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High level expression of a synthetic gene encoding *Peniophora lycii* phytase in methylotrophic yeast *Pichia pastoris*

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Abstract Phytase is widespread in nature. It has been used as a cereal feed additive that can enhance the phosphorus and mineral absorption in monogastric animals to reduce the level of phosphorus output in manure. Phytase of *Peniophora lycii* is a 6′-phytase, which owns high specific activity. To achieve a high expression level of 6′-phytase in *Pichia pastoris*, the 1,230-bp phytase gene of *P. lycii* was synthesized and optimized for codon usage, G+C content, as well as mRNA secondary structures. The gene constructs containing wild type or modified phytase gene coding sequences under the control of the highly-inducible alcohol oxidase gene (AOX1) promoter, the synthetic signal peptide (designated MF4I), which is a codon-modified *Saccharomyces cerevisiae* mating factor α-prepro-leader sequence, were used to transform *P. pastoris*. The *P. pastoris* strain that expressed the modified phytase gene (*phy-pl-sh*) with MF4I sequence produced 12.2 g phytase per liter of fluid culture, with the phytase activity of 10,540 U ml^{-1}. The yield of the modified phytase gene, with bias codon usage and MF4I signal, is 4.4 times higher than that of the wild type gene with MF4I signal and 13.6 times higher than that of the wild type gene with wild type *S. cerevisiae* signal. The recombinant phytase had one optimum pH (pH 4.5) and an optimum temperature of 50°C. The *P. pastoris* strain expressed the modified 6-phytase gene, with the MF4I signal peptide showing great potential as a commercial phytase production system.

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Introduction

Salts of phytic acid are called phytates (*myo-inositol* hexakisphosphate or *myo-inositol* 1,2,3,4,5,6-hexakis dihydrogen phosphate) (Reddy et al. 1982; Haefner et al. 2005). In many plant feeds, phosphorus digestibility is exceptionally low because a high proportion is in the form of a compound phytate (Stahl et al. 2000). Phytate is the major form of phosphorus storage in the cereal grains and legumes that are used in commercial animal feeds. Furthermore, phytate acts as an antinutrient by chelating cations, and it prevents the absorption of mineral, therefore reducing their availability (Graf 1983; Lei et al. 1993; Hurrell 2004). Furthermore, monogastric animals are incapable of utilizing the phosphorus bound in phytate due to low levels of phytase activity in the digestive organ (Jendza et al. 2005). Inorganic phosphorous, supplemented into animal feed, can solve this nutritional problem, but excess phosphorus excreted in animal manure poses a serious environmental problem (Lei and Stahl 2001).

Adding phytases to the feed of monogastric animals not only enhances the phosphorus and mineral absorption, but it also reduces the level of phosphorus output in the manure (Ullah and Phillippy 1994). Phytase is a widespread enzyme existing in nature including fungi (Wodzinski and Ullah 1996; Wyss et al. 1999a,b), bacteria (Rodriguez et al. 1999; Cho et al. 2005), and many plant seeds (Mitchell et al. 1997). Phytases mainly belong to the family of histidine acid phosphatases (Mitchell et al. 1997; Ullah

and Gibson 1987). Most of the known phytase are either 3- or 6-phytase (EC 3.1.3.8 or EC 3.1.3.26, respectively), depending on the position of the phosphomonoester group on the phytate molecule from where hydrolysis is initiated (Lassen et al. 2001). The use of microbial phytase as dietary supplement in the poultry and swine industry has been established.

A number of phytase genes have been isolated and characterized from plants (Reddy et al. 1982; Mullaney and Ullah 1998), bacteria (Rodriguez et al. 1999; Greiner et al. 1993) and fungi (Wodzinski and Ullah 1996; Wyss et al. 1999a,b; Pasamontes et al. 1997a,b; Wyss et al. 1998). During the last decades, many researches have been carried out on the modification and expression of those phytase genes in different host organisms, such as yeasts and plants (Lei and Porres 2003; Vohra and Satyanarayana 2003; Lee et al. 2005). Phytase gene from *Peniophora lycii* has been cloned by Lassen et al. (2001). The phytase owned high specific enzyme activity (1080 U/mg) and high heat stability (Residual activity was 62% after preincubation of the phytase for 60 min at 80°C). The *P. lycii* phytase has high specific activity, with a high affinity for phytic acid; however, the low expression level of the phytase in *P. lycii* suggests that the organism is not most suitable as a phytase supplement in animal feed.

Pichia pastoris is a methylotrophic yeast, which can utilize methanol as sole carbon source. *P. pastoris* can grow in simple defined media and can reach very high cell density and can accumulate extremely high concentration of intracellular protein under the control of a methanol-regulated alcohol oxidase (AOX1) promoter (Gellissen 2000; Waterham et al. 1997). We have expressed a recombinant thermostable phytase and two recombinant acidic phytases in *P. pastoris* and obtained the yield of 5.6, 4.2, and 6.1 g l^{-1} (Peng et al. 2002; Xiong et al. 2004a, 2005).

