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# Recombinant HAP Phytase of the Thermophilic Mold *Sporotrichum thermophile*: Expression of the Codon-Optimized Phytase Gene in *Pichia pastoris* and Applications

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**Abstract** The codon-optimized phytase gene of the thermophilic mold *Sporotrichum thermophile* (*St-Phy*) was expressed in *Pichia pastoris*. The recombinant *P. pastoris* harboring the phytase gene (*rSt-Phy*) yielded a high titer of extracellular phytase (480 ± 23 U/mL) on induction with methanol. The recombinant phytase production was ~40-fold higher than that of the native fungal strain. The purified recombinant phytase (rSt-Phy) has the molecular mass of 70 kDa on SDS-PAGE, with $K_m$ and $V_{max}$ (calcium phytate), $k_{cat}$ and $k_{cat}/K_m$ values of 0.147 mM and 183 nmol/mg s, $1.3 \times 10^3$/s and $8.84 \times 10^6$/M s, respectively. $Mg^{2+}$ and $Ba^{2+}$ display a slight stimulatory effect, while other cations tested exert inhibitory action on phytase. The enzyme is inhibited by chaotropic agents (guanidinium hydrochloride, potassium iodide, and urea), Woodward's reagent K and 2,3-bunatedione, but resistant to both pepsin and trypsin. The rSt-Phy is useful in the dephytinization of broiler feeds efficiently in simulated gut conditions of chick leading to the liberation of soluble inorganic phosphate with concomitant mitigation in antinutrient effects of phytates. The addition of vanadate makes it a potential candidate for generating haloperoxidase, which has several applications.

**Keywords** Thermophilic mold · *Pichia pastoris* · Recombinant phytase · Broiler feeds · Dephytinization · Haloperoxidase

## Introduction

The inability of monogastric nonruminant animals to digest feed phytates has necessitated supplementation with inorganic phosphate, which has led to phosphate pollution of water bodies in the areas of intensive animal rearing [22, 36, 57]. Phytate degrading enzyme (phytase) has been included in broiler diets for more than 20 years mainly to liberate phytate-bound P and to mitigate antinutrient effects of phytates [29, 42]. Approximately 60 % of the marketed enzymes used for animal nutrition are phytases that account for annual sales of US$ 350 million. About 70 % of animal feeds meant for monogastric animals are supplemented with phytase. The enzyme manufacturers predicted annual growth rate of phytase sales of 7.1 % during 2013–2018 in North American markets (https://www.asdreports.com).

Unfortunately, phytases from most of the microorganisms are sensitive to pH and heat, which limits their application in the feed industry [41, 54]. Moreover, phytases display low specific activities with low stability that results in high manufacturing costs [35, 37, 58]. Since phytase production levels by the native microbial strains are very low, attempts are being made to clone phytase-encoding genes and overexpress them heterologously in bacteria (e.g., *Escherichia coli*) or yeast (e.g., *Pichia pastoris*). *Pichia pastoris* is a methylotrophic yeast, in which heterologous proteins can be expressed either constitutively or inducibly [11]. By using an expression plasmid with the secretory signal sequence of α-factor,

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heterologous proteins are secreted into the medium [28]. Until now, a large number of genes have been successfully expressed in *P. pastoris* [5, 7]. Several successful strategies have also been followed in order to improve the expression of heterologous genes, like increasing the copy number [30], introducing effective transcriptional promoters [8, 14], optimizing the culture conditions [17, 51, 56] and replacing the secretory signal in the vector [1, 30, 32]. Unfortunately, these approaches do not always result in the predictable high levels of expression of recombinant proteins [59]. Codon optimization is a beneficial exercise for enhancing the expression of heterologous proteins, because there is a considerable difference in codon usage in host cells. There are a few reports on improving protein expression by using codon-optimized genes [19, 33, 44].

The thermophilic mold *Sporotrichum thermophile* produces very low titers of phytase extracellularly in both solid state and submerged fermentations [45–50]. The phytase of *S. thermophile* (St-Phy) has been shown to have the requisite characteristics such as acid stability and thermostability, broad substrate spectrum and resistance to proteolytic cleavage [50]. Since there was no expression of the wild-type phytase gene of *S. thermophile* when cloned into *P. pastoris*, this investigation has been undertaken to clone and express the codon-optimized gene and characterization of the recombinant phytase and to test its applicability in dephytinization of chick feeds and as a haloperoxidase.

## Materials and Methods

### *P. pastoris* Strain and Vectors

*Pichia pastoris* X-33 (Invitrogen, USA) was used as the host to express recombinant phytase (rSt-Phy) protein. The expression cassette with inducible promoter (*AOX1*) was used for protein expression. The plasmid pPICZαA was used to construct the methanol-inducible St-Phy expression vector. *Eco*RI and *Kpn*I restriction sites were added to sense and antisense primers, respectively, for cloning *St-Phy* in the pPICZαA vector. Cloning steps were performed in *Escherichia coli* (DH5α).

