Appl Biochem Biotechnol (2010) 162:2157–2165
DOI 10.1007/s12010-010-8990-4

High Level Expression of an Acid-Stable Phytase from *Citrobacter freundii* in *Pichia pastoris*

Wei Zhao · Aisheng Xiong · Xiaoyan Fu · Feng Gao · Yongsheng Tian · Rihe Peng

Received: 9 October 2009 / Accepted: 11 May 2010 /
Published online: 17 June 2010

Abstract To obtain a high level expression of phytase with favorable characteristics, a codon-optimized phytase gene from *Citrobacter freundii* was synthesized and transferred into *Pichia pastoris*. Small-scale expression experiments and activity assays were used to screen positive colonies. After purified by Ni^{2+}–NTA agarose affinity column, the characterizations of the recombinant phytase were determined. The recombinant phytase (r-phyC) had two distinct pH optima at 2.5 and 4.5 and an optimal temperature at 50 °C. It retained more than 80% activity after being incubated under various buffer (pH 1.5–8.0) at 37 °C for 1 h. The specific activity, *K*m, and *V*max values of r-phyC for sodium phytate were 2,072±18 U mg^{-1}, 0.52±0.04 mM, and 2,380±84 U mg^{-1} min^{-1}, respectively. The enzyme activity was significantly improved by 1 mM of K^{+}, Ca^{2+}, and Mg^{2+}. These characteristics contribute to its potential application in feed industry.

Keywords *Citrobacter freundii* · Codon optimization · *Pichia pastoris* · pH-stable · Phytase

Introduction

Phosphorus is a fundamental mineral nutrient in the growth and development of animals. In most feed sources used in animal production, such as cereals grains and legumes, phytate (myo-inositol hexakisphosphate) is regarded as the principal storage form of phosphorus [1]. However, because of digestive enzyme deficiency, non-ruminant animals such as poultry, pigs, and fish do not utilize the phosphorus combined with phytate efficiently [2]. Animal manures with excess amounts of undigested phytates can accelerate eutrophication

Electronic supplementary material The online version of this article (doi:10.1007/s12010-010-8990-4) contains supplementary material, which is available to authorized users.

W. Zhao · A. Xiong · X. Fu · F. Gao · Y. Tian · R. Peng (✉)
Biotechnology Research Institute, Shanghai Academy of Agricultural Sciences,
2901 Beidi Rd, Shanghai 201106, China
e-mail: yaoquanhong_sh@yahoo.com.cn

in water and led to other massive environmental problems [3]. Moreover, phytates can prevent nutrition absorption in animals by binding to proteins and by chelating with calcium, magnesium, and zinc or other minerals [4].

Phytase (myo-inositol hexakisphosphate phosphohydrolases), the enzyme that catalyzes hydrolysis of phytate to inositol and inorganic phosphate, has a wide distribution in many organisms, such as plants [5], bacteria [6], yeasts [7], and particularly fungi [8, 9]. These enzymes can be subdivided into two classes, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26), according to the respective position of the initial hydrolysis of phytate [10]. Many studies have shown that supplementing phytase in feeds is an effective method to enhance phosphorus and mineral absorption in monogastric animals, in addition to minimizing environmental pollution significantly by reducing phosphorus excretion [11, 12].

The methylotrophic yeast *Pichia pastoris* has proved to be a powerful system for phytase expression. As a eukaryote, *P. pastoris* has many advantages mainly including the ability to produce foreign proteins at high levels, the capability of performing many eukaryotic posttranslational modifications [13]. Previously, we have succeeded in expressing a heat-stable phytase [14] and acidic phytases [15] in *P. pastoris*.

In this study, the phytase gene from *Citrobacter freundii* was synthesized according to codon preference and secreted and expressed in *P. pastoris*. The recombinant phytase, designated as r-phyC, showed a great application potential as a feed additive due to its relatively high specific activity under acidic conditions.

Materials and Methods

Strains, Vectors, and Chemicals

P. pastoris strain GS115 ($His^{-}Mut^{+}$) was purchased from Invitrogen Corporation. Vector pYPX88 (GenBank accession no. AY178045) was constructed in our laboratory previously according vector pPIC9K (Invitrogen) [15]. Sodium phytate were purchased from Sigma. Taq DNA polymerase, T4 DNA ligase and restriction endonucleases were purchased from Takara. Endoglycosidase H (Endo H) was purchased from New England Biolabs.