In this report, our objective was to achieve a high level expression of *P. lycii* phytase in *P. pastoris*, so it can be applied for large-scale production. We synthesized the phytase gene of *P. lycii* and the mating factor (α-factor) prepro-leader of *Saccharomyces cerevisiae*. To maximize the overexpression of phytase, the synthesized gene and signal sequence was optimized according to codon preference of *P. pastoris* (Sharp et al. 1986; Zhao et al. 2000). Optimized fragments were then assembled into a secreting expression vector of *P. pastoris* (Sreekrishna et al. 1997). We have successfully produced the phytase of *P. lycii* with very high yield using this heterologous expression system. To our knowledge, this research shows the highest reported expression level of the 6′-phytase in *P. pastoris*.

Materials and methods

Chemicals, enzymes, plasmids, and yeast strains

Phytic acid, sodium phytate, and G418 were purchased from Sigma Chemical (St Louis, MO, USA). *pfu Taq* DNA polymerase, T4 DNA ligase, and restriction endonucleases were purchased from Promega (Madison, WI, USA). DNA sequencing kit was purchased from Applied Biosystems (Foster City, CA, USA). Protein markers were purchased from Watson Biotech (Shanghai, China). QLAquick gel extraction kit was purchased from Qiagen (Stanford, CA, USA). Endoglycosidase H (Endo H_f) was purchased from New England Biolabs (Beverly, MA, USA). *P. pastoris* strain GS115 (his4) and vector pPIC9K were purchased from Invitrogen (San Diego, CA, USA). Oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology and Service (Shanghai, People's Republic of China). Plasmid pPIC9K (GenBank accession no. Z46234), containing the promoter and terminator of the methanol-inducible *P. pastoris* AOX1 gene, the α-mating factor prepro-secretion signal from *S. cerevisiae*, G418 resistance, and the HIS4 auxotrophic selection marker for transforming *P. pastoris* GS115 (his4), was used as a vector for expression of the phytase genes in *P. pastoris*.

Synthesis of the recombinant and wild-type phytase genes of *P. lycii*

To obtain a high level of expression, we designed and synthesized a recombinant gene, designated as *phy-pl-sh*, coding to phytase based on the original DNA sequence of the wild-type gene (GenBank accession no. AJ310696) from *P. lycii* using optimized genetic codons bias of yeast *P. pastoris* (Sharp et al. 1986; Zhao et al. 2000). Twenty oligonucleotide primers were synthesized (Supplement Table 1) and were joined together in a single polymerase chain reaction (PCR) step to synthesize this recombinant gene by using 1.5 μM of each inner primer (sh2–sh17) and 30 μM of each outer primer (sh1 and sh18) (Fig. 1a). The joined product was further amplified by a successive PCR method (Xiong et al. 2004b; Peng et al. 2003). The PCR conditions were as follows: 94°C for 30 s, 65°C for 40 s, and 72°C for 1.5 min for first five cycles, and then 94°C for 30 s, 70°C for 40 s, and 72°C for 1.5 min for 25 cycles. For comparison purposes, we also synthesized the wild-type phytase gene, *phy-pl-wt*, in a different fashion: 20 oligonucleotide primers (Supplement Table 2) were used in a simple, high-fidelity, and cost-effective PTDS method (Xiong et al. 2004b). In brief, two subsequent PCRs were conducted, and each reaction contained 1.5 μM of each inner oligonucleotide and 30 μM of each outer oligonucleotide. Conditions of the first PCR were: 25 cycles of 90°C for 30 s, 60°C for 45 s, 72°C for 50 s, with 2.5 U *pyrobest Taq* polymerase. To obtain the full-length *phy-pl-wt* gene, two approximate 600 bp products from the first PCR were mixed and used as the templates for the second PCR reaction, with the two outermost oligonucleotides as primers (wt1 and wt30) (Fig. 1b). The conditions of the second PCR were: one cycle at 94°C for 1 min, and then 25 cycles of 94°C for 30 s, 58°C for 35 S, and 72°C for 1.5 m. The sequences of the synthetic genes were confirmed by DNA sequencing. Errors in the synthetic