### Checking the Suitability of the *St-Phy* Gene for Expression in *P. pastoris*

*Sporotrichum thermophile* phytase gene coding sequence (accession number KF535924.1) was optimized by https://eu.idtdna.com/CodonOpt for expression in *P. pastoris*. The unusual codons were replaced with more frequently used ones in *P. pastoris*, and the GC content and solidity of possible secondary RNA structures were reduced. The assessment and comparison of the secondary structures and their free energy ($\Delta G$) in native sequence and the newly synthesized one were done using the web-free software Centroid fold (http://www.ncrna.org/centroidfold) [39]. This tool predicts RNA secondary structures with the $\gamma$-centroid estimator which is a kind of posterior decoding method based on statistical assessment. The relative adaptation of the synthetic *St-Phy* toward the codon usage of highly expressed genes in *P. pastoris* was assessed by codon adaptation index (CAI), using unusual codon analysis tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). This numerical value is the ratio of the usage of each codon to that of the most copious codons for the same amino acid and ranges from 0 to 1. A high value of relative adaptation indicates a high percentage of the most abundant codons, while a low value points to a high chance that the desired gene will be expressed poorly [43]. Codon-optimized gene was supplied by Bio-Link India Private Limited, New Delhi.

### Construction of Recombinant Phytase (rSt-Phy)

The codon-optimized gene was amplified using the primers P1 and P2 having flanking regions of *Eco*RI and *Kpn*I restriction sites. PCR conditions: denaturation at 95 °C for 5 min; 15 cycles (denaturation at 95 °C for 30 s, annealing 60 °C for 30 s, elongation 72 °C for 75 s) and final elongation at 72 °C for 5 min. The primers used are given in Table 1. The final 1464 bp PCR product thus obtained was digested with *Eco*RI and *Kpn*I and cloned into plasmid pPICZαA vector to make *rSt-Phy*-pPICZαA construct. In this construct, the rSt-Phy ORF was fused in-frame with the secretory signal sequence of α-factor, and the *myc* epitope and 6× His-tag from the vector at the N- and C-termini, respectively. Confirmation of the construct was done by double digestion with *Eco*RI and *Kpn*I. The presence and precise positioning of the insert were confirmed by DNA sequencing at the Central Instrumentation Facility, University of Delhi South Campus, New Delhi.

**Table 1** Primers used in this investigation; underlines indicate restriction sites added to the primers

| Primers | Oligonucleotide sequence (5′-3′) |
|---|---|
| *P*1 | CGGAATTCATGACTGGGCTGGGTGTTA |
| *P*2 | GGTTGGTACCTTGGCGAAACACAAGTCCC |
| *F*′ (For Q-PCR) | TGGCATCAAGTTTTACAGAAG |
| *R*′ | (For Q-PCR) AAGTGTCTTGAGCGTCATCTC |

GAATTC, restriction site for *Eco*RI; GGTACC, restriction site for *Kpn*I

### Bioinformatic Analysis

The nucleotide and protein sequences were compared with the NCBI's nucleotide/protein database using BLASTN and BLASTP programs, respectively. The sequence alignment of codon-optimized and wild-type phytase gene was performed using DNAMAN software.

### Transformation of *P. pastoris* with *St-Phy*

The *rSt-Phy*-pPICZαA was linearized with *Sac*I for transformation of *P. pastoris*. The competent *P. pastoris* cells were prepared by combining chemical transformation with electroporation. Approximately 10 μg of linearized plasmid was mixed with competent cells, and the mixture was directly transferred to a 0.2-cm pre-chilled electroporation cuvette and incubated on ice for 5 min. The electroporation was accomplished with voltage of 1.5 kV, capacitance of 25 μF and resistance of 200 Ω. To 1.0 mL aliquot, 1.0 M ice-cold sorbitol was immediately added to the cuvette after electroporation, and the mixture was spread on YPD agar (g/L: yeast extract 10, peptone 20 and dextrose 20, agar 20) medium supplemented with 1.0 M sorbitol containing different concentrations of Zeocin (100, 200, 500, 1000 and 1500 μg/mL). The plates were incubated at 30 °C until single distinct colonies appeared. Colony PCR was performed to identify positive clones harboring phytase gene. Spheroplasting of the *P. pastoris* was done before proceeding to colony PCR by spinning 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 and PCR was performed as described above using primers P1 and P2. The transformants with the fast growth rate on YPD agar plates (supplemented with 1.0 M sorbitol) containing the highest concentration of Zeocin were screened for the production of recombinant phytase (rSt-Phy). These clones were first grown in YPD broth, and then, biomass was transferred to yeast extract-peptone (YP) medium containing 0.5 % (v/v) methanol [17]. The production of rSt-Phy was checked by quantitative phytase assay.

### Quantitative Phytase Assays

The reaction mixture containing 0.1 mL appropriately diluted rSt-Phy, 0.4 mL sodium acetate buffer (100 mM, pH 5.0), and 0.5 mL of 4 mM calcium phytate was incubated at 60 °C for 10 min. Two milliliters of acetone-ammonium molybdate solution (acetone, 5 N $H_2SO_4$, 100 mM ammonium molybdate in a 2:1:1 ratio) was added to the reaction mixture, followed by 0.1 mL of 1 M citric acid to stop the reaction. The phosphate released was determined according to Heinonen and Lahti. One unit (U) of phytase is defined as the amount of enzyme that liberates 1 nmol of inorganic phosphate $s^{-1}$ under the defined assay conditions.

