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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Bioprocess for efficient production of recombinant *Pichia anomala* phytase and its applicability in dephytinizing chick feed and whole wheat flat Indian breads

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Abstract The phytase of the yeast *Pichia anomala* (PPHY) is a suitable biocatalyst as a food and feed additive because of its adequate thermostability, acid stability, protease insensitivity and broad substrate spectrum. The cell-bound nature and low phytase titres are the main bottlenecks for its utility in food and feed industries. In this investigation, we have overcome the problems by constitutive secretory expression of PPHY under glyceraldehyde phosphate dehydrogenase (GAP) promoter. A ~44-fold increase in rPPHY titre has been achieved after optimization of cultural variables by one-variable-at-a-time approach and two factorial statistical design. The use of GAP promoter makes the cultivation of the recombinant *P. pastoris* straight forward and eliminates the requirement of methanol for induction and hazards associated with its storage. Among metal-phytate complexes, Ca^{2+} phytate is hydrolyzed more efficiently by rPPHY than Co^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+} phytates. The enzyme is effective in dephytinizing whole wheat unleavened flat Indian breads (naan and tandoori) and different broiler feeds, thus mitigating anti-nutritional effects of phytates.

Keywords *Pichia anomala* · Recombinant phytase · Anti-nutrient factor · Dephytinization · Unleavened flat wheat breads

Introduction

Since ancient times, wheat flour is used in making flat breads which are consumed by people in India and other Asian countries. Different types of breads are prepared depending on the type of wheat flour. For example, finely milled white wheat flour is used in making pizza, pita, focaccia, naan and others. While high bran and fiber rich flour is used in making chapathi, paratha, roti, poori, tandoori and others. Whole wheat flour contains up to 4 mg g^{-1} of phytic acid [6]. Most of the phosphorous in plant-based animal diets is in the form of phytic acid [21]. Phytic acid is a potent anti-nutrient factor (ANF) as it chelates nutritionally important metal ions, and forms complexes with dietary proteins and carbohydrates which are difficult to digest. The phosphorus from phytates remains unavailable to monogastrics (humans, poultry birds, swine, and fishes) because of inadequate levels of phytic acid hydrolyzing enzymes in their gastrointestinal tract (GIT) [1]. In the areas of intensive animal rearing, undigested phytates enter into the environment through feces and cause eutrophication of aquatic bodies and environmental pollution [23]. The adverse nutritional and environmental effects of the phytates are a major concern for food and feed industries. The supplementation of foods and feeds with phytase aids in overcoming aforementioned problems. Phytase is one of the industrially important animal feed additive enzymes in swine and poultry [20]. The global feed enzyme market is expected to increase to $1193 million by 2018, with a compound annual growth rate of 7.3 %

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(https://www.asdreports.com). The inclusion of phytase in the diets of monogastrics mitigates anti-nutrient effects of phytastes, ameliorates the assimilation of phytate phosphorus and other nutrients and growth, besides reducing the addition of phosphate to their diets. The recombinant *Pichia pastoris* strains have been extensively used for the production of industrially important enzymes and other products [5, 40, 41]. During the last few decades, inducible alcohol oxidase (AOX1) and constitutive GAP promoters have been used for recombinant protein expression in *P. pastoris* [10, 13, 19, 38]. Although inducible promoters permit a continuous control of gene expression, their applications are limited by transcriptional heterogeneity at the single cell level, inducer toxicity and inducer-mediated pleiotropic effects [30]. AOX1 is one of the stringently regulated promoters, but it is repressed by glucose, glycerol and ethanol, and also poses fire hazard due to use of inflammable methanol. To overcome the problems, there has been an emphasis on developing a promoter library based on a constitutive promoter that permits steady-state regulation of gene expression and ensures transcriptional homogeneity without an inducer.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in glycolysis. Its' promoter (GAP) provides strong constitutive expression on glucose at a level comparable to that of AOX1 [39], and has been widely used for constitutive expression of heterologous proteins in *P. pastoris* [7, 13, 19]. The additional advantage of using GAP for industrial fermentations is that methanol is not required for induction. This makes microbial growth straightforward without the need of shifting the culture from a medium containing one carbon source to the other, and thus, eliminating the hazards and costs associated with the storage and delivery of large volumes of methanol. Moreover, the size of GAP (477 bp) is smaller than AOX1 (960 bp), making the former suitable for generating efficient expression cassettes or mutant libraries [24].

The production of recombinant *P. anomala* phytase (rPPHY) has been reported recently in *P. pastoris* under AOX1 promoter [11]. In this investigation, a higher secretory PPHY expression has been achieved using *P. pastoris* integrant harboring a *PPHY* gene under GAP promoter. A ~44-fold increase in PPHY expression has been attained through optimization of nutritional and physiological parameters. The recombinant phytase thus produced is efficient in dephytinizing metal-phytate complexes, broiler feeds and whole wheat dough used in making unleavened flat Indian breads.

Materials and methods

Construction of r*PPHY*-pGAPZαA

Gene-specific primers (PPHY *Eco*RI F′ and PPHY *Xba*I R′) were used for amplifying phytase ORF using genomic DNA of *P. anomala* as template (Table 1). The phytase ORF thus obtained was cloned in pGAPZαA (Invitrogen, Carlsbad, CA, USA) between *Eco*RI and *Xba*I restriction sites. Positive clones harboring *rPPHY*-GAPZαA (Fig. 1) were selected according to Joshi and Satyanarayana [11].

Generation of rPPHY producing *P. pastoris* integrants

The electrocompetant *P. pastoris* cells were prepared and transformed according to the manufacturers' protocol (Invitrogen, Carlsbad, CA, USA). Recombinant *P. pastoris* colonies were screened by colony PCR and positive integrants were checked for rPPHY production. Primary cultures of these integrants were grown in 5 mL Yeast extract-Peptone-Dextrose (YPD) broth at 30 °C and 250 rpm for 24 h. Fresh 50 mL YPD was inoculated with 2 mL of primary culture and grown at 30 °C and 250 rpm for 120 h. For the time course study of rPPHY production by various clones, aliquots were withdrawn at every 24 h for phytase assay.