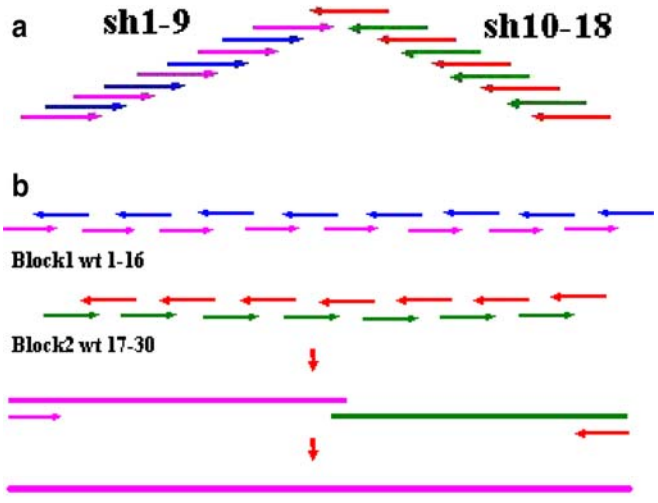

Fig. 1 **a** The strategy for the synthesis and assembly of the *phy-pl-sh* gene using successive PCR. Oligonucleotides of ~90 bp were assembled by single-step PCR using 1.5 μM of inner primers and 30 μM of external primers, which contained suitable restriction cleavage sites for cloning. **b** The strategy for the synthesis of the *phy-pl-wt* gene using the PTDS method. Oligonucleotides of ~60 bp were assembled using two-step PCR with 1.5 μM of inner primers and 30 μM of external primers, which contained suitable restriction cleavage sites for cloning

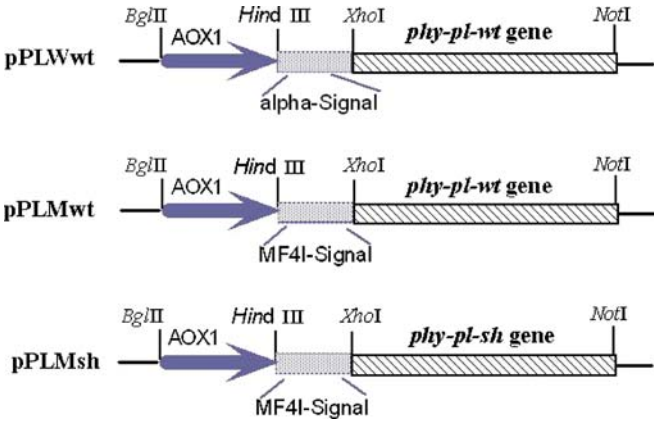

Fig. 2 Phytase gene constructs, pPLWwt, pPLMwt, and pPLMsh. The basic expression vector was pPIC9K. The *phy-pl-wt* or *phy-pl-sh* gene was cloned into the *Xho*I and *Not*I sites. The wild-type signal or MF4I signal sequence was cloned into the *Hin*dIII and *Xho*I sites

genes were corrected by overlap extension PCR method (Xiong et al. 2004b; Stemmer et al. 1995).

Construction of expression plasmids and recombinant DNA techniques

Standard DNA manipulation methods (Sambrook et al. 1989) were used in plasmid construction, *Escherichia coli* transformation, and Western blotting. To enhance the expression, a 357-bp of the α-factor prepro-leader MF4I (GenBank accession no. AY145833) was synthesized using *P. pastoris*-preferred codon usage (Xiong et al. 2003). The modified signal sequence, MF4I, was inserted into the *P. pastoris* expression vector pPIC9K between the unique *Hin*dIII and *Xho*I sites, after the wild-type α-signal sequence was excised. The 1,230-bp *phy-pl-wt* and *phy-pl-sh* genes were inserted between the *Xho*I and *Not*I sites, producing the constructs pPLMwt and pPLMsh, respectively. The *phy-pl-wt* gene was inserted between the *Xho*I and *Not*I sites of pPIC9K, producing the construct pPLWwt (Fig. 2).

Transformation of *P. pastoris* and selection of recombinant clones

Transformation of the *P. pastoris* strain GS115 (his4) was achieved using the electroporation method (Xiong et al. 2004a, 2005). Approximately 500 transformants were recovered and replica-plated onto MD medium and MD medium containing 0.5 g l^{-1} G418. To obtain the transformants containing a single copy of phytase expression unit, ten transformants were further selected from those ectypal transformants that grew normally on the MD medium but showed little or no growth on the MD medium containing 0.5 g l^{-1} G418. The clone screening was performed as described by Xiong (Xiong et al. 2004a, 2005).

High cell density fermentation of *P. pastoris*

From ten pPLMsh transformants that exhibited the enzymatic activities, three samples with high phytase yield and activity were selected for further fermentation and characterization. Similarly, three pPLMwt and pPLWwt transformants, respectively, showing high enzymatic activity, were selected for comparison. Those *P. pastoris* fermentations follow a well-established protocol described by Xiong (Xiong et al. 2004a, 2005), with some modifications as follows. The culture was then used to inoculate a 5-l fermentor (B.Braun, Melsungen AG, Melsungen, Germany). To favor optimal cell growth, temperature was controlled at 30°C (using tap water as the coolant), pH was controlled at 5.5 (using ammonium hydroxide, 30% solution), and oxygen was controlled at 30% (using 300–1,000 rpm agitation, 5–10 l/m airflow and combining with pure oxygen), responding automatically to oxygen demand. After exhaustion of the initial glycerol in approximate 20 h, as judged from the drop in O_2 consumption, 50% (v/v) glycerol containing 12 ml of PTM1 (Clare et al. 1991) solution per liter and 3 mg of biotin per liter was used as feed. The shift rate was started at approximate 10 ml h^{-1} of culture, in prophase 20 h, the feed rate was accelerated from 10 up to 50 ml h^{-1}, and then the feed was kept at 50 ml h^{-1} continuously. This phase was continued for 20 to 60 h. After all the glycerol was consumed, a brief starvation phase was given. The feeding was switched to 100% methanol solution with 12 ml l^{-1} of

PTM1 for additional 80 h to induce phytase expression. The complete fermentation took approximately 140 h.

