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A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase

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Abstract

The *Escherichia coli* phytase gene *appA* was highly expressed in the methylotrophic yeast *Pichia pastoris* under the control of the *AOX1* promoter. Replacement of culture medium with fresh medium in order to remove repressing glycerol and metabolic wastes prior to methanol induction significantly improved phytase expression. The phytase activity level was enhanced from 118 to 204 U/ml at the flask scale and 1880–4946 U/ml for high cell-density fermentation, respectively, by appropriately modifying the medium composition and fermentation strategy. Most of the protein in the culture supernatant was recombinant phytase, the enzyme characteristics of which were similar to native *E. coli* phytase.

Keywords: *appA*; Methanol induction; *Pichia pastoris*; Phytase

1. Introduction

Phytic acid (*myo*-inositol hexakisphosphate), the major storage form of phosphorous in plant seeds [1–3], is regarded as an antinutrient factor since it forms insoluble complexes with proteins and a variety of nutritionally important metal ions such as calcium, zinc, magnesium, and iron, and decreasing the bioavailability of phourous [4,5]. Monogastric animals are unable to utilize phytic acids due to the low levels of phytase activity in their digestive tracts and inorganic phosphate is commonly added into the feed for the purposes of phosphorous supplementation [6]. Phytases (*myo*-inositol hexakisphosphate 3- or 6-phosphohydrolases; EC 3.1.3.8 or EC 3.1.3.26) are typically found in plants, certain animal tissues and many microorganisms [7–11]. Supplemental microbial phytase present in corn- or soybean-based feed for pigs or poultry has been demonstrated to effectively improve phosphorous utilization and reduce fecal phosphorous excretion of such animals [6]. In order to obtain phytases with a high activity or thermostability, phytase genes from *Escherichia coli* [9,12], *Bacillus sp.* [11], *Aspergillus niger* [13,14], *Emericella nidulans*, *Talaromyces thermophilus* [15], *Aspergillus terreus*, *Myceliophthora thermophila* [16] were cloned and expressed. Among these phytase genes, the *E. coli* phytase gene (*appA*) has been reported to demonstrate the greatest specific activity compared to those from other microorganisms [17,18].

The *appA* gene has been successfully expressed in *E. coli* [19,20], *Pichia pastoris* [12,21]and *Streptomyces lividans* [21]. For several years now, the methylotrophic yeast *P. pastoris* has been successfully developed for the heterologous expression of foreign proteins [22–24]. Alcohol oxidase (AOX1), involved in the first step of methanol metabolism, is strongly induced by methanol [6]. A protein of interest cloned under the control of the *AOX1* promoter is highly expressed when methanol is used as the sole carbon source and is repressed by most other carbon sources [6]. In 2003, Stahl et al. [21] reported that *appA* was expressed in *S. lividans* at 950 U/ml. Such a level was not sufficient for commercial production for feed additive applications. In this paper, we demonstrate that the production of *E. coli* phytase in *P. pastoris* was greatly improved by adjusting the culture medium composition and by altering the fermentation process. The biochemical properties of the recombinant phytase were also determined.

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2. Materials and methods

2.1. Strains and plasmids

The *appA* gene was amplified by PCR using an *appA*-containing vector as the template [25]. An upstream primer 5′-GCGAATTCCAGAGTGAGCCGGAGCTG and a downstream primer 5′-GCTCTAGATACGCATTAGACAG-TTCTTCGTT were used, while *Eco*RI and *Xba*I restriction sites (underlined) were designed for flanking the PCR product at the 5′- and 3′-terminus, respectively. The PCR fragment was cloned into the *Eco*RI/*Xba*I site of pPICZαA (Invitrogen, Carlsbad, CA, USA) in order to produce the expression plasmid pPICZαA:appA. The phytase expression plasmid was transformed into *E. coli* DH5α (GIBCOBRL, Grand Island, NY, USA) and selected under the pressure of 25 μg/ml zeocin (Invitrogen). The plasmid was purified and linearized by *Pme*I for transformation into the expression host *P. pastoris* KM71 (*his4*, *aox1::ARG4*, *arg4*, Mut^s; Invitrogen). One transformant, KM71-61 was found to feature the most pronounced phytase activity and was used as the phytase-producing strain for this study.

