HETEROLOGOUS EXTRACELLULAR EXPRESSION OF BACTERIAL PHYTASE (*AppA*) IN *Pichia pastoris* AND ITS BIOCHEMICAL CHARACTERIZATION

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ABSTRACT

Phytase, used as an animal feed additive, catalysesphytic acid hydrolysis with sequential release of phosphate and reduces feeding costs. In this work, we amplified the *Escherichia coli* acid phosphatase gene *appA* and inserted it into an expression vector pPICZαA. *appA* was under the control of inducible promoter of alcohol oxidase *AOX1* with an α-factor signal peptide, which provides secretory protein expression. The recombinant plasmid was purified and linearized with the restriction enzyme *Pme*I, followed byits transformation into the host strain *Pichia pastoris* GS115,using electroporation. The transformed *P.pastoris* GS115 grew well on YPDS containing 100 µg/mL zeocin. PCR results confirmed the integration of *appA* into the genome of *P. pastoris.* SDS-PAGE analysis revealed that phytase was over expressed and secreted into the culture supernatant. The maximal extracellular activity and optimum temperature of phytase was reported to be 1510 U/mL and 50°C, respectively. Phytase was active at pH 2.0-7.0, with optimum activity at pH 5.0. The recombinant protein was thermostable and retained 80% of its activity after incubation at 60°C for 10 min. Protein glycosylation was confirmed with endoglycosidase H. The recombinant yeast strain *P. pastoris* GS115 pPICZαA/AppA exhibits potential industrial applications.

Keywords: phytase, *Escherichia coli, Pichia pastoris,* heterologous expression.

INTRODUCTION

Phytase is an enzyme that has the ability to specifically catalyze the step-wise cleavage of phosphate groups from phytic acid. Phytate or phytic acid is the main storage form of phosphorus in grains and oil seeds[1]. These enzymes have been used in animal feed to improve phosphorus nutrition, because monogastric animals such as swine and poultry require extrinsic phytase to digest phytate.

Supplementation of feeds destined to these animals with inorganic phosphorus is not only expensive, but also potentially polluting and non-sustainable [2]. Moreover phytate presence in feeds has an anti nutrient effect, as chelator of divalent cations like calcium, magnesium, iron, and zinc, thereby, decreasing the bioavailability of phosphorus [3].

Currently, four different classes of phytases are recognized: β-propeller phytase, histidine acid phosphatase, cysteine phosphatase and purple acid phosphatase. Phytases belonging to histidine acid phosphatasefamily have been used successfully as a feed additive and most widely studied and utilized today[4].

During the last decades, phytases from various microorganisms such as fungi (*Aspergillus niger*, *Candida krusei*),yeast(*Pichiaanomala*)and bacteria (*Bacillus subtilis*, *Escherichia coli*)have been isolated, cloned and characterized in different hosts[5-9]. Since phytases from various sources have different biochemical characteristics like temperature and pH optimum, not all are suitable for industrial applications. Among many phytases, *E.coli*AppAphytaseis more promising because of its thermostability, acidic pH optimum, higher specific activity for phytate, greater resistance to pepsin and trypsin digestion and better catalytic efficiency[10].

The methylotrophic yeast *Pichia pastoris* has been developed as a host for heterologous gene expression producing high level of foreign proteins. Benefits of the *P. pastoris* expression system include: growth to very high cell densities using a simple and cheap media, strongly inducible and constitutive promoters, high levels of protein expression and secretion into the growth medium providing simple purification. Another important advantage is the ability to produce properly post-translation modification including glycosylation, disulfide bond formation and proteolytic processing [11].

Protein glycosylation in eukaryotic cells is important for proper protein folding, transport, and protein stability. Also, glycosylation is reported to be vital for phytasethermostability, but does not have a significant impact upon the pH optimum or temperature of enzymes' activity[12]. Increased level of

glycosylation of phytase from *E. coli* expressed in *P. pastoris* improved the thermotolerance of the protein over the deglycosylated form[13]. The glycosylated alkaline phytase r-PHY from *B. subtilis* US417 showed higher activity than the wild type without glycosylation[9].

Our group previously has successfully expressedgene*appA*in*E.coli* strainArcticExpress(DE3) [14].Considering the high potential of recombinant phytaseAppA for using as a feed supplement, in present article, we describe molecular cloning of the phytase gene from *E.coli appA* and extracellular expression in *P. pastoris* strains GS115 under the control of the inducible AOX1 promoter. The biochemical properties of the recombinant phytase were also determined.The recombinant enzyme showed desirable characteristics for biotechnological application and thus may be potentially useful as a feed additive.

