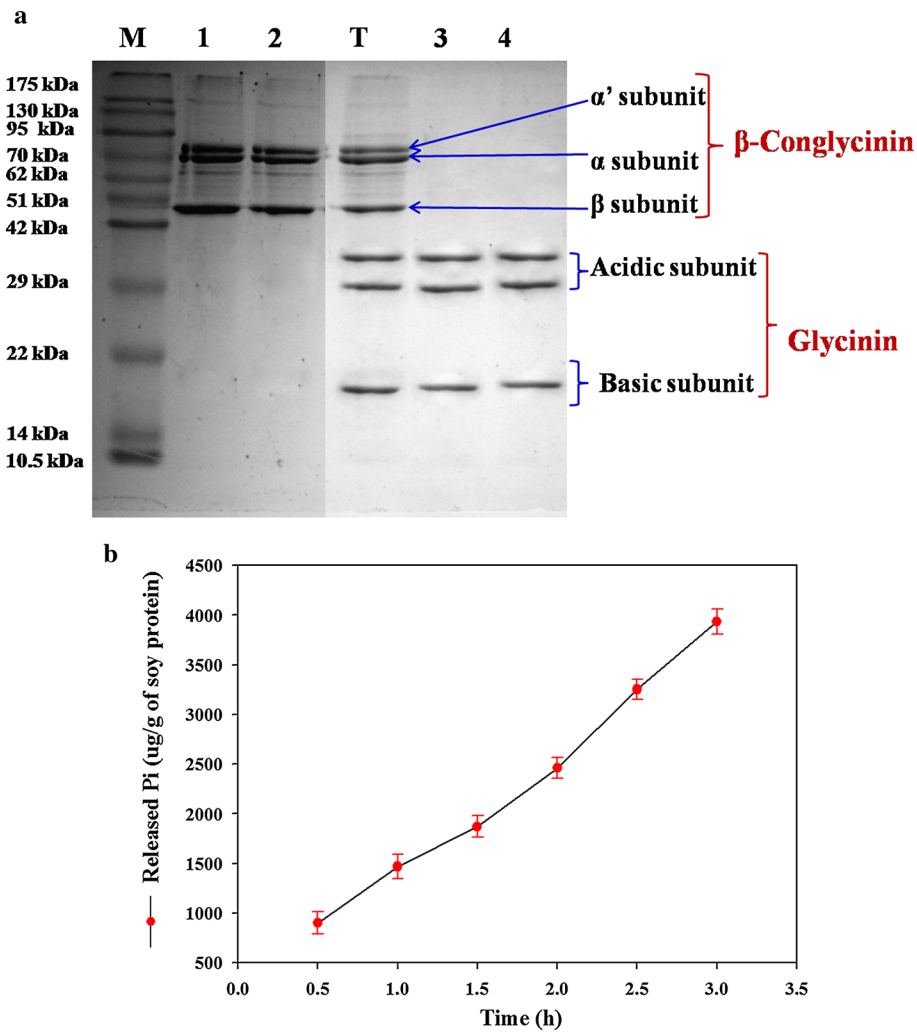

Fig. 6 Fractionation of β-conglycinin and glycinin. **a** SDS-PAGE profile of separated glycinin and β-conglycinin by the action of rPPHY. *Lane M* molecular weight markers. *Lanes 1–2* β-conglycinin fraction. *Lane T* total soy protein. *Lanes 3, 4* glycinin fraction. **b** Dephytinization of soy protein

The molecular mass of the purified rPPHY determined by SDS-PAGE and gel filtration are ~70 (Fig. 5b) and ~420 kDa, respectively (Fig. 5c), suggesting that this is a homohexamer. Phytase of *A. niger* had also been reported to be oligomeric in nature (pentamer of 353 kDa with a monomer of 66 kDa) [26]. Upon deglycosylation, rPPHY was reduced from ~70 to ~53 kDa (Fig. 5b), which is close to its theoretical molecular weight as reported in the *Debaryomyces castellii* CBS 2923 [19]. This confirms that rPPHY is a glycoprotein with 24.3 % N-linked carbohydrate. Glycoprotein nature of rPPHY was further confirmed by oxidizing carbohydrate with periodic acid followed by staining with Schiff's reagent that turned the band pink (Fig. 5a). This staining method was used for confirming glycoprotein nature of the phytase of *Sporotrichum thermophile* [23]. Glycosylation is considered to be essential for the secretion of proteins. According to Verivouri et al. [27], glycosylation is required for thermal and pH stability of secreted cellulases and for protection against proteolysis. Partial or complete removal of carbohydrate rendered cellulases less heat- and pH stable.