### Quantitative Real-Time PCR (Q-PCR) for Gene Copy Number Determination

Q-PCR using Fast SYBR® Green dye was employed to determine *rSt-Phy* gene copy number. The experiments were conducted with the Roche Light Cycler® 96 instrument (Roche Life Science). Pure genomic DNA and plasmid harboring phytase gene (*rSt-Phy*-pPICZαA) were used as the template in all Q-PCR analysis. Primers were designed according to Fast SYBR® Green Master Mix protocol. The melting temperature ($T_m$) of all primers was kept between 59 and 61 °C. Primers were designed in such a way that the amplified products are of 284 bp. The primers used in this investigation are listed in Table 1. The concentration of each primer in the reaction mix was kept at 50 nM. The specificity of amplification was confirmed by melt curve analysis after 35 cycles. In addition, the amplified Q-PCR products were analyzed on agarose gel (1 %) to ensure the presence of single amplified PCR product of 284 bp in each case. Each reaction contained 2× Fast SYBR green dye (5 μL), forward and reverse primers (50 nM each) and genomic DNA as well as *rSt-Phy*-pPICZαA (a tenfold dilution series) in a total volume of 10 μL. All experiments were performed in triplicate with the procedure recommended by the program (stage 1: 95 °C for 2 min, stage 2: 35 cycles of 95 °C for 10 s and 60 °C for 20 s, 72 °C for 30 s followed by stage 3, i.e., 95 °C for 15 s and 65 °C for 60 s, 97 °C for 1 s). To construct the standard curves for genomic DNA as well as *rSt-Phy*-pPICZαA, tenfold serial dilutions of genomic DNA and *rSt-Phy*-pPICZαA ranging from $3 \times 10^7$ to $3 \times 10^2$ copies/μL were used and the $C_T$ values were plotted against log (copies of genomic DNA and *rSt-Phy*-pPICZαA). The concentration of the genomic DNA and *rSt-Phy*-pPICZαA was determined with a Nanodrop spectrophotometer, and corresponding DNA copies were calculated as follows [27, 40]:

$$\text{DNA (Copy)} = \frac{6.023 \times 10^{23}\ (\text{copies/mol}) \times \text{DNA amount(g)}}{\text{DNA length (bp)} \times 660\ \text{g/mol/bp}}$$

The length of *P. pastoris* genome is 9.43 Mbp [40]. Q-PCR amplifications were conducted at each dilution in triplicate, and average $C_T$ values were plotted against the logarithm of DNA copies. Q-PCR amplification efficiency ($E$) was calculated from slopes of the standard curves based on following equation [27]:

$$E = 10^{-1/\text{slope}} - 1$$

The absolute copy number was determined by dividing genomic DNA copies of *rSt-Phy* by the plasmid DNA copies of the reference gene, i.e., *rSt-Phy*-pPICZαA.

### Purification of the Recombinant Phytase (rSt-Phy)

The cell-free culture supernatant was passed through 10 kDa ultrafiltration membrane cartridge (Millipore); desalting and reconcentration were carried out for three cycles using the same membrane cartridge with 20 mM Tris–HCl buffer (pH 8.0). The concentrated enzyme was purified by fast protein liquid chromatography (FPLC) system (ÄKTA prime PLUS, GE Healthcare, Bio-Sciences, Uppsala, Sweden) using Resource Q column (Pharmacia Biotech) and eluting the bound protein with a linear gradient of 0–1 M NaCl in Tris–HCl buffer (20 mM, pH 8.0) at 0.6 mL/min. The fractions with phytase activity were pooled and dialyzed against the sodium acetate buffer (20 mM, pH 5.0) and concentrated by Speed Vac (Thermo Scientific, USA). The concentrated enzyme was loaded on gel filtration chromatography column [Sephacryl S-200HR(16/60)] and eluted with 20 mM sodium acetate buffer (pH 5.0) containing 50 mM NaCl at a flow rate of 0.4 mL/min. The fractions having phytase activity were collected and used to check for purity on SDS-PAGE and its characterization.

### Deglycosylation of rSt-Phy by Endoglycosidase $H_f$

The glycosylation of rSt-Phy was confirmed by deglycosylation using 10.0 U of endoglycosidase $H_f$ (*Endo*$H_f$, NEB) for 3 h at 37 °C. The electrophoretic mobility of glycosylated and deglycosylated phytases was checked on SDS-PAGE to observe the difference in molecular weight.

### Proteolytic Resistance of the rSt-Phy

In order to check the resistance of the rSt-Phy for proteolytic cleavage, the purified rSt-Phy was pre-incubated with 0.1 mg/mL trypsin (20 mM Tris–HCl buffer, pH 7.5) and pepsin (20 mM Glycine-HCl buffer, pH 3). The rSt-Phy was kept at both pH values without proteolytic enzyme as control at 37 °C, and phytase activities were recorded till 1 h. The relative activities were calculated from the control at the respective pH values.

### Biochemical Characterization of the rSt-Phy

The optimum pH for the activity of rSt-Phy was determined by conducting enzyme assays at different pH [Glycine-HCl buffer 20 mM (pH 2.0, 3.0), sodium acetate buffer 20 mM (pH 4.0, 5.0), Tris–HCl buffer 20 mM (pH 6.0–8.0) and glycine-NaOH buffer 20 mM (pH 9.0)] at 60 °C. Similarly, optimum reaction temperature was determined by rSt-Phy assays at various temperatures (30–80 °C) at pH 5.0. The effect of various modulators on recombinant phytase activity was assessed by performing enzyme assays in the presence of the modulators into the reaction mixtures.

