Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Expression, purification and characterization of a *phyA*^{m}-*phyCs* fusion phytase*

Li-kou ZOU[†1,2], Hong-ning WANG[†‡1], Xin PAN[3], Guo-bao TIAN[1], Zi-wen XIE[1], Qi WU[4], Hui CHEN[4], Tao XIE[4], Zhi-rong YANG[1]

([1]*College of Life Science, Bioengineering Research Center for Animal Disease Prevention and Control, Sichuan University, Chengdu 610065, China*)

([2]*Faculty of Biotechnology of Dujiangyan Campus, Sichuan Agricultural University, Dujiangyan 611830, China*)

([3]*College of Earth Science, Chengdu University of Technology, Chengdu 610059, China*)

([4]*College of Life Science, Sichuan Agricultural University, Ya'an 625014, China*)

[†]E-mail: zoulkcn@hotmail.com; whongning@163.com

Received Dec. 7, 2007; revision accepted Apr. 26, 2008

Abstract: The *phyA*^{m} gene encoding acid phytase and optimized neutral phytase *phyCs* gene were inserted into expression vector pPIC9K in correct orientation and transformed into *Pichia pastoris* in order to expand the pH profile of phytase and decrease the cost of production. The fusion phytase *phyA*^{m}-*phyCs* gene was successfully overexpressed in *P. pastoris* as an active and extracellular phytase. The yield of total extracellular fusion phytase activity is (25.4±0.53) U/ml at the flask scale and (159.1±2.92) U/ml for high cell-density fermentation, respectively. Purified fusion phytase exhibits an optimal temperature at 55 °C and an optimal pH at 5.5~6.0 and its relative activity remains at a relatively high level of above 70% in the range of pH 2.0 to 7.0. About 51% to 63% of its original activity remains after incubation at 75 °C to 95 °C for 10 min. Due to heavy glycosylation, the expressed fusion phytase shows a broad and diffuse band in SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After deglycosylation by endoglycosidase H (EndoH_f), the enzyme has an apparent molecular size of 95 kDa. The characterization of the fusion phytase was compared with those of *phyCs* and *phyA*^{m}.

Key words: Expression, *PhyA*^{m}, *PhyCs*, Fusion phytase, *Pichia pastoris*
doi:10.1631/jzus.B0720006 **Document code:** A **CLC number:** Q55

INTRODUCTION

Phytate (*myo*-inositol hexakisphosphate) is the predominant form of storage of phosphorus in cereals, oilseeds and vegetables which are the major ingredients of animal feed (Reddy *et al*., 1982; Wodzinski and Ullah, 1996). Monogastric animals such as swine, poultry and fish are unable to utilize phytate due to the low levels of phytate-degrading enzyme activity in their digestive tracts and inorganic phosphate is added to the feed for the purpose of phosphorous supplementation (Bitar and Reinhold, 1972; Common, 1989). Hence non-metabolized phytate and unabsorbed phosphate pass through the intestinal tract and are excreted in manure causing phosphorous eutrophication. Furthermore, phytate also acts as an antinutritional factor in monogastric animals by chelating various metal ions needed by the animal, such as calcium, copper and zinc, therefore decreasing the dietary availability of these nutrients (Applegate *et al*., 2003; Bohn *et al*., 2008; Veum *et al*., 2006).

Phytase (*myo*-inositol hexaphosphate phosphohydrolase, EC 3.1.3.8 or EC 3.1.3.26) hydrolyzes phytate, thereby releasing inorganic phosphate (Liu *et al*., 1998; Wodzinski and Ullah, 1996). Supplemental microbial phytase in meal diets for swine, poultry and fish effectively improves phytate phosphorus utilization by these animals, decreasing phosphorus excre-

‡ Corresponding author

* Project supported by the National Key Technologies R & D Program of China during the 10th Five-Year Plan Period (No. 2002BA514A-12), the Education Department of Sichuan Province (No. 2006B014) and the Innovative Fund for Distinguished Young Scholars of Sichuan Agricultural University, China

tion pollution (Augspurger *et al*., 2003; Rutherfurd *et al*., 2002; Olukosi *et al*., 2007).

There have been many reports on the cloning and expressions of *phyA*, *phyC* and *appA* phytases (Chen *et al*., 2004; Rodriguez *et al*., 2000; Kim *et al*., 1999; Zinin *et al*., 2004; Rodriguez *et al*., 1999; Wang *et al*., 2005; Wu *et al*., 2004a; 2004b; Han *et al*., 1999). Recently *phyA* was site-mutated to *phyA*^{m} and *phyCs* was synthesized without altering the protein sequence of *phyC* according to the *Pichia pastoris* codon usage bias because of the low productions of wild-type *phyA* and *phyC* phytases in *P. pastoris*, and we overexpressed them in yeast as an active and extracellular enzyme (Chen *et al*., 2005; Zou *et al*., 2006). The *phyA* (*phyA*^{m}) phytase exhibits an optimum pH at 2.5 to 5.5, having a pH-activity profile ideally suited for maximal activity at low pH point (Chen *et al*., 2003; Kim *et al*., 2006). The *phyC* (*phyCs*) phytase exhibits an optimum pH around 6 to 9, suitable for neutral tracts and is more thermostable (Janne *et al*., 1998; Kim *et al*., 1998). However, the digestive tract of monogastric animals reveals pH between 2.0 to 7.0. For example, the stomach pH in many species is around 3.5, which happens to be the lowest activity point in the pH profile of *phyC*. And the small intestine pH is around 7.0, which is the lowest activity point in the pH profile of *phyA*. Thus a relatively high level of phytase supplementation is required in animal diets for adequate hydrolysis of phytate-phosphorus.

Therefore the objective of the present study is to expand the pH profile of phytase. The aim can be circumvented by fusion of *phyA*^{m} and *phyCs* phytases, which can be a feasible undertaking. Hence we report the overexpression of *phyA*^{m}-*phyCs* fusion phytase in *P. pastoris*. The enzyme was purified to homogeneity to study the thermostability and the effect of pH and temperature on its activity.