Protein analysis and enzyme activity assays

One phytase unit was defined as the activity that releases 1 ìmol of inorganic phosphorus from sodium phytate per minute at 37°C (Engelen et al. 1994). The phytases produced from transgenic *P. pastoris* strains were purified according to Lassen's procedure (Lassen et al. 2001). The optimum pH, optimum temperature, and thermostabilities of the expressed phytases were determined according to Xiong's procedure (Xiong et al. 2004a, 2005). All assays were repeated three times.

Total solvable proteins from culture supernatants of the fermentation were run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels on the Mini-protein electrophoresis system (Bio-Rad, Hercules, CA, USA). The proteins were stained with Coomassie brilliant blue R-250 and destained for 12 h in 30% methanol and 10% acetic acid. Western blot analysis was performed using a rabbit polyclonal immunoglobulin G against phytase as the primary antibody (diluted 1:5,000 before application). A goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad Laboratories) was used as the secondary antibody for the final colorimetric detection (Sambrook et al. 1989).

Due to heavy glycosylation, the expressed phytases were found to have variable molecular sizes of 64, 67, 87, 110, and 120 kDa. Endo H_f was used to deglycosylate the expressed phytases (Han and Lei 1999). The reaction was carried out by incubating fermented samples with 0.3 IU of Endo H_f for 4 h at 37°C according to the manufacturer's instruction (New England Biolabs, Beverly, MA, USA).

Genetic stability of phytase expression

To determine the inheritance and stability of the clones expressing various levels of phytase in the progenies, ten clones of the strain containing pPLMsh were grown on rich (YPD) and SD selective medium for 10 cycles (After the first culture reached the stationary phase of growth on YPD or SD medium, 0.1 ml aliquot of the final culture was inoculated into 100 ml new YPD or SD medium for the next cycle of growth. All 10 cycles were cultured in the similar way). Phytase enzyme activities and gene expression levels were determined in the strain after ten generations of culture.

Results

Synthesis of *phy-pl-sh* (successive method) and *phy-pl-wt* genes (PTDS method)

All codons in *phy-pl-sh* were designed to be preferential for methylotrophic yeast *P. pastoris* (Supplement Fig. 1). Eighteen of 90 nt oligonucleotides were used (Supplement Table 1) to synthesize the recombinant gene by a single-step successive PCR procedure. These errors were corrected using overlap extension PCR method. The wild-type phytase gene (*phy-pl-wt*, GenBank accession no. AJ310696) was also synthesized and used for comparison. Thirty of 60 nt oligonucleotides were used for its synthesis with the PTDS method (Supplement Table 2). The errors were corrected using overlap extension PCR method (Xiong et al. 2004b; Stemmer et al. 1995).

The synthesized 1,230-bp recombinant phytase gene, *phy-pl-sh*, was submitted to GenBank (accession no. AY137198), and BLAST search showed it was 74.96% identical to the wild type phytase gene *phy-pl-wt* (GenBank accession no. AJ310696). To improve the efficiency of gene transcription and RNA stability, the G+C content of the synthesized phytase gene was decreased to around 50%, from 59.27 to 49.59%, and all hairpin structures and motifs containing consecutive ATs were eliminated by using degenerate codons (Supplement Fig. 1).

High level expression of the synthetic gene coding for phytase of *P. lycii* in *P. pastoris*

Approximately 500 transformants of *P. pastoris* containing the constructs pPLMsh, pPLMwt, and pPLWwt, respectively, which grew normally on the MD medium but showed little or no growth on the MM medium, were recovered onto MD medium and MD medium plates that contained G418 (0.5 g l^{-1}). With this method, only the transformants containing a single copy of the phytase gene could not grow normally on the MD medium containing 0.5 g l^{-1} G418 (Xiong et al. 2003; Scorer et al. 1994). Ten transformants were selected from each constructs for further analysis. Small-scale cultures of those three groups of transformants of *P. pastoris* were screened for their ability to secrete phytase of *P. lycii*. Phytase was detected by SDS-PAGE, and the enzyme activity was measured. According to results of the enzyme activity assay and level of expression, there were no significant differences among those ten transformants.