Gene Cloning and Construction of Expression Vector

Based on GenBank accession no.AY390262.1, the phytase gene of *C. freundii*, with an additional 6× histidine tag sequence at 3′-end, was optimized using codon usage bias [16] and synthesized by successive polymerase chain reaction (PCR) method [17]. Errors found upon DNA sequencing were corrected by using overlap extension PCR method. The modified gene was cloned into the *P. pastoris* expression vector pYPX88 between the unique *XhoI* and *SacI* sites. Vector pYPX88 contains a 357-bp fragment of α-factor prepro-leader MF4I (GenBank accession no. AY145833) with *P. pastoris*-preferred codon usage, which substituted for the wild-type α-signal sequence to enhance the expression level [15]. Our previous study has demonstrated that the use of the optimized signal sequence, MF4I, could increase production and activity of phytase in *P. pastoris* [15]. The expression of the inserted gene was controlled by the tightly regulated and highly inducible alcohol oxidase 1 (*AOX 1*) promoter. Routine DNA manipulations were performed by standard recombinant method [18].

Transformation and Screening of Transformants

The recombinant plasmid was linearized with *BglII* and transformed into *P. pastoris* by electroporation method. The cells were plated on SD-his medium and incubated at 30 °C for 3 days. The transformants were screened for their ability to grow on histidine-deficient medium. Small-scale expression experiments were performed to detect expression of the recombinant protein. The his^+ transformants were streak cultivated on BMGY (1% yeast extract, 2% peptone, 1.34%YNB, 0.000004% biotin, and 1% glycerol) agar plates for 24 h at 28 °C. Cells were inoculated into 96-well plates containing 50 μl BMMY medium (1% methanol), respectively, and incubated for 8 h at 28 °C, 225 rpm. Subsequently, the positive colonies were selected by enzymatic activity determination. For a large-scale protein production and purification, the single isolated colony with a high level expression was cultured in 50 ml BMGY until the OD_{600} reached 3.0. The cells were harvested and resuspended in equal volume of BMMY. Methanol was added every 24 h to a final concentration of 1%.

Purification, Deglycosylation, and SDS-PAGE

The culture was induced with methanol for 72 h in the BMMY medium until the OD_{600} reached 6.0 and then the culture supernatant was collected by centrifugation at 12,000 rpm for 30 min and concentrated by ammonium sulfate precipitation method. Ni^{2+}–NTA agarose affinity column (Sigma) was used to purify the recombinant protein with an additional six histidine residues at the C-terminal. The concentration of purified protein was determined using Bradford assay kit. Ten micrograms purified protein was deglycosylated using 100 U endoglycosidase H for 2 h at 37 °C. Its production was analyzed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue R-250.

Assay of Phytase Activity

Phytase activity was determined by the Vanadium–Molybdenum Yellow spectrophotometric method [19]. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 μmol inorganic phosphate per minute from 5 mM of a sodium phytate solution under the assay conditions.

Characterization of Phytase

The pH profile of r-phyC was determined using the following buffers at intervals of 0.5:0.2 M glycine–HCl (pH 1.0–3.5), 0.2 M HAc-NaAc buffer (pH 4.0–5.5), and 0.2 M Tris–HCl (pH 6.0–8.0) and glycine–NaOH (pH 9.0–10.0). For pH stability the enzyme was pre-incubated during 1 h at 37 °C in different buffers, and later, the activity was assayed at optimal pH. To determine the optimum temperature, enzyme activity was measured over a range of temperature (from 20–80 °C). The thermal stability was ascertained by measuring the residual activity of the enzyme after being incubated at 80 °C for 1, 2.5, 5, 7.5, 10, 15, and 30 min. The effect of metal ions (Pb^{2+}, Zn^{2+}, K^+, Fe^{3+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Al^{3+}, Fe^{2+}, and Cr^{2+}) and other reagents (SDS, EDTA) on enzyme activity was assessed by adding 1 mM concentration of different reagents in the reaction. Kinetic parameters were determined by Lineweaver–Burk method. The reaction was carried out in 2.5 mM HAc-NaAc buffer at 37 °C for

10 min using 0.125 to 5 mM sodium phytate as substrates. All the results were the means of three independent experiments.