Table 1 Primers used in this investigation

Primer	Polynucleotide sequence 5′ → 3′	T_m (°C)	Product size (bp)	Application
PPHY *Eco*RI F′	CCCGAATTCATGGTCGCTATACAAAAAGCTCTCGTTC	65	~1400	Primers for PPHY amplification
PPHY *Xba*I R′	CCCTCTAGAAATCTCGTAATTCGCTCGGCGGTGGCATTGTA CTG	71		
GAP F′	GTCCCTATTTCAATCAATTGAA	48.8	~1929	Sequencing primers
AOX R′	GACTGGTTCCAATTGACAAGC	60		
qPHY F′	GTTGACCGAGGGTAACGAAG	55.4	131	Primers used for RT-qPCR
qPHY R′	CCAATTCGTCTTGGGTAAAGA	52.6		
qGAP F′	CAAGTACGACTCTACCCACAAGGC	59.1	179	
qGAP R′	CCTCCAAAGTGGTGAAAACACCG	57.1		

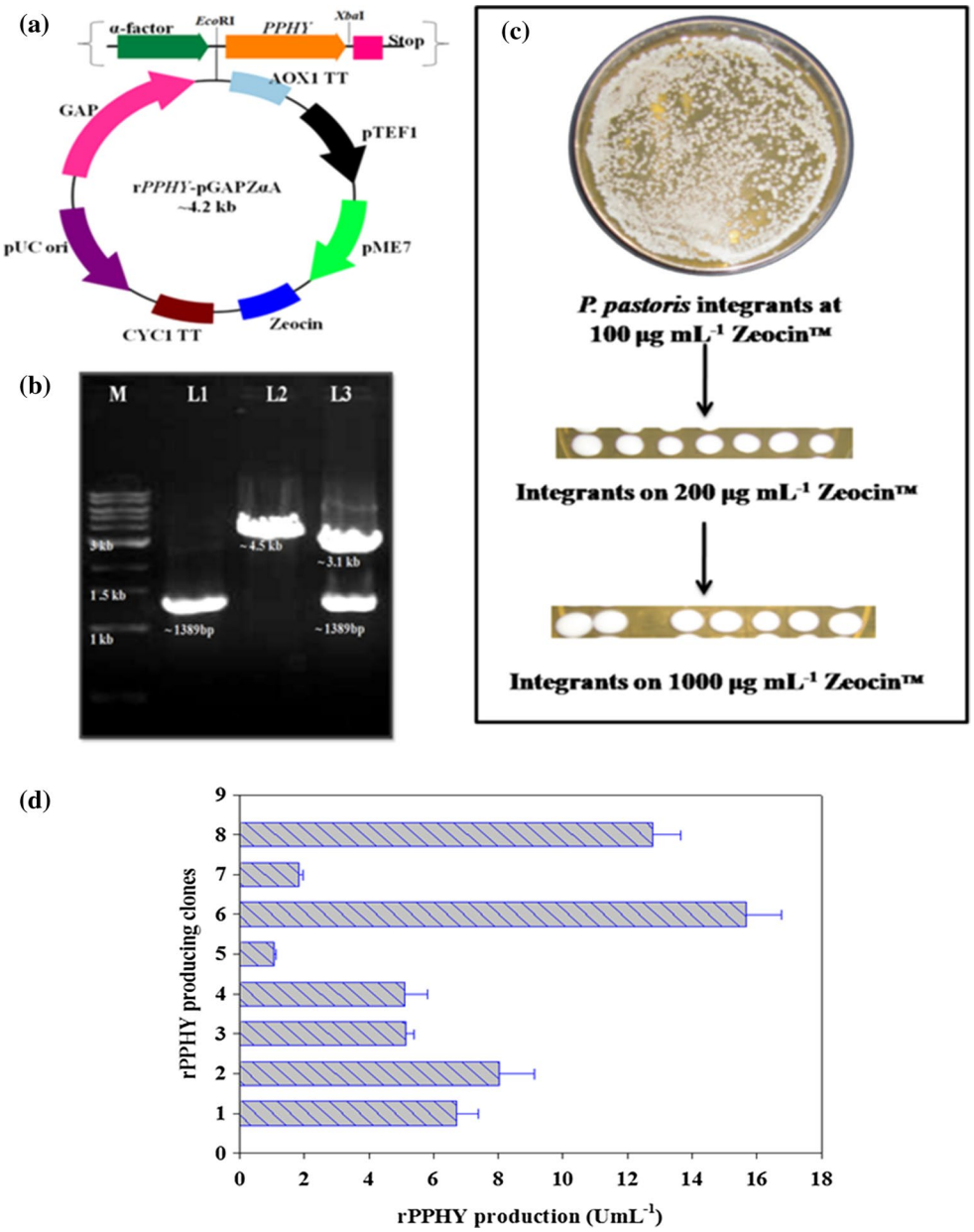


Fig. 1 Construction of r*PPHY*-pGAPZαA and generation of recombinant *P. pastoris*, r*PPHY*-pGAPZαA construct (**a**). Confirmation of r*PPHY*-pGAPZαA construction by double digestion (**b**) where L1 is double digested *PPHY*, L2 is single digested r*PPHY*-pGAPZαA and L3 is double digested r*PPHY*-pGAPZαA showing rPPHY fallout. Recombinant *P. pastoris* transformants have been grown at different concentrations of Zeocin™ (**c**). The production of rPPHY by various *P. pastoris* integrants while screening (**d**)

Non-integrant *P. pastoris* was taken as the negative control in all the cases. All the experiments were performed in triplicates and the average values are presented with ±SD values.

Quantitative assay for phytase

Phytase was assayed according to Joshi and Satyanarayana [11]. The phosphate liberated by the action of rPPHY was determined according to Heinonen and Lahti [9]. One unit (U) of phytase is defined as the amount of enzyme that liberates 1 nmol of inorganic phosphate per second under the defined enzyme assay conditions.