Three colonies transformed with pPLMsh exhibiting the high phytase activity among the colonies were separately selected for fermentation. Because our aim was to produce phytase of *P. lycii* at industrial scale, fermentation was performed in a three-step process including an initial batch phase in 5% glucose as the sole carbon source. After 20 h of batch culture when OD_{600} reached 48 nm, 50% (v/v) glycerol containing 12 ml of PTM solution per liter and 3 mg of biotin per liter was used as feed. The glycerol fed-batch process was initiated. After the 40-h growing phase, the OD_{600} reached 385 nm, a half-hour carbon-source starvation period was given. The production phase (methanol feeding) was started after 60 h of cell growth. The production feed medium consisted of methanol and PTM1. As shown in Fig. 3c, after 80 h of methanol induction (140 h total culture time), the fermentation culture achieved a final OD_{600} nm=392. This culture yielded 12.2 g l^{-1}

phytase with an activity of 10,540 U ml^{-1}. The clones with constructs pPLMwt and pPLWwt were fermented using the same methods and reagents, respectively. After 80 h of methanol induction (140 h total culture time), as shown in Fig. 3a,b, the fermentation culture achieved final OD_{600} of 387 and 392 nm, respectively, yielding 2.8 and 0.9 g l^{-1} phytase and with activities of 2, 650 and 905 U ml^{-1}, respectively. The final culture concentrations (reading at OD_{600}), phytase yields, and enzymatic activities are shown in Table 1.

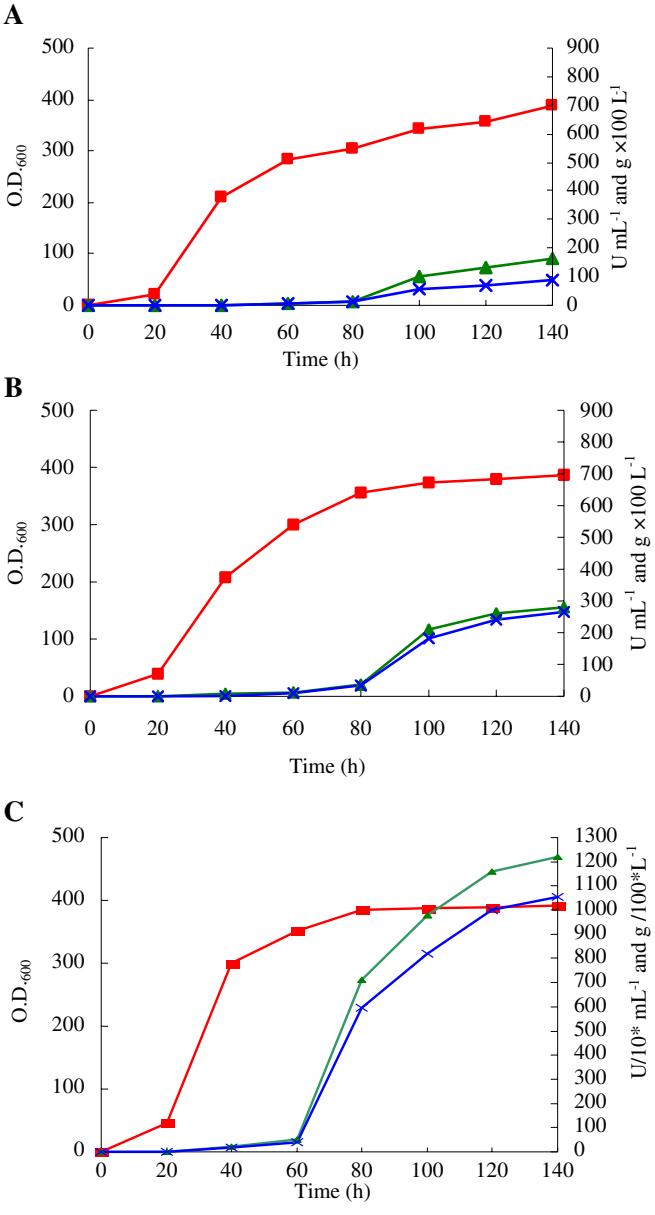

Fig. 3 Fermentation process of **a** pPLWwt, **b** pPLMwt, and **c** pPLMsh. OD_{600}(*filled squares*, *red*); phytase expression level (*filled triangles*, *green*); activity of phytase (*x marks*, *blue*). *Left Y-axis*, OD_{600}; *right Y-axis*, U ml^{-1} and g×100 l^{-1}

Quantities, activities and properties of phytases of *P. lycii* produced from *P. pastoris*strains that contained *phy-pl-sh* or *phy-pl-wt* genes

The three strains grew very similar with readings at OD_{600} about 390 nm. The use of the modified signal peptide sequence, MF4I, resulted in a big increase (3.1 times) in phytase yield compared to the one with wild-type signal peptide. However, the use of the modified coding sequence of the phytase gene led to 4.4 times of phytase yield compared to the wild-type gene. The use of the combination of the modified coding sequence and modified signal peptide sequence led to the highest yield of phytase, 12.2 g l^{-1}, which was 13.6 times of the wild-type gene with the wild-type signal peptide. The phytase activity from pPLMsh was 11.6 times and 4.0 times higher than those from pPLWwt and pPLMwt, respectively (Table 1).