2.2. Media and culture conditions

E. coli strains were cultured in Luria-Bertani (LB) medium (Difco, Detroit, MI, USA) or low-salt LB when using zeocin as the selection antibiotic. Different media used for *P. pastoris* cultivation are listed in Table 1. The *P. pastoris* strain KM71-61 was cultured in a 500 ml Hinton flask containing 100 ml standard rich medium BMGY or modified medium mBMGHY, respectively, supplemented with 1% (v/v) glycerol as a carbon source. Cells were grown at 30 °C and shaken at 250 rpm until an OD_{600} value of approximately 20 had been reached, then harvested by centrifugation at 3000 × g and 4 °C for a period of 5 min, followed by removal of the supernatant. After washing the biomass with potassium phosphate buffer (100 mM, pH 6.0), the pellet was resuspended in 100 ml standard induction medium BMMY, modified induction medium mBMMHY, or minimal media FBSH in separate 500 ml Hinton flasks as the protocol dictated. Methanol (0.5%, v/v) was added to the Hinton flask every 24 h in order to induce phytase production during the induction period.

2.3. Fermentation

P. pastoris KM71-61 was cultured in a 500 ml Hinton flask containing 100 ml BMGY at 30 °C until an OD_{600} value of around 20 had been reached, following which 10 ml of the seed culture was added into a 5 l jar fermentor (B. Braun Biotech International, Germany) containing 2 l of mBMGHY supplemented with 4% (v/v) glycerol. The temperature and pH during fermentation were maintained at 30 °C and 6.0, respectively. The stirring speed was set to 800 rpm with airflow being maintained at approximately 2–3 vvm. This batch culture was maintained until the glycerol in the medium had been completely exhausted as indicated by a sudden increase in the level of dissolved oxygen. Cells were then collected by centrifugation and resuspended in 2 l of fresh mBMMHY containing 0.5% (v/v) methanol and then returned to into the fermentor for phytase induction. During the induction period, methanol feeding (100% methanol with 12 ml/l PTM_1 trace salts, Invitrogen) was controlled and the methanol concentration was maintained at 0.5% using the MC-168 controller (PTI Instruments, Inc., Lincoln, NE, USA). Samples were taken periodically throughout this phase for phytase and protein analyses. For high cell-density fermentation, *P. pastoris* KM71-61 was cultured in a volume of 2 l FBSH (pH 5.0) supplemented with PTM_1 trace salts and 4% glycerol. Prior to the exhaustion of glycerol, a solution of 50% (v/v) glycerol containing 12 ml/l PTM_1 trace slats was fed for 20 h at a rate of 25 ml/h. Subsequently, the cells were harvested and resuspended in 2 l of fresh FBSH or mBMMHY for phytase induction. The methanol concentration was also maintained at 0.5% by means of the MC-168 controller during this period.

2.4. Enzyme assay

Phytase activity was determined according to the 1999 report of the technique of Bae et al. [26] with minor modification. Briefly, 75 μl of enzyme solution was incubated with 300 μl substrate solution (1.5 mM sodium phytate in 0.1 M sodium acetate buffer, pH 5.0) at 37 °C for 20 min. The reaction was stopped by adding a volume of 375 μl of 5% (w/v) trichloroacetic acid. The released inorganic phosphate was analyzed by adding 375 μl of a coloring reagent (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) and the solution's absorbance at 700 nm was measured by means of a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA). One unit of phytase activity was defined as the amount of activity that releases 1 μmol of phosphate per min at 37 °C. The protein concentration of the medium was determined by means of a BCA protein-assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) using bovine serine albumin as a standard.