Results

Gene Cloning and Construction of Expression Vector

The synthesized phytase gene showed 78% homology with the wild-type gene according to the alignment results given by a BLAST search. The codon-optimized gene was subcloned into the expression vector pYPX88, which contains the inducible promoter from *AOX1* gene, the chemical synthesized signal MF4I, and the native transcription termination and polyadenylation signal from *AOX1* gene. Sequencing data proved that the expression plasmid was constructed correctly.

Functional Expression of Phytase in *Pichia*

Multiple plasmid integration events occur spontaneously in *Pichia* at a frequency between 1% and 10% of all His^+ transformants [20]. Because different ways of recombination can affect expression, positive transformants with enzyme activity should be screened for expression levels [21]. Small-scale expression experiments and activity assay was an efficient method to analyze expression levels of the transformants. After induction for 8 h in 96-well plates, 81 (22.5%) of the tested transformants showed phytase activity (1.8–5.2 U/ml). Three single colonies with higher activity were selected for further induction with methanol in shake flasks to improve the phytase yield. After 72 h, they produced recombinant phytase with activity of 179 U ml^{-1}, 183 U ml^{-1}, and 193 U ml^{-1}, respectively.

Purification and Deglycosylation

According to SDS-PAGE (Fig. 1), the purified r-phyC migrated as smear bands with apparent molecular mass of 58 to 60 kD, which were greater than the predicted values (47 kD). This discrepancy is probably caused by glycosylation to varying degrees at six putative sites. After *N*-deglycosylation with Endo H, the molecular mass of the enzyme approximately decreased to 50 kD.

Properties of the Recombinant Phytase

The pH profile for r-phyC activity showed two peaks, the lower one at pH 2.5 (90% of the maximum activity) and the other at pH 4.5 (Fig. 2a). r-phyC was stable over a broad range of pH from 1.5 to 8.0. More than 80% activity remained after pre-incubated r-phyC under various buffers (pH 1.5–8.0) at 37 °C for 1 h. r-phyC showed optimum activity at 50 °C (Fig. 3) and was sensitive to temperature. It retained 50% activity after 5 min incubation at 80 °C, but that activity decreased to 11% after further incubation for 25 min (Fig. 4).

As shown in Fig. 5, r-phyC activity was partially inhibited by Pb^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Fe^{2+}, and Al^{3+} and completely abolished by SDS in a concentration of 0.1 mM. The enzyme activity was significantly activated by K^+, Ca^{2+}, and Mg^{2+}.

The specific activity, K_m, and *V*max values for r-phyC was 2, 072±18 IU mg^{-1}, 0.52± 0.04 mM, and 2,380 IU±84 mg^{-1} min^{-1}, respectively.

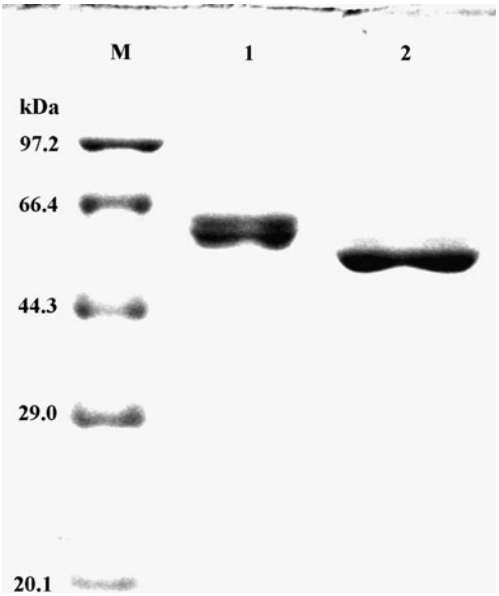

Fig. 1 SDS-PAGE patterns of the purified and deglycosylated recombinant phytase. *M* molecular mass standard, *lane 1* purified recombinant phytase by Ni^{2+}-NTA agarose affinity column, *lane 2* deglycosylated recombinant phytase with Endo H