MATERIALS AND METHODS

Primers, plasmids and strains

The genes were amplified using pUC18-*phyA*^{m} and pUC18-*phyCs* vectors as templates (Chen *et al*., 2005; Zou *et al*., 2006). To amplify the *phyA*^{m} gene, the upstream primer 5′-TACGTACTGGCAGTCC CCGCCTCGAG-3′ and the downstream primer 5′-CCTAGGTGGAGCAAAACACTCCGCCCAAT-3′ were designed with *Sna*BI and *Avr*II restriction enzyme sites at 5′ and 3′ terminals, respectively. To amplify the *phyCs* gene, an upstream primer 5′-CCT AGGGGTGGCGGGGGTTCTGGTGGCGGTGGTT CCGGTGGCGGGGGTTCCAAGCACAAGTTGTC TGACCCTTACCACTTCA-3′ was synthesized with *Avr*II restriction enzyme site and linker $(Gly_4Ser)_3$ at the 5′ terminal, and a downstream primer 5′-GCGG CCGCTTACTTACCAGATCTGTCAGTCAACTTT CTTGGGTCGACTTGCTTGTTGAC-3′ was designed with *Not*I restriction enzyme site. The two PCR (polymerase chain reaction) fragments were cloned into pUCm-T vector (Sangon Bioengineering Co., Ltd., Shanghai, China), named pUCm-*phyA*^{m} and pUCm-*phyCs*, respectively. Vector pPIC9K was from Introgen (Carlsbad, CA, USA) for expression. *P. pastoris* GS115 (Introgen, Carlsbad, CA, USA) was used as the host for expression, and *Escherichia coli* JM109 was used as the host for subcloning. *P. pastoris* PP-NP^m-8 (Chen *et al*., 2005) and *P. pastoris* PP9KCs3 (Zou *et al*., 2006) were positive strains for *phyA*^{m} and *phyCs* phytase expressions.

Chemicals, enzymes and medium

Sodium phytate was purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Enzymes (*Taq*DNA polymerase, restriction endonucleases), protein markers, Agarose Gel DNA Purification Kit Version 2.0 and DNA Ligation Kit Version 2.1 were purchased from TaKaRa (Dalian, China). Endoglycosidase H ($EndoH_f$) was from New England Biolabs (Beverly, MA, USA). All other materials were from the Sangon Bioengineering Co., Ltd. (Shanghai, China) and of analytical reagent grade.

E. coli was cultured in Luria-Bertani (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) or on LB agar plate. Yeast was cultivated in YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar) medium. WBEG (wheat bran extract grown) and WBEI (wheat bran extract induction) media (Zou *et al*., 2006) were used for shake-flask growing and induction. The expression strains were screened in MD (minimal dextrose) (1.34% (w/v) YNB (yeast nitrogen base), 4×10^{-5}% (w/v) biotin, 2% (w/v) dextrose) and MM (minimal methanol) (1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 0.5% (v/v) methanol). The WBSG (wheat bran silkworm grown) (100 g/L malt powder and 15 g/L wheat

bran prepared as WBEG, 8 g/L silkworm chrysalis, 17.8 ml H_3PO_4, 0.62 g/L $CaSO_4$, 12.1 g/L K_2SO_4, 9.9 g/L $MgSO_4 \cdot 7H_2O$, 2.75 g KOH) medium was used for phytase fed-batch fermentation (Xie and Wang, 2007).

Subcloning, construction of expression vector and transformation

The DNA manipulations were carried out by using standard procedures. The *phyCs* gene was obtained by digestion of pUCm-*phyCs* with restriction endonucleases *Avr*II and *Not*I, followed by agarose gel electrophoresis resolution and purification using TaKaRa Agarose Gel DNA Purification Kit Version 2.0. The purified *phyCs* fragment was ligated to the purified *Avr*II-*Not*I double-digested secretory expression vector pPIC9K using TaKaRa DNA Ligation Kit Version 2.1. The recombinant vector, pPIC9K-*phyCs*, was prepared by a plasmid miniprep kit (Ω), identified by *Avr*II-*Not*I double-digested restriction endonucleases analysis and sequenced. The $phyA^m$ gene was inserted into the vector pPIC9K-*phyCs* at *Sna*BI and *Avr*II sites. The construct, pPIC9K-$phyA^mCs$, was transformed into JM109 which was plated on LB medium containing 80 μg/ml kanamycin. The positive colonies were then grown to prepare DNA for transformation.

P. pastoris GS115 strain was grown in the YPD medium and prepared for transformation according to the manufacturer instructions. Ten μg of plasmid DNA was linearized using *Bsp*EI and then transformed into yeast by electroporation. Roughly 10 μg linearized DNA pPIC9K-$phyA^mCs$ and 80 μl competent GS115 cells were used for each transformation. Transformants were plated onto an MD plate and incubated at 28 °C for 3~4 d. All plates should be checked daily. Using a sterile toothpick, pick one colony and patch the His^+ transformant in a regular pattern on both MM and MD plates, making sure to patch the MM plate first. For testing the effectiveness of expression conditions, GS115 albumin and GS115 β-Gal were grown as control.

Shake-flask cultivation

Single colonies were inoculated in 10 ml WBEG medium and grown at 28 °C and vigorously shaken at 250 r/min in a 100-ml flask for 16~20 h. Next, 3% (v/v) cells were inoculated into 100 ml WBEG medium. Cells were grown at 28 °C for 17~20 h and shaken at 250 r/min, then harvested by centrifugation at 5000×*g* and 4 °C for 5 min. The supernatant was decanted, and the pellet was resuspended and cultivated in a 500-ml flask containing 100 ml induction medium WBEI. Yeast growth was monitored by measuring the optical density at 600 nm (OD_{600}). 1.5% (v/v) methanol was added every 12 h in order to induce phytase production. The culture supernatant after induction for 0, 24, 48, 60, 72, 84, 96 and 120 h obtained by centrifugation was assayed for phytase activity.

