Recombinant *Pichia pastoris* overexpressing bioactive phytase*

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Abstract Phytase gene *phyA*2, whose signal peptide encoding sequence and intron sequence had been removed, was modified. The Arg-encoding codons CGG and CAG in *phyA*2 were mutated into synonymous codon AGA. The modified *phyA*2 was fused behind α-factor signal sequence under the control of *AOX*1 promoter in plasmid pPIC9, then introduced into the host *Pichia pastoris* by electroporation. The results of Southern blotting analysis and Northern blotting analysis demonstrated that the *phyA*2 gene had integrated into the genome of *P. pastoris* and transcribed. The result of SDS-PAGE of the phytase expressed by *P. pastoris* showed that the modified *phyA*2 had been overexpressed and secreted. The concentration of the phytase expressed by *P. pastoris* with modified *phyA*2 exceeded 15 000 U/mL, which had a 3 000-fold increase over that of origin *Aspergillus niger* 963 and was 37 times higher than that of recombinant *P. pastoris* with non-modified *phyA*2.

Keywords: **phytase gene *phyA*2, recombinant *Pichia pastoris*, overexpression.**

Phosphorous is an essential element for the growth and development of all animals, playing key roles in skeletal structure and in vital metabolic pathways. Up to 80% of the total phosphorous in feedstuffs of plant origin is the phytate phosphorous. It is poorly available to monogastric animal due to the lack of digestive enzyme capable of hydrolyzing the phytate phosphorous[1]. Bioavailability value for phosphorous in plant feed ingredients is from 0 to 40%. Phytate phosphorus passes through the intestinal tract and ends up in the feces causing environmental problems in areas of intensive livestock production. In addition, phytate forms complexes with multivalent metal ions such as iron, zinc and calcium and also binds to proteins, thereby decreasing the bioavailability of nutritionally important minerals and proteins[2].

Phytase (*myo*-inositol hexaphosphate phosphohydrolase, EC 3.1.3.8) are enzymes which catalyze the hydrolysis of phytate into *myo*-inositol and inorganic phosphate. Increase in phosphorus availability by 60% and decrease in the excretion of phosphorus in the feces by 40% have been proven after the addition of phytase to monogastric animal feed[3]. Additionally, such a treatment can also counteract the anti-nutritional properties associated with unhydrolyzed phytate. Although a broad range of microorganisms can produce phytase, the expression levels of phytase in them are too low for economic considerations. Inorganic phosphorous supplement of monogastric animal feedstuffs is still the method of choice in many cases. Obviously, any commercially viable procedure for the production of a large quantity of phytase would be of tremendous value both from the feed-conversion and an environment point of view.

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Towards the ultimate goal of improving the production of phytase and reducing its production cost, here we report the modification of phytase gene *phyA2*, construction of recombinant *P. pastoris* and overexpression of bioactive phytase in recombinant *P. pastoris*.

1 Materials and methods

1.1 Strains and plasmids

Phytase gene *phyA2* was cloned in our laboratory. *E. coli* $DH_{5\alpha}$ and plasmid pUC18 were purchased from Promega Inc. Yeast *Pichia pastoris* GS115 (his^-mut^+) and plasmid pPIC9 from Invitrogen Inc. were kindly provided by Dr. D. Luo.

1.2 Modification of *phyA2* and construction of recombinant *P. pastoris* expression vectors

Restriction enzyme, T_4 DNA ligase, Mung bean nuclease and Taq DNA polymerase were purchased from Boehringer Inc. T_7 DNA sequencing kit was purchased from Pharmacia Inc. *In vitro* mutagenesis system was purchased from Promega Inc.

1.3 Transformation of *P. pastoris* GS115 and screening for transformants

1.3.1 Material. Media YPD: 1.5% yeast extract, 2% peptone, 2% dextrose. RDB: 18.6% sorbitol, 2% dextrose, 1.34% yeast nitrogen base w/o amino acids (YNB), 0.000 04% biotin, 0.005% L-glutamic acid, 0.005% L-lysine, 0.005% L-leucine, 0.005% L-methionine, 0.005% L-isoleucine, 2% Agarose. MM: 1.34% YNB, 0.000 04% biotin, 0.5% methanol, 1.5% agarose. MD: 1.34% YNB, 0.000 04% biotin, 2% dextrose, 1.5% agarose.