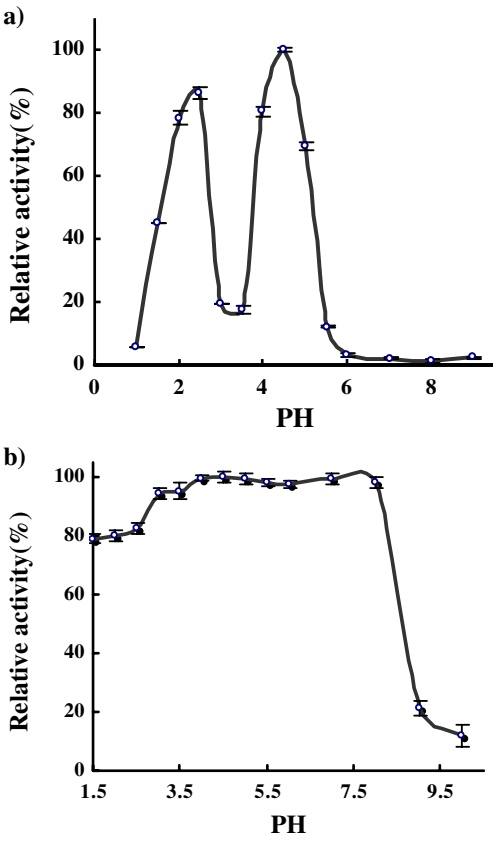

Fig. 2 Optimal pH (**a**) and pH stability (**b**) of the recombinant phytase

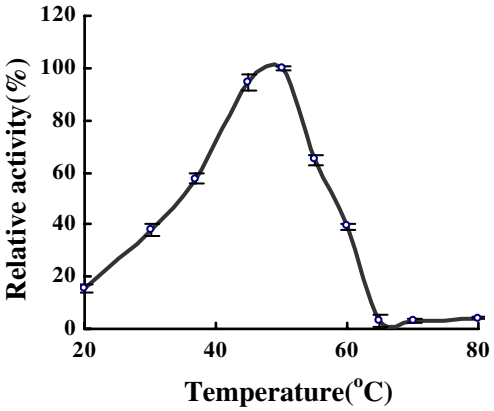

Fig. 3 Optimal temperature of the recombinant phytase

Discussion

An 'ideal phytase' should be effective in hydrolyzing phytate in the digestive tract, stable to heat from feed processing and storage, and cheap to produce. Although many phytases gene have been identified, none of them possesses all of the favorable characteristics.

Yi and Kornegay [22] have shown that the stomach is the major site of exogenous microbial phytase activity for dephosphorylation from phytate. The pH in the animal stomach is normally very low. For example, the stomach pH of an adult pig varies from 1.0 to 4.5 [23]. Because most of the described microbial phytases have pH optima range from 4.0 to 4.5 [24], they cannot perform their full function in the stomachs. Phytase gene from *Citrobacter braakii* have been expressed in *P. pastoris* previously and the recombinant phytase showed optimum activity at 4.5 [25]. In contrast, r-phyC in this study has two optima at 2.5 and 4.5 and has great stability under acidic conditions. The recovery of enzyme activity following incubation with difference buffers (pH 1.5–4.5) was significantly higher for r-phyC (>80%) than that of *Pedobacter nyackensis* phytase (<25%) [26]. These profiles contribute to maintain relative high activity of r-phyC in the gastrointestinal tract.

Thermostability is another important character of phytase. r-phyC lost 90% activity after being incubated at 80 °C for 30 min indicated that it is sensitive to heat. Kim et al. [27] reported that the glycosylated phytase retained about 66% of the initial activity after it was heated for 30 min at 70 °C, whereas the non-glycosylated phytase retained 15% of its