### Enzyme Kinetics and Thermal Deactivation of the rSt-Phy

The enzyme was assayed at different concentrations of calcium phytate (0.3–4.0 mM). The $K_m$ and $V_{max}$ values were graphically determined from the Lineweaver–Burk plot. Activation energy ($E_a$) was calculated according to Kumar and Satyanarayana [25]. The efficiency of liberation of inorganic phosphate (Pi) by the enzyme action was calculated as $V_{max}/K_m$ ratio. The energy of deactivation ($E_d$) of the enzyme was calculated from residual activity at different temperatures by incubating the enzyme solution in 20 mM sodium acetate buffer (pH 5.0) at various temperatures (60–80 °C) in the absence of substrate. The aliquots were drawn at the desired intervals, cooled on ice for 30 min and performed phytase assays for calculating the residual activities. Calculations for determining inactivation rate constants ($K_d$) and energy of deactivation ($E_d$) are given below:

Thermal denaturation is a reaction in which the rate of enzyme deactivation ($dC/dt$) follows first-order kinetics in relation to the concentration of the active enzyme ($C$):

$$dC/dt = -k_d C \quad (1)$$

which can also be expressed as

$$\ln[C_t/C_0] = -k_d t \quad (2)$$

where $C_0$ is the initial concentration of the enzyme and $C_t$ is the concentration of the enzyme at time $t$. Since the residual enzyme activity ($E$) is directly proportional to the concentration of the active enzyme ($C$); thus,

$$E_t/E_0 = C_t/C_0$$

Now, Eq. (2) can be written as:

$$\ln[E_t/E_0] = -k_d t$$

or

$$2.303 \log[E_t/E_0] = -k_d t \quad (3)$$

$K_d$ is the deactivation rate constant, which is calculated from the slope of the plots of $\log[E_t/E_0]$ versus $t$. The half-life ($t_{1/2}$) of the enzyme is defined as the time required for the enzyme to lose half its initial activity. It can be expressed as follows:

$$t_{1/2} = 2.303 \log 2/K_d \quad (4)$$

Energy required for deactivation ($E_d$) has been calculated by Arrhenius plot using the Arrhenius equation:

$$K_d = Ae^{(-E_d/RT)} \quad (5)$$

So that

$$\ln[K_d] = -E_d/RT + \ln A \quad (6)$$

where $E_d$ represents energy of deactivation, $R$ is the universal gas constant (8.314 J/K mol) and $T$ is the absolute temperature. Deactivation energy ($E_d$) involved in the deactivation process is calculated from the slope of a linear plot of $\ln[K_d]$ versus $T^{-1}$. The effect of temperature on the rate of reaction was expressed in terms of temperature quotient ($Q_{10}$), which is the factor by which the rate increases due to a raise in the temperature by 10 °C. Temperature quotient ($Q_{10}$) was calculated by rearranging the equation of Dixon and Webb [9]:

$$Q_{10} = \text{antilog}\left(E_a \times 10/RT^2\right) \quad (7)$$

where $E_a$ = activation energy, $R$ is universal gas constant and $T$ is absolute temperature.

### Thermodynamic Parameters of the rSt-Phy

Thermodynamics of irreversible inactivation of the rSt-Phy was determined by rearranging the Eyring's absolute rate equation derived from the transition state theory:

$$K_d = (K_b T/h)\, e^{(-\Delta H/RT)} e^{(-\Delta S/RT)}$$

$\Delta H$ (change in enthalpy of deactivation), $\Delta G$ (change in free energy of inactivation) and $\Delta S$ (change in entropy of inactivation) for irreversible inactivation were calculated as follows:

$$\Delta H = E_d - RT \quad (8)$$

$$\Delta G = -RT \ln(K_d h/K_b T) \quad (9)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (10)$$

where $K_b$ is the Boltzmann's constant ($R/N$) = $1.38 \times 10^{-23}$ J/K, $T$ is the absolute temperature (K), $h$ the Planck's constant = $6.626 \times 10^{-34}$ J s, $N$ is the Avogadro's number = $6.02 \times 10^{23}$ $mol^{-1}$ and $R$ is the gas constant = 8.314 J/K mol.

### Dephytinization of Various Poultry Feeds Under the Gastrointestinal Tract (GIT) Condition of Chick Using rSt-Phy

Hydrolysis of different autoclaved broiler feeds [Broiler Pre-Starter Feed (BPSF) is made of very small granules, which makes easy for the chicks to feed faster, which is fed to 0- to 10-day-old chicks. BPSF has low energy (2800–2900 kcal/kg) and high protein requirement (22–24 %). Broiler Starter Feed (BSF) is made of small granules, which is fed to 10- to 20-day-old chicks having protein (21.5 %) and energy (29,50 kcal/kg) requirements. Broiler Finisher Feed (BFF) is designed with balanced protein energy ratio to attain faster and maximum weight gain at the lowest feed conversion ratio (FCR) and fed when the body weight of chicks reaches to approximately 500 g (Table S1)] was initiated by adding 10 ml of sodium acetate buffer (0.02 M, pH 5) in 0.2 g of feed containing 100 U of rSt-Phy at 40 °C in a shaking incubator at 100 rpm [The standard body temperature of poultry bird is 40 °C (http://www.poultryhub.org/production/husbandry-management/housing-environment/climate-in-poultry-houses/)]. Aliquots were taken at different time intervals, and the amounts of liberated inorganic phosphate were determined.

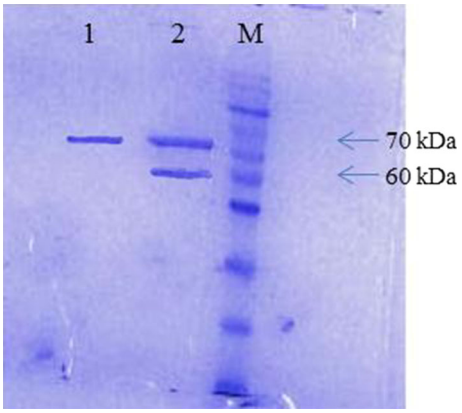


**Fig. 1** SDS-PAGE analysis of the purified rSt-Phy: *Lane 1* untreated rSt-Phy, *Lane 2* deglycosylated with *Endo*$H_f$ rSt-Phy, *Lane M* protein marker

### Haloperoxidase Activity Using rSt-Phy

The haloperoxidase activity of the rSt-Phy was confirmed by a qualitative assay [16]. One milliliter reaction mixture contained 500 µL enzyme, 300 mM potassium phosphate buffer (pH 7), 20 mM KBr, 10 µM metavanadate, 50 µL of 0.3 % $H_2O_2$ and 100 µL of 0.2 % phenol red (prepared in 95 % ethanol). The reaction was carried out at 37 °C while observing periodically for change in color from red–orange to blue–violet.