1.3.2 Transformation and screening. 500 mL of *P. pastoris* GS115 was cultured in a 2-L flask at 30℃ overnight to $A_{600}=1.5$. The GS115 cells were spun down at 4℃ and washed with 500 mL and 250 mL of ice-cold sterile water successively. The cell pellet was then resuspended in 20 mL of ice-cold 1 mol/L sorbitol. The cells were spun down again and were then resuspended in ice-cold 1 mol/L sorbitol to a final volume of approximately 0.5 mL. 40 μL of the cells was mixed with 1.5 μL of linearized recombinant plasmid DNA and was transferred to an ice-cold cuvette and incubated on ice for 5 min. The cells were pulsed with electroporation parameters of 0.8 kV, 11.5 μF. 0.5 mL of ice-cold 1 mol/L sorbitol was immediately added to the cuvette. 200 μL of the aliquots was spread on RDB plates. The plates were incubated at 30℃ until colonies appeared. With sterile toothpicks, transformants (his^+) were picked onto both an MM plate and an MD plate from RDB plates. The MM and MD plates were incubated at 30℃ for 2 d. The transformants (his^+ mut^-), in which the *AOXI* gene had been disrupted, were found by looking for patches that grew normally on the MD plates but a little bit slowly or did not grow on MM plates.

1.4 Induced expression of *phyA2* in recombinant *P. pastoris*

The recombinants were inoculated in 10 mL BMGY medium (1% yeast extract, 2% peptone, 13.4% YNB, 0.000 04% biotin, 1% glycerol) at 30℃ with vigorous shaking. The cells were cultured to saturation ($A_{600}=10$—20) and harvested by centrifugation for 10 min at room temperature at 4 000 *g*. Then the cell pellet was resuspended in 10 mL of BMGY medium (1% yeast extract, 2% peptone, 13.4% YNB, 0.000 04% biotin, 0.05% methanol) and continued to culture for 2 d.

1.5 Southern blotting analysis and Northern blotting analysis

Southern blotting analysis and Northern blotting analysis of transformants were carried out as described by Sambrook[4]. The *Bam*HI fragment (+ 570— + 1 551 bp) of *phyA*2 was used as probe for Southern blotting analysis and Northern blotting analysis.

1.6 SDS-PAGE analysis and characterization of the phytase expressed by *P. pastoris*

Recombinants were induced in BMMY at 30℃ for 36 h. 3 μL of cell-free culture was analyzed by SDS-PAGE.

The assay methods of phytase activity were performed as described by Ullah[5].

One unit of phytase was defined as the amount of enzyme which was able to liberate 1 nmole of inorganic phosphate from substrate per minute.

1.7 Time-course for phytase secretion in *P. pastoris*

P. pastoris recombinants were induced in BMMY at 30℃ for 108 h. The phytase production was determined at an interval of 12 h.

1.8 Genetic stability of *P. pastoris* expressing phytase

After a round of growth and expression of recombinant *P. pastoris*, 0.1 mL of the final culture aliquots was inoculated in 10 mL BMGY for the next round of growth and expression. All 10 rounds of that were carried out successively. The cell densities and the expression levels of phytase were determined in each run. Additionally, the genome DNA of recombinant *P. pastoris* after 10 rounds was used as PCR template to amplify *phyA*2.