A comparison of biochemical properties of rPPHY with endogenous phytase is given in Table 6. The recombinant phytase exhibited optimum activity at 60 °C and pH 4.0 like the endogenous phytase [29]. The K_m of rPPHY is 0.2 mM ± 0.03 (for calcium phytate), which is also similar to K_m of the endogenous phytase, indicating that both the enzymes have equal affinity for phytate. The V_{max} of rPPHY is 78.2 ± 1.43 nmol mg^{-1} s^{-1}, while it is 105.67 ± 2.84 nmol mg^{-1} s^{-1} for the endogenous phytase. Turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) of rPPHY are 65,655 s^{-1} ± 10.92 and 328.28 ± 3.12 μM^{-1} s^{-1}, respectively.

Soy protein contains around 1.47 % phytate [25]. The phytate content may affect solubility, fractionation, and the related function of soy protein components in various soy foods [3]. Soybean has been categorized as one of the 'big 8' allergens [22]. Most of the allergic reactions in the soy proteins are caused by two of its components, glycinin and β-conglycinin, which are oligomeric proteins [7]. In this investigation, the treatment of soy protein with rPPHY for 3 h led to the separation of glycinin (Fig. 6a) and liberation of phytate-bound phosphorous (Fig. 6b). Upon treatment with rPPHY, glycinin was precipitated due to the hydrolysis and removal of phytate. The removal of phytate reduced the solubility of glycinin that led to its precipitation, while β-conglycinin fraction remained soluble in the supernatant due to its glycoprotein nature [21]. This finding may pave the way for the use of PPHY in producing improved quality as well as special foods for people who are allergic to the specific components of soy protein.

Conclusions

The cloning and expression of the phytase of *P. anomala* in *P. pastoris* extracellularly is being reported for the first time. The recombinant clone contains three copies of the *PPHY* gene integrated into the genome. By optimization of medium components, 21.8-fold enhancement in enzyme production was achieved over the endogenous yeast. The rPPHY is a homohexameric glycoprotein with 24.3 % N-linked carbohydrate, which is useful in separating glycinin from the soy protein besides dephytinization. Our efforts are underway to bring down the cost of enzyme production using cheaper industrial by-products such as molasses and corn steep liquor in the fermentation medium, and selection of mode and scale-up of fermentation.

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References

1. Arjmand S, Lotfi AS, Shamsara M, Mowla SJ (2013) Elevating the expression level of biologically active recombinant human alpha 1-antitrypsin in *Pichia pastoris*. Electron J Biotechnol. doi:10.2225/vol16-issue1-fulltext-4
2. Bai J, Swartz DJ, Protasevich II, Brouillette CG, Harrell PM, Hildebrandt E, Gasser B, Mattanovich D, Ward A, Chang G, Urbatsch IL (2011) A gene optimization strategy that enhances production of fully functional P-glycoprotein in *Pichia pastoris*. PLoS ONE 6(8):e22577
3. Chen BHY, Morr CV (1985) Solubility and foaming properties of phytate-reduced soy protein isolate. J Food Sci 50:1139–1142
4. Cregg JM, Tolstorukov I, Kusari A, Sunga J, Madden K, Chappell T (2009) Expression in the yeast, *Pichia pastoris*. Methods Enzymol 463:69–189
5. Harland BF, Morris ER (1995) Phytate: a good or bad food component. Nutr Res 15:733–754
6. Heinonen JK, Lahti RJ (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal Biochem 113:313–317
7. Holzhauser T, Wackermann O, Ballmer-Weber BK, Bindslev-Jensen C, Scibilia J, Perono-Garoffo L, Utsumi S, Poulsen LK, Vieths S (2009) Soybean (*Glycine max*) allergy in Europe: Gly m 5 (β-conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. J Allergy Clin Immunol 123(2):452–458
8. Inan M, Aryasomayajula D, Sinha J, Meagher MM (2005) Enhancement of protein secretion in *Pichia pastoris* by over expression of protein disulfide isomerase. Biotechnol Bioeng 93:771–778
9. Kaur P, Kunze G, Satyanarayana T (2007) Yeast phytase: present scenario and future perspectives. Crit Rev Biotechnol 27:93–109
10. Kaur P, Satyanarayana T (2005) Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: optimization using statistical tools. Process Biochem 40:3095–3102
11. Kaur P, Satyanarayana T (2010) Improvement in cell-bound phytase activity of *Pichia anomala* by permeabilization and