Phytase activity assay

Phytase activity was assayed by measuring the rate of increase in inorganic orthophosphate (P_i) using the method as previously described (Janne *et al*., 1998; Kim *et al*., 1999; Tye *et al*., 2002; Kim and Lei, 2005). One unit of enzyme activity was defined as the activity that releases 1 μmol of inorganic phosphorus per minute from sodium phytate at pH 5.5 and 37 °C. Briefly, the crude enzyme was diluted in a suitable volume with 50 mmol/L sodium acetate buffer (2 mmol/L $CaCl_2$, 10% (v/v) glycerol, pH 5.5). A reaction mixture containing 250 μl diluted enzyme preparation and 1 ml 2 mmol/L sodium phytate in 100 mmol/L sodium acetate buffer (2 mmol/L $CaCl_2$, pH 5.5) was incubated at 37 °C for 30 min. Then the reaction was stopped by adding 1.25 ml of 15% (w/v) trichloroacetic acid (TCA). Blanks were run by incubating the enzyme samples with TCA for 30 min before adding the substrate. The released inorganic phosphate was measured by adding 2.5 ml of a color reagent. The color reagent was freshly prepared by mixing four volumes of 1.5% (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric-acid solution and one volume of a 2.7% (w/v) ferrous sulfate solution. Following a 10 min color-development period, the liberated inorganic phosphate was measured at 700 nm.

Fermentation

A single colony of transformant, found to produce the highest level of fusion phytase, was used for inoculation. A 30 ml YPD medium inoculated with fusion phytase-producing strain was grown in a 200-ml flask at 28 °C for 18 h. The 30 ml cultivated cells were then inoculated into a 2-L flask containing

270 ml YPD medium and grown at 28 °C for a further 16 h, following which 300 ml of the seed culture was added into a 5-L fermentor (East Biotech Equipment and Technology Co., Ltd., Zhenjiang, China) containing 2.7 L WBSG medium supplemented with 4% (v/v) glycerol as the sole carbon source. The temperature during fermentation was maintained at 28 °C and the pH was adjusted to 5.5 with 50% (w/w) NH_4OH. Dissolved oxygen was measured and maintained at over 20% air saturation by regulating agitation to between 300 and 800 r/min. Aeration was maintained at 1.5~2 vvm. The fermentation was carried out in two phases, a glycerol batch culture phase for approximately 18~19 h, followed by a glycerol and methanol fed-batch induction culture phase for 96 h. The glycerol batch culture phase was maintained until the glycerol had been completely exhausted, as indicated by a sudden increase in the level of dissolved oxygen. During the fed-batch induction culture phase the 50% (v/v) glycerol was supplemented with a feeding rate of 12 ml/(L·h) for 12 h. The methanol feeding (100% (v/v) methanol with 12 ml/L PTM1 trace salts) was controlled and the methanol rate was maintained at 8 ml/(L·h). The fermentation product was taken periodically after induction for 0, 24, 48, 60, 72, 84 and 96 h for phytase activity assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Purification of recombinant fusion phytase

All purification steps were carried out at 4 °C unless otherwise stated (Janne *et al*., 1998; Zou *et al*., 2006; Zhang *et al*., 2007). The culture supernatant obtained by centrifugation at 5000×*g* for 10 min was supplemented with $CaCl_2$ to a final concentration of 2 mmol/L. Initial purification was achieved by mixing the culture supernatant with 3 volumes of cold (−20 °C) enthanol and the enzyme was precipitated overnight at −20 °C. The precipitate was pelleted by centrifugation at 5000×*g* for 20 min, and washed once with cold (−20 °C) ethanol and once with cold (−20 °C) acetone. Protein pellets were air-dried at room temperature and re-dissolved in 10 mmol/L Tris-maleate buffer (2 mmol/L $CaCl_2$, 10% (v/v) glycerol, pH 6.0). The dissolved enzyme was performed by Chromatography Systems BioLogic LP and BioFrac^{TM} Fraction Collector (Bio-Rad, California, USA). An aliquot of the dissolved sample was applied to a Q-sepharose fast flow (FF) anion-exchange column (Amersham Biosiences) equilibrated with Buffer A (10 mmol/L Tris-maleate, 2 mmol/L $CaCl_2$, pH 6.0). The bound proteins were eluted with a linear NaCl gradient from 0 to 0.5 mol/L using Buffer B (50 mmol/L Tris-maleate, 2 mmol/L $CaCl_2$, pH 6.0). A flow rate of 5 ml/min was used and protein elution was monitored by absorbance at 280 nm. The peak fractions were pooled and then dialyzed against 10 mmol/L sodium acetate buffer (2 mmol/L $CaCl_2$, 10% (v/v) glycerol, pH 5.5). The portion after dialysis was concentrated by PEG (polyaethylene glycol) 20000 and stored at −20 °C for the next step analysis. The *phyA*^{m} acid phytase and *phyCs* neutral phytase were purified as we reported previously (Chen *et al*., 2005; Zou *et al*., 2006).

Characterization of recombinant fusion phytase

Enzyme activity assays were performed in defined buffers for various pH and temperature. The optimal pH was determined at 37 °C using buffers of 0.1 mol/L glycine-HCl (pH 2.0, 2.5 and 3.0), 0.1 mol/L sodium acetate (pH 3.5, 4.0, 4.5, 5.0 and 5.5), 0.1 mol/L Tris-maleate (pH 6.0 and 6.5), 0.1 mol/L Tris-HCl (pH 7.0, 7.5, 8.0 and 9.0) and 0.1 mol/L glycine-NaOH (pH 10.0, 11.0, 12.0 and 13.0). All buffers contained 2 mmol/L sodium phytate and 2 mmol/L $CaCl_2$. The optimal temperature at pH 5.5 was measured at 16, 26, 37, 45, 50, 55, 60, 65, 70, 75, 80, 90 and 100 °C. The sample was incubated in a water bath for 30 min, then the reaction was quenched with 1.25 ml of 15% (w/v) TCA and the phytase activity was monitored as previously outlined. For enzyme thermostability tests the enzyme was incubated for 10 min in a water bath at seven different temperatures (37, 75, 80, 85, 90 and 95 °C) in a buffer of 50 mmol/L sodium acetate (2 mmol/L $CaCl_2$, 10% (v/v) glycerol, pH 5.5) and then cooled to room temperature. The remaining phytase activity was measured at 37 °C, as described above.

Deglycosylation

EndoH_f was used to deglycosylate the fusion phytase expressed. Deglycosylation of purified enzyme was done according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). After the reaction the mixture was subjected to SDS-PAGE, using 8% (w/v) separating gels with a

4.4% (w/v) stacking gel. Gels were stained using Comassie brilliant blue R-250, methanol and glacial acetic acid.