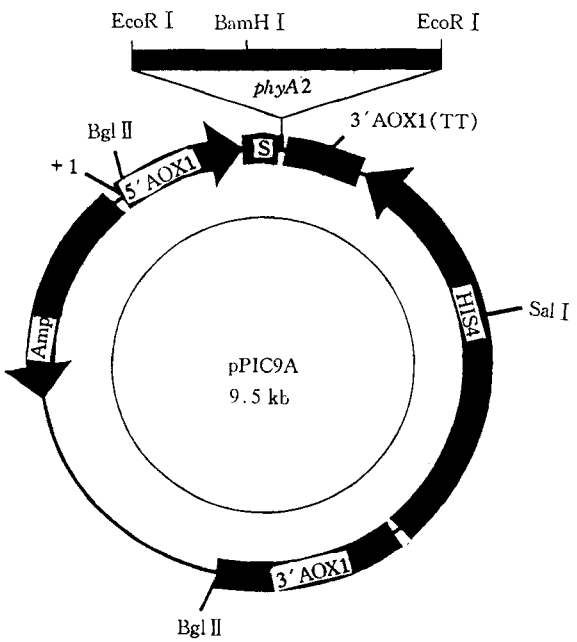

Fig. 1. Physical map of recombinant plasmid pPIC9A. Amp, ampicillin resistance gene; 5′ AOX1, *AOX*1 promoter; S, α-factor secretion signal; 3′ AOX1 (TT), *AOX*1 transcription termination fragment; His4, histidinol dehydrogenase gene; 3′ AOX1, 3′ flanking sequence of *AOX*1.

2 Results

2.1 Modification of *phyA*2

In the *phyA*2, the codons encoding Arg at positions + 85, + 146, + 159 and + 446 were CGG or CGA, which were not used in highly expressed genes in yeast, were mutated into AGA which was optimal synonymous codon for Arg in yeast[6]. The modified *phyA*2 was confirmed by sequencing.

2.2 Construction of recombinant plasmid of *P. pastoris*

The modified *phyA*2 was inserted in plasmid pPIC9 behind the α-factor signal sequence by the control of *AOX*1 (alcohol oxidase 1) promoter. The recombinant plasmid was designated as pPIC9A (fig. 1). The non-modified *phyA*2, but without signal sequence and intron sequence, was also inserted in pPIC9 and used as a control to study the expression

of *phyA2*.

2.3 Transformation of host *P. pastoris* GS115

One—five μg of recombinant plasmid DNA digested with Bgl II was electroporated into the host *P. pastoris* GS115. 108 transformants (his^+) were screened in RDB plates. Furthermore, 78 transformants (his^+ mut^-) were screened in MD plates. The transformants with modified *phyA2* were designated as *P. pastoris* pPIC9A-1, 2, 3…, 41. The transformants with non-modified *phyA2* were designated as *P. pastoris* pPIC9B-1, 2, 3…37.

2.4 Southern blotting analysis and Northern blotting analysis

The results (fig. 2) of Southern blotting analysis showed that the *phyA2* had integrated in *P. pastoris* genome. The integrated *phyA2* copy number in *P. pastoris* pPIC9A-6, 7, 9 was 1, 2, 1, respectively.

The results (fig. 3) of Northern blotting analysis showed that the *phyA2* had transcribed. No notable difference at mRNA level was observed between recombinant *P. pastoris* with modified *phyA2* and that with non-modified *phyA2*.

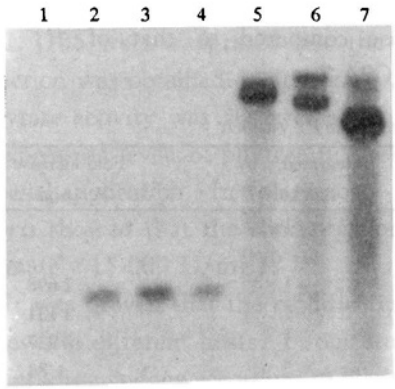

Fig. 2. Southern blotting analysis of recombinant *P. pastoris*. Lane 1, Host *P. pastoris* GS115; lanes 2—4, recombinant *P. pastoris* pPIC9A-6, 7 and 9 digested with *EcoR*I, showing a 1.4 kb specific hybridization band, respectively; lanes 4—6, recombinant *P. pastoris* pPIC9A-6, 7 arnd 9 digested with *BamH*I, showing one, two and one specific hybridization bands, respectively.