The activity of the phytases from the colonies that contained pPLWwt, pPLMwt, and pPLMsh were determined at different pH levels, temperatures, and durations, as described in "Materials and methods." One single phytase activity peak was found at pH 4.5. The activity at pH 3.0 was 32% lower than that at pH 4.5, and the activity at pH 7.0 was 23% lower than that at pH 4.5 (Fig. 4a). The optimal temperature for phytase activity tested at pH4.5 was 50°C (Fig. 4b). The thermostability of the expressed phytase was tested, and the result revealed that the enzyme retained 25% activity when heated at 80°C for 10 min, but that activity decreased significantly after heat treatment for 15 min or more (Fig. 4c).

In this study, the phytase of *P. lycii* with high expression in *P. pastoris* exhibited some different properties from the phytase expressed in *Aspergillus oryzae* (Lassen et al. 2001; Ullah and Sethumadhavan 2003). Those activities are shown in Table 2.

Because of heavy glycosylation, the expressed phytase of *P. lycii* had molecular sizes ranged from ~70 to ~110 kDa measured by SDS-PAGE (Fig. 5a). However, after deglycosylation with Endo H_f, the phytase had an apparent molecular size of ~60 kDa (Fig. 5c). Western blot using rabbit polyclonal immunoglobulin G against the purified phytase further demonstrated that phytase was primarily the protein isolated from fermentation fluid (Fig. 5b).

Genetic stability of phytase expression

After ten successive generations of culture, the strains that expressed the modified version of the *P. lycii* phytase showed 97 and 96.5% of the phytase yield and activity, respectively, in comparison with the original colony (data not shown). There was no significant difference in yield and activity for the phytases produced from the strains growing in the rich (YPD) medium and the SD selective medium (data not shown). These results indicated that the strains reengineered with pPLMsh were stably inherited.

Table 1 The comparison of the expression level among three constructs

Construct	Signal sequence	Codon usage	Reading at OD_{600}[a]	Phytase Yield (g l^{-1})	Increase of yield[b] (fold)	Phytase activity U ml^{-1}	Increase of activity[c] (fold)
pPLWwt	Wild type	–	392	0.9	Base	905	Base
pPLMwt	MF4I	Optimized	387	2.8	3.1	2,650	2.9
pPLMsh	MF4I	Optimized	385	12.2	13.6	10,540	11.6

The numbers are average of three samples
[a]OD_{600} reading was measured at the end of 140-h culture period
[b]Increase in yield of phytase was calculated by using the phytase yield of the pPLWwt as the base
[c]Increase in enzymatic activity of phytase was calculated by using the phytase activity of the pPLWwt as the base