All experiments have been conducted in triplicate, and the mean values are presented with standard deviation.

## Results and Discussion

In order to achieve the expression of phytase, differences in the relative codon frequency between *S. thermophile* and *P. pastoris* were analyzed. The *St-Phy* gene is of 1464

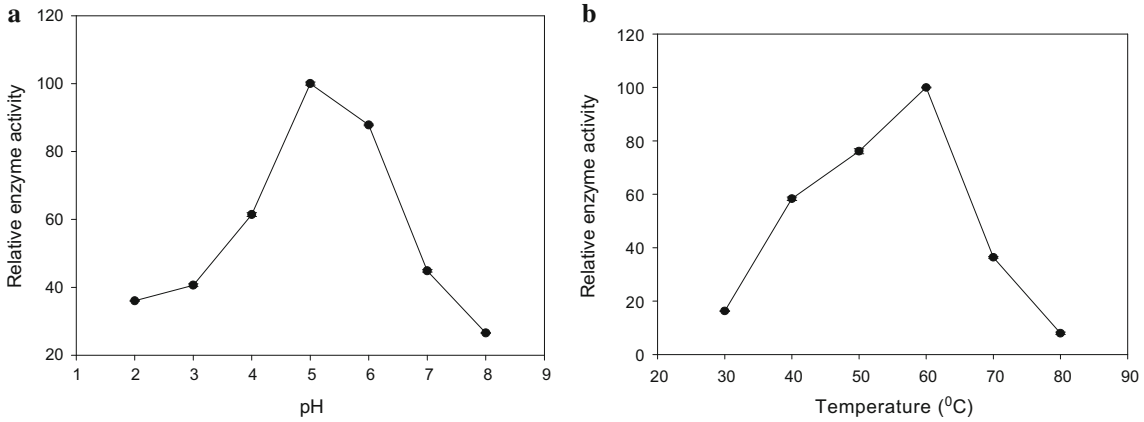


**Fig. 2** Influence of pH (**a**) and temperature (**b**) on rSt-Phy

nucleotides that translate into a protein of 487 amino acids. The optimized phytase gene was compared with the wild-type phytase gene sequence, and the sequence alignment has been done (data not shown). Considering the codon bias of *P. pastoris*, 26.63 % nucleotides were replaced in the synthesized gene. The CAI value increased in *P. pastoris* from 0.48 for native phytase gene sequence to 0.76 for *rSt-Phy* gene. A high value of relative adaptation indicates a high proportion of the most abundant codons, while a low value of this measurement suggests a high possibility that the anticipated gene will be expressed poorly [43]. The codon usage of the phytase gene was optimized by means of the most frequently used triplet codons in *P. pastoris* such that the codon usage of phytase looks like that of the host strain (Table S2). Codon usage by host *P. pastoris* for wild-type gene and optimized gene has been compared, which are 18 and 52 %, respectively (Table S2). The value of 100 is set for the codon with the maximum usage frequency for a given amino acid in *P. pastoris*. Codons with the values below 30 are likely to obstruct the expression efficiency (http://www.genscript.com/). A similar observation was made in the present case because the native sequence has percent distribution of codons of only 18 % for *P. pastoris*. Besides codon bias, RNA secondary structure is another significant factor reorganized by codon change and represents remarkable effects on heterologous gene expression and hence should be placed under great surveillance during codon optimization. Lower folding energies (higher stability) slow down the translation efficiency [26, 52]. Optimization caused changes in mRNA folding and subsequently in its total free energy ($\Delta G$) which reduced after codon optimization from −553.40 to −272.92 kcal/mol. Mol% G+C content of optimized gene also changed from 69.16–48.11 %. Ideal G+C content should be in the range of 30–70 %; away from this range will adversely affect transcriptional and translational efficiency [2].

The construction of *rSt-Phy*-pPICZαA (Fig. S1) was confirmed by colony PCR (data not shown) and double digestion with *Eco*RI and *Kpn*I (data not shown). Among 100 clones screened, clone 22 producing highest extracellular rSt-Phy titer of 480 ± 23 U/mL was selected for further investigations. No cell-bound phytase activity was observed in clone 22, which reveals the efficient functioning of the secretory signal sequence of α-factor.

Clone 22 harbors three copies of *rSt-Phy*, as confirmed from the absolute Q-PCR. $R^2$ value for the genomic DNA copies of *rSt-Phy* and *rSt-Phy*-pPICZαA was ∼0.99, and these were with 96.78 and 95.68 % efficiency, respectively (Fig. S2).

In shake flasks, ∼40-fold improvement in rSt-Phy production has been attained over that by the native host. The rSt-Phy was purified to apparent homogeneity, and the purity was confirmed by SDS-PAGE. The band corresponds to approximately 70 kDa, while in the wild type, it is approximately 90 kDa which might be due to difference in the level of glycosylation in native and recombinant hosts. The enzyme assay confirmed proper folding of rSt-Phy.