MATERIALS AND METHODS

Strains, plasmid, oligonucleotides, growth conditions and media

The phytase gene was amplified from genomic DNA of *Escherichia coli* BL-21(DE3) by PCR using specific primers: 5'-EcoRI-appA-PM (5'CCGGAATTCCGGATGAAAGCGATCTTAATCCC3') and 3'-NotI-appA (5' TTTTCCTTTTGCGGCCGCCAAACTGCACGCCGG-3').The *Escherichia coli* BL-21(DE3) strain was cultivated in LB-medium at 37°C. Genomic DNA of *Escherichia coli* BL-21(DE3) was obtained from laboratory stock using PureLink® Genomic DNA Mini Kit (Invitrogen).Restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific (USA). Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA) was used for the amplification of the target gene. For expression in eukaryoticsystem, *P. pastoris*GS 115 and pPICZαA expression vector(EasySelect Pichia Expression Kit) were purchased fromInvitrogen, San Diego, CA. Plasmid pPICZαA contain tightly regulatedmethanol-inducible alcohol oxidase (*AOX1*) promoter which provided exceptionally high levels of heterologous protein expression. Plasmid contains α-factor secretion signal from *S. cerevisiae* for secretion of the recombinant protein into the growth medium.

Construction of the expression vector and cloning in *Pichia pastoris*

PCR conditions were as follows: one cycle of 95°C for 3 minutes, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. *AppA* gene was cloned into pPICZαA vector using *Eco*RI and *Not*I restriction enzymes. The ligation reaction was transformed into DH5α cells plated on LB medium containing 25 µg/mL zeocin. Colonies with integrated inserts were selected for further purification of plasmids. Resulting recombinant vector was named pPICZαA/AppA. *Pichia pastoris* strainGS115 (his4) was used as the host for protein expression (EasySelect Pichia Expression Kit, Invitrogen). 6.5 µg of linearized plasmid pPICZαA/AppA by restriction enzyme *Pme*Iwere transformed into GS115 strains by the electroporation. Selection of positive colonies was performed on YPDS agar (1% yeast extract, 2% peptone, 2% dextrose, and 1 M sorbitol) containing antibiotic zeocin(100 µg/mL).To select the multi-resistant clones, transformed cells were grown on YPDS plates containing 0.5-1 mg/mL zeocin. Integration of an expression cassette into the genome of *P. pastoris* was checked out by PCR using recombinant Taq DNA polymerase[15]and the 5' AOX1 and 3' AOX1 primers according to the instructions of the *Pichia* expression kit (Invitrogen, San Diego, CA, USA).

Expression of recombinant phytase AppA in *Pichia pastoris*

Pichia transformants were incubated in 10 ml of YPD (Yeast Extract Peptone Dextrose: 1% yeast extract, 2% dextrose (glucose), 100 µg/ml Zeocin) media for 16 h at 30°C with vigorous shaking at 250 rpm. Next day 1 ml of culture was transferred into 50 ml of BMGY (Buffered Glycerol-Complex Medium: 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) and cells were grown for 24 h at 30°C with vigorous shaking at 250 rpm. Centrifugation of cells was performed at: 3000×g for 5min. Protein expression was induced after resuspending cells in 50 ml of BMMY media (Buffered Methanol-complex Medium: 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin) with 1,5% methanol, and culture was incubated for 168 h with vigorous shaking at 250 rpm. The culture was centrifuged at 12000 rpm for 30 min, 4°C and supernatant was collected for measuring phytase activity.

Deglycosylation of AppA by endoglycosidase H

Deglycosylation of AppA was done using endoglycosidase H (Promega,Madison, WI, USA). Briefly, 1µl of 10X denaturing solution was added to 10 µg of recombinant phytaseAppA. 10X denaturing solution is composed of 5% SDS, 0.4M DTT. The mixture was heated at 95°C for 5 min and then cooled to room temperature.Then 2µl of 10X Endo H Reaction Buffer and 2µl of endoglycosidase H (1,000 U) were added to the sampleandadjustedwith distilled water to final volume of 20 µL.Reaction wasincubated at 37°C for 3 h. Deglycosylated protein was analyzed by SDS–PAGE.

SDS-PAGE analysis and protein concentration assay

SDS-PAGE analysis of crude phytase extracts at different induction time was performed in12% polyacrylamide gel. After electrophoresis, the gel was stained with coomassie brilliant blue R 250 and then destained. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard[16].

Phytasezymogram analysis

Zymograms were prepared by soaking the gels first in 1% Triton X-100 for 1 h at room temperature and then in 0.1 M sodium acetate buffer (pH 5.0) for 1 h at 4°C. Phytase activity was detected by incubating the gels for 16 h in a 0.1 M sodium acetate buffer (pH 5.0) containing 0.4% (w/v) sodium phytate. Activity bands were visualized by immersing the gel in a 2% (w/v) aqueous cobalt chloride solution. After a 5 min incubation at room temperature the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Phytase activity was evident as zones of clearing in an opaque background[17].

