# Cloning and over Expression of an *Aspergillus niger* XP Phytase Gene (phyA) in *Pichia pastoris*

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***Abstract***—*A. niger* XP isolated from Vietnam produces very low amount of acidic phytase with optimal pH at 2.5 and 5.5. The phytase production of this strain was successfully improved through gene cloning and expression. A 1.4 - kb DNA fragment containing the coding region of the *phyA* gene was amplified by PCR and inserted into the expression vector pPICZαA with a signal peptide α-factor, under the control of AOX1 promoter. The recombinant plasmid was transformed into the host strain *P. pastoris* KM71H and X33 by electroporation. Both host strains could efficiently express and secret phytase. The multicopy strains were screened for over expression of phytase. All the selected multicopy strains of *P. pastoris* X33 were examined for phytase activity, the maximum phytase yield of 1329 IU/ml was obtained after 4 days of incubation in medium BMM. The recombinant protein with MW of 97.4 KW showed to be the only one protein secreted in the culture broth. Multicopy transformant *P. pastoris* X33 supposed to be potential candidate for producing the commercial preparation of phytase.

***Keywords***—*Aspergillus niger* XP; cloning; over expression; *Pichia pastoris*; phyA , phytase.

## I. INTRODUCTION

DURING the last 20 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology. Phytate, *myo*-inositol hexophosphate, is the major storage form of phosphorus in food or feeds of plant origin [5], [12], [17]. Phytases, a specific group of phosphatases, are required to initiate the release of phosphorus from phytate. Single-stomached animals such as swine and poultry, as well as humans lacked phytase activity in their gastrointestinal tracts. Thus, nearly all of the dietary phytate phosphorus ingested by these species is excreted into the environment, resulting in phosphorus pollution in areas of intensive animal production [2], [16], [17].

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Supplemental phytase in diets for swine and poultry effectively improves phytate phosphorus utilization by these animals and reduces their fecal phosphorus excretion by up to 50% [4]. Moreover, additions of phytases not only enhance utilization of phosphorus and reduce phosphate output in manures but also increase mineral uptake [10].

Phytases have a wide distribution in plants, microorganisms, and in some animal tissues [3], [8]. Recent research has shown that microbial phytases are most promising for a biotechnological application. Although phytases from several species of bacteria, yeast and fungi have been characterized [8], commercial production currently focuses on the fungus *Aspergillus. Aspergillus niger* phytase (EC 3.1.3.8) has been well characterized and the gene encoding it, *phyA*, has been cloned and sequenced [6], [15,] [11]. There are 10 potential *N*-glycosylation sites in the primary structure of *A. niger* phytase [3], and the sugar moieties of the protein seems to be vital for the functional expression of the gene. *PhyA* is unable to be expressed in *E.coli* because this transformant produces a nonglycosylated, intracellular inclusion protein. *PhyA* has been expressed in active forms in tobacco seeds or leaves and soybean seeds [8]. Although there are differences in glycosylation among these hosts, the expressed phytase enzymes are all active, and the sizes of the deglycosylated proteins are similar. However, these systems express only moderate levels of phytase activity. The methylotrophic yeast, *Pichia pastoris,* has been widely used as a host organism to produce heterologous proteins, and the expressed proteins are generally hyperglycosylated [2]. Our objective was using gene cloning and over expression to obtain a potential phytase as a feed additive to upgrade the nutritional quality of phytate-rich seed-based animal feed for Vietnam husbandry.

## II. MATERIALS AND METHODS

### *Bacterial strains and plasmids*

*A. niger* XP strain obtained from Microbiology and Biotechnology Department, Faculty of Biology, Hanoi National University of Education (Hanoi, Vietnam) was a primary material for isolation of a phytase gene.

Plasmid vectors, pPICZαA (Invitrogen) were used as intermediate and principal vectors for cloning and expression of phyA gene into *E. coli* DH5α and *P. pastoris* KM71H and X33, respectively.

### Isolation of genomic DNA from A. niger XP

Strain *A. niger* XP was grown on YMA (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar, pH 6.0) slants for 5 days at 30°C. Sterilized solution of Tween 80 (0.5%) was added, mixed well and spore suspension was added to 2% malt extract broth at ratio 1/10 and incubated overnight at 30°C in a shaking 200 rpm. Cells were collected, washed with 2×SSC, boiled (99°C) in 2×SSC for 20 min. Cells were washed with distilled water. Glass beads, water and phenol-chloroform were added. Cells were disrupted using bead beater for 1 min. The tubes were centrifuged and the upper layer was used directly as template for PCR.

### PCR amplification

The coding region of *phyA* gene was amplified by PCR using two primers:

5'GCTGAATTCCTGGCAGTCCCCGCCTCGAGA3'(T-phyA-EcoRI- F) and

5'TGTTCTAGATCAAGCAAAACACTCCGCCCAATC3'(T-phyA-XbaI-Stop-R); The *Eco*RI restriction site was introduced into the forward primer, and the *Xba*I restriction site was introduced into the reverse primer in order to make an advantage for ligation with pPICZαA.