The SDS-PAGE analysis of the rSt-Phy showed an apparent molecular size of approximately 70 kDa before N-deglycosylation. After $\text{EndoH}_f$ treatment, the band has been visualized with a molecular mass of ∼60 kDa (Fig. 1), suggesting the occurrence of N-glycosylation of the protein. This molecular weight was still larger than the calculated one from the sequence (54 kDa). As there are three potential O-glycosylation sites at positions 181, 185, and 268, as predicted by http://www.cbs.dtu.dk/services/NetOGlyc-4.0, it may be possible that the enzyme has one or more O-glycosylation.

The purified rSt-Phy showed resistance to proteases, since 96 % of the initial activity was recovered after 1.0 h of incubation in pepsin (pH 3.0) and trypsin (pH 7.5) (Fig. S3), as reported for the native phytase [50].

**Table 2** Effect of different metal ions, organic solvents, detergents, inhibitors and chaotropic agents on rSt-Phy

| Effect | Relative enzyme activity | |
|---|---|---|
| Metal ions | | |
| Control | 100 ± 0.4 | |
| | 1 mM | 5 mM |
| $Ca^{+2}$ | 97 ± 0.6 | 95.6 ± 0.5 |
| $Mg^{+2}$ | 103.2 ± 0.5 | 106.2 ± 0.8 |
| $Co^{+2}$ | 98.2 ± 0.4 | 89.8 ± 0.9 |
| $Cu^{+2}$ | 91.6 ± 1.3 | 81.2 ± 0.7 |
| $Fe^{+2}$ | 94.2 ± 0.4 | 87.6 ± 0.9 |
| $Fe^{+3}$ | 90.9 ± 0.7 | 81.4 ± 0.6 |
| $Zn^{+2}$ | 64.3 ± 0.9 | 39 ± 0.7 |
| $Al^{+3}$ | 85.1 ± 1.7 | 76.6 ± 0.7 |
| $Na^{+2}$ | 98.9 ± 0.3 | 92.4 ± 0.8 |
| $Ba^{+2}$ | 104.7 ± 0.6 | 105.4 ± 0.7 |
| $Mn^{+2}$ | 93.8 ± 0.5 | 87.6 ± 1.6 |
| Organic solvents (10 % v/v) | | |
| Acetone | | 95.8 ± 0.8 |
| Butanol | | 115.6 ± 2.6 |
| Isoamyl alcohol | | 105.4 ± 0.8 |
| Benzyl alcohol | | 79.7 ± 0.6 |
| Toluene | | 87.1 ± 0.8 |
| Ethanol | | 79 ± 0.7 |
| Hexane | | 101.5 ± 1.5 |
| Detergents | | |
| Detergents | 0.1 % | 0.5 % |
| Tween-20 | 128.3 ± 1.4 | 139.4 ± 0.9 |
| Tween-40 | 137.4 ± 0.5 | 143.2 ± 0.7 |
| Tween-80 | 159.4 ± 1.0 | 170.5 ± 1.2 |
| SDS | 85.6 ± 0.5 | 80.4 ± 1.9 |
| Inhibitors | | |
| | 1 mM | 5 mM |
| EDTA | 100 ± 1.1 | 98.1 ± 0.4 |
| B-ME | 98.8 ± 0.7 | 96.4 ± 1.4 |
| *N*-Ethylmaleimide | 95.2 ± 0.4 | 93.1 ± 1.2 |
| Dithiothretol | 96.4 ± 1.3 | 91.5 ± 1.4 |
| *N*-Bromosuccinimide | 94.2 ± 1.2 | 92.4 ± 0.5 |
| PMSF | 85.3 ± 1.1 | 71.5 ± 0.4 |
| Iodoacetate | 97.4 ± 0.6 | 96.2 ± 0.8 |
| Woodward's reagent K | 82.3 ± 1.2 | 41.1 ± 1.4 |
| 2,3-Butanedione | 64.3 ± 1.2 | 35.1 ± 0.4 |

| Chaotropic agents | | | | | |
|---|---|---|---|---|---|
| | 0.5 M | 1 M | 2 M | 4 M | 8 M |
| KI | 95 ± 1.4 | 91.5 ± 0.7 | 53.8 ± 1.5 | 28.5 ± 1.3 | 1.2 ± 1.2 |

The optimum temperature and pH for the activity is 60 °C and pH 5, respectively (Fig. 2), which is similar to that reported for the native phytase of *S. thermophile* [50] and other fungal and yeast phytases [3, 24, 31, 57]. Among various metal ions tested, the enzyme activity was marginally stimulated by $Mg^{2+}$ and $Ba^{2+}$, while others inhibited the activity (Table 2). The formation of insoluble complexes from metal ions and phytates decreases the availability of the substrate [24]. $Mg^{2+}$ and $Ba^{2+}$ ions enhance the enzyme activity because these may interact with the enzyme leading to a conformational change and increase activity as in the phytases of *Aspergillus niger* [38], *S. thermophile* [50], *Rhizopus oligosporus* [4] and *Aspergillus niger* [55].

Amongst all organic solvents tested, butanol, isoamyl alcohol and hexane slightly stimulated the phytase activity, while others inhibited, signifying that hydrophobic residues do not appear to play a substantial role in the catalytic activity as in the phytase of *A. niger* [55]. The activity of rSt-Phy was strongly inhibited by SDS, while Tweens stimulated (Table 2). Detergents bind to the protein and induce structural changes resulting in the stimulation in nonionic (Tweens) and inhibitory in anionic (SDS) detergents [50].