2.5. Electrophoresis and phytase zymogram analysis

SDS-PAGE and native-PAGE were performed on slab gels containing 10% (w/v) polyacrylamide. For phytase zymogram analysis, the denaturing temperature selected was 70 °C rather than 100 °C to prevent enzyme inactivation. The SDS-PAGE gel was soaked in a 0.1 M sodium-acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 for a period of 1 h at room temperature and then moving it to a 0.1 M sodium-acetate buffer solution (pH 5.0). Phytase activity was detected by incubating the gel in a 4 mM sodium-phytate solution in 0.1 M sodium-acetate buffer for a period of 20 min.

Table 1
Media for *P. pastoris* cultivation

Media	Composition
BMGY	1% Glycerol[a], 1% yeast extract (YE), 2% peptone, 0.34% yeast nitrogen base w/o amino acids and ammonium sulfate (YNB), 1% $(NH_4)_2SO_4$ (w/v) and 4×10^{-5}% biotin in pH6.0 100 mM potassium phosphate buffer
BMMY	0.5% Methanol, 1% YE, 2% peptone, 0.34% YNB, 1% $(NH_4)_2SO_4$ (w/v) and 4×10^{-5}% biotin in 100 mM potassium phosphate buffer
mBMGHY	1% Glycerol, 1% YE, 1% $(NH_4)_2SO_4$ (w/v) and 4×10^{-5}% biotin, 0.004% histidine in 100 mM potassium phosphate buffer
mBMMHY	0.5% methanol, 0.1% YE, 1% $(NH_4)_2SO_4$ (w/v) and 4×10^{-5}% biotin, 0.004% histidine in 100 mM potassium phosphate buffer
FBSH	26.7 ml/l H_3PO_4 (85% stock), 0.93 g/l $CaSO_4$, 18.2 g/l K_2SO_4, 14.9 g/l $MgSO_4{\cdot}7H_2O$, 4.13 g/l KOH and adjusted to pH 5.0 by NH_4OH. Add 0.004% histidine and 0.435 ml of PTM1 trace metal solution (6 g/l $CuSO_4{\cdot}5H_2O$, 0.08 g/l KI, 3 g/l $MgSO_4{\cdot}H_2O$, 0.2 g/l Na_2MoO_4, 0.02 g/l H_3BO_3, 0.5 g/l $CoCl_2$, 20 g/l $ZnCl_2$, 65 g/l $FeSO_4{\cdot}7H_2O$, 0.2 g/l biotin and 5 ml H_2SO_4)

[a] The concentration of carbon source may be varied.

Subsequent to two washings with water, the phytase bands were detected by immersing the gel in a coloring reagent (freshly prepared by mixing 18 ml of 2.5 N HCl, 18 ml of 2.6% (w/v) ammonium molybdate, 13 ml of dH_2O and 1 ml of a 0.126% (w/v) malachite-green solution) for a period of 1–2 h until the relevant visible green band(s) appeared.

2.6. Deglycosylation of the enzyme

Recombinant phytase was treated with 0.5 IU of endoglycosylase H (Endo H_f, New England Biolabs, Beverly, MA, USA) for 1 h at 37 °C according to the manufacturer's instructions. For samples used for zymogram analysis, the denaturing process that was conducted prior to the Endo H_f treatment was eliminated.

3. Results

3.1. Flask level expression

The phytase production of the *P. pastoris* KM71-61 construct grown in different media in flasks is illustrated in Fig. 1. When grown in BMGY for 24 h and induced by the addition of 0.5% methanol every 24 h without medium replacement, no phytase activity was detected for the first 48 h. By contrast, phytase activity was detected immediately, and its relative level increased with time, when the existing culture medium was replaced by fresh BMMY prior to induction.