PCR was carried out with 1 μl genomic DNA as a template, 0.2 mM dNTPs, 1 pmol of each primer, 1.25 mM MgCl$_2$, 10X *Taq* polymerase buffer and 2 U of *Taq* polymerase in a total volume of 50 μl. The reaction mixture was preheated to 94°C for 3 min and then subjected to 30 cycles of 94°C for 40 sec, 52°C for 40 sec and 72°C for 1 min; followed by one final cycle of 72°C for 7 min. PCR products were analysed by 1% agarose gel electrophoresis and purified by QIAEX (Roche) according to the manufacturer's instruction.

### Cloning of phyA into pPICZαA

Purified PCR products were ligated into pPICZαA (Invitrogen) according to manufacture's protocol. The ligated products were transformed into competent cells *E. coli* DH5α by heat-shock protocol [1] and 200 μl of the cell suspensions were plated on LB low salt (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) with 25 mg/l zeocin. Colonies PCR screening was conducted with factor α sequencing primer and T-PhyA-R.

Selected colonies were picked up and transferred on fresh plates containing 25mg/l zeocin and grown in 50 ml Erlenmeyer flask containing 10 ml of LB low salt-zeocin with vigorous shaking at 37°C. On the next day, cells were collected and plasmids were extracted using NucleoSpin® Plasmid extraction kit (Macherey-Nagel, Germany).

### Transformation into P. pastoris

Recombined plasmid pPICZαA/PhyA was lineared by restriction enzyme *Pme*I at 37°C for 3 hours and then inactivated by heating at 75°C for 10 min. 10 μg of digested pPICZαA/PhyA were purified using NucleoSpin® Plasmid extraction kit and transformed in to *P. pastoris* KM71H *and* X33 prepared by electroporation method [1]. Various volumes

of cell suspension were spread on YPDS agar plate (Yeast Extract Peptone Dextrose Medium:1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 100 mg/l zeocin and incubated at 30°C for 3 days. Positive colonies were selected, purified to extract DNAs. These DNAs were used as templates for PCR screening with primers α-factor and T-PhyA-R.

### Expression of recombinant Pichia strain

The experiments were carried out according to *Pichia* expression kit manuafacture. The transformants were incubated in a 1 litre baffled flask containing 100 ml of BMM (Buffered Minimal Methanol : 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10-5% biotin, 0.5% methanol) at 28°C in a shaking incubator (250 rpm). Methanol was added to a final concentration of 0.5% every 24 hours to maintain induction. After 4 days, cells were eliminated by centrifuging at 3000 x g for 10 minutes. The supernatant was used as extracellular enzyme sample. Enzyme assays were preformed as described by Shimizu [14]. One phytase unit was defined as the activity that releases 1 μmol of inorganic phosphorus from sodium phytate per min under assay conditions.

### Selection of over expressed phytase strains

After the transformation, all the resistant zeocin colonies were collected and speared on YPD plates with different zeocin concentration at 100 mg/l; 1000 mg/l and 2000 mg/l. The resistant colonies at 2000 mg/l zeocin were selected and transffered to the other fresh agar-plates. Then the phytase expression was carried out in BMM medium with 0.5% methanol.

## III. RESULTS AND DISCUSSION

### Cloning of phyA gene into pPICZαA

Base on designed primers, the DNA fragment containing the coding region of *phyA* was amplified with Fermentas *Taq* for 50μl reactions. The result was showed in Fig.1. The nucleotide sequence of *A. niger* XP *phyA* PCR product was determined and it was 97% homomology with sequences of *A. niger* NRRL 3135 *phyA* (Z16414) .

After digestion, purified PCR product were ligated into pPICZαA and transformed into *E. coli* DH5α. Several positive clones were collected and the presence of phyA gene was determined by using conoly PCR method with primers α-factor and T-PhyA-R. The positive clone, pPICZαA containing a DNA fragment that encodes for phytase activity, was used for DNA plasmid extraction. For efficient integration into the *Pichia* genome at the *AOX1* locus, pPICZAα/*PhyA* was lineared by restriction enzyme before the transformation.

The digested pPICZAα/*PhyA* were then purified and transformed into the host strains *P. pastoris* KM71H and X33. Both transformants *P. pastoris* KM71H and X33 were grown well on YPDS containing 100 mg/l zeocin. To determine the presence of *phyA* gene in transformants, the DNA from several colonies were isolated and used as templates for the PCR screening with primers α-factor and T-PhyA-R. The

result showed that the recombined plasmid was successfully transformed into *P. pastoris* KM71H and X33 (Fig. 2).

### Expression of *phyA* in *P. pastoris* KM71H and X33

The expression of phyA in *P. pastoris* was successful in both KM71H and X33 strains. In total of 152 sub-clones from *P. pastoris* KM71H and 190 sub-clones from *P. pastoris* X33 were collected. After screening 15 colonies of recombinant KM71H and 15 colonies of recombinant X33 with activities ranged from 21.3 to 86.4 IU/ml were selected. The vigorous expression of the phytases of those recombinant strains was found at 28h and maximum phytase yield was attained at 96h.