Phytase activity assay

Phytase activity was determined in the culture supernatant of the transformed *P. pastoris*GS115 cells. The phytase activity was measured by standard method based on phytase ability to release inorganic phosphate and subsequent reaction of inorganic phosphate with ammonium heptamolybdate/ammonium vanadate reagents. The release of P_i from sodium phytate was determined colorimetricly with an ammonium molybdate/ascorbic acid solution[18]. 0.1 ml of sample, diluted with 0.1 M sodium acetate buffer, pH 5.5, prewarmed in a water bath (37°C) for 5 min, was mixed with 2 ml of sodium phytate(5 µmol/ml in 0.1 M sodium acetate buffer, pH 5.5) and incubated at 37°C for 30 min. The reaction was stopped by adding 2 ml of color/stop solution, containing 2.5% ammonium heptamolybdate solution and 0.06% ammonium vanadate with 27% nitric acid solution. The resulting yellow complex was measured at 415 nmby spectrophotometer Pharmacia Ultrospec III and the released inorganic phosphate was quantified with a phosphate standard curve. The standard curve was prepared using 0.5-2.0 µmol/ml $KH_2PO_4$and one phytase unit (FTU) was defined as the amount of enzyme that releases 1 µmol of inorganic phosphorus from sodium phytate per minute at pH 5.5 and 37°C. A control without enzyme was performed at the same conditions. All enzyme assays were done in triplicate.

Effect of pH on enzyme activity

The pH optimum was determined by measuring enzymatic activity at pH 1.5-8.0 in the following buffers: 0.1M glycine-HCl for pH 1.5-3.0; 0.1M sodium acetate for pH 3.5-6.0; 0.1M Tris-HCl for pH 6.5-8.0. These assays were performed at 37°C for 30 minutes using 5 µmol/ml sodium phytate as substrate.The optimum pH was determined from the curve of relative enzyme activity versus pH.

Effect of temperature on enzyme activity

The optimal temperature was determined in 0.1 M sodium acetate pH 5.5 at 10°C, 20°C, 30°C, 40°C, 50°C, 60°C and 70°C.The optimum temperature was determined from the curve of relative enzyme activity versus temperature. Thermostability of the recombinant phytase was determined by assaying residual activity after 10 to 60 min incubating the enzyme in 0.1 M acetate buffer (pH 5.5) at temperatures ranging from 50 to 80°C.Immediately after the heat treatment, the enzymes were placed on ice for 5 min. The remaining phytase activity was measured at 37°C and pH 5.5 as described previously.

Pepsin and trypsin tolerance tests

To determine the resistance to proteolytic activity, the phytase was incubated at 37°C for 1 h with pepsin (0.1mg/ml) in 80 mM glycine-HCl pH 2.0 and trypsin(0.1mg/ml) in 80 mM $NH_4H_2CO_3$ pH 7.5 at protease/phytase (w/w) ratios of 1/1000, 1/500, 1/200, 1/100 and 1/50. Theresidual activity was measured by the method described above.

Effects of metal ions on phytase activity

To study the effect of metal ionson phytase activity, reactions were performed by pre-incubation of the enzyme in 0.1 M sodium acetate buffer (pH 5.5) containing 5mMand 10 mMmetal ions (Co^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, K^{+}, Na^{+}, Li^{+}, Mn^{2+}, Cd^{2+}, Cs^{2+}, Ni^{2+}, Ba^{2+})for 30 min at 37°C before performing the enzymatic activity assay. Reaction in the absence of any additive was used as a control.

RESULTS AND DISCUSSION

Construction of pPICZαA/AppA Expression Vector

The gene *appA* was cloned intopPICZαAexpression vector between *Eco*RI and *Not*I restriction sites. The pPICZαA vector is a powerful expression vector, which uses the *AOX1* promoter to inducible express

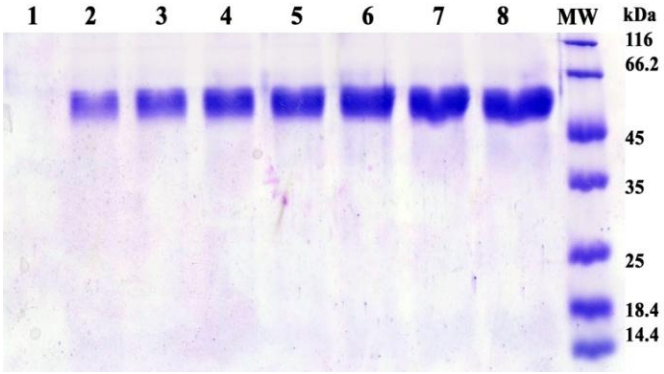
recombinant proteins in *P. pastoris.*Then chemically competent cells *E. coli* DH5α strain were transformed by recombinant vector and grown in low salt LB plate with 25 μg/mL of zeocin.Therecombinant plasmid pPICZαA/AppA includesappA-expression cassette consisting of *appA* gene in frame with *S. cerevisiae* α-factor secretion signal, flanked by AOX1 promoterand terminator sequences. Transformation *P. pastoris* GS115 strain by linearizedpPICZαA/AppA resultedin about 100 his^+transformants. The integration of appA-expression cassette into the hostgenome was confirmed by PCR using 5' and 3' AOX1 primers. Colony-PCR amplification products ofabout ~ 1.8kb (with AOX region) and ~ 2.2 kb (*AOX1* gene) indicated the integration of appA-expression cassette into the genome of*P. pastoris* GS115 strain.