Selection of the efficient rPPHY producing *P. pastoris* integrant

The production of rPPHY by recombinant *P. pastoris* integrants was studied in YPD medium. The clone that produced highest PPHY titre was selected for further investigation.

Determination of gene copy number by quantitative real-time PCR (qRT-PCR)

For determining r*PPHY* gene copy number, qPCR was performed with the clone selected in previous step. The qPCR experiments were conducted with the Applied Biosystems

7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA). The data were analyzed using SDS 2.0 software. RNA-free genomic DNA was used as the template. Primers were designed according to Fast SYBR®Green Master Mix protocol in such a way that length of amplified products is between 150 and 200 bp. The melting temperature (T_m) of primers was kept between 58 and 60 °C. The primer sets employed in this investigation are listed in Table 1. The concentration of each primer in the reaction mix was kept 50 nM. The specificity of amplification was confirmed by melt-curve analysis after 45 cycles. All experiments were performed in triplicates with the software recommended program (stage 1: 50 °C for 2 min; stage 2: 95 °C for 2 min; stage 3: 40 cycles of 95 °C for 15 s and 60 °C for 1 min followed by stage 4, i.e., 95 °C for 15 s and 60 °C for 15 s). To construct the standard curves for GAPDH, a tenfold dilution series of genomic DNA ranging from 3×10^7 to 3×10^2 copies μL^{-1} was used and the C_T values were plotted against logarithm of genomic DNA copies. The concentration of the genomic DNA was determined in a Nanodrop spectrophotometer and corresponding genomic DNA copies were calculated according to Schutter et al. [28].

The length of *P. pastoris* genome is 9.43 Mbp [28]. The efficiency (E) of qPCR amplification and absolute copy number of *PPHY* were calculated according to Lee et al. [14].

Optimization of rPPHY production

Selection of the basal medium

Medium M1, M2 and M3 were tested for their suitability for rPPHY production [Medium M1 (YPG) %: yeast extract 1.0, peptone 2.0, glycerol 2.0, pH 6.0; Medium M2: YPG + *Pichia* trace metal solution (PTM) 0.44 % (v/v), pH 6.0; Medium M3 (YPD + PTM) %: yeast extract 1.0, peptone 2.0, dextrose 2.0, PTM 0.44, pH 6.0; PTM solution contained (g L^{-1}): $CuSO_4.5H_2O$ 6.0, KI 0.09, $MnSO_4.H_2O$ 3.0, H_3BO_3 0.02, $MoNa_2O_4.2H_2O$ 0.2, $CoCl_2$ 0.5, $ZnCl_2$ 20.0, $FeSO_4.7H_2O$ 65.0, biotin 0.2 and H_2SO_4 5.0 mL].

One-variable-at-a-time (OVAT) approach

The effect of temperature, agitation, different carbon sources, surfactants and nitrogen sources were studied by single factor experiments (Supplementary data). In each experiment, one factor was varied, while keeping other factors constant.

Two factorial designs with five factors

As statistical methods are known to aid the production of microbial enzymes [12, 35], factorial design was employed to study the effect of following five factors (independent variables) on the rPPHY production: Tween-80 (A), ammonium chloride (B), concentration of glycerol (C), sorbitol (D) and yeast extract (E). The levels of selected factors were chosen based on preliminary experiments and the levels used include: Tween-80 (0.3, 0.5 and 0.7 % v/v), ammonium chloride (0.2, 0.3 and 0.4 %), glycerol (0.75, 1.0 and 1.25 % v/v), sorbitol (0.75, 1.0 and 1.25 %) and yeast extract (0.75, 1.0 and 1.25 %). Factorial design of type 2^5 with four center points was used to study the effect of selected factors within a specified range (Supplementary Tables A1, A2).

Recombinant phytase production was determined by assaying phytase at every 24 h over 4 days. The experiment was performed in twenty runs including 4 center points, as suggested by the software. All twenty combinations were run in triplicate and their mean values were used for analysis. All the experiments were run in random fashion to reduce the effect of unexpected variability in the experiential responses. The observations were evaluated by analysis of variance (ANOVA) to study the effect of selected factors and their potential interaction that affects rPPHY production. The Design-Expert 6.0 software (StatEase, Inc., Minneapolis, MN, USA) was used for data analysis.

To fit the experimental data, polynomial model (2FI) for regression can be given as following equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum \sum_{i<j} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y is the response, β_0 is the intercept of the model, β_i is the linear coefficient in the regression model, β_{ij} is the interaction coefficient between different factors and ϵ represents the error term.

Validation and scale-up of phytase production

The model was validated by performing a set of suggested solutions by the software. The rPPHY production was also carried out in 7 L laboratory fermenter (Applicon, Netherlands) under the optimized conditions. The fermenter containing 3 L production medium [0.75 % (w/v) yeast extract, 0.2 % (w/v) NH_4Cl, 1.25 % (v/v) glycerol, 1.25 % (w/v) sorbitol, 0.7 % (v/v) Tween-80] was operated at 30 °C, 300 rpm with 1 vvm of aeration. The samples were withdrawn aseptically at desired time intervals for determination of rPPHY activity, total soluble protein and yeast biomass.

Dephytinization of metal-phytate complexes

To study the hydrolysis of metal-phytates, 100 mM stock solutions of metal ions (Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}

and Zn^{2+}) were prepared by dissolving $CaCl_2{\cdot}2H_2O$, $CoCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, $MgSO_4{\cdot}7H_2O$ and $ZnCl_2{\cdot}7H_2O$ in distilled water [32, 33]. Equal volumes (0.8 mL each) of 100 mM salt solution and 10 mM sodium phytate were mixed and incubated overnight at 4 °C. The precipitated salts were centrifuged at 2000 rpm at 4 °C for 1 min. The supernatants were decanted and the precipitated salts were washed thrice with distilled water and finally suspended in 0.5 ml of 0.1 M Na-acetate buffer (pH 4.0) [32, 33].The hydrolysis reactions were initiated by incubating each phytate salt with recombinant phytase (10 U) at 45 °C. Aliquots of the mixture were drawn at the desired intervals, and the salts were sedimented by centrifugation. The amount of inorganic phosphate in the supernatant was quantified.