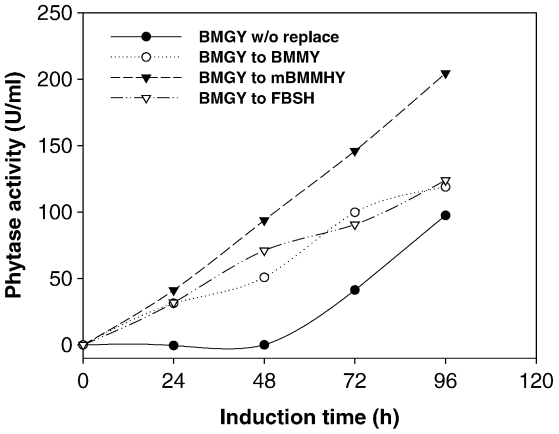

Fig. 1. Effect of induction media on phytase activity. *P. pastoris* KM71-61 was grown in 100 ml BMGY for 24 h and 0.5% methanol was added for induction. Solid circle represents the induction without medium replacement; open circle, open triangle and solid triangle represent the induction with medium replacement by BMMY, FBSH and mBMMHY, respectively.

We noticed that the protein concentration had barely changed during either the cell-growth in BMGY or the induction periods in BMMY, indicating that peptone, YNB and YE might be in excess in the medium during these periods. In order to utilize the culture medium more efficiently, we modified the medium composition by reducing the concentrations of peptone, YNB and YE. Cells cultured in mBMGHY medium, as modified from BMGY medium by replacement of peptone and YNB with histidine, showed little difference in viable-cell concentration and OD_{600} when compared to those cultured in BMGY (data not shown). For media used for induction, we noted no significant difference in relative phytase activity for situations when cells were induced with BMMY or FBSH, reaching levels of 118 and 123 U/ml respectively after 96 h induction. The phytase production was increased when a modified mBMMHY medium was used. The mBMMHY medium was modified from BMMY medium by replacing peptone and YNB with histidine and the YE concentration was reduced from 1 to 0.1%. The phytase activity in mBMMHY was 204 U/ml after an induction period of 96 h, virtually twice the level that it achieved when induction was conducted in BMMY or FBSH.

3.2. Phytase production at high cell-density

The high cell-density culturing was performed by feeding 500 ml of 50% glycerol (25 ml/h) after the initial amount of glycerol had been exhausted as evidenced by the abrupt increase in the level of dissolved oxygen. For this glycerol-fed batch culture, the viable-cell concentration and the OD_{600} were $2.59 \pm 0.21 \times 10^{10}$ cfu/ml and 321 ± 13, respectively. After the glycerol-fed batch cultivation, cells were centrifuged and resuspended in fresh FBSH or mBMMHY and returned to the fermentor.

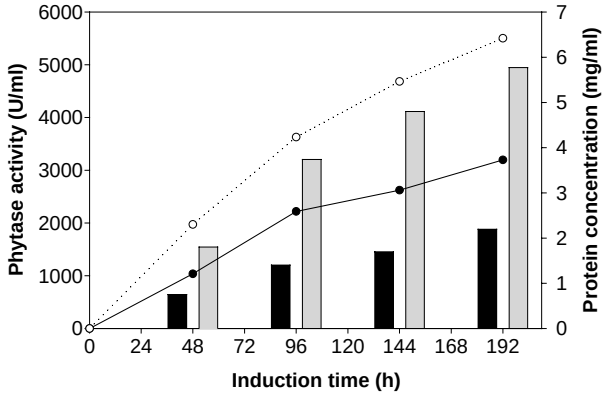

Fig. 2. Protein and phytase production of *P. pastoris* KM71-61 in the high cell-density fermentation. Solid and open circles represent the protein concentrations in response to the induction with medium replacement by FBSH or mBMMHY, respectively. Black and gray bars represent the phytase activities in response to the induction with medium replacement by FBSH or mBMMHY, respectively.