RESULTS

Construction of expression vector pPIC9K-*phyA^{m}Cs* and its expression in *P. pastoris*

To create the construction of pPIC9K-*phyCs*, the *phyCs* gene was amplified by primers containing the linker $(Gly_4Ser)_3$ before subcloning into the expression vector pPIC9K. The final expression vector pPIC9K-*phyA^{m}Cs*, with the expected cloning junction sequence *phyA^m-phyCs* gene, was selected and sequenced before transformation into *P. pastoris*. The expression vector containing the *phyCs* and fusion *phyA^m-phyCs* genes in pPIC9K is shown in Fig.1. The *Bsp*EI-linearized pPIC9K-*phyA^{m}Cs* was transformed into competent *P. pastoris* strain GS115. By linearizing the recombinant vector at the unique site located in HIS_4 region, Mut^+ recombinants were generated. In this study a total of 40 transformants appeared and one colony, which exhibited the highest phytase activity among the colonies examined, was selected for shake-flask expression. The fusion phytase amino acid sequence is shown in Fig.2. As shown in Fig.3, after 96 h of methanol induction, the yield of total extracellular phytase activity reached its highest value at (25.4±0.53) U/ml in WBEI medium at the flask scale.

Purification and deglycosylation of the recombinant fusion phytase

The purification of recombinant phytase from WBEI medium by Q-sepharose FF anion-exchange chromatography is shown in Fig.4. When applied to an anion-exchange chromatography column at pH 6.0, the fusion phytase was eluted as a single peak at about 33 ml. The active protein fraction which showed higher phytase activity was pooled and combined. The sample was then dialyzed against a 10 mmol/L sodium acetate buffer (2 mmol/L $CaCl_2$, 10% (v/v) glycerol, pH 5.5) and concentrated by PEG 20000, prior to the measurement of enzyme properties. The expressed purified fusion phytase showed a broad and diffuse band rather than a protein with an apparent molecular size (Fig.5c).

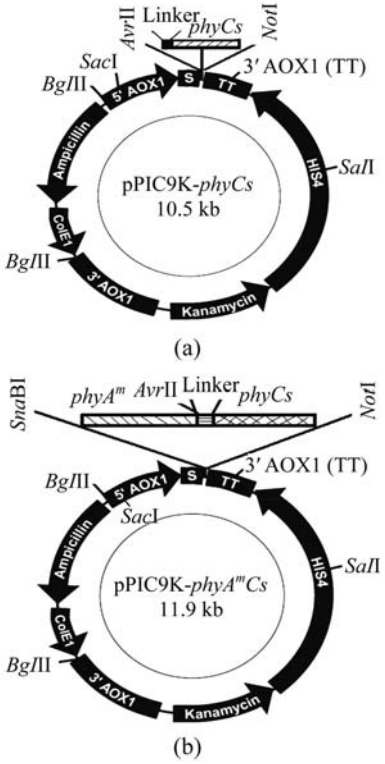

Fig.1 Maps of recombinant expression plasmids pPIC9K-*phyCs* (a) and pPIC9K-*phyA^{m}Cs* (b)

phyA^m →
YVLAVPASRNQSTCDTVDQGYQCFSETSHLWGQYAPFFSLANESAISPDVPAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEI
QQNATTFDGKYAFLKTYNYSLGADDLTPFGEQELVNSGIKFYQRYESLTRNIIPFIRSSGSSRVIASGKKFIEGFQSTKLKDPRAQP
GQSSPKIDVVISEASSSNNTLDPGTCTVFEDSELADAVEANFTATFVPTIRQRLENDLSGVSLTDTEVTYLMDMCSFDTISTSTVD
TKLSPFCDLFTHDEWINYDYLQSLKKYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDDTSSNHTLDSNSATFPLNSTLYADF
SHDNGIISILFALGLYNGTKPLSTTTVQNITQTDGFSSAWTVPFASRLYVEMMQCQAEQEPLVRVLVNDRVVPLHGCPVDALGR
linker *phyCs* →
CTRDSFVKGLSFARSGGDWAECFAP**PR***GGGGSGGGGSGGGGS*KHKLSDPYHFTVNAAAETEPVDTAGDAADDPAIWLDPKNP
QNSKLITTNKKSGLVVYSLEGKTLHSYHTGKLNNVDIRYDFPLNGKKVDIAAASNRSEGKNTIEIYAIDGKNGTLQSITDPDRPI
ASAIDEVYGFSLYHSQKTGKYYAMVTGKEGEFEQYELNADKNGYISGKKVRAFKMNSQTEGMAADDEYGSLYIAEEDEAIW
KFSAEPDGGSNGTVIDRADGRHLTPDIEGLTIYYAADGKGYLLASSQGNSSYAIYERQGQNKYVADFQITDGPETDGTSDTDGI
DVLGFGLGPEYPFGLFVAQDGENIDHGQKVNQNFKMVPWERIADKIGFHPQVNKQVDPRKLTDRSGK

Fig.2 Amino acid sequence of fusion phytase. The restriction enzyme sites amino acids are shown in bold, the linker is shown in italic, and the *phyA^m* and *phyCs* genes are shown by the arrows

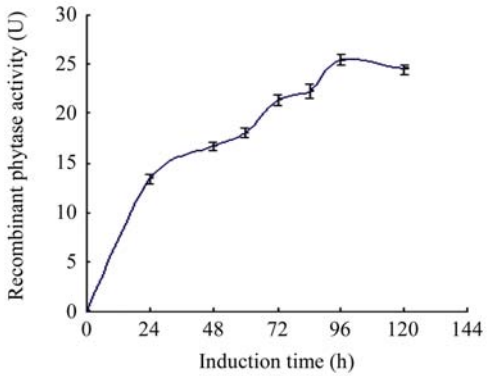

Fig.3 Effect of induction time on secretory expressed fusion phytase in flask scale. *Pichia pastoris* transformants were grown in wheat bran extract induction (WBEI) and 1.5% (v/v) methanol was added for induction. The phytase activity was expressed as mean±*SD* (*n*=3)

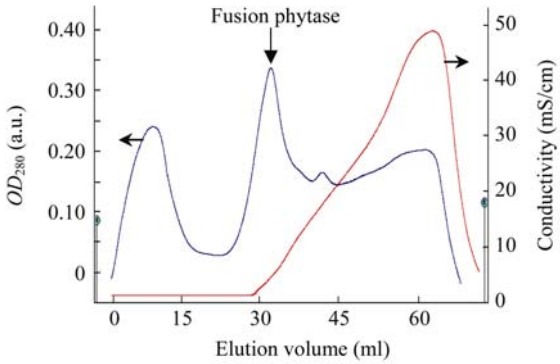

Fig.4 Purification of fusion phytase by anion-exchange chromatography. Five ml of dissolved samples containing fusion phytase were applied to a Q-sepharose FF anion-exchange chromatography column and eluted with a linear sodium chloride gradient. The fusion phytase was eluted as a single peak at an elution volume of approximately 33 ml