Applicability of rPPHY in dephytinization of unleavened flat wheat breads (tandoori and naan)

The rPPHY was used for preparation of non-fermented tandoori and naan (unleavened flat Indian breads). Five units of rPPHY were added to per gram of atta/maida flour, kneaded with luke warm tap water. The dough was then covered with a wet cotton cloth and was kept aside at room temperature for 5 h. For preparation of tandoori bread, the clay oven was preheated for 30 min such that the temperature reaches ~230 °C. The atta dough weighing ~85 g was flattened and cooked on the wall of preheated oven for 40–50 s. Similar procedure was followed for preparation of Naan using maida dough weighing ~123 g. The breads prepared without addition of rPPHY were treated as controls. The reducing sugars in the breads were determined using dinitrosalicylic acid (DNSA) reagent [18]. The protein content was determined according to Lowry et al. using BSA as the standard [16]. The wet and dry weights of the breads were determined for calculating the moisture content. Inorganic phosphate content was determined as mentioned earlier.

Dephytinization of wheat bran, rice bran and various poultry feeds under simulated gut condition

Wheat bran, rice bran (which are ingredients of poultry feeds) and different broiler feeds used at different developmental stages of poultry birds [Broiler Pre-Starter Feed (BPSF), Broiler Starter Feed (BSF), Broiler Finisher Feed (BFF) (supplementary data)] were autoclaved at 15 lb psi and 120 °C for 20 min to inactivate any phytase present in these samples. The average body temperature of a poultry bird is 40 °C (http://www.poultryhub.org/production/husbandry-management/housing-environment/climate-in-poultry-houses/). The pH of crop and proventriculus remains ~5.0 and ~3.5 (http://www.thepoultrysite.com/articles/978/maintaining-gut-integrity) and transit time of the food in crop and proventriculus are 50–90 min, respectively. The feed hydrolysis assay was carried out based on this information. One gram of the autoclaved sample was suspended in 20 mL of acetate buffer (0.1 M, pH 4.0) and the suspension was incubated with pure recombinant phytase (225 U mL^{-1} of feed suspension) at 40 °C on a rotary shaker at 100 rpm (Kuhner, Switzerland). Aliquots were drawn periodically and the amount of liberated inorganic phosphate was determined as described earlier.

Results and discussion

The PCR-amplified *PPHY* gene was cloned into pGAPZαA. As *PPHY* gene was flanked with *Eco*RI and *Xba*I restriction sites, the construction of *rPPHY*-pGAPZαA (Fig. 1a) was confirmed by double digestion (Fig. 1b). Among several integrants generated from *Avr*II linearized *rPPHY*-pGAPZαA (Fig. 1c), nine displayed phytase production. The clone 6 exhibited higher rPPHY production than others, and therefore, was selected for further studies (Fig. 1d). The PPHY has been expressed extracellularly under GAP promoter in *P. pastoris* for the first time in this investigation. There are several studies on protein expression under methanol-induced AOX promoter, but the safe and robust constitutive GAP promoter has not been exploited fully at industrial level. The pGAPZαA vector was chosen because it allows the ORF fusion to α-factor mating signal sequence which has been used successfully in secretion of foreign proteins [10, 11, 37].

The clone 6 harbors single copy of *PPHY*, as determined by absolute qRT-PCR. R^2 value, for the housekeeping gene (GAP) and *PPHY* was ~0.99 and these were amplified with 109 and 100 % efficiency, respectively (Fig. 2). To increase the recombinant phytase titres, cultivation parameters were optimized. Among different media tested, complex medium M1 supported constitutive expression of rPPHY (Fig. 3a), and therefore, was selected for further optimization. Other complex media containing trace metals did not support rPPHY production. The major difference in M1 and other media tested is the absence of *Pichia* trace metals, which could hinder the quantitative estimation of phytase from the culture supernatant.

Among carbon sources tested, glycerol supported high rPPHY titres, followed by oleic acid, methanol and sorbitol, while the production was very low in lactose containing medium. Among the combinations of carbon sources tested, glycerol–sorbitol supported a higher production of rPPHY (Fig. 3b). A combination of glycerol–sorbitol was, therefore, selected for rPPHY production instead of single carbon source. A variety of carbon sources have been used for GAP promoter-driven expression of recombinant

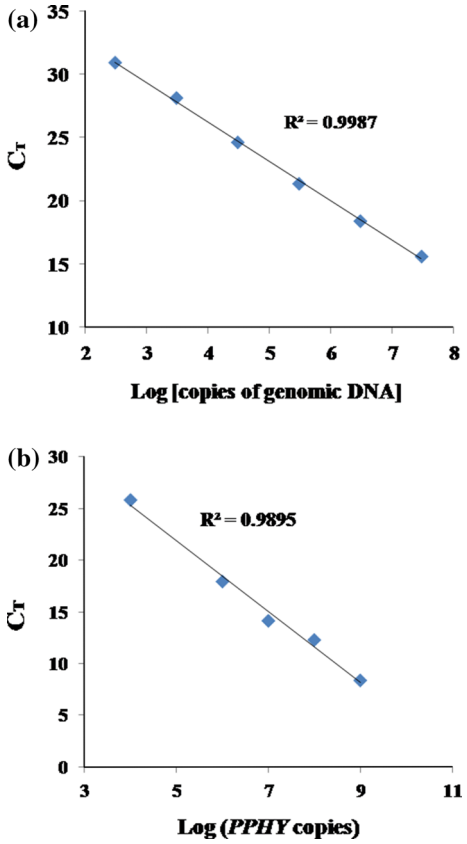


Fig. 2 qPCR standard curves. qPCR standard curve for *GAP* gene **(a)**. C_T values were determined by qPCR and plotted against logarithm of template copies. RNA-free genomic DNA from *P. pastoris* integrant was isolated as described in the Materials and methods section. The standard curve was constructed with tenfold dilutions of genomic DNA ranging from 3×10^7 to 3×10^2 copies μL^{-1}. All PCR amplifications were conducted in triplicate and average values are presented in the graph. qPCR standard curve for *PPHY* gene **(b)**

proteins [4, 22]. The choice of carbon source is dependent on the nature of the protein, as different carbon sources are suitable for expressing different proteins [29, 34]. Glycerol supported a high yield of human angiostatin [34], while glucose played a similar role in other cases [4, 39]. Glycerol decreased the production of recombinant L2 lipase under GAP promoter [29]. Sorbitol is known to enhance cell viability and decrease proteolytic degradation of proteins that leads to improvement in recombinant protein production [37].