The inhibition by Woodward's reagent K confirms the role of carboxyl groups and suggests the chemical modification of aspartic acid residues involved in catalysis [6, 23, 34]. The inhibition by 2,3-butanedione suggests the role of arginine in catalysis [50]. The reducing agents such as β-mercaptoethanol have no effect on the enzyme activity, signifying that –SH groups have no role in the catalytic activity or these enzymes do not have any free and accessible –SH groups [50, 60]. The chelating agent EDTA has no discernible effect on the activity of rSt-Phy, indicating that it does not require any cations for activity. Inhibition by chaotropic agents suggests the role of non-covalent forces (H-bonds and van der Waals forces) in retaining the active conformation of the enzyme [13, 55].

The $K_m$ and $V_{max}$ of rSt-Phy (for calcium phytate) are 0.147 mM and 183 nmol/mg s. The $k_{cat}$ and $k_{cat}/K_m$ for phytase from different organisms are presented in Table 3. The $k_{cat}$ of the rSt-Phy is significantly higher ($1.3 \times 10^3$/s) than that of the native phytase [50], and this could be due to the difference in glycosylation that affected the enzyme activity. A larger $k_{cat}$ value signifies that the least amount of enzyme can be used for its applications. The catalytic efficiency ($k_{cat}/K_m$) of rSt-Phy is higher than that of the native phytase of *S. thermophile* (Table 3). The activation energy ($E_a$) for phytate hydrolysis by rSt-Phy and native phytase is 43.6 and 48.6 KJ/mol, respectively. The temperature quotient ($Q_{10}$) calculated for rSt-Phy is 1.64 and that of native phytase is 1.66 [50]. The characteristics of

**Table 3** Comparison of $K_m$, $k_{cat}$ and catalytic efficiency ($k_{cat}/K_m$) of rSt-Phy with other phytases

| Source of enzyme | $K_{m\ (mM)}$ | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}\ s^{-1}$) | References |
|---|---|---|---|---|
| rSt-Phy | 0.147 | $1.3 \times 10^3$ | $8.84 \times 10^6$ | This study |
| *S. thermophile* | 0.156 | 37.8 | $2.4 \times 10^5$ | [50] |
| *Aspergillus ficuum* | 0.270 | 628 | $6.0 \times 10^6$ | [10, 53] |
| *Yersinia enterocolitica* (*YeappA*) | 0.19 | 5.71 | $3.0 \times 10^4$ | [12] |

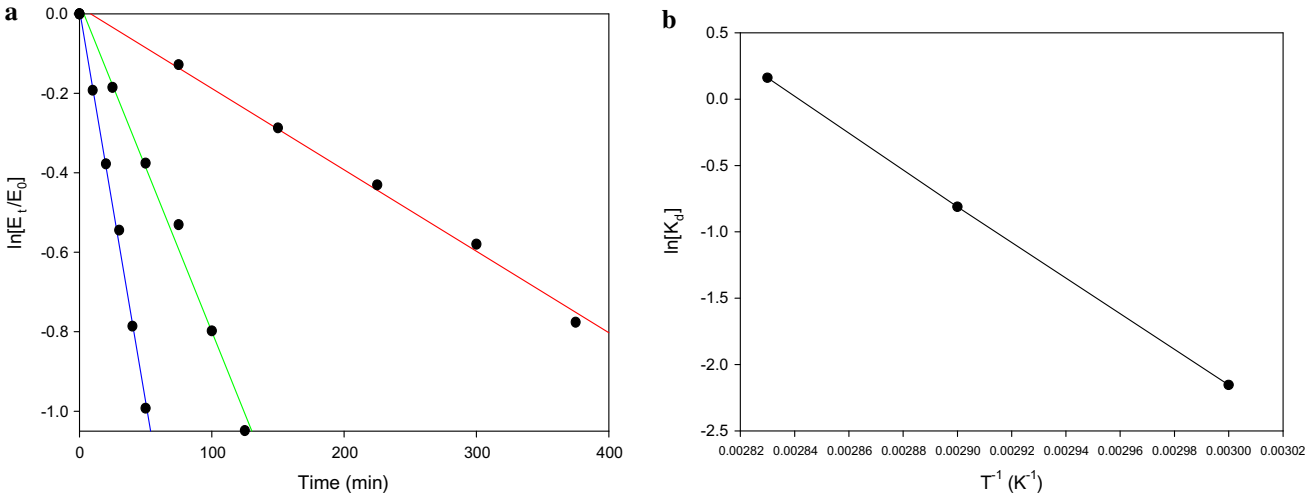


**Fig. 3** **a** Plot of $\ln[E_t/E_0]$ versus time (min) for the calculation of deactivation constant ($K_d$) and $t_{1/2}$ of rSt-Phy at different temperatures [60 °C (*red line*), 70 °C (*green line*), 80 °C (*blue line*)]. **b** Arrhenius plot of rSt-Phy for the calculation of deactivation energy ($E_d$) (Color figure online)

**Table 4** The characteristics of the rSt-Phy

| Properties of phytase | *S. thermophile* (native) | rSt-Phy |
|---|---|---|
| Specific enzyme activity (U/mg) | 52.4 | 46,000 |
| Molecular weight (kDa) | 90 | 70 |
| Optimum pH | 5 | 5 |
| Optimum temperature (°C) | 60 | 60 |
| Thermostability ($T_{1/2}$ at 60 and 80 °C) | 16 and 1.5 h | 6 and 0.59 h |
| Deactivation energy ($E_d$) | ND | 127.55 KJ/mol |
| Temperature quotient ($Q_{10}$) | 1.66 | 1.64 |
| Activation energy ($E_a$) | 48.6 KJ/mol | 43.6 KJ/mol |