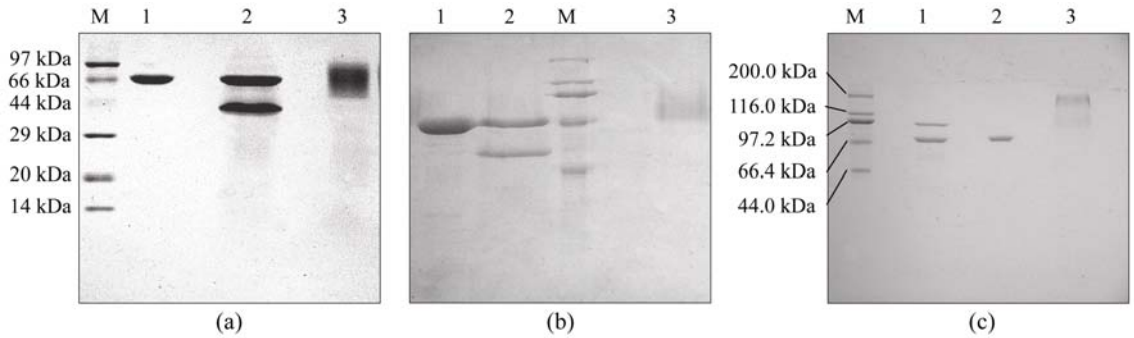

Fig.5 SDS-PAGE analysis of deglycosylated recombinant phytase. (a) Deglycosylation of *phyCs* phytase. 7.5 μl of purified *phyCs* phytase was loaded. Concentrations of protein were 0.968 μg/μl (Lane 1), 1.177 μg/μl (Lane 2, upper), 1.029 μg/μl (Lane 2, lower) and 0.837 μg/μl (Lane 3). Lane M: Lower molecular weight protein marker from TaKaRa; Lane 1: $EndoH_f$; Lane 2: $EndoH_f$ (upper) and deglycosylated phytase (lower); Lane 3: Purified *phyCs* phytase; (b) Deglycosylation of $phyA^m$ phytase. Six μl of purified phytase was loaded. Concentrations of protein were 1.036 μg/μl (Lane 1), 0.549 μg/μl (Lane 2, upper), 0.545 μg/μl (Lane 2, lower) and 0.312 μg/μl (Lane 3). Lane M: Higher molecular weight protein marker from TaKaRa; Lane 1: $EndoH_f$; Lane 2: $EndoH_f$ (upper) and deglycosylated phytase (lower); Lane 3: Purified $phyA^m$ phytase; (c) Deglycosylation of $phyA^m$-*phyCs* fusion phytase. Six μl of purified phytase was loaded. Concentrations of protein were 0.3 μg/μl (Lane 1, upper), 0.407 μg/μl (Lane 1, lower), 0.327 μg/μl (Lane 2) and 0.3 μg/μl (Lane 3). Lane M: Higher molecular weight protein marker from TaKaRa; Lane 1: Deglycosylated phytase (upper) and $EndoH_f$ (lower); Lane 2: $EndoH_f$; Lane 3: Purified $phyA^m$-*phyCs* fusion phytase

To prove that the fusion phytase gene was expressed successfully in *P. pastoris*, the $phyA^m$ and *phyCs* phytases were also purified and deglycosylated by $EndoH_f$. As shown in Figs.5a, 5b and 5c, after deglycosylation by $EndoH_f$ the *phyCs*, $phyA^m$ and fusion $phyA^m$-*phyCs* phytases had an apparent molecular size of 42, 51 and 95 kDa, respectively. The linker sequence encoding $(Gly_4Ser)_3$ had a molecular size of about 2 kDa. Therefore the fusion $phyA^m$-*phyCs* phytase gene was expressed successfully in *P. pastoris* as an active and extracellular enzyme.

Characterization of recombinant fusion phytase

Fig.6a shows the effect of temperature on fusion phytase activity in defined buffers and the optimum temperature proved to be 55 °C. The relative activities of fusion phytase were 0.39, 0.50, 0.68, 0.75, 0.83, 1.00, 0.86, 0.78, 0.40, 0.25, 0.17, 0.10 and 0.06 at 16, 26, 37, 45, 50, 55, 60, 65, 70, 75, 80, 90 and 100 °C, respectively. As shown in Fig.6b, the purified fusion phytase had the optimum pH at 5.5 to 6.0, but the over 70% of enzyme activity remained in the range of pH 2.0 to 7.0 including three peaks at pH 2.0 to 2.5, pH 5.5 to 6.0 and pH 7.0. The relative activities of the

fusion phytase were 0.67, 0.73, 0.49, 0.53, 0.60, 0.78, 0.94, 1.00, 0.99, 0.73, 0.74, 0.25, 0.08, 0.05, 0.02, 0, 0 and 0 at pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0, 11.0, 12.0 and 13.0, respectively. To test the thermostability, the activity of the fusion phytase was measured after incubation at various temperatures for 10 min. The relative activity of the enzyme remains at the levels of 1.00, 0.63, 0.63, 0.57, 0.56 and 0.51 at 37, 75, 80, 85, 90 and 95 °C, respectively. In conclusion, 51% to 63% of its original activity remained after incubation at 75 to 95 °C for 10 min, as shown in Fig.6c. The properties of *phyA*^{m} acid phytase and *phyCs* neutral phytase were measured as we reported previously (Chen *et al*., 2003; 2005; Zou *et al*., 2006).

Fermentation

The high cell-density culturing was performed by feeding 50% (v/v) glycerol (12 ml/h for 12 h) after the initial amount of glycerol had been exhausted, as evidenced by the sudden increase in the level of dissolved oxygen. As shown in Fig.7b, for the glycerol batch phase the OD_{600} was 129.8±4.25. The OD_{600} reached 143.3±2.8 after the 24 h fed-batch induction cultivation. Maximum activity occurred within 84 h of post-induction and SDS-PAGE analysis indicated that phytase comprised the major protein present in the broth (Fig.7a). The enzyme secreted into the medium was approximately (159.1±2.92) U/ml, with the cell density reaching approximately OD_{600}=178.4±2.08.