A high rPPHY production was attained in the medium containing PEG 20,000, which could be due to enhanced secretion of protein from permeabilized cell membrane. Non-ionic surfactant Tween-80 exerted a marked effect on rPPHY production as compared to Triton-X100 (Fig. 3c). Tween-80 enhanced rPPHY production at 0.7 % concentration. Membrane permeabilization facilitates protein secretion, where a combination of both hydrophobic and ionic interactions plays a major role in binding of membrane proteins with lipids [21]. Alteration of microenvironment by adding salts, polyalcohols, surfactants, sugars and polymers is also a strategy for increasing the stability of enzymes [3]. Tween-80 interacts with cell membranes and other surfaces that alter permeability facilitating the release of exoenzymes from the cell [25]. Calado et al. reported enhanced secretion of chitinases in *Saccharomyces cerevisiae* in the presence of Tween-80 in the production medium [2].

As methylotrophic yeasts are capable of utilizing a variety of nitrogenous compounds, various organic and inorganic nitrogen sources were tested to study their effect of rPPHY production. The choice of nitrogen source appears to vary from protein to protein, and therefore, needs to be optimized individually. Among nitrogen sources tested, ammonium chloride, ammonium sulfate and triammonium citrate supported high rPPHY titres (Fig. 3d). Triammonium citrate and ammonium chloride supported almost similar level of rPPHY production. NH_4Cl along with yeast extract supported a higher rPPHY production than other combinations.

Increase in the initial absorbance (A_{600}) of the production medium did not have a marked effect on rPPHY production (Fig. 3e) and a high enzyme titre was attained even at a low initial absorbance (A_{600}:0.8). The levels of the important factors identified by OVAT methodology have been further optimized by 2^5 factorial design. The factorial central composite design (CCD) is routinely used in various optimization studies [26].

Among the factors tested, two (Tween-80 and yeast extract) were observed to critically influence rPPHY production by *P. pastoris*. The results of 20-run factorial design including the input factors, their levels and the observed values of response are presented in supplementary Table A2. The ANOVA analysis of data suggested that yeast extract and ammonium chloride interact positively resulting in enhanced rPPHY production (supplementary Table A3). This suggests that yeast extract, ammonium chloride, glycerol, and sorbitol not only work independently, but their effect depends on the levels of other factors.

Lower concentration of factor E (yeast extract) caused a high response. With an increase in factor B (NH_4Cl), the observed response was even higher (Supplementary Fig. A1). Similarly, upon increase in the concentration of factor D (sorbitol), the response increased, but the effect was more pronounced with higher concentration of factor C (glycerol). Their interaction is, therefore, more significant than the effect of these individual factors (Supplementary Fig. A1).