*ND* not determined

**Table 5** The thermodynamic parameters of rSt-Phy measured during thermal deactivation at various temperatures

| Temp. (K) | $K_d$ ($h^{-1}$) | $T_{1/2}$ (h) | $\Delta H$ (KJ/mol) | $\Delta G$ (KJ/mol) | $\Delta S$ (J/mol K) |
|---|---|---|---|---|---|
| 333.15 | 0.116 | 6.0 | 124.78 | 87.88 | 110.76 |
| 343.15 | 0.444 | 1.56 | 124.69 | 86.77 | 110.50 |
| 353.15 | 1.175 | 0.59 | 124.62 | 86.52 | 107.88 |

the rSt-Phy are comparable with those of the native phytase, as reported for the recombinant and native phytases of *Pichia anomala* [21].

The deactivation constant ($K_d$) of rSt-Phy was calculated from the plot of $\ln(E_t/E_0)$ versus time (Fig. 3a). This value of $K_d$ has been substituted in Eq. (4) to calculate $T_{1/2}$ of the rSt-Phy (Table 4). The $T_{1/2}$ values of the rSt-Phy at 60 and 80 °C are 6 and 0.59 h, respectively. Arrhenius plot of the deactivation constant (Fig. 3b) at different temperatures was plotted for calculating the deactivation energy ($E_d$) of rSt-Phy, which is 127.55 KJ/mol. Thermodynamic parameters of thermal inactivation of rSt-Phy have been

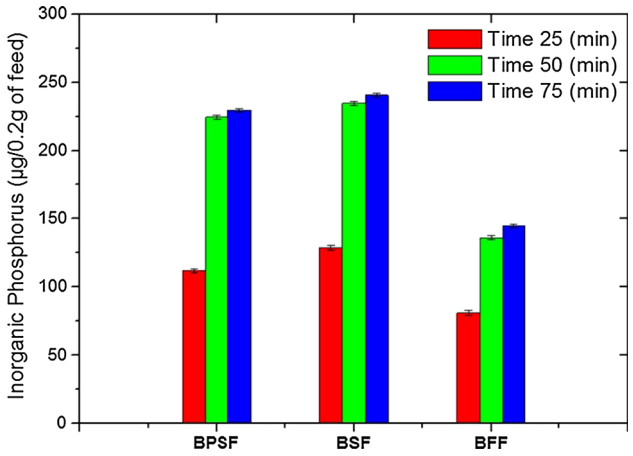


**Fig. 4** Liberation of inorganic phosphate from poultry feeds (BPSF, BSF, BFF) at different time intervals

calculated by applying the first-order kinetics to the thermal inactivation data. The overall $\Delta G$, $\Delta H$ and $\Delta S$ values for thermal inactivation of rSt-Phy are positive (Table 5). The enthalpy change ($\Delta H$) represents the energy required for thermal denaturation of protein. Large $\Delta H$ value signifies that high energy is required for breaking covalent bonds in the thermal inactivation of rSt-Phy. Free energy change ($\Delta G$) is positive for rSt-Phy, which indicates that the thermal denaturation of recombinant enzyme is non-spontaneous as reported for the recombinant phytase of *P. anomala* [18]. Denaturation of the enzyme is accompanied by the increase in the disorder of the enzyme structure which can be measured as entropy change ($\Delta S$) which decreases with increasing enzyme stability.

Liberation of inorganic phosphate by rSt-Phy after 25, 50 and 75 min from BPSF, BSF and BFF is given in Fig. 4. In chick GIT, initially feed passes through crop (http://www.thepoultrysite.com/articles/978/maintaining-gut-integrity/) in which transit time is 50 min and pH ~5 which is favorable condition for rSt-Phy and liberates most of the inorganic phosphate in 50 min. Under virtual gut conditions, the rSt-Phy is efficient in dephytinizing poultry feeds used at different growth phases of poultry birds.

The rSt-Phy brings out a change in color of phenol red from red–orange to blue–violet in the presence of meta-vanadate ions, $H_2O_2$ and KBr in the reaction mixture, which confirms the bromoperoxidation of phenol red (data not shown). It is known that only histidine acid phosphatases with the active site sequence RHGXRXP can function as haloperoxidase [57], when vanadate ion is incorporated into the active site. Vanadate is a phosphate analog, which is generally considered to bind as a transition state analogue to the phosphoryl transfer enzymes and inhibits their activities. The vanadium chloroperoxidase shares structural similarities with some membrane-bound acid phosphates [15]. Applications of haloperoxidase are in the field of iodination of proteins, diagnostics to determine the concentration of organic metabolites, immunoassays to quantitatively detect and determine certain antigens, estimation of chloride in liquid samples and as an antimicrobial agent due to the formation of singlet oxygen [20]. In view of the diverse applications of vanadate haloperoxidases, further investigations are called for assessing the efficacy of haloperoxidase derived from rSt-Phy.

## Conclusions

The codon-optimized phytase gene of *S. thermophile* has been successfully expressed in *P. pastoris*. The recombinant *P. pastoris* harboring 3 copies of phytase gene (*rSt-Phy*) secreted 40-fold higher phytase than the native fungal strain. The thermostability, acid stability, high catalytic efficiency, resistance to both pepsin and trypsin and broad substrate spectrum make rSt-Phy a good biocatalyst for application as an animal feed additive in poultry and aquaculture industries and deriving haloperoxidase.

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**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no competing interests.

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