applicability of permeabilized cells in soymilk dephytinization. J Appl Microbiol 108:2041–2049
12. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K (2000) Addition of oleic acid increases expression of recombinant human serum albumin by the AOX2 promoter in *Pichia pastoris*. J Biosci Bioeng 89(5):479–484
13. Leach BS, Collawan JF Jr, Fish WW (1980) Behaviour of glycopolypeptides with empirical molecular weight estimation methods. 1. Sodium dodecyl sulphate. Biochemistry 19:5734–5741
14. Ledeboer AM, Edens L, Maat J, Visser C, Bos JZ, Verrips CT, Janowicz Z, Eckart M, Roggenkamp R, Hollenberg CP (1985) Molecular cloning and characterization of a gene coding for methanol oxidase in *Hansenula polymorpha*. Nucleic Acids Res 13:3063–3082
15. Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, van Loon AP (1997) The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiol 143:245–252
16. Pereira GG, Hollenberg CP (1996) Conserved regulation of the *Hansenula polymorpha* MOX promoter in *Saccharomyces cerevisiae* reveals insights in the transcriptional activation by Adr1p. Eur J Biochem 238:181–191
17. Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 37:305–325
18. Prielhofer P, Maurer M, Klein J, Wenger J, Kiziak C, Gasser B, Mattanovich D (2013) Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*. Microb Cell Fact 12:5
19. Ragon M, Neugnot-Roux V, Chemardin P, Moulin G, Boze H (2008) Molecular gene cloning and over expression of the phytase from *Debaryomyces castellii* CBS 2923. Protein Expr Purif 58:275–283
20. Sambrook J, Russell DW (2006) Southern blotting: capillary transfer of DNA to membranes. Cold Spring Harbor Protocols pdb.prot4040-pdb.prot4040
21. Satio T, Kohno M, Tsumura K, Kugimiya W, Kito M (2001) Novel method using phytase for separating soyabean β-conglycinin and glycinin. Biosci Biotechnol Biochem 65(4):884–887
22. Sicherer SH, Sampson HA (2006) Food allergy. J Allergy Clin Immunol 117(2):470–475
23. Singh B, Satyanarayana T (2013) Characterization of a HAP-phytase from a thermophilic mould *Sporotrichum thermophile*. Biores Technol 100:2046–2051
24. Stahl CH, Han YM, Roneker KR, House WA, Lei XG (1999) Phytase improves iron bioavailability for haemoglobin synthesis in young pigs. J Anim Sci 77:2135–2142
25. Tajoddin MD, Shinde M, Lalitha J (2011) In vivo reduction the phytic acid content of Mung Bean (*Phaseolus aureus* L) cultivars during germination. J Agric Environ Sci 10(1):127–132
26. Vats P, Banerjee UC (2005) Biochemical characterisation of extracellular phytase (myo-inositol hexakisphosphate phosphohydrolase) from a hyper-producing strain of *Aspergillus niger* van Teighem. J Ind Microbiol Biotechnol 32:141–147
27. Verivouri H, Seigler KM, Sands JA, Montenecourt BS (1985) Regulation of cellulose biosynthesis and secretion in fungi. Biochem Soc Trans 13:411–414
28. Verma D, Satyanarayana T (2012) Phytase production by the unconventional yeast *Pichia anomala* in fed-batch and cyclic fed-batch fermentations. Afr J Biotechnol 11:13705–13709
29. Vohra A, Satyanarayana T (2002) Purification and characterization of a thermostable and acid-stable phytase from *Pichia anomala*. World J Microbiol Biotechnol 18:687–691
30. Vohra A, Satyanarayana T (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol 23:29–60
31. Wang Y, Wang Z, Du G, Hu Z, Liu L, Li J, Chen J (2009) Enhancement of alkaline polygalacturonate lyase production in recombinant *Pichia pastoris* according to the ratio of methanol to cell concentration. Biores Techol 100:1343–1349
32. Woo JH, Liu YY, Neville DM Jr (2006) Minimization of aggregation of secreted bivalent anti-human T cell immunotoxin in *Pichia pastoris* bioreactor culture by optimizing culture conditions for protein secretion. J Biotechnol 121:75–85
33. Xuan NT, Hang MT, Thanh VN (2009) Cloning and over expression of an *Aspergillus niger* XP Phytase Gene (phyA) in *Pichia pastoris*. WASET 56:750–753
34. Yu P, Yan Y, Tang YP (2011) Medium optimization for endochitinase production by recombinant *Pichia pastoris* ZJGSU02 using response surface methodology. Afr J Biotechnol 10(1):75–84