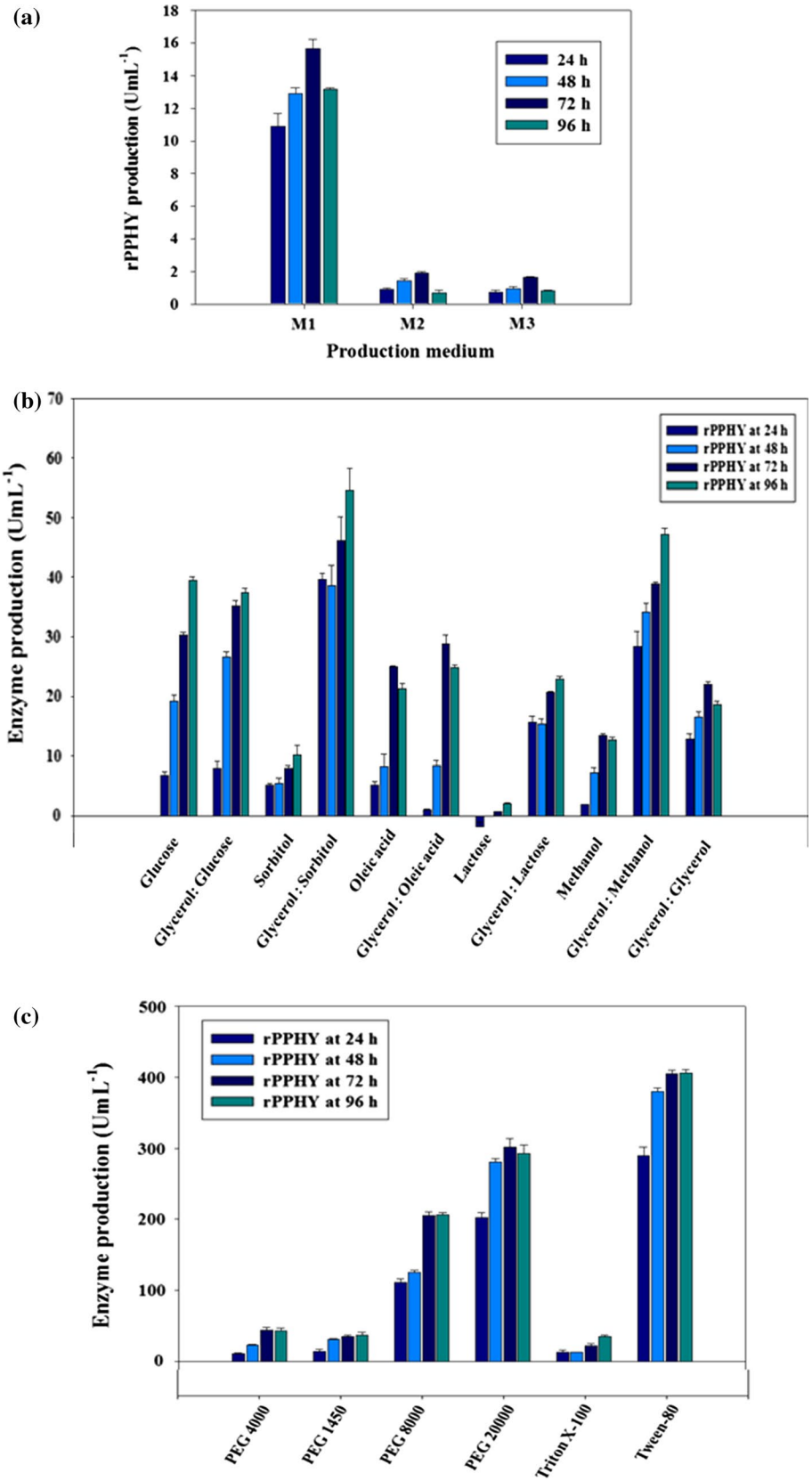
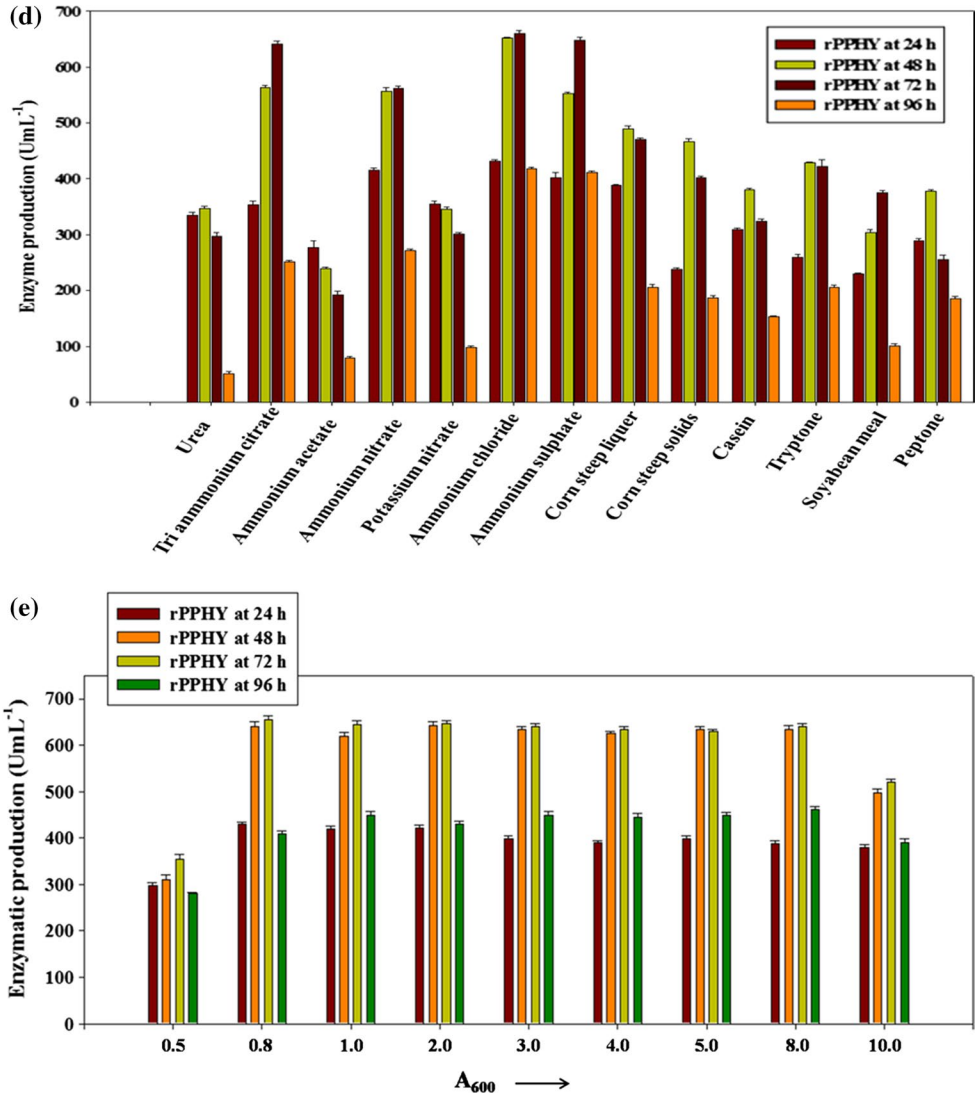


Fig. 3 rPPHY production profile in different media by clone 6, where medium M1 supported rPPHY production (**a**). Effect of different carbon sources, and in combination with glycerol (**b**) and surfactants (**c**) on phytase production. Effect of various organic and inorganic nitrogen sources on rPPHY production (**d**) and initial absorbance (A_{600}) (**e**) on rPPHY production

Fig. 3 continued



The regression model for rPPHY production can be explained by the following model equation:

$$\text{Response 1} = +382.81 + 86.88 * \text{A} + 53.46 * \text{B} - 13.58 * \text{C} + 57.45 * \text{D} - 144.66 * \text{E} + 42.05 * \text{A} * \text{B} + 64.42 * \text{B*} \text{E} + 49.29 * \text{C} * \text{D} \quad (2)$$

where Response 1 is rPPHY (U mL^{-1}), while A, B, C, D, E, AB, BE, and CD are different variables studied in the model. The regression model in Eq. fitted the data adequately since the value of coefficient of determination R^2 for rPPHY production is 0.9059 which is quite high. The F value of the model is 12.03 and the 'Prob > F' value is <0.0003, suggesting that the model is highly significant. The predicted R^2 (0.7257) and adjusted R^2 (0.8306) are in reasonable agreement with each other (Supplementary Table A4). 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 recorded for the model was 10.546. Very close experimental and predicted levels of rPPHY production in validation experiments suggest that model can be used for rPPHY production (supplementary Table A5).