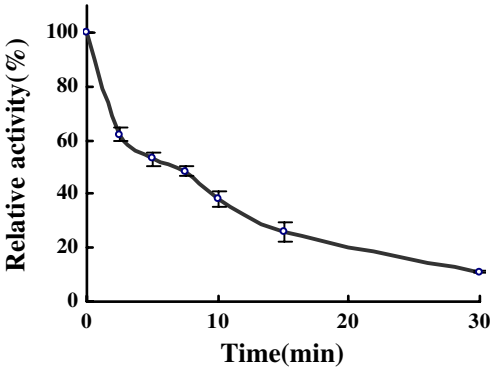

Fig. 4 Thermal stability (80 °C) of the recombinant phytase

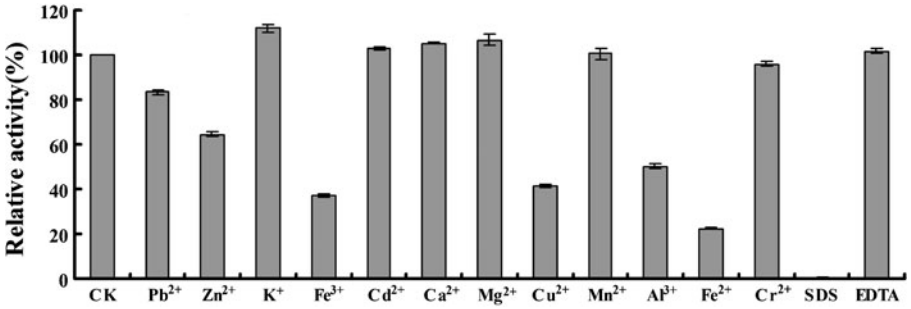

Fig. 5 Effects of chemical reagents on the recombinant phytase activity

activity under the same treatment condition. Stahl et al. have shown a similar role of glycosylation in protein thermostability [28]. In our study, we found *N*-glycosylated r-phyC increased thermostability compared to original non-glycosylated enzyme from *C. freundii*, which was inactivated by 4 min incubation at 70 °C [29]. The 50-kDa molecular size of the deglycosylated r-phyC is still larger than the putative value (47 kDa). The difference in molecular size maybe resulted by *o*-glycosylation and/or other modifications, since Endo H can only remove N-linked glycans.

Analysis of the effect of various metal ions on r-phyC activity revealed that K^+, Ca^{2+}, and Mg^{2+} had a stimulatory effect. This is in agreement with phytase from *Pedobacter nyackensis* [26] and *Hansenula fabianii* [30]. The inhibitory effect by Pb^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Fe^{2+}, and Al^{3+} can be owing to a conformational change or formation of poorly soluble complexes of the metal ions with phytic acid, which may decrease the active concentration of phytic acid in the assay [31].

As shown in Table 1, the specific activity of r-phyC (2,072±18 U mg^{-1}) is lower than that of the wild enzyme. The differences between the recombinant enzyme and the wild enzyme in their specific activities have also been reported in other research [27]. This phenomenon may be due to a decrease in the affinity of the enzyme for its substrate caused by hyperglycosylation in *P. pastoris* [27]. Moreover, enzyme purity and differences between assay methods can also affect the results [32]. However, the specific activity of r-phyC is nearly 20-fold higher than that of *Aspergillus niger* phytase (100 U mg^{-1}, two pH

Table 1 Source and properties of phytase.

Phytase source	Production strain	Specific activity (U mg^{-1})	pH optima	Reference
Citrobacter freundii	*Pichia pastoris*	2, 072	2.5, 4.5	This study
Citrobacter freundii	–	13,200	4.0–4.5	Luo et al. [29]
Pedobacter nyackensis MJ11 CGMCC 2503	*E. coli*	24	4.5	Huang et al. [26]
Citrobacter braakii	*E. coli*	1,122	–	Kim et al. [27]
Peniophora lycii	*Pichia pastoris*	864	4.5	Xiong et al. [15]
A. niger NRRL 3135	–	103	2.5, 5.5	Ullah et al. [33]
A. niger SK-57	–	158	2.5, 5.5	Tadashi et al. [34]
Dickeya paradisiaca	*E. coli*	666	4.5, 5.5	Gu et al. [35]

optima at 2.5 and 5.5), which is currently in widespread use [33]. Tadashi et al. [34] and Gu et al. [35] have also reported phytases with two pH optima. To our knowledge, r-phyC possesses the highest specific activity among those acidic phytases which have two pH optima (Table 1). In this research, the maximum yield of the recombinant phytase produced in shake flask culture reached to 193.2 U ml^{-1} by the modified phytase gene. A well-established high cell density fermentation protocol described by Xiong et al. [15] can be used to further increase the yield.