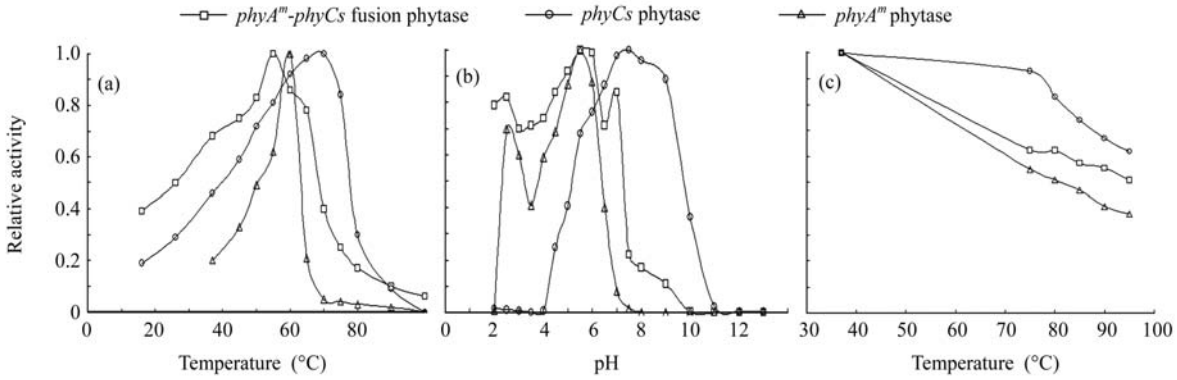

Fig.6 Effects of temperature (a), pH (b) and temperature stability (c) on phytase activity. To test the thermostability, the enzyme was pre-incubated at various temperatures for 10 min, and residual activity was measured at 37 °C. The data were presented as relative activity at each pH and temperature (*n*=3)

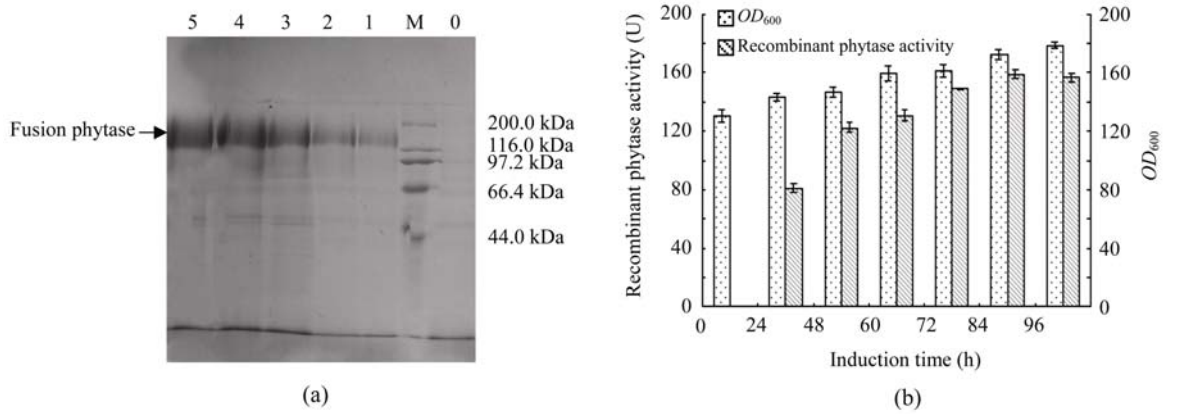

Fig.7 Fusion phytase production in fermentation. (a) SDS-PAGE analysis. One μl of fermentation product was loaded. Concentrations of protein were 0.585, 0.738, 0.949, 1.326 and 1.335 μg/μl (from Lane 1 to Lane 5). Lane M: Higher molecular weight protein marker from TaKaRa as the same as that in Fig.5c; Lane 0: Culture supernatant before induction; Lanes 1~5: Culture supernatant after induction for 24, 48, 72, 84 and 96 h, respectively; (b) The correlation of culture concentration and activity of fusion phytase with induction time. The data were presented as mean±*SD* (*n*=3)

DISCUSSION

The most striking success in the study was in proving the feasibility of expanding the pH profile of phytase to enhance its catalytic efficiency in the digestive tract. As shown in Fig.6a, the *phyA*^{m} acid phytase exhibited an optimum pH at 2.5 or 5.5, but it lost most of its activity when the pH was below 2.0 or above 6.5. The *phyCs* neutral phytase exhibited an optimum pH at 7.5 but it lost most of its activity below pH 4.0 or above 10.0. The optimum pH of the fusion phytase was at 5.5 to 6.0 and over 70% of relative activity of the enzyme remained in the range of pH 2.0 to 7.0 including three peaks at pH 2.0 to 2.5, pH 5.5 to 6.0 and pH 7.0. Only 1%, 40% and 8% relative activity of the *phyA*^{m} phytase remained at pH 2.0, 6.5 and 7.0, respectively. The wild-type *phyA* phytase showed optimum pH at 4.6 and lost most of its activity at pH 2.0 and 6.5 (Chen *et al*., 2003). But 79%, 71% and 84% of relative activity of the fusion phytase remained at pH 2.0, 6.5 and 7.0, respectively, higher than that of *phyA*^{m} and wild-type *phyA*. Between pH 2.0 and 4.5, 70% to 92% of relative activity of the fusion phytase remained, which was higher than that of *phyCs* (0% to 25% of relative activity). Therefore the two phytases may have an interactive effect which we explored by crystal structure comparison (data not shown). However, the fusion phytase activity declined suddenly at pH 7.5, which was unexpectedly different from that of *phyCs*. Thus the fusion phytase could be not only suitable for the acid but also for the neutral digestive tracts of animals. Characterization of the recombinant fusion phytase revealed its higher relative activity and broader pH optimum, which was considered to be important for the application.