For the production of rPPHY, GAP promoter is more efficient than AOX1 promoter, since single copy harboring integrant with the former produced higher rPPHY titre than that harboring three copies of *PPHY* under the latter. Similarly, Wang et al. reported improved expression of *Yarrowia lipolytica* lipase (YlLIP2) with constitutive expression under GAP promoter than that under AOX1 [38].

A high titre of recombinant phytase was attained in both shake flasks and fermenter in 72 h. There was a marginal increase in rPPHY production titre in the lab fermenter (756 U mL^{-1}) as compared to shake flasks (731 U mL^{-1}) (Fig. 4). After optimization, a ~44-fold enhancement in

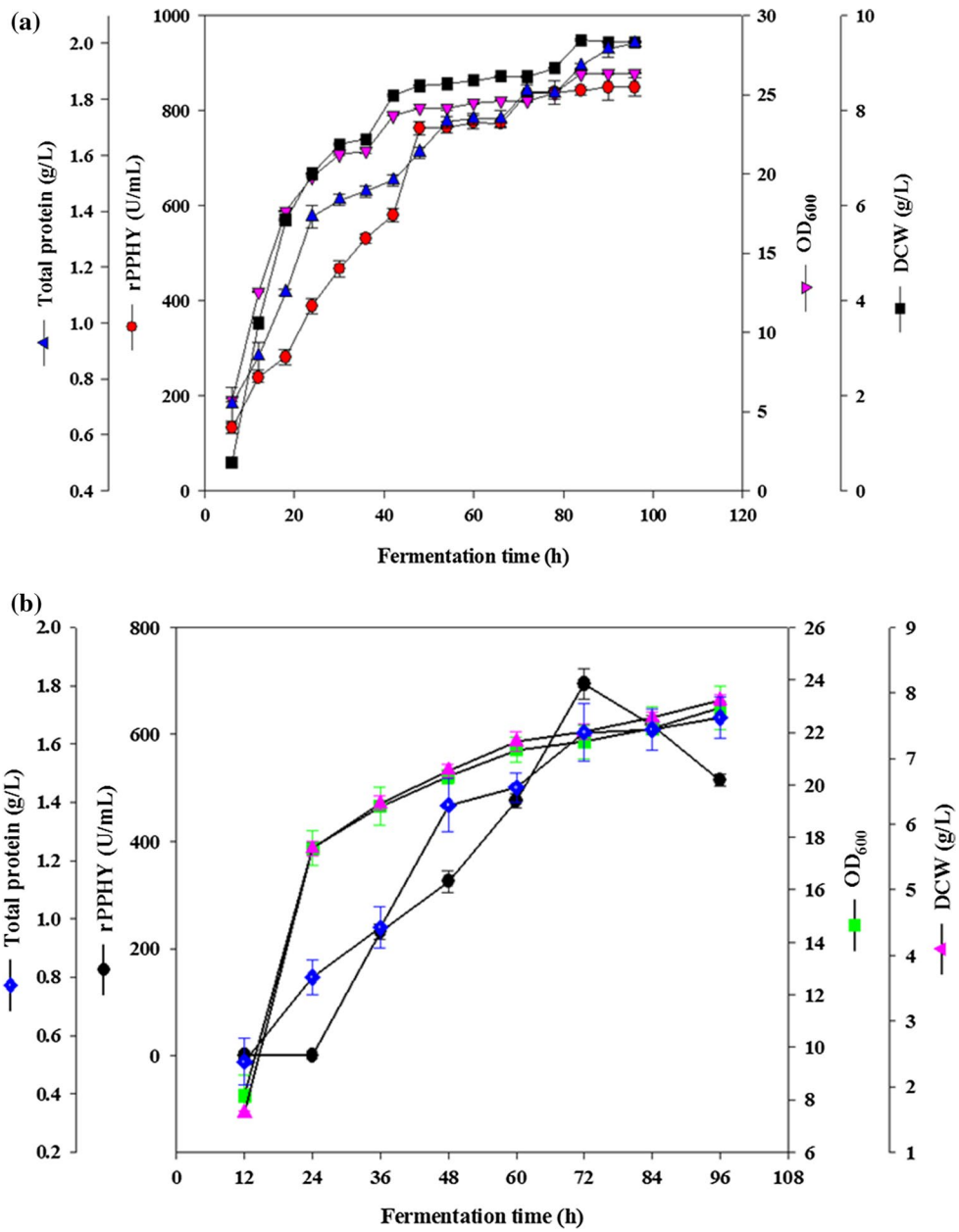


Fig. 4 The rPPHY production profile in a 7 L laboratory fermenter by the recombinant *P. pastoris* under GAP promoter (**a**). Production profile of rPPHY in batch fermentation conducted in 0.25 L shake flasks under the optimized conditions (**b**)

Table 2 Improvement in rPPHY production under GAP promoter upon optimization

Organism	Production condition	Phytase production (UmL^{-1})	Yield ($mg\ L^{-1}$)	Productivity ($U\ L^{-1}\ h^{-1}$)	Fold improvement in phytase production	Fold improvement in productivity	References
P. anomala MTCC 4133	–	~17.0	–	710	1.0	1.0	Kaur and Satyanarayana [12]
Recombinant *P. pastoris*	Under AOX promoter	~371.0	447	5152	~21.8	7	[11]
P. pastoris	In lab scale fermenter under GAP promoter	~756	910	10,500	~44	14.7	This investigation

rPPHY production has been achieved over that of the native host (Table 2).

As hydrolysis of metal-phytate complexes is critically important improving the availability of nutritionally important cations, we tested the potential of rPPHY in hydrolyzing them. Among metal-phytate complexes tested, calcium phytate was rapidly hydrolyzed in comparison with others (Fig. 5a), which could be due to innocuous nature of Ca^{+2} as compared to other heavy metals. Similar observation was recorded with the phytase of *Sporotrichum thermophile* [31].