Methanol was fed to the medium in order to maintain the methanol concentration at 0.5% using the methanol controller. Fig. 2 reveals the phytase and total extracellular protein production by *P. pastoris* KM71-61 in different media. Both phytase and protein production increased with time regardless of the medium used for culturing. Cells induced in FBSH produced 3.7 g of protein/l of culture supernatant with a phytase activity level at 1880 U/ml following induction for a period of 192 h. The protein concentration of cells induced in mBMMHY medium reached 6.4 g/l of culture supernatant and the phytase activity was noted to be 4946 U/ml after an induction period of 192 h.

3.3. Electrophoresis and phytase zymogram analysis

The glycosylation of *P. pastoris* phytase was visualized by SDS-PAGE analysis and three major bands were found at 56.6, 60.3 and 64.4 kDa (Fig. 3A). The intensity of these bands increased with induction time, indicating that the level of recombinant phytase production was proportional to the induction level. According to the results of non-denaturing PAGE analysis suggested that the native phytase exists as a dimer (Fig. 3B). Subsequent to deglycosylation by treatment with Endo H_f, only a single 45 kDa band was visualized, this being the predicted molecular mass of a native phytase gene (Fig. 4).

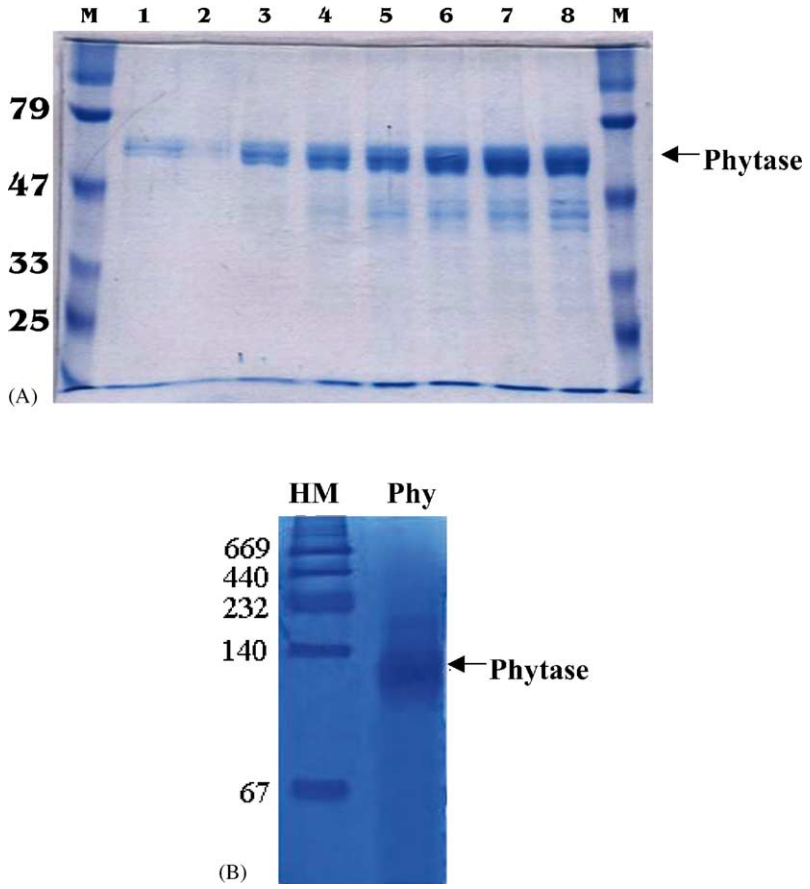

Fig. 3. PAGE analysis of the recombinant phytase. (A) SDS-PAGE of culture supernatant at different induction time. Lane 1–8: 7.5 μl of culture supernatant after 24–192 h induction was loaded. Lane M, Pre-stained protein marker. (B) Native PAGE of the phytase separated with 10% non-denaturing gel. Lane HM, high molecular weight protein marker. Phy: phytase.