### Selection of over express phytase strains

Like in *Saccharomyces cerevisiae*, linear DNA can generate stable transformants of *Pichia pastoris* via homologous recombination between the transforming DNA and regions of homology within the genome. Multiple insertion events occur spontaneously at about 1-10% of the single insertion events [1].

Multiple gene insertion events at a single locus in a cell will generate multicopy recombinant strains which have a lot of expression cassettes in genome. Genetically, the number of expression cassettes is integrated to genome of *P. pastoris* leading recombinant strains produce high protein concentration. In order to screen multicopy recombinant strains for phytase over expression, all zeocin -resistant transformant colonies were collected and spreaded on YPD plates with zeocin concentration of 100 mg/l; 1000 mg/l and 2000 mg/l. After 2 days incubation, on the plate with 2000 mg/l of zeocin there were only several colonies of *P. pastoris* X33 appeared (Fig. 3).

The expression of the multicopy recombinant strains *P. pastoris* X33 was investigated at flask scale by using the BMM medium (100 mM Postassiumphosphate, 1.34% Yeast Nitrogen base and 0.5% Methanol), and then 0.5% methanol was added to the culture broth at every 24. The aliquots of the culture broth were taken at the intervals for determination of phytase activity. The expression gradually increased and reached maximum in 4 days with the maxium phytase yield of 1329 IU/ml.

Interestingly, the recombinant enzyme from culture broth of *P. pastoris* X33 was quite purified. A clear single band protein with MW of 97 kDa was shown on SDS–PAGE (Fig. 4). Thus, it has an great advantage in production of phytase used for animal feeds, since no purification process is needed.
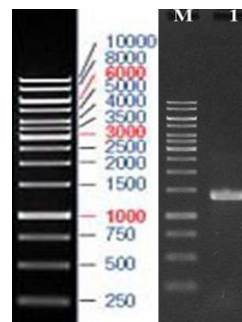


Fig. 1 *PhyA* PCR product

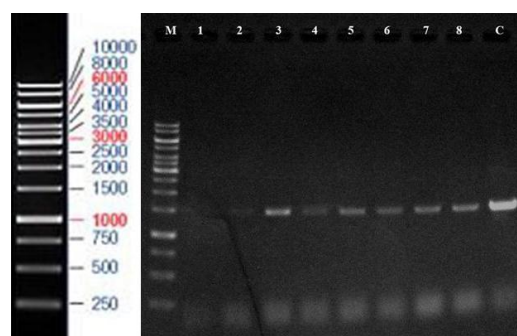M – Invitrogen 1Kb PlusLadder; 1 - *PhyA* PCR products



Fig. 2 PCR screening of transformant *Pichia pastoris*

M - Invitrogen 1Kb PlusLadder; 1→ 4 - transformants KM71H; 5→8- transformants X33; C- positive control



a. YPD 100 mg/l zeocin   b. YPD 1000 mg/l Zeocin   c. YPD 2000 mg/Zeocin

Fig. 3 Growth of *Pichia pastoris* X33 transformants on high zeocin concentration
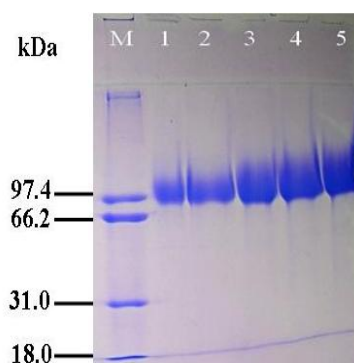


Fig. 4 SDS-PAGE of culture supernatant at different induction time

M: Protein Marker; 1→5: induction time (days)

Wild microbe strain produce phytase in very low amount that could not be satisfied the practical demand of this enzyme. Therefore, genetically engineered strains are constructed by genetic recombination technology in order to enhance the yield.

*P. pastoris* has received tremendous attention for the expression of eukaryotic proteins since it has become commercially available. The advantages of *P. pastoris* as an expression host include a strongly inducible promoter and post-translational modification activity. Chen, C.C. *et al.*, 2004 reported the successful expression of *E. coli appA* gene in *P. pastoris* with the maximum phytase activity after an induction period of 96 h was 118 to 204 IU/ml at the flask scale and 1880 – 4946 IU/ml for high cell-density fermentation [3]. There were several other reports on the successfully cloning *Aspergillus niger* phytases for over expression to date [6], [11], [15], [18]. However, In this work, we have constructed a modified plasmid for phytase expression without *C-myc* epitope and polyhistidine tag in order to eliminate the "none nature factor" from recombinant phytase.

Commercial preparation of 5000 IU/g phytase now is available in the market. Thus, to attain the preparation of the same phytase activity is easy achieved from culture broth of *P. pastoris* X33. It will be prospective strain for enzyme feed production at industrial scale.

## IV. CONCLUSION

phyA gene from *A. niger* XP has been expressed as an active, extracellular phytase in *P. pastoris* X33 under the control of AOX1 promoter and α-factor signal peptide. The recombinant phytase could be produced of 1329 IU/ml in culture broth. *P. pastoris* X33 will be prospective strain for enzyme feed production at industrial scale.

## ACKNOWLEDGMENT

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