In conclusion, we have successfully expressed functionally active phytase in *P. pastoris*. r-phyC appears to be a potential candidate for feed industry because it possessed favorable characteristics, such as acidic-stability, relative high activity under acidic stress, and thermostability.

Acknowledgments The research was supported by the International Scientific and Technological Cooperation of Shanghai-Canada (Alberta-08540706500); Shanghai Rising-Star Program and Natural Science Foundation (08QH14021), High-tech Research, and Development Program of China (2006AA06Z358; 2008AA10Z401).

References

1. Reddy, N. R., Sathe, S. K., & Salunkhe, D. K. (1982). Phytates in legumes and cereals. *Advanced Food Research, 28*, 1–92.
2. Bitar, K., & Reinhold, J. G. (1972). Phytase and alkaline phosphate activities in intestinal mucosa of rat, chicken, calf and man. *Biochimica et Biophysica Acta, 268*, 442–452.
3. Abulkalam, M. S. (2008). Demonizing phytate. *Nature Biotechnology, 26*, 496–497.
4. Nayani, N. R., & Markakis, P. (1983). Effects of inositol phosphates on mineral utilization. *Federation Proceedings, 45*, 819–826.
5. Peers, F. G. (1953). The phytase of wheat. *Biochemical Journal, 53*, 102–110.
6. Yanke, L. J., Bae, H. D., Selinger, L. B., & Cheng, K. J. (1998). Phytase activity of anaerobic ruminal bacteria. *Microbiology, 144*, 1565–1573.
7. Mélanie, R., André, A., Patrick, C., Santiago, G., Guy, M., & Hélène, B. (2008). Complete hydrolysis of myo-inositol hexakisphosphate by a novel phytase from *Debaryomyces castellii* CBS 2923. *Applied Microbiology and Biotechnology, 78*, 47–53.
8. Gargova, S., Roshkova, Z., & Vancheva, G. (1997). Screening of fungi for phytase production. *Biotechnology Techniques, 11*, 221–224.
9. Vats, P., & Banerjee, U. C. (2002). Studies on the production of phytase by a newly isolated strain of *Aspergillus niger van Teigham* obtained from rotten wood-logs. *Process Biochemistry, 38*, 211–217.
10. Harland, B. F., & Oberleas, D. (1999). Phytic acid complex in feed ingredients. In M. B. Coelho & E. T. Kornegay (Eds.), *Phytase in animal nutrition and waste management, 2nd review* (pp. 69–76). Mexico: BASF.
11. Pillai, U. P., Manoharan, V., Lisle, A., Li, X., & Bryden, W. (2009). Phytase supplemented poultry diets affect soluble phosphorus and nitrogen in manure and manure-amended soil. *Journal of Environmental Quality, 38*, 1700–1708.
12. Stefan, H., Anja, K., Edzard, S., Joerg, B., Markus, L., & Oskar, Z. (2005). Biotechnological production and applications of phytases. *Applied Microbiology and Biotechnology, 68*, 588–597.
13. Cereghino, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews, 24*, 45–66.
14. Peng, R. H., Xiong, A. S., Li, X., Fan, H. Q., Yao, Q. H., Guo, M. J., et al. (2002). High expression of a heat-stable phytase in *Pichia pastoris*. *Acta Biochimica et Biophysica Sinica, 34*, 725–730.
15. Xiong, A. S., Yao, Q. H., Peng, R. H., Zhang, Z., Xu, F., Liu, J. G., et al. (2006). High level expression of a synthetic gene encoding *Peniophora lycii* phytase in methylotrophic yeast *Pichia pastoris*. *Applied Microbiology and Biotechnology, 72*, 1039–1047.