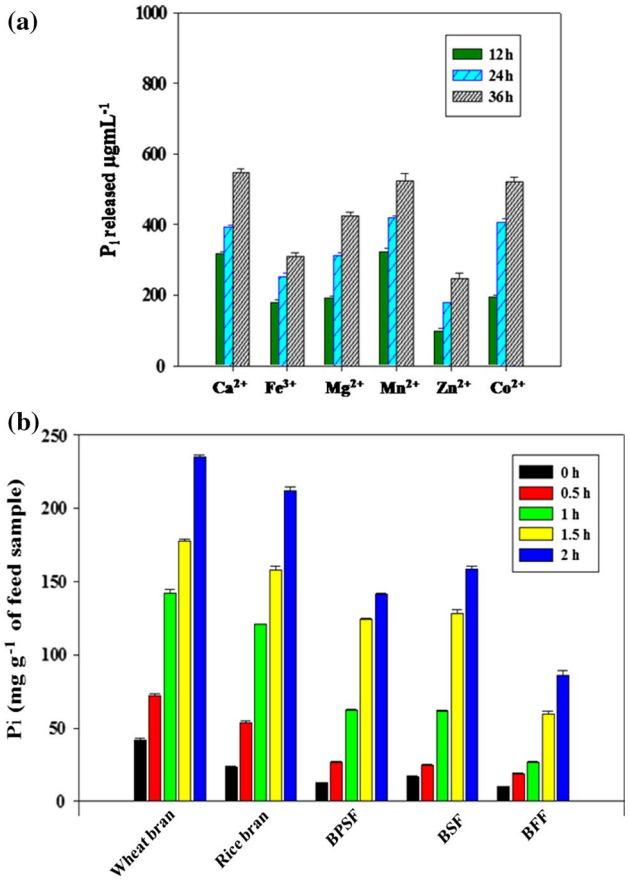


Fig. 5 Hydrolysis of various phytates by rPPHY that led to the liberation of phytate phosphorous (**a**). The liberation of phytate phosphorous by the hydrolysis of different poultry feeds by rPPHY under simulated gut conditions (**b**)

Poultry birds are monogastric animals and lack adequate levels of phytic acid hydrolyzing enzymes in their GIT. Dephytinization of feed materials by addition of an exogenous phytase is highly desirable to mitigate anti-nutrient effects of phytic acid. The PPHY is efficient in dephytinizing wheat bran and rice bran. Within 2 h, phytase liberated 193, 189, 128.2, 142 and 75.6 mg inorganic phosphorous per gram of wheat bran, rice bran, BPSF, BSF and BFF, respectively (Fig. 5b). Among poultry feeds, broiler starter feed was more rapidly dephytinized followed by pre-starter and finisher feeds. This suggests that ingredients of BPSF and BSF are not resistant to dephytinization, but may need a slight modification or pretreatment for efficient phytate hydrolysis. As the addition of rPPHY liberated phytic acid-bound phosphorous from various broiler feeds, this can be used as a promising feed additive. The addition of phytase to the feeds of monogastric animals not only reduces the production cost of poultry but also helps in environmental protection by reducing the release of phytic acid into water bodies [15, 17, 36].

Greiner and Konietzny reported that the average daily intake of phytate is around 2.0–2.6 g for vegetarian diets mainly in rural regions of developing nations, and 0.3–1.3 g for mixed diet [8]. Mechanical reduction in phytic acid content by removing outer layers of grain lowers the nutritional value of the food, as most of the vitamins, minerals and dietary fibers are bound to phytate. The enzymatic reduction of phytic acid will lead to the retention of the nutrients, and thus, result in a significant improvement in mineral absorption. The supplementation of tandoori and

Table 3 Comparison of the characteristics of the test and control unleavened flat breads

Properties of Indian bread	Tandoori		Naan	
	Test[a]	Control[b]	Test[a]	Control[b]
Weight of the dough	84 ± 2.1	83.5 ± 1.87	122 ± 3.21	124 ± 2.1
Weight of bread (g)	69.32 ± 1.21	70.32 ± 1.54	99.56 ± 2.31	102 ± 1.78
Bread moisture (%)	31.21 ± 0.78	31.23 ± 1.1	31.21 ± 0.67	31.1 ± 0.8
Soluble protein content (mg g^{-1} bread)	37.2 ± 1.54	25.4 ± 0.7	34.8 ± 0.9	25.4 ± 1.1
Reducing sugars (mg g^{-1} bread)	51.6 ± 2.31	43.4 ± 1.87	29.98 ± 0.78	23.02 ± 1.22
Inorganic phosphate (P_i) (mg g^{-1} bread)	4.15 ± 0.23	2.1 ± 0.13	0.89 ± 0.1	0.25 ± 0.03
Pi from phytic acid (mg g^{-1} bread)	2.0 ± 0.1	–	0.64 ± 0.11	–
Phytic acid reduction (%)	67.5 ± 2.1	–	22.23 ± 1.21	–

[a] Bread made by supplementing the dough with rPPHY

[b] Bread made of the dough without rPPHY supplementation

naan dough with rPPHY increased the inorganic phosphate, reducing sugars and soluble protein in the bread as compared to the control breads. The addition of rPPHY resulted in 67.5 and 23.2 % reduction in phytic acid content in tandoori and naan, respectively (Table 3). The texture of the test breads remained as good as the control breads. Sadananda et al. exploited *Candida versatilis* mutants (exhibiting phytase activity) to bring about reduction in phytic acid content in wheat-based dough [27].

Conclusions

Due to efficient expression of PPHY under constitutive GAP promoter, a very high titre of PPHY has been achieved as compared to that under AOX1 promoter. This process simplifies the production strategy and eliminates the use of hazardous methanol, and therefore, can be adopted for the production of proteins of nutritional significance. The optimization of cultivation variables led to an overall ~44-fold improvement in rPPHY production over that of the native yeast. The phytase hydrolyzes calcium phytate more efficiently than other metal-phytate complexes. Under simulated gut conditions, the phytase is effective in dephytinizing wheat bran, rice bran and poultry feeds used at different growth phases of poultry birds. The supplementation of recombinant phytase to the dough of whole wheat flour ameliorated nutrient availability in breads by increasing the amount of soluble phosphate, sugars and proteins, besides mitigating anti-nutritional effects of phytates.

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