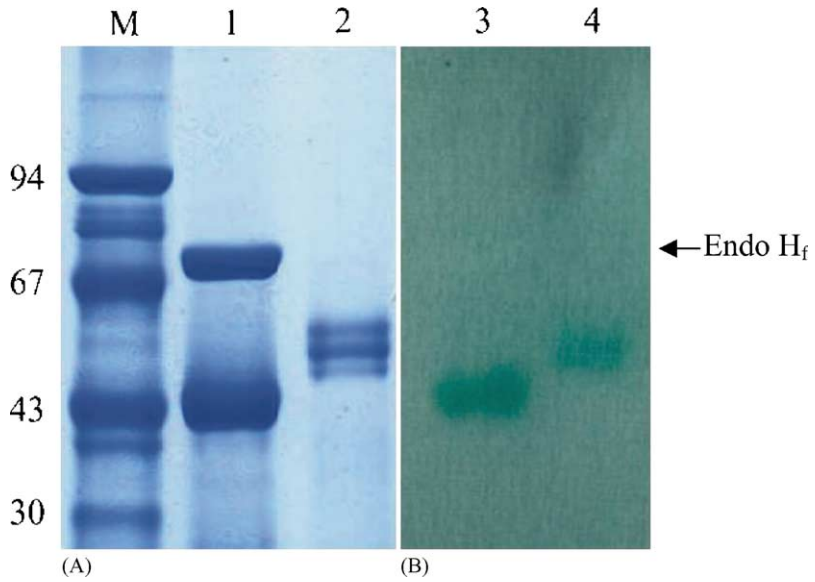

Fig. 4. SDS-PAGE (A) and zymogram (B) analysis for phytase activity of the recombinant phytase before (Lane: 2, 4) and after (Lane: 1, 3) deglycosylation by Endo H_f.

4. Discussion

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. In 1999, Rodriguez et al. [12] reported that the expression of *E. coli appA2* gene in *P. pastoris* and that the maximum phytase activity after an induction period of 72 h was 114 U/mL. In our study, we have successfully expressed the *appA* gene in *P. pastoris* and produced phytase at fermentor-scale. The level of phytase activity was tremendously enhanced in a high cell-density fermentation as a consequence of our modifying the medium composition and the fermentation strategy. We also found that culture-medium replacement was crucial to achieving high levels of phytase production. This might be attributed to the complete removal of glycerol or metabolic wastes. Some earlier studies have demonstrated that the gradual addition of methanol prior to the glycerol depletion in the culture medium might have facilitated the de-repression of the *AOX1* promoter [24,27]. Use of a methanol monitor/controller also significantly improved the enzyme production. FBSH appears to be an excellent medium for batch culture of *P. pastoris*. However, it does not appear to be suitable for protein induction. To the best of our knowledge, our results suggest the highest level of expression of phytase in *P. pastoris* that has been reported to date. Although in 1999, Mayer et al [28] did claim a greater expression of phytase was achieved using *Hansenula polymorpha* featuring a high copy number of the phytase gene.

Most of the *P. pastoris*-secreted protein in the culture medium was recombinant phytase as evidenced by the results of the SDS-PAGE assay. According to *appA* gene-sequence analyses, there exist three predicted *N*-glycosylation sites [12,29]. The molecular weight of recombinant phytase subsequent to deglycosylation by Endo H_f appeared to be similar to that of the native form of phytase [12,29]. Some minor bands did appear for phytase subsequent to the longer induction time, suggesting that some proteolytic enzymes may be produced by *P. pastoris* during the induction period. This observation is similar to chitinase expression by *P. pastoris* [30]. In this study, we have demonstrated that the productivity of *E. coli* phytase in *P. pastoris* fermentation peaked at 4946 U/ml. This high yield would thus suggest that phytase production by *P. pastoris* was both economical and feasible.

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