16. Sharp, P. M., Tuohy, T. M. F., & Mosurski, K. R. (1986). Codon usage in yeast: Cluster analysis clearly differentiates highly and lowly expression genes. *Nucleic Acids Research, 14*, 5125–5143.
17. Xiong, A. S., Yao, Q. H., Peng, R. H., Li, X., Fan, H. Q., Li, Y., et al. (2004). A simple, rapid, high fidelity and cost-effective PCR based two-step DNA synthesis (PTDS) method for long gene sequences. *Nucleic Acids Research, 32*, e98.
18. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (2nd ed.). New York: Cold Spring Harbor.
19. Heeft, D. (1995). Phytase activity, vanadate manual method. *Gist-brocades method Nr, 61696*, 2832.
20. Romanos, M., Scorer, C., Sreekrishna, K., & Clare, J. (1998). The generation of multicopy recombinant strains. In D. R. Higgins & J. M. Cregg (Eds.), *Pichia protocols* (pp. 55–72). Totowa: Human Press.
21. Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K., & Romanos, M. A. (1991). High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Biotechnology, 9*, 455–460.
22. Yi, Z., & Kornegay, E. T. (1996). Sites of phytase activity in the gastrointestinal tract of young pigs. *Animal Feed Science and Technology, 61*, 361–368.
23. Chesson, A. (1987). Supplementary enzymes to improve the utilization of pig and poultry diets. In W. Haresign & D. J. A. Cole (Eds.), *Recent advances in animal nutrition* (pp. 71–89). London: Butterworths.
24. Vohra, A., & Satyanarayana, T. (2003). Phytases: microbial sources, production, purification and potential biotechnological applications. *Critical Reviews in Biotechnology, 23*, 29–60.
25. Huang, H. Q., Luo, H. Y., Bai, Y. G., Wang, Y. R., & Yao, B. (2006). Overexpression of *Citrobacter braakii* phytase with high specific activity in *Pichia pastoris*. *Acta Microbiologica Sinica, 46*, 945–950.
26. Huang, H. Q., Shao, N., Wang, Y. R., Luo, H. L., Yang, P. L., Zhou, Z. G., et al. (2009). A novel beta-propeller phytase from *Pedobacter nyackensis* MJ11 CGMCC 2503 with potential as an aquatic feed additive. *Applied Microbiology and Biotechnology, 83*, 249–259.
27. Kim, Y. O., Kim, H. W., Lee, J. H., Kim, K. K., & Lee, S. J. (2006). Molecular cloning of the phytase gene from *Citrobacter braakii* and its expression in *Saccharomyces cerevisiae*. *Biotechnology Letters, 28*, 33–38.
28. Stahl, C. H., Wilson, D. B., & Lei, X. G. (2003). Comparison of extracellular *Escherichia coli* AppA phytases expressed in *Streptomyces lividans* and *Pichia pastoris*. *Biotechnology Letters, 25*, 827–831.
29. Luo, H. Y., Shi, P. J., Li, J., Wang, Y. R., & Yao, B. (2006). Purification and properties of *Citrobacter freundii* phytase. *Acta Microbiologica Sinica, 46*, 139–142.
30. Takashi, W., Hiroko, I., Kazuo, M., Tsutomu, F., & Haruyuki, I. (2009). Cloning and characterization of a novel phytase from wastewater treatment yeast *Hansenula fabianii* J640 and expression in *Pichia pastoris*. *Journal of Bioscience and Bioengineering, 108*, 225–230.
31. Wang, H. L., Savain, W., & Hesseltine, C. W. (1980). Phytase of molds used in oriental food fermentation. *Journal of Food Science, 45*, 1261–1266.
32. Henry, B. M. (1926). The effect of enzyme purity on the kinetics of tryptic hydrolysis. *The Journal of General Physiology, 10*, 217–225.
33. Ullah, A. H. J., & Gibson, D. M. (1987). Extracellular phytase (E.C. 3.1.3.8) from *Aspergillus ficuum* NRRL 3135: Purification and characterization. *Preparative Biochemistry & Biotechnology, 17*, 63–91.
34. Tadashi, N., Tatsuya, T., & Hideharu, A. (1999). Dephosphorylation of phytate by using the *Aspegillus niger* phytase with a high affinity for phytate. *Applied and Environmental Microbiology, 65*, 4682–4684.
35. Gu, W. N., Huang, H. Q., Meng, K., Yang, P. L., Fu, D. W., Wang, Y. R., et al. (2009). Gene cloning, expression, and characterization of a novel phytase from *Dickeya paradisiaca*. *Applied Microbiology and Biotechnology, 157*, 113–123.