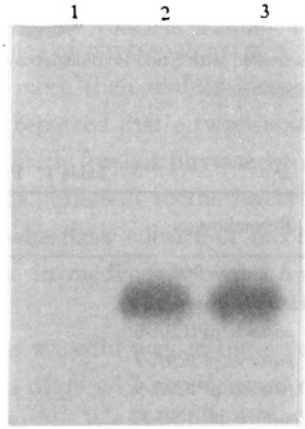

Fig. 3. Northern blotting analysis of recombinant *P. pastoris*. Lane 1, Host *P. pastoris* GS115; lane 2, recombinant *P. pastoris* pPIC9B-12, an about 2.0 kb specific hybridization band; lane 3, recombinant *P. pastoris* pPIC9A-7, an about 2.0 kb specific hybridization band.

2.5 SDS-PAGE analysis of the phytase expressed by *P. pastoris*

The result (fig. 4) showed that the expressed and secreted phytase had a single apparent molecular weight of about 85 ku, while the deglycosylated phytase treated with Endo H (Endo-β-N-acetylglycosminidase H) had an apparent molecular weight of about 64 ku. The result indicated that the phytase expressed by recombinant *P. pastoris* could be modified by glycosylation, which was essential for bioactivity of the phytase. The expression level of phytase protein exceeded 1 mg/mL. The phytase secreted into the media was greater than 90% homogenous.

2.6 Activity assays of the phytase expressed by *P. pastoris*

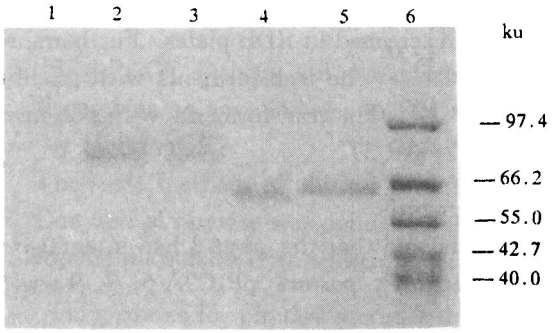

Fig. 4. SDS-PAGE analysis of the phytase expressed by recombinant *P. pastoris*. 1, Untransformed host *P. pastoris*; 2 and 3, recombinant *P. pastoris* pPIC9A-7, 9, respectively; 4 and 5, recombinant *P. pastoris* pPIC9A-7, 9, respectively, the phytase protein was treated with Endo H to deglycosylate; 6, standard protein molecular weight.

The recombinants were induced in BMMY for 36 h and the activity of phytase was measured at 37℃, pH5.5. The results (table 1) showed that the levels to which phytase accumulated in the media were different for individual recombinant. The expression levels of *P. pastoris* pPIC9A-6, 7, 9 were higher up to 13 488, 15 656 and 14 636 U/mL, respectively, which had a 3 000-foldincrease compared to that of origin strain *Aspergillus niger* 963. The observations also showed that the expression level of phytase of *P. pastoris* pPIC9B-12, which had the highest activity in all recombinant *P. pastoris* with non-modified *phyA2*, was only 420 U/mL, which had a 37-fold decrease compared to that of *P. pastoris* pPIC9A-7.

Table 1 Production of phytase by recombinant *P. pastoris*

Recombinants	Phytase activity /U·mL^{-1}	Integrated copy number	Fold increase over *A. niger* 963
A. niger 963	5	1	-
Host *P. pastoris*	-	-	-
P. pastoris pPIC9A-6	13 488	1	2 698
P. pastoris pPIC9A-7	15 656	2	3 131
P. pastoris pPIC9A-9	14 636	1	2 927
P. pastoris pPIC9B-12	420	2	84

2.7 Time-course for phytase secretion from *P. pastoris*

The result (fig. 5) of the time-course for phytase secretion showed that the production of phytase secreted into media was increased significantly during 36 h in the methanol induced-culture phase. After 36 h, the production increased slightly.

In addition, 50 mg/mL wet cells (equal to a cell density of $A_{600} = 20$) were obtained in the growth phase of about 24 h in BMGY. During 108 h of the methanol induced-culture in BMMY, the cell yield without remarkable increase was observed, while the pH values of media decreased gradually from a starting 5.0 to a final 1.5 during this period.