The calculated molecular mass of the protein was about 45 kDa. PhytaseAppA contained the conserved N-terminal sequence motif RHGXRXP and C-terminal HDTN at the active sites, which are features of phytase belonging to the HAP family [19]. The expression of the protein from *P. pastoris* produces the recombinant AppA with three potential N-glycosylation sites that were predicted by the NetNGlyc 1.0 (position:141 NVTD, 206 NVSL and 319 NWTL). Cysteine residues analysis using DiANNA software indicated that 8 cysteine residues may form pairs 79-110, 135-180, 190-393 and 384-410 that were predicted to form disulphide bonds.

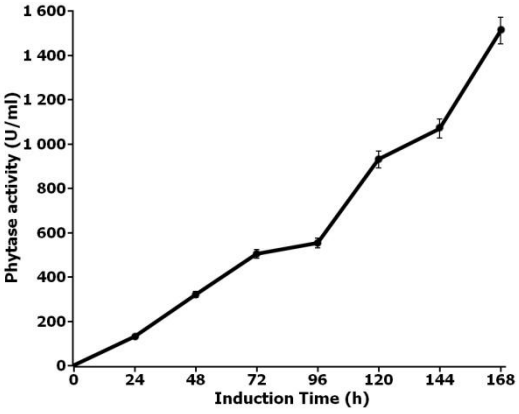
Expression of the recombinant phytase AppA in *Pichia pastoris*

P.pastoris was transformed bypPICZαA/AppA and zeocin-resistant clones were picked for further studies. A quick, direct way to select putative multi-copy is to plate the transformation mix on high concentrations of zeocin[20]. As higher production of recombinant proteins by resistance to high concentration of zeocin has been established earlier, clones were subsequently grown on high concentrations of zeocin(500-2,000 μg/ml) [21]. Clones GS115 pPICZαA/AppA were able to survive up to 2,000 μg/ml of zeocin. These clones resistant to high zeocin concentration were further checked for shake flask expression.

After methanol induction, the selected *P. pastoris*GS115 pPICZαA/AppA strain produced and secreted N-glycosylated form of the phytase AppA. This strain produced phytase up to 130 U/mL phytase in the medium after 24 h of induction with1.5% methanol. After 168 h of methanol induction, production of extracellular recombinant phytase reached 1510 U/mL with specific activity of 5.47 U/mg and extracellular protein concentration of 276mg/L. The time-course of phytase expression levels is presented in figure 1.



a



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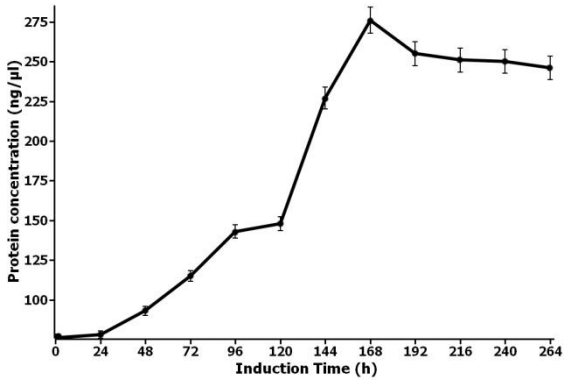


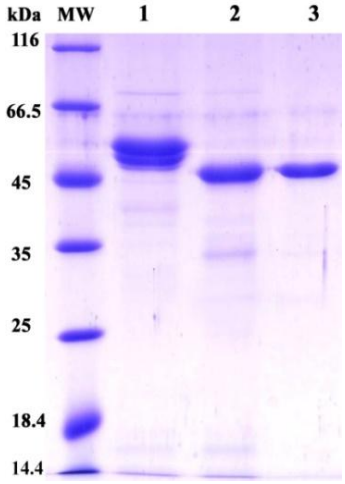
c

Lanes:1 – without induction; 2 – induction for 24 h; 3 – induction for 48 h; 4 – induction for 72 h; 5 – induction for 96 h; 6 – induction for 120 h; 7 – induction for 144 h; 8 – induction for 168 h; MW – protein marker (b). Time dependence of the activity of phytaseAppA and protein concentration dependence (c)

Fig. 1.SDS-PAGE and time dependence of the activity of phytaseAppA(a).SDS-PAGE analysis of AppA expressed in *P. pastoris*GS115/pPICZαA/AppA grown in BMMY

Deglycosylation of AppA by endoglycosidase H

SDS-PAGE analysis of the yeast culture supernatant showed two protein bands of approximate molecular mass 47-55 kDa, but deglycosylation of AppA by Endo H deglycosylase resulted in single band of molecular mass about 45kDa. This suggested that the phytase protein was partially glycosylated in *P. pastoris* and the observed molecular weights were higher than the molecular weight predicted from the amino acid sequence alone(45 kDa). Results are shown in figure 2.