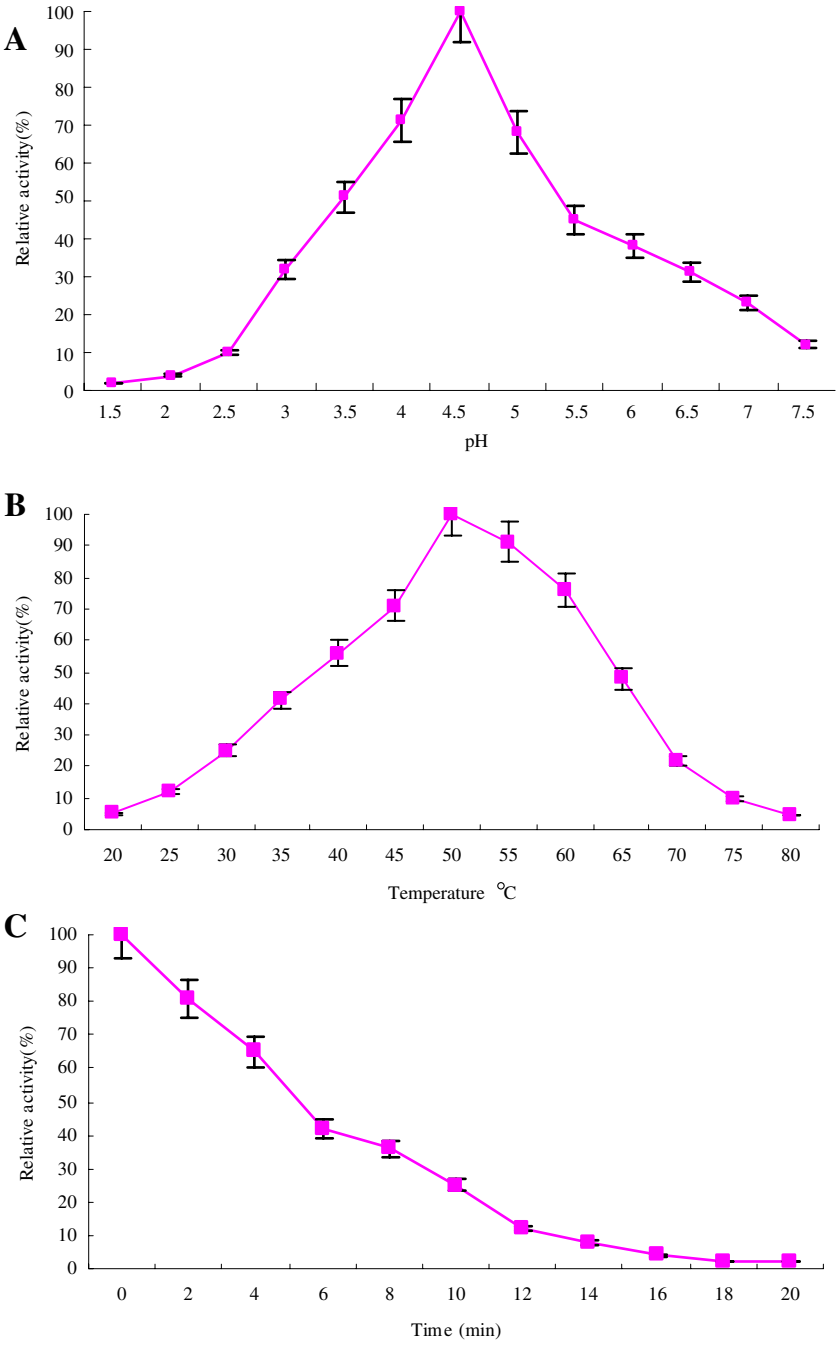

Fig. 4 The activity of expressed phytase of *P. lycii* produced from strain that expressed the *phy-pl-sh* gene with the MF4I sequence at different pH, temperatures and different times. **a** pH dependence of enzyme activity. **b** Temperature dependence of enzyme activity. **c** Heat stability of enzyme activity at 80°C

Table 2 Characterizations among those *P. lycii* phytases from different researches of optimal temperature, optimal pH, and thermal stability

Host source	Optimal pH	Optimal temperature (°C)	Thermal stability	Reference
A. oryzae	5.0	58	70°C for 10 min, retained 40%	Ullah and Sethumadhavan 2003
A. oryzae	4.0–4.5	50–55	80°C for 60 min, retained 62%	Lassen et al. 2001
P. pastoris	4.5	50	80°C for 10 min, retained 25%	This research

Discussion

In the former study, we have demonstrated that the use of *P. pastoris* preferred codons in some different kind of phytase gene and the signal peptide sequences could increase production and activity of phytase in *P. pastoris*

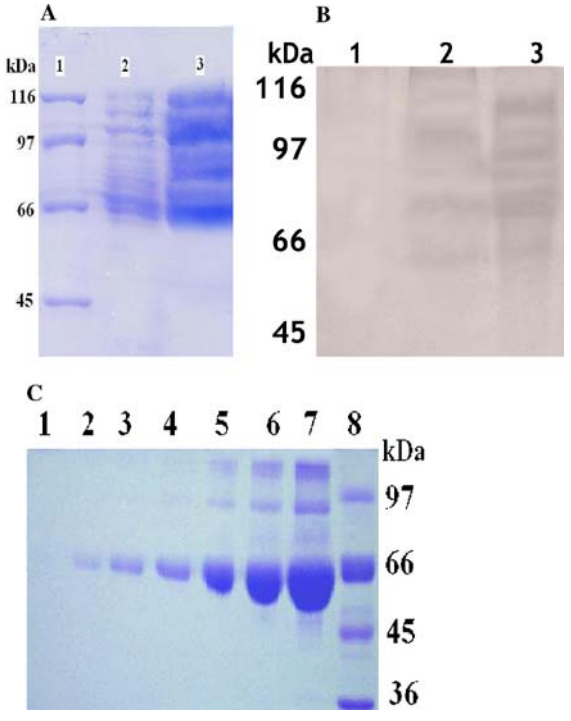

Fig. 5 Analysis of the expressed wild type and modified phytases of *P. lycii* in *P. pastoris*. **a** Expressed phytase ranging from ~70 to 120 kDa in size due to glycosylation. *Lane 1* Protein marker, *Lane 2* phytase expressed by the *phy-pl-wt* gene with MF4I signal in *P. pastoris*, *Lane 3* phytase expressed by the *phy-pl-sh* gene with MF4I signal in *P. pastoris*. **b** Western blot analysis of the phytase protein expressed in *P. pastoris*. *Lane 1* Protein marker, *Lane 2* phytase expressed in a strain that was transformed with the *phy-pl-wt* gene, *Lane 3* phytase expressed in a strain that was transformed with the *phy-pl-sh* gene. **c** Expressed phytase after deglycosylation by Endo H_f. *Lane M* Protein marker, *Lane 1–7* phytase expression after methanol induction of 10, 20, 40, 60, 80, 100, and 140 h

(Peng et al. 2002; Xiong et al. 2003–2005). In this report, we obtained a high expression 6′-phytase of *P. lycii* in *P. pastoris*. The phytase yield was achieved as high as 12.2 g l^{-1}, which was 4.4 times higher than the yield of wild-type phytase gene and 13.6 times higher than the yield of wild-type phytase gene with wild-type signal peptide that was expressed in *P. pastoris*. To our knowledge, the high level expression of phytase in *P. pastoris* is first reported here.

