Advanced Journal of Microbiology Research Vol. 2011
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

The surface display of phytase on yeast cell and activity assay of the displayed protein

Shumin Yao*, Jing Huang and Jingli Tan

College of Life Science, Qufu Normal University, Qufu 273165, People's Republic of China.

Accepted 05 June, 2011

Phytase is a new-style enzyme used in animal feed additive. It can increase phosphorus availability, decrease environmental phosphorus pollution and improve the performance of animals. Phytase gene was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using first strand cDNA as template after reverse transcripting *Aspergillus niger* total RNA. The phyA gene was cloned into plasmid pYD1 which allows regulated expression, secretion and detection of expressed proteins on the surface of *Saccharomyces cerevisiae* cells using immunofluorescence. The construct was propagated in *Escherichia coli* DH5α and then was transformed into the yeast strain EBY100. After induction, the phytase activity was measured every 12 h. The results indicated that the activity of the fusion protein reached the highest level after being induced with 2.0% galactose for 48 h. This enzyme had pH optima (pH 7) and its optimum temperature was about 65°C.

Key words: Yeast surface display, *Aspergillus niger*, phytase.

INTRODUCTION

Phytate (myoinositol hexakisphosphate or myoinositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate), which is also known as phytic acid, is a form of phosphate storage in plants such as soybeans, cottonseeds, and other legumes and cereals. Therefore, phytase can be incorporated into commercial poultry, swine, and fish diets and has a wide range of applications in animal and human nutrition as it can reduce phosphorus the excretion of monogastric animals by replacing inorganic phosphates in the animal diet. Phytase A (PhyA) from *Aspergillus niger* is known to be the most active enzyme, and is frequently used in animal feeds to improve the availability of phosphorous and minerals because it has two pH optima (2 to 2.5 and 5 to5.5) and a temperature optimum between 55 and 60°C (Han et al., 1999, Lim et al., 2001).

The low optimum pH and high thermo-stability of phytase provide the additional advantage of being able to smoothly pass through the stomach acid and the heat denaturation (60 to 80°C) during feed pelleting, respectively (Wyss et al., 1999). It combines the advantages of both a prokaryotic system, such as high expression levels and easy scale-up, as well as a eukaryotic system to conduct most of the post-translational modification. The yeast-based expression system is unique compared to other expression systems. Moreover, the yeast is non-conventional hemiascomycetous yeast and is able to grow on hydrophobic substrates; it has been used in several industrial processes and is non-pathogenic (GRAS, generally regarded as safe) (Madzak et al., 2004). Consequently, it is used in livestock feeds for fish, poultry and fur-bearing animals, and as a food supplement for consumption by humans.

Recently, a number of heterologous proteins of varied size have been displayed on yeast cell surface using a genetic engineering technique (Colby et al., 2004). The yeast cell-surface display allows peptides and proteins to be displayed on the surface of yeast cells by fusing them with the anchoring motifs. The characteristics of anchoring motif, displayed protein and host cell, and fusion method all affect the efficiency of surface display of proteins. The yeast cell surface display has many potential applications, including live vaccine development, peptide library screening, bioconversion using whole cell biocatalyst and bioadsorption (Kondo et

*Corresponding author. E-mail: yaoshumin299@163.com.

al., 2004). Yeast surface display used in combination with directed evolution is a robust platform for protein engineering, allowing the discrimination of subtle phenotype differences using fluorescence activating cell sorter (FACS), immunofluorescence, and the characterization of protein kinetics and thermal stability measured directly on the cell surface. Yeast surface display also confers a eukaryotic expression bias, resulting in posttranslational assembly such as that mediated by foldases and chaperones, and posttranslational modification such as glycosylation.

A large variety of proteins have been successfully engineered using yeast surface display. In addition to protein engineering, more recent applications of this platform include enzyme engineering (Antipov et al., 2008), epitope mapping (Chao et al., 2006), and cell panning for the discovery of novel surface receptors (Wang et al., 2007). In this study, we attempted to achieve the expression of PhyA from *A. niger* on the cell surface of *S. cerevisiae* to apply it as a new candidate for dietary yeast supplementation and as a whole cell bio-catalyst that can hydrolyze the phytate in animal feed and waste, respectively.

MATERIALS AND METHODS

Experimental strains

The *Escherichia coli* strain used in this study was DH5α[F− endA1 hsdR17(rK−/mK+) supE44 thi-1λ− recA1 gyr96ΔlacU169(φ80lacZΔM15)] kept in this laboratory. *A. niger* 424-1 was used for the cloning of phyA and *S. cerevisiae* EBY100(MATa ura 3-52 trp 1 leu2Δ1 his3Δ200 pep4:HIS3 prb1Δ1.6R can1 GAL) was used as the recipient cell for phytase production. Plasmid pYD1 for phyA production in EBY100 was purchased from Invitrogen. *S. cerevisiae* EBY100 was maintained in a YPD medium (1% yeast extract, 2% peptone, and 2% dextrose), and a tryptophan-deficient selective medium (0.67% yeast nitrogen base without amino acids, and 1.0% ammonium sulfate, 2.0% glucose, 0.01% leucine, 1.5% agar) was used at 30°C to screen the transformants.

Cloning of phyA genes from *A. niger* 424-1

Genomic RNA from *A. niger* 424-1 was prepared according to the methods described by Tritol manual. According to PrimeScript™ 1st Strand cDNA Synthesis Kit instructions on reverse transcription, the first strand cDNA. PhyA ds cDNA was amplified by one pair of primer (P1: 5′-CGGAATTCATGGGCAGTCCCAGACTCGAG-3′; P2:5'-CGGCGGCCGCCTAAGCAAAACACTCC-3′) designed according to the phyA gene sequence (XM_001401676) in *A. niger* and multiple cloning sites on pYD1. The phyA genes were amplified from the genomic RNA with the pair of primers by PCR with PrimeSTAR^{TM} HS DNA Polymerase, respectively. The PCR procedure was as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 45 s at 94°C, 40 s at 55°C, 2 min at 72°C, followed by additional 10 min at 72°C. The resultant 1404 bp PCR products were digested with EcoR I and Not I and cloned into pYD1 vector, respectively. The resultant plasmids were named as pYD-phyA. These plasmids were transformed into *E. coli* DH5α and phyA genes on the plasmids purified from the transformants were sequenced, respectively.

Transformation of yeast strain

The competent cells of the yeast strain EBY100 were prepared and pYD1- phyA, and pYD1 were transformed into the competent cells according to the manufacturer instructions of pYD1 Yeast Display Vector Kit (Invitrogen), respectively. The transformants were grown on the selective medium which contained 0.67% YNB without amino acids and 1.0% ammonium sulfate, 2.0% glucose, 0.01% leucine, 1.5% agar at 30°C for 48 h. The derived transformants containing pYD1- phyA and pYD1 were confirmed by colony PCR technique and named EBY100/pYD1-phyA, and EBY100/pYD1, respectively.

phyA genes expression and bioassay of surface displayed protein

EBY100/pYD1- phyA display on the yeast cells were carried out according to the manufacturer's instruction of pYD1 Yeast Display Vector Kit (Invitrogen). The cells were induced by growing the cells in YNB-CAA medium containing 2.0% galactose for 0, 12, 24, 36 and 48 h, respectively. The cell cultured over a 48-h time period (0, 12, 24, 36, and 48 h) was assayed to determine the optimal induction time for maximum display. The staining of displayed proteins on the yeast cells was performed according to the methods described in pYD1 Yeast Display Vector Kit (Invitrogen). The stained cells with fluorescence were suspended in 40 µL of phosphate buffered saline (PBS) buffer and observed under microscope with ultraviolet (UV) light, and the percentage of the cells displaying phyA were calculated. A volume of the yeast cells equivalent to 2.0 OD 600 nm units were collected and washed with PBS buffer by centrifugation after EBY100/pYD1-phyA, EBY100(negative control), EBY100/PYD1 (positive control) were induced in YNB-CAA medium containing 2.0% galactose for 0, 12, 24, 36 and 48 h.