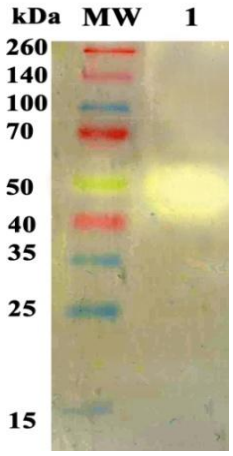


Lanes: MW – protein molecular weight standards №26610 (Thermo Scientific); 1 – culture supernatant from recombinant *P. pastoris* GS115/pPICZαA/AppA; 2 – culture supernatant from recombinant *P. pastoris* GS115/pPICZαA/AppA after Endo H treatment; 3 – AppA expressed in *E. coli*

Fig. 2.SDS-PAGE analysis of expressed AppA.SDS-PAGE analysis of glycosylated and deglycosylatedAppA

Phytasezymogram analysis

As shown in zymogram recombinant enzyme phytaseAppA has phosphohydrolase activity against substrate - sodium phytate during the enzymatic reaction forming zones of clearing in an opaque background polyacrylamide gel near the estimated mass of protein ~ 50 kDa. Results are shown in figure 3.



Lanes: MW – protein molecular weight standards Spectra BR; 1 – culture supernatant from recombinant *P. pastoris* GS115/pPICZαA/AppA

Fig. 3. Phytasezymogram analysis

Because phytase is intended for use as an animal feed supplement, its activity and stability will be affected by the pH, temperature, and the presence of metal ions and proteases in the animal's digestive tract.The optimum temperature for the activity of phytase was 50°C thatis similarforphytases from*A.niger, E.parvum and E. coli*[6, 12, 22](fig. 4a).Thermostability is important since animal feed is usually produced by pelletization or extrusion, which exposes the feed to high heat. The thermostability of the recombinant protein was investigated by incubating at 50-80°C for 10 to 60 min.The results showed the protein retained more than 80% residual activity after exposure to 60°C for 10 min. Meanwhile, almost 60% residual activity was retained from the initial activity when exposed to 70°C for 10 min and more than 40% retained when exposed to 80°C for 10 min,respectively (fig.

4b).Consequently, the phytaseAppA enzyme would be good for use with pelletization where high temperatures areemployed for 30-60 sec.

For optimal pH, the enzyme was incubated at 37°C for 30 min at different pHs from 1.5 to 8.0. AppAphytase hasoptimum pH 5.0, similar to AppA2 and phytasefrom *K.terrigena*but different fromphytases of*B. subtilis*, *P.simplicissimum*and*A.niger* (PhyB) whose optimum pH are 7.5, 4.0, and 2.5, respectively[23, 24, 25].

At pH 7.0 or higher, AppAphytase was completely inactivated, as was the case for most phytases in previous reports[26]. In acidic conditions, AppAphytase was more stable. Its activity was reduced along with decreasing pH, and was completely inactivated at pH 2(fig. 4c).

The proteolytic resistance is important property, since an effective phytaseshould resist hydrolytic breakdown by digestive proteinases in the digestive tract[26]. At a pepsin/phytase ratio of 0.001-0.002, more than 80% of the initial AppAphytase activity was retained after 1 h of pepsin digestion, but at a higher pepsin/phytase ratio (0.01) only 38% of the initial activity was retained.

When trypsin was used for proteolytic digestion similar results with pepsin were obtained: trypsin/phytase ratios of 0.001 and 0.002 for 1 h, *E. coli* phytase AppA activity retained 86% and 79%, respectively. However recombinant phytaserevealed remarkable stability against proteolytic digestion with high trypsin concentration (0.01), more than 55% of the initial AppAphytase activity was retained. Thereby, phytaseAppA has a sufficiently high resistance to the action of proteolytic enzymes (fig. 4d).

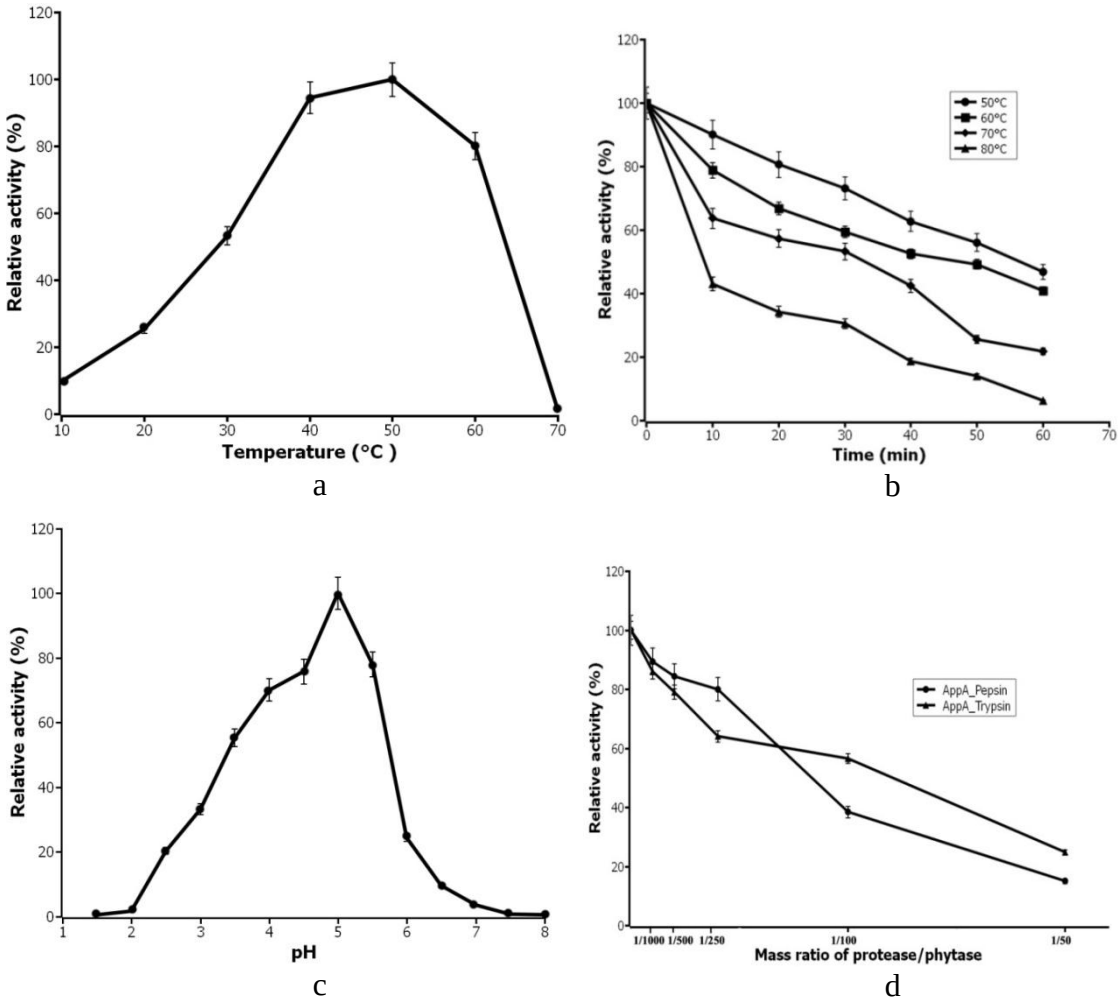


Fig. 4. Biochemical characterization of recombinant phytaseAppA.The optimal temperature (a) of the recombinant phytaseAppA. The effects of thermal stability (b), optimal pH (c) and effects of proteases: pepsin and trypsin (d) on the stability of recombinant phytaseAppA

The effect of metal ions on the enzyme activity was tested. The results showed that the phytaseAppA was moderately inhibited by Co^{2+}, Cs^{2+} and Ni^{2+} and the activity was significantly inhibited in the presence ofFe^{3+}. It was activated by Ba^{2+}, Ca^{2+} (table 1). The inhibitory effect metal ions of Fe^{3+}might be due to the formation of insoluble complex between phytate and metal ions, leading to a decrease of available substrates for the enzyme[22]. $Ba^{2+}$and Ca^{2+}ions enhance the enzyme activity

becauseprobably they can interactwith the enzyme leading to a conformational change and increase activity like it was shown before for phytases of *A.niger*, *S. thermophile*, *R.oligosporus*[27].Results are shown in table 1.

Table 1. Effect of metal ions on recombinant *E. coli* phytase from *P. pastoris* GS115 pPICZαA/AppA

Metal ions	Relative Activity (%)	
	5mM	10mM
Control	100	100
Ba^{2+}	113.78	111.78
Ca^{2+}	104.07	102.07
Mn^{2+}	102.50	95.50
Mg^{2+}	100.29	93.29
Na^{+}	98.28	90.28
K^{+}	96.25	89.25
Li^{+}	95.98	84.98
Co^{2+}	85.22	80.22
Cs^{2+}	82.65	76.65
Ni^{2+}	80.72	75.72
Fe^{3+}	50.74	43.74

CONCLUSION

A phytaseappA gene was isolated from *E. coli*BL21(DE3) andsuccessfully expressed in *P. pastoris* GS 115 strain.In this study, the biochemical properties of the phytaseAppAwere investigated. The maximal extracellular phytase activity level was 1510U/ml. The optimal temperature of the phytase was 50°C andthe enzyme was active at pH 2.0-7.0, with optimum activity at pH 5.0.Protein glycosylation was confirmed by using endoglycosidase H. Recombinant yeast strains may be suitable for use in industrial applications.

ACKNOWLEDGMENTS

This work was supported by the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan in the framework of the "Development of technological platforms for the production of recombinant enzyme in yeast culture."

REFERENCES

1. Reddy N.R., Sathe S.K., Salunkhe D. K. Phytates in legumes and cereals. *Adv Food Res*, 1982, vol. 28, pp. 1-92. PMID:6299067.

2. Lei X.G., Porres J.M. Phytase enzymology, applications, and biotechnology .*Biotechnol Lett*, 2003, vol. 25, no. 21, pp. 1787-1794. PMID: 14677699.

3. Hurrell R.F. Phytic acid degradation as a means of improving iron absorption.*Int J VitamNutr Res*, 2004, vol. 74, no. 6, pp. 445-452. PMID: 15743020.

4. Fonseca-Maldonado R., Maller A., Bonneil E., Thibault P., Botelho-Machado C., Ward R.J., PolizeliMde L. Biochemical properties of glycosylation and characterization of a histidine acid phosphatase (phytase) expressed in Pichia pastoris. *Protein Expr Purif*, 2014, vol. 99, pp. 43-49. PMID: 24680732.

5. Golovan S., Wang G., Zhang J., Forsberg C.W. Characterization and overproduction of the Escherichia coli appA encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. *Can. J. Microbiol*., 2000, vol.46, no. 1, pp. 59-71.PMID:10696472.

6. Promdonkoy P., Tang K., Sornlake W., Harnpicharnchai P., Kobayashi R.S., Ruanglek V., Upathanpreecha T., Vesaratchavest M., Eurwilaichitr L., Tanapongpipat S. Expression and characterization of Aspergillus thermostable phytases in Pichia pastoris. *FEMS Microbiol Lett*, 2009, vol. 290, no. 1, pp. 18-24. PMID: 19025560.

7. Quan C.S., Fan S.D., Zhang L.H., Wang Y.J., Ohta Y. Purification and properties of a phytase from Candida krusei WZ-001. *J BiosciBioeng*, 2002, vol. 94, no. 5, pp. 419-425. PMID: 16233328.

8. Vohra A., Kaur P., Satyanarayana T. Production, characteristics and applications of the cell-bound phytase of Pichia anomala. *Antonie Van Leeuwenhoek*, 2011, vol. 99, no. 1, pp. 51-55. PMID: 20730601.

9. Hmida-Sayari A., Elgharbi F., Farhat A., Rekik H., Blondeau K., Bejar S. Overexpression and biochemical characterization of a thermostable phytase from Bacillus subtilis US417 in Pichia pastoris. *MolBiotechnol*, 2014, vol. 56, no. 9, pp. 839-848. PMID: 24859267.

10. Chen C.C., Wu P.H., Huang C.T., Cheng K.J. A Pichia pastoris fermentation strategy for enhancing the heterologous expression of an Escherichia coli phytase.*Enzyme and Microbial Technology*, 2004, vol. 35, no. 4, pp. 315-320.

11. Cregg J.M., Vedvick T.S., Raschke W.C. Recent advances in the expression of foreign genes in Pichia pastoris. *Biotechnology (N Y)*, 1993, vol. 11, no. 8, pp. 905-910. PMID: 7763913.

12. Fugthong A., Boonyapakron K., Sornlek W., Tanapongpipat S., Eurwilaichitr L., Pootanakit K. Biochemical characterization and in vitro digestibility assay of Eupenicilliumparvum (BCC17694) phytase expressed in Pichia pastoris. *Protein Expr Purif*., 2010, vol. 70, no. 1, pp. 60-67. PMID: 19818856.

13. Yao M.Z., Wang X., Wang W., Fu Y.J., Liang A.H. Improving the thermostability of Escherichia coli phytase, appA, by enhancement of glycosylation.*Biotechnol Lett.*,2013,vol. 35, no. 10, pp. 1669-1676. PMID:23794051.

14. Abeldenov S., Kirillov S., Nurmagambetova A., Kiribayeva A., Silayev D., Khassenov B. Expression, purification and biochemical characterization of recombinant phosphohydrolaseAppA in Escherichia Coli. *Biotechnology theory and practice*, 2014, vol. 3, pp. 61-64.

15. Abeldenov S.K., Khassenov B. Cloning, expression and purification of recombinant analog of Taq DNA polymerase. *Biotechnology theory and practice*, 2014, vol. 1, pp. 12-16.

16. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 1976, vol. 72, pp. 248-254. PMID: 942051.

17. Bae H.D., Yanke L.J., Cheng K.J., Selinger L.B. A novel staining method for detecting phytase activity.*J Microbiol Methods*, 1999, vol. 39, no. 1, pp. 17-22. PMID:10579503.

18. Gizzi G., Thyregod P., von Holst C., Bertin G., Vogel K., Faurschou-Isaksen M., Betz R., Murphy R., Andersen B.B. Determination of phytase activity in feed: interlaboratory study. *J AOAC Int*, 2008, vol. 91, no. 2, pp. 259-267. PMID:18476336.

19. Lim D., Golovan S., Forsberg C.W., Jia Z. Crystal structures of Escherichia coli phytase and its complex with phytate. *Nat StructBiol*, 2000, vol. 7, no. 2, pp. 108-113. PMID: 10655611.

20. Yang S., Kuang Y., Li H., Liu Y., Hui X., Li P., Jiang Z., Zhou Y., Wang Y., Xu A., Li S., Liu P., Wu D. Enhanced production of recombinant secretory proteins in Pichia pastoris by optimizing Kex2 P1' site. *PLoS One*, 2013, vol. 8, no. 9, pp. e75347. PMID: 24069404.

21. Joshi S., Satyanarayana T. Optimization of heterologous expression of the phytase (PPHY) of Pichia anomala in P. pastoris and its applicability in fractionating allergenic glycinin from soy protein. *J IndMicrobiolBiotechnol*, 2014, vol. 41, no. 6, pp. 977-987. PMID:24668018.

22. Tai H.M., Yin L.J., Chen W.C., Jiang S.T. Overexpression of Escherichia coli phytase in Pichia pastoris and its biochemical properties. *J Agric Food Chem*., 2013, vol. 61, no. 25, pp. 6007-6015. PMID:23738921.

23. Oh B.C., Choi W.C., Park S., Kim Y.O., Oh T.K. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. *ApplMicrobiolBiotechnol*, 2004, vol. 63, no. 4, pp. 362-372. PMID: 14586576.

24. Lei X.G., Weaver J.D., Mullaney E., Ullah A.H., Azain M.J. Phytase, a new life for an "old" enzyme. *Annu Rev AnimBiosci*., 2013, vol. 1, pp. 283-309. PMID: 25387021.

25. Greiner R., Haller E., Konietzny U., Jany K.D. Purification and characterization of a phytase from Klebsiellaterrigena. *Arch BiochemBiophys*, 1997, vol. 341, no. 2, pp. 201-206. PMID: 9169005.

26. Pandee P., Summpunn P., Wiyakrutta S., Isarangkul D., Meevootisom V. A Thermostable phytase from Neosartoryaspinosa BCC 41923 and its expression in Pichia pastoris.*J Microbiol*, 2011, vol. 49, no. 2, pp. 257-264. PMID: 21538247.

27. Ranjan B., Satyanarayana T. Recombinant HAP Phytase of the Thermophilic Mold Sporotrichum thermophile: Expression of the Codon-Optimized Phytase Gene in Pichia pastoris and Applications. *MolBiotechnol*, 2016, vol. 58, no. 2, pp. 137-147. PMID: 26758064.

БАКТЕРИЯЛЫҚ ФИТАЗАНЫҢ (*AppA*) *Pichia pastoris*-ТА ГЕТЕРОЛОГИЯЛЫҚ ЖАСУШАДАН ТЫС ЭКСПРЕССИЯСЫ ЖӘНЕ БИОХИМИЯЛЫҚ СИПАТТАМАСЫ

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ТҮЙІН

Фитаза ферменті әрі қарай фосфаттың бөлінуімен жалғасатын фитин қышқылын ыдыратумен айналысады және де жануарлар үшін жемдік қоспалар ретінде қолданылады. Ал ол болса жемнің шығынын азайтады. Бұл жұмыста қышқыл фосфатазаның appA гені *E. coli* геномынан бөлініп, көбейтіліп, AOX1 алкогольоксидазалы белсендіретін промоторы мен ақуыздың секреттік экспрессиясын қамтамасыз ететін α-фактор сигналдық пептидінің бақылауымен pPICZαA экспрессиялық векторына енгізілді. Құрастырылған pPICZ A/appA векторы ДНҚ кассетасы түзілуі үшін PmeI эндонуклеазасымен ыдыратылды. Pichia pastoris штаммының GS115 компетентті жасушалары ДНҚ кассетасымен электропорация әдісімен трансформацияланды. Трансформациядан соң P. pastoris GS115 культурасын 100 мкг/мл зеоцин антибиотигі бар YPDS қатты қоректік ортасына отырғызды. Алынған трансформанттар ПТР әдісімен ашытқының хромосомалық ДНҚ-сына мақсатты appA генінің енгізілгені-енгізілмегеніне тексерілді. Фитазаның культуралық сұйықтыққа бөлінетінін электрофореграмма талдауы көрсетті. Фитазаның белсенділігі кешенді ортасында 168 сағат инкубациядан соң культуралық сұйықтық үшін 1510 ед/мл болатыны анықталды. Белсенділікті зерттеу AppA фитазасы 50°C, pH диапазоны 2.0 - 7.0, pH-тың оптималды белсенділігі 5,0 болганда максималдық фитаздық белсенділік көрсететінін көрсетті. Рекомбинантты ақуыздың термотұрақтылығы 10 мин бойы 60°C–қа дейін қыздырудан соң қалдықтық белсенділіктен 80%-дан аса сақталынды. Ақуыздың гликозилденгені H эндогликозидазасын қолданумен дәлелденді. AppA ақуызының жоғары деңгейлі өнімділігі алынған P. pastoris GS 115/appA рекомбинантты штамын рекомбинантты фитазаның өндірістік продуценті ретінде қолдану мүмкіншілігін көрсетеді.

Негізгі сөздер: фитаза, *Escherichia coli, Pichia pastoris*, гетерологиялық экспрессия.