It was reported that Natuphos from BASF Corporation is 1.71 times more bioefficacious than phytase from *P. lycii*. On average, 1.59 times more phytase of *P. lycii* than Natuphos is required to reach the same effect (information found from http://www.basf.com). However, due to the high specific activity of phytase of *P. lycii*, the 6′-phytase has a greater commercial value. Moreover, the phytase of *P. lycii* has been reported to be more heat stable and effective under the conditions of the gastrointestinal tract of the monogastric animal. We have produced several recombinant phytases, such as a recombinant acid phytase, an *Aspergillus niger* 113 phytase, a high thermostable phytase, in *P. pastoris*. In this report, we also produced the new 6′-phytase, which exhibited some different properties, for example 6′ cleavage site, high specific activity, high thermostability, and can be acquired from different sources. It is believed that the application of an effective blend of enzymes should further improve phytate utilization efficiency by some animals. Multienzyme preparations could increase the applicable bound and could be used effectively to reduce nitrogen and phosphorus excreted by animals, while maintaining an enhanced productivity.

We chose the methylotrophic yeast *P. pastoris* as the heterologous host to express *P. lycii* phytase, as it has been previously used to express a wide variety of proteins (Cregg et al. 1993). There ware many reasons for obtaining 12.2 g l^{-1} phytase per liter in this study. We believed that the use of *P. pastoris* system was the primary reason. The use of methanol inducible promoter, AOX1, also played a critical role for the high-level expression. Another important reason for the high-level expression was the redesign of the phytase gene with yeast-favored genetic codons. Our previous research indicated that gene modification could increase by several times the expression of different phytase in *P. pastoris* (Peng et al. 2002; Xiong et al. 2004a, 2005). Thirdly, the replacement of the native signal peptide of *S. cerevisiae* á-factor use of a synthetic MF4I signal peptide (Genbank no. AY145833) for secretion of the recombinant phytase also made a contribution for such a high-level expression. This signal peptide has been used for the secretion of numerous heterologous proteins in both *S. cerevisiae* and *P. pastoris* (Kjeldsen et al. 1999; Xiong et al. 2004a). To optimize the translation efficiency of heterologous proteins, a ten-residue spacer peptide (EEAEA EAEPK) was inserted between the prepro-leader and the endoprotease processing site of the mating factor. Three residues (AIP), which were taken from the AOX1 protein in *P. pastoris*, were added behind ATG of the mating factor prepro-leader. A *Bam*HI site between the AOX1 promoter and the signal sequence was deleted. These changes proved to be effective in increasing phytase

secretion (Xiong et al. 2003, 2004a). The highly efficient signal peptide was an important foundation of the high-level expression of the *P. lycii* phytase in *P. pastoris*.

The commonly used culture media for recombinant *P. pastoris* strains are based on simple synthetic components. A suitable carbon source must be added in appropriate time, rate, and concentration to achieve maximal biomass. This was the last reason of obtaining 12.2 g l^{-1} expression. For obtaining high cell density, a variable feed means was used in this research. Production of the cell was the foundation of the high expression of the heterologous phytase. In this study, under optimal growth conditions, the cell density achieved OD_{600} 390 nm, and the phytase expression level achieved 12.2 g l^{-1}.

For unknown reasons, the 6′-phytase, which is produced with a high yield from *P. pastoris*, was not thermostable in our lab (80°C, 10 min retained 25%), as shown by Ullah and Sethumadhavan (2003) (70°C, 10 min retained 40%). However, Lassen et al. (2001) showed that the 6′-phytase was more thermostable (80°C, 60 min, retained 62%). It is the key for commercial phytases with high thermostability. Some thermostable phytase genes were found and cloned, but the specific activities of those phytases were low (Wyss et al. 1998, 1999a; Pasamontes et al. 1997b; Ha et al. 2000; Mullaney et al. 2000). We have obtained some highly thermostable phytase candidates using DNA shuffling method, and the results will be reported soon. There were some properties different from other reported phytases (e.g., optimal pH, optimal temperature, and thermostability). Some researches indicated that glycosylation was essential for the biosynthesis of *phyA* phytase in *P. pastoris* and the establishment of its thermostability (Han and Lei 1999). The phytase of *P. lycii* produced in *P. pastoris* was highly glycosylated, as there were nearly ten *N*-glycosylation sites (Bagger et al. 2003). Sagt et al. (2000) reported that the use of *N*-glycosylation could significantly enhance heterologous protein secretion.

We have demonstrated that the PTDS method offers a simple, rapid, high fidelity and low cost alternative to synthesize and assemble long DNA fragments (Xiong et al. 2004b). For *phy-pl-sh*, we used successive extension PCR method in year 2002. The error rate was 4.2/1,000. For *phy-pl-wt*, we used PTDS method in year 2004. The error rate was only 1.6/1,000.

To our knowledge, we are the first group that conducted the study using high-level expression system of 6′-phytase constructs in *P. pastoris*. The phytase of *P. lycii* has high specific activity character, and is highly expressed in *P. pastoris*. The results showed extremely high potentials for industrial production of the 6′-phytase.

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