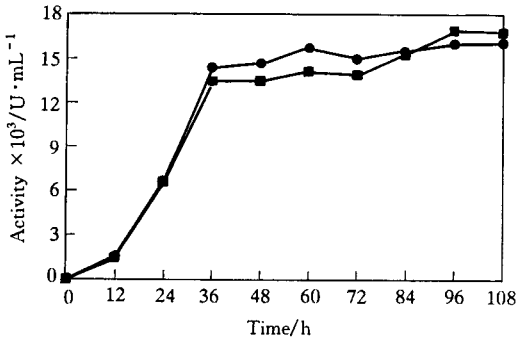

Fig. 5. Time-course for phytase secretion from recombinant *P. pastoris*. ●, *P. pastoris* pPIC9A-7; ■, *P. pastoris* pPIC9A-9.

2.8 Genetic stability of *P. pastoris* expressing phytase

The recombinant *P. pastoris* was continually incubated through 10 rounds of growth phase and induction expression phase. The results showed that no notable differences were observed in the cells growth densities and the expression levels of phytase in each round. The genome DNA of recombinant *P. pastoris* after 10 rounds was used as PCR template to amplified *phyA*2. The result showed that the *phyA*2 was still kept in the genome of *P. pastoris*, it was proven that the recombinant *P. pastoris* had excellent genetic stability in the properties of growth and expression of phytase.

3 Discussion

The addition of microbial phytase to feedstuffs of monogastric animal has been shown to increase the availability of phosphorus[3]. However, the high cost of microbial phytase production was still a key limitation factor for its application. Obviously, the genetic engineering techniques would be a useful tool for the production of great quantities of phytase. Piddington et al.[7] reported that the expression level of phytase gene *phy* of *A. niger* var. *awamori* was up to 329 U/mL in *A. niger* ALK 02268, exhibiting a three—sevenfold increase over untransformed hosts. Van Hartingsveldt et al.[8] reported that up to 15 copies of phytase gene *phy* of *A. niger* NRRL 3135 were integrated into the genome of itself and more than tenfold increased phytase production was obtained. Additionally, Moore et al.[9] also reported that a two—sixfold increase in phytase activity was observed in transformed *A. oryzae* with foreign phytase gene. Overall, the expression levels of phytase in these reports might not be sufficient to the levels relevant for industrial application. In this report, the results from a shake-flask culture of recombinant *P. pastoris* showed that the concentration of expressed phytase in media was over 1 mg/mL (approximately 15 000 U/mL).

It was proven that the modification of foreign genes was a useful way to improve its expression level in different hosts. In our studies, the modification of *phyA*2 was focused on mutating Arg-encoding codons according to bias in codon choice in yeast[6]. We found that four Arg-encoding codons in origin *phyA*2 are CGA or CGG, which were not used in highly expressed genes in yeast. Consequently, in order to improve the expression level of *phyA*2 in yeast, we changed the four codons into another Arg-encoding synonymous codon AGA, whose usage frequency in yeast higher expression genes was up to 86.6%. This modification of *phyA*2 resulted in a 37-fold increase of phytase production. Because the result of Northern blotting analysis confirmed that no differences were found in level of transcription between mutated *phyA*2 and non-modified *phyA*2, it was suggested that the difference of phytase production should result from the difference of translation efficiency of the two genes. The modification to *phyA*2 was evaluated to be successful.

Pichia pastoris has many of the advantages of higher eukaryotic expression system such as protein processing, protein folding, and posttranslation modification[10]. Our studies showed that the secreted phytase from recombinant *P. pastoris* can be modified by glycosylation, which was necessary for the bioactivity of the phytase.

In our research, the recombinant *P. pastoris* secreting phytase did not carry any antibiotic-resistant marker, so the produced phytase need not be purified and can be added to feed directly with the form of yeast culture. It would decrease the cost of production of phytase.

During the 1970s, *P. pastoris* was investigated for potential use as a source of single-cell protein[11]. As a result, fermentation techniques were developed for maintaining the organism in large-volume continuous culture and at cell densities in excess of 100 g/L dry cell weight[12,13]. The growth medium, a defined mixture of salts, trace elements, biotin and carbon source are inexpensive and can be formulated free toxins and pyrogens. It is a safe prediction that the *P. pastoris* overexpressing phytase would be used for more efficient and economical phytase production.

This is the first report of recombinant *P. pastoris* expressing a high level of bioactive phytase.

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