The fusion phytase had the same optimum temperature at 55 °C as the wild-type *phyA* phytase, but its residual activities at 16 °C and 37 °C were higher than those of *phyA*^{m} acid phytase and *phyCs* neutral phytase (Chen *et al*., 2003; 2005; Zou *et al*., 2006). As shown in Fig.6a, the *phyA*^{m} and *phyCs* phytases revealed optimum temperatures of 60 and 70 °C, respectively. Thermostability is a particularly important issue since feed pelleting is commonly performed at temperatures between 65 °C and 95 °C. About 51% to 63% of the original activity of fusion phytase remained after incubation at 75 °C to 95 °C for 10 min without a protective agent, which was more thermostable than *phyA*^{m} phytase (Fig.6c) and *phyA* mutant which retained 20% activity after being heated at 80 °C for 10 min (Zhang *et al*., 2007).

The expressed *phyA*^{m}, *phyCs* and fusion *phyA*^{m}*-phyCs* phytases showed a broad and diffuse band in SDS-PAGE due to the heavy glycosylation (Fig.5) as described by Markus *et al*.(1999) and Zou *et al*.(2006). After deglycosylation by EndoH_f, the *phyA*^{m}, *phyCs* and fusion *phyA*^{m}*-phyCs* phytases had an apparent molecular size of 51, 42 and 95 kDa, respectively. The glycosylation may have a number of effects on the properties of an enzyme, and it may have a positive effect on the thermostability (Yoshimasu *et al*., 2004; Han and Lei, 1999). As we previously reported, the glycosylation of *P. pastoris* phytase appeared to have increased with induction time and the glycosylated phytase resulted in the enhancement of thermostability (Zou *et al*., 2006), so we deduced that deglycosylation may lead to the decrease of fusion phytase thermostability. The purified phytase showed streaking in SDS-PAGE, but after deglycosylation the streaking was eliminated as shown in Fig.5.

Vuolanto *et al*.(2001) reported recombinant *phyC* neutral phytase activity of 48 U/ml with *Bacillus subtilis* expression system at a 2-L fermentor using a fed-batch protocol, which was lower than the *phyCs* phytase activity of 54 U/ml expressed in *P. pastoris* (data not shown). The modified *phyA-sh* acid phytase activity expressed in *P. pastoris* only reached 125 U/ml at a 5-L fermentor reported by Xiong *et al*. (2005). In this paper, after an induction period of 84 h, the fusion phytase activity reached (159.1±2.92) U/ml at a 5-L fermentor using a fed-batch protocol. This is the first report of a fusion phytase gene in *P. pastoris* which has been shown to be suitable for high-level expression of various heterologous proteins, either intracellular or secretory. Under fermentation conditions *P. pastoris* has been shown to be able to grow to high cell densities capable of giving high levels of expressed protein with levels greater than 10 g/L reported (Cregg and Higginns, 1995; Romanos, 1995; André *et al*., 2006). So there is a huge potential for the fermentation of fusion phytase if optimizing the cultivation conditions. According to our calculation, the cost of the BMMY (buffered methanol-complex medium), BSM (basal salts medium) and WBSG media

was 7.5, 2.5 and 0.5 Yuan/L, respectively. Hence compared with the expensive BMMY and BSM (Xiong *et al*., 2005) media, WBSG is an economical medium for the growth of *P. pastoris* and phytase expression.

In conclusion, the *phyA*^{m}*-phyCs* gene was successfully expressed as an active and extracellular phytase in *P. pastoris*. The fusion phytase shows an extended pH profile and favourable thermostability suitable for production and application.

References

André, N., Cherouati, N., Prual, C., Steffan, T., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R., Reinhart, C., 2006. Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. *Protein Science*, **15**(5):1115-1126. [doi:10.1110/ps.062098206]

Applegate, T.J., Webel, D.M., Lei, X.G., 2003. Efficacy of a phytase derived from *Escherichia coli* and expressed in yeast on phosphorus utilization and bone mineralization in turkey poults. *Poultry Science*, **82**(11):1726-1732.

Augspurger, N.R., Webel, D.M., Lei, X.G., Baker, D.H., 2003. Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. *J. Anim. Sci*., **81**(2):474-483.

Bitar, K., Reinhold, J.G., 1972. Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf, and man. *Biochem. Biophys. Acta*, **268**(2):442-452.

Bohn, L., Meyer, A.S., Rasmussen, S.K., 2008. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. *J. Zhejiang Univ. Sci. B*, **9**(3):165-191. [doi:10.1631/jzus.B0710640]

Chen, C.C., Wu, P.H., Huang, C.T., Cheng, K.J., 2004. A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase. *Enzyme and Microbial Technology*, **35**(4):315-320. [doi:10.1016/j.enzmictec.2004.05.007]

Chen, H., Zhao, H.X., Wang, H.N., 2003. The research of phytase's characteristics from gene engineering yeast. *Microbiology*, **30**(3):38-41 (in Chinese).

Chen, H., Zhao, H.X., Wang, H.N., 2005. Increasing expression level of phytase gene (*phyA*) in *Pichia pastoris* by changing rare codons. *Chinese Journal of Biochemistry and Molecular Biology*, **21**(2):171-175 (in Chinese).

Common, F.H., 1989. Biological availability of phosphorus for pigs. *Nature*, **143**:370-380.

Cregg, J.M., Higginns, D.R., 1995. Production of foreign proteins in the yeast *Pichia pastoris. Can. J. Bot.*, **73**(Suppl.):5891-5987.

Han, Y., Lei, X.G., 1999. Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (*phyA*) in *Pichia pastoris*. *Archives of Biochemistry and Biophysics*, **364**(1):83-90. [doi:10.1006/abbi.1999.1115]

Han, Y., Wilson, D.B., Lei, X.G., 1999. Expression of an *Aspergillus niger* phytase gene (*phyA*) in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **65**(5):1915-1918.

Janne, K., Marko, L., Paivi, N., Nisse, K., Juha, A., 1998. Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. *Applied and Environmental Microbiology*, **64**(6):2079-2085.

Kim, T., Mullaney, E.J., Porres, J.M., Roneker, K.R., Crowe, S., Rice, S., Ko, T., Ullah, A.H.J., Daly, C.B., Welch, R., Lei, X.G., 2006. Shifting the pH profile of *Aspergillus niger phyA* phytase to match the stomach pH enhances its effectiveness as an animal feed additive. *Applied and Environmental Microbiology*, **72**(6):4397-4403 [doi:10.1128/AEM.02612-05]

Kim, T.W., Lei, X.G., 2005. An improved method for a rapid determination of phytase activity in animal feed. *J. Anim. Sci.*, **83**:1062-1067.