Phytase assay

In brief, 1 ml of the cell culture was centrifuged at 5,000×g for 10 min. The supernatant obtained was used as the crude extracellular phytase preparation. The phytase activity was assayed as follows: 0.8 ml of sodium phytate solution (5.0 mM sodium phytate in 0.2 M sodium acetate pH 5.0) was pre-incubated at 65°C for 5 min, and 0.2 ml of the crude extracellular phytase preparation was added and mixed well. The mixture was incubated at 65°C for 30 min and afterward, the reaction was stopped by the addition of 1.0 ml of 50.0 g/L trichloroacetic acid. The inorganic phosphate liberated was quantitatively determined by using the ammonium molybdate method (Chi et al., 1999) spectrophotometrically at 700 nm. One unit of phytase activity was defined as the amount of enzyme causing the release of 1.0 µM of inorganic phosphate per minute under the assay conditions.

Effects of pH and temperature on phytase activity and stability

The effect of pH on the recombinant enzyme activity was determined by incubating the recombinant enzyme between pH 3.0 and 9.0 using the standard assay conditions. The buffers used were 0.1 M citric acid buffer(pH 3.0 to 6.0) and 0.1 M barbital sodium buffer (pH 7.0 to 10.0). The pH stability was tested via a 6-h pre-incubation of the recombinant enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3.0 to 10.0 at 0°C. The remaining activities of phytase were

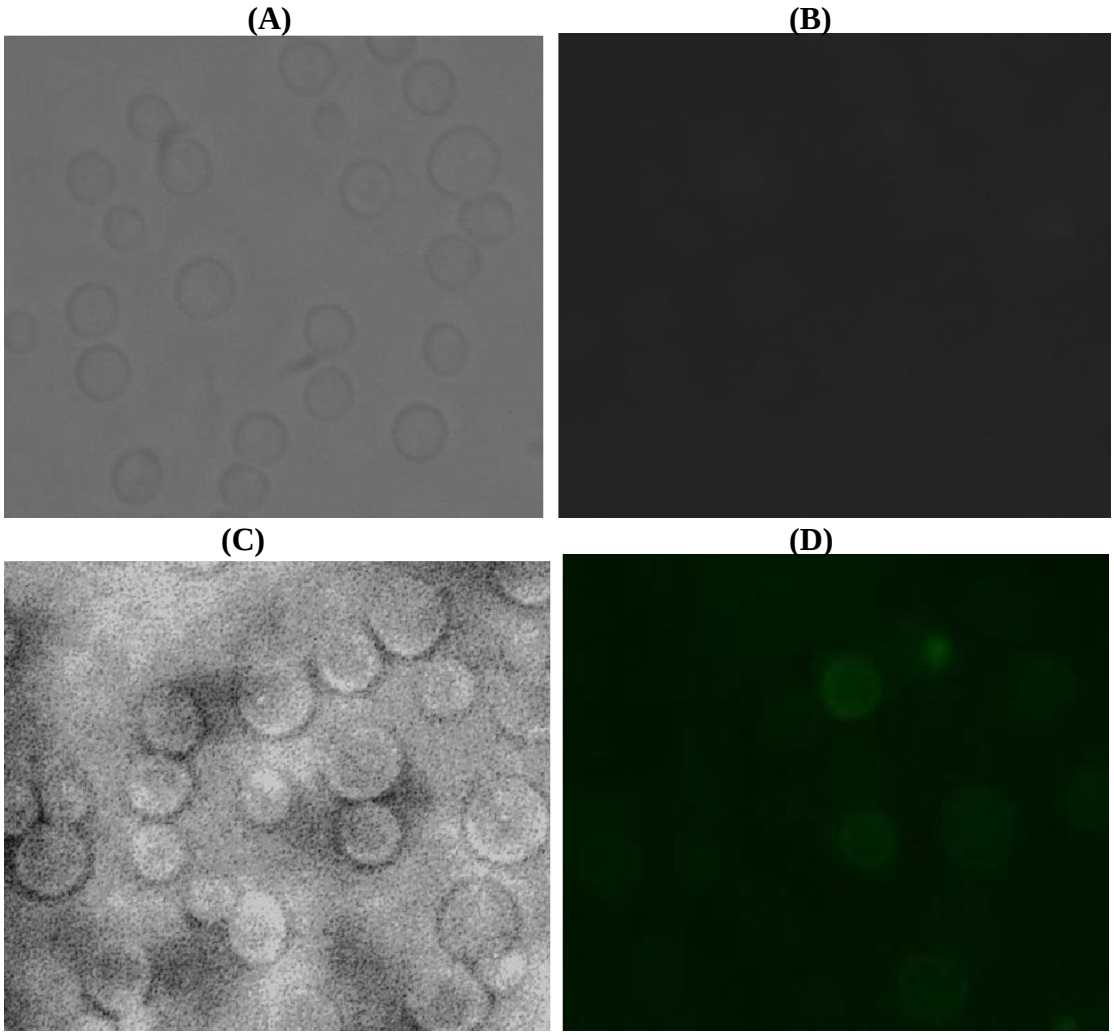

Figure 1. Detection of phyA on the cell surface by confocal laser scanning microscopy. (A-B) *S. cerevisiae* EBY100 under normal white light and the FITC filter (492 nm); (C-D) *S. cerevisiae* EBY100 carrying pYD1-phyA under normal white light and the FITC filter(492nm). Magnification: 100×10.

measured immediately after this treatment with the standard method as mentioned earlier.

The optimal temperature for activity of the enzyme was determined at 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85°C in the same buffer as described in the preceding text. Temperature stability of the purified enzyme was tested by pre-incubating the enzyme at different temperatures (20, 30, 40, 50, 60, 65, 70, and 80°C) for 1 h; the residual activity was measured immediately as described in the preceding text. Here, a pre-incubated sample at 0°C was used as reference to calculate the residual activity.

Effects of different metal ions on phytase activity

To examine effects of different metal ions on phytase activity, an enzyme assay was performed for 1 h in the reaction mixture as described in the preceding text with various metal ions at final concentrations of 1.0 and 5.0 mM. The activity assayed in the absence of metal ions was defined as the control. The metal ions tested included zinc (Zn^{2+}), copper (Cu^{2+}), magnesium (Mg^{2+}), iron (Fe^{3+}), calcium (Ca^{2+}), potassium (K^{+}), manganese (Mn^{2+}), mercury (Hg^{2+}), lithium ($Li^{+)}$,Fe^{2+}, gold (Ag^{+}), sodium (Na^{+}), barium (Ba^{2+}), and cobalt (Co^{2+}).

RESULTS AND DISCUSSION

Display of phyA on yeast- cell surface

We selected the method of displaying phytase on the yeast cell surface because of its convenience and ease of handling enzyme with a highly homogeneous quality without purification. In order to obtain maximal displayed phytase on yeast cells, the transformed cells were induced by galactose for 0, 12, 24, 36 and 48 h. Immunofluorescence analysis showed that the largest amount of phytase was displayed on the yeast cells after induction for 48 h (Figure 1). Under this condition, about one-third of the yeast cells harboring pYD1-phyA had the

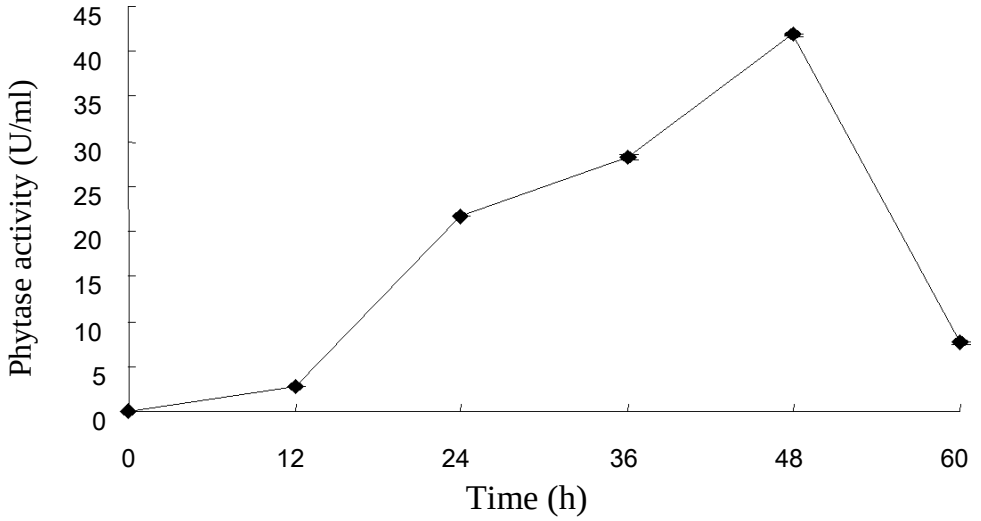

Figure 2. Time course of phytase production by the recombinant yeast. All the data are given as means ±SD, n = 3.

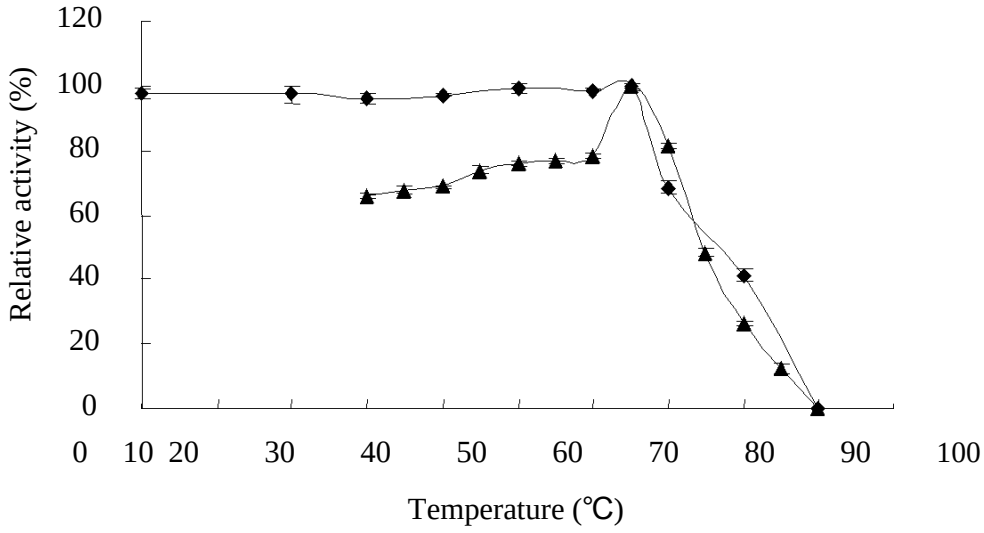

Figure 3. Effects of different temperature on activity (▲) and stability (◆) of the phytase. Temperature stability of the recombinant enzyme was tested by pre-incubating the enzyme at different temperatures (0, 20, 30, 40, 50, 60, 65, 70, 80, 90°C) for 1 h; the residual activity was measured immediately as earlier described. Here, pre-incubated sample at 0°C was used as reference to calculate the residual activity. Data are given as means ±SD, n = 3.

displayed phytase. It was found that most of the intense fluorescence as first localized in the small bud, then observed on the entire cell wall.

Induced expression of the recombinant yeast

After being induced, the phytase activity was measured every 12 h. The results indicated that the activity of the fusion protein reached the highest level after induced for 48 h. The results in Figure 2 indicate that 42.1 U/ml of phytase activity could be reached within 48 h of the induction. These results demonstrate that the *recombinant* yeast strain could produce high yield of extracellular phytase.

Optimum temperature and thermal stability

The phytase activity measured as a function of temperature from 30 to 90°C shows that the activity was highest at 65°C (Figure 3). Thermostability is considered

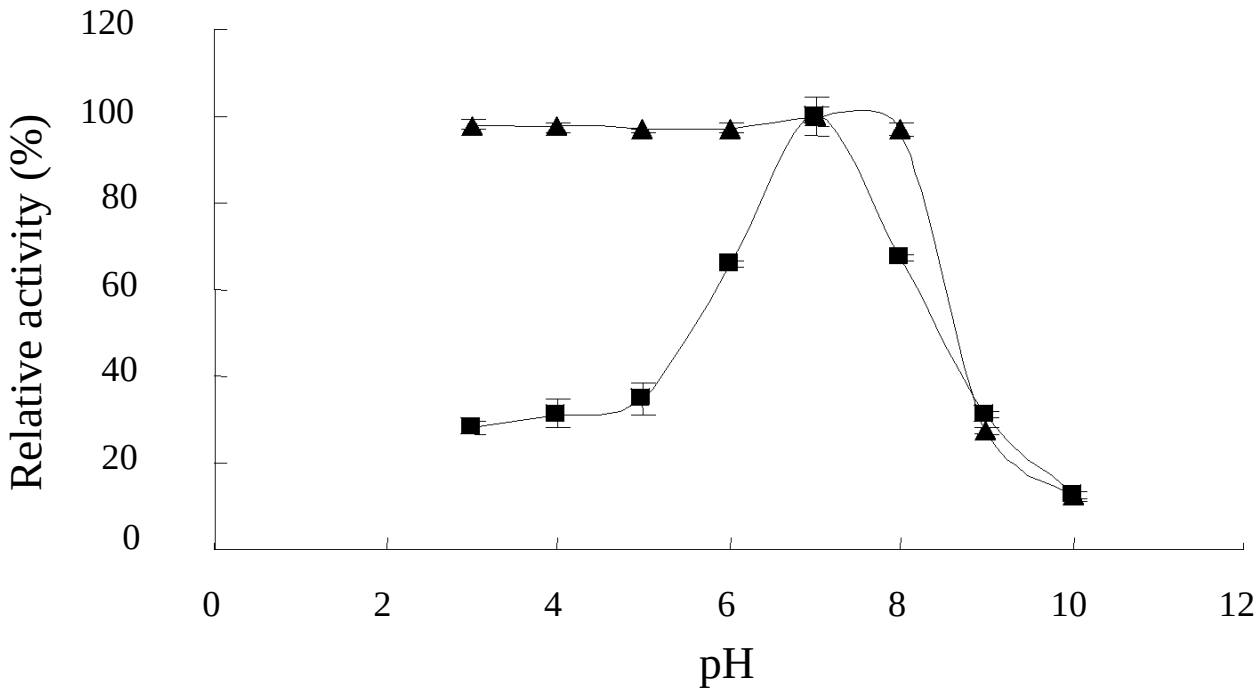

Figure 4. Effects of different pH on activity (■) and stability (▲) of the phytase. The pH stability was tested via a 6-h pre-incubation of the purified enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3.0 to 10.0 at 4°C. The remaining activities of phytase were measured immediately after this treatment with the standard method as earlier described. The phytase activity of the finally concentrated elute without pre-incubation was regarded as 100%. Data are given as means ± SD, n = 3.

an important and useful criterion for industrial application of phytase. For example, thermostability is a prerequisite for the successful application of enzymes in animal feeds that are exposed to 60 to 90°C during the pelleting process. Therefore, thermostability was investigated by pre-incubating the enzyme in the same buffer as earlier described for 1 h, and the remaining activity was determined. As shown in Figure 3, the residual phytase activity still maintained 99.5% of the control after treatment at 65°C for 1 h, indicating that the enzyme was stable up to 65°C. Figure 3 also reveals that the recombinant enzyme was inactivated rapidly at temperatures higher than 65°C and was almost inactivated at 90°C within 1 h.

From these results, the phytase seemed to have considerable thermostability. For example, the phytase produced by *Saccharomyces castellii* exhibited an uncommon preference for high temperatures, with optimum activity at 77°C and thermostability up to 74°C (Segueilha et al., 1992; Pandey et al., 2001). This means that the thermostability of phytase from the recombinant yeast was not higher than that from *S. castellii*.

Optimum pH and pH stability

Phytase activity was measured at various pH values in buffers with the same ionic concentrations. The results (Figure 4) show that the maximum activity was observed at pH 7.0. pH stability was tested via 6 h pre-incubation of the purified enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3.0 to 10.0 at 0°C. The remaining activities of phytase were measured immediately after this treatment with the standard method as mentioned earlier. It can be seen from the results in Figure 4 that the activity profile of the enzyme was stable from pH 3.0 to pH 8.0, and greater than 97.0% the residual activity was maintained after treatment at pH from 3.0 to 8.0 and 0°C for 6 h. These results suggest that the enzyme was very stable in the pH range of 3.0 to 8.0, and at pH 9, the activity fell sharply.

Effects of different cations and enzyme inhibitors on the phytase activity of the recombinant yeast

Ca^{2+}, Na^{+}, Li^{+}, Ba^{2+} Cu^{2+} and Mg^{2+} (at concentrations of 5.0 mM) stimulated the phytase activity of the recombinant yeast. However, K^{+}, Mn^{2+}, Fe^{2+}, Li^{+}, Fe^{3+}, Ag^{+}, $Co^{2+}$and Zn^{2+}(at concentrations of 5.0 mM) acted as inhibitors in decreasing the phytase activity, with Fe^{3+}(at a concentration of 5.0 mM) showing the lowest level (15%) (Table 1). The results show that phytase activity have a certain relationship with some metal ions, the specific activation of the principles will be investigated further in

Table 1. Effect of different cations on the recombinant yeast phytase activity.

Metal ions	Final concentration in reaction mix (mM)	Relative phytase activity (%)
Ca^{2+}	1.0	92.8 ± 0.8
	5.0	104.6 ± 2.3
Na^{+}	1.0	92.2 ± 0.6
	5.0	98.3 ± 2.5
K^{+}	1.0	125.0 ± 1.2
	5.0	96.4 ± 1.7
Mn^{2+}	1.0	129.2 ± 1.0
	5.0	100.3 ± 1.5
Fe^{2+}	1.0	96.4 ± 0.2
	5.0	35.4 ± 2.5
Li^{+}	1.0	91.1 ± 1.8
	5.0	86.3 ± 1.6
Fe^{3+}	1.0	87.7 ± 1.1
	5.0	15.1 ± 0.4
Ag^{+}	1.0	139.2 ± 0.4
	5.0	96.0 ± 1.7
Cu^{2+}	1.0	89.8 ± 1.2
	5.0	101.2 ± 1.3
Co^{2+}	1.0	133.5 ± 2.8
	5.0	68.4 ± 1.6
Mg^{2+}	1.0	90.1 ± 0.8
	5.0	93.6 ± 1.7
Zn^{2+}	1.0	139.2 ± 0.5
	5.0	98.0 ± 1.1

Data are given as means ±SD, n = 3. The phytase activity in the absence of metal ions is regarded as 100%.

future studies.

Conclusion

Here, we explored the potential of a *S. cerevisiae* expression and display