Kim, Y.O., Lee, J.K., Kim, H.K., Yu, J.H., Oh, T.K., 1998. Cloning of the thermostable phytase gene (*phy*) from *Bacillus* sp. DS11 and its expression in *E. coli*. *FEMS Microbiology Letters*, **162**(1):185-191. [doi:10.1111/j.1574-6968.1998.tb12997.x]

Kim, Y.O., Lee, J.K., Oh, B.C., Oh, T.K., 1999. High level expression of a recombinant thermostable phytase in *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.*, **63**(12):2205-2207. [doi:10.1271/bbb.63.2205]

Liu, B.L., Rafiq, A., Tzeng, Y.M., Rob, A., 1998. The induction and characterization of phytase and beyond. *Enzyme and Microbial Technology*, **22**(5):415-424. [doi:10.1016/S0141-0229(97)00210-X]

Markus, W., Luis, P., Arno, F., Roland, R., Michek, T., Alexandra, K., Anke, M., Martin, L., Line, S., Ursro, T., *et al*., 1999. Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. *Applied and Environmental Microbiology*, **65**(2):359-366.

Olukosi, O.A., Sands, J.S., Adeola, O., 2007. Supplementation of carbohydrases or phytase individually or in combination to diets for weanling and growing-finishing pigs. *J. Anim. Sci.*, **85**5(7):1702-1711. [doi:10.2527/jas.2006-709]

Reddy, N.R., Sathe, S.K., Salunkhe, D.K., 1982. Phytates in legumes and cereals. *Adv. Food Res.*, **28**:1-92.

Rodriguez, E., Han, Y., Lei, X.G., 1999. Cloning, sequencing, and expression of an *Escherichia coli* acid phosphatase/phytase gene (appA2) isolated from pig colon. *Biochem. Biophys. Res. Commun.*, **257**(1):117-123. [doi:10.1006/bbrc.1999.0361]

Rodriguez, E., Mullaney, E.J., Lei, X.G., 2000. Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. *Biochemical and Biophysical Research Communications*, **268**(2):373-378. [doi:10.1006/bbrc.2000.2121]

Romanos, M., 1995. Advances in the use of *Pichia pastoris* for high-level expression. *Curr. Opin. Biotechnol.*, **6**(5):

527-533. [doi:10.1016/0958-1669(95)80087-5]

Rutherfurd, S.M., Chung, T.K., Moughan, P.J., 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in the broiler chicken. *Br. Poultry Sci*., **43**(4):598-606. [doi:10.1080/0007166022000004516]

Tye, A.J., Siu, F.K.Y., Leung, T.Y.C., Lim, B.L., 2002. Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and *B. licheniformis*. *Appl. Microbiol. Biotechnol*., **59**(2):190-197. [doi:10.1007/s00253-002-1033-5]

Veum, T.L., Bollinger, D.W., Buff, C.E., Bedford, M.R., 2006. A genetically engineered *Escherichia coli* phytase improves nutrient utilization, growth performance, and bone strength of young swine fed diets deficient in available phosphorus. *J. Anim. Sci.*, **84**:1147-1158.

Vuolanto, A., von Weymarn, N., Kerovuo, J., Ojamo, H., Leisola, M., 2001. Phytase production by high cell density culture of recombinant *Bacillus subtilis*. *Biotechnology Letters*, **23**(10):761-766. [doi:10.1023/A:1010369325558]

Wang, H.N., Wu, Q., Zhao, H.X., Zou, L.K., Liu, S.G., 2005. Secretory expression of *Bacillus subtilis* phytase *phyC* in *Pichia pastoris*. *Journal of Zhejiang University* (*Agric. & Life Sci*.), **31**(5):621-627 (in Chinese).

Wodzinski, R.J., Ullah, A.H., 1996. Phytase. *Adv. Appl. Microbiol.*, **42**:263-302.

Wu, Q., Wang, H.N., Zou, L.K., Zhao, H.X., Liu, S.G., 2004a. Expression of phytase *phyC* from *B. subtilis* in *E. coli* and its effect in enzymetic thermostability. *High Technology Letters*, **14**(5):23-27 (in Chinese).

Wu, Q., Wang, H.N., Zou, L.K., Zhao, H.X., Liu, S.G., 2004b. Intrcellular expression of *Bacillus subtilis* phytase *phyC* in *Pichia patoris*. *Journal of Jishou University (Natural Science Edition)*, **25**(1):36-41 (in Chinese).

Xie, Z.W., Wang, H.N., 2007. Preliminary study on flask fermentation condition of producing phytase by engineering bacterium. *China Feed*, **3**:13-16 (in Chinese).

Xiong, A.S., Yao, Q.H., Peng, R.H., Han, P.L., Cheng, Z.M., Li, Y., 2005. High level expression of a recombinant acid phytase gene in *Pichia pastoris*. *Journal of Applied Microbiology*, **98**(2):418-428. [doi:10.1111/j.1365-2672.2004.02476.x]

Yoshimasu, M.A., Tanaka, T., Ahn, J.K., Yada, R.Y., 2004. Effect of N-linked glycosylation on the aspartic proteinase porcine pepsin expressed from *Pichia pastoris*. *Glycobiology*, **14**(5):417-429. [doi:10.1093/glycob/cwh024]

Zhang, W., Mullaney, E.J., Lei, X.G., 2007. Adopting selected hydrogen bonding and ionic interactions from *Aspergillus fumigatus* phytase structure improves the thermostability of *Aspergillus niger* phyA phytase. *Applied and Environmental Microbiology*, **73**(9):3069-3076. [doi:10.1128/AEM.02970-06]

Zinin, N.V., Serkina, A.V., Gelfand, M.S., Shevelev, A.B., Sineoky, S.P., 2004. Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*. *FEMS Microbiology Letters*, **236**(2):283-290. [doi:10.1111/j.1574-6968.2004.tb09659.x]

Zou, L.K., Wang, H.N., Pan, X., Xie, T., Wu, Q., Xie, Z.W., Zhou, W.R., 2006. Design and expression of s synthetic *phyC* gene encoding the neutral phytase in *Pichia pastoris*. *Acta Biochimica et Biophysica Sinica*, **38**(11):803-811. [doi:10.1111/j.1745-7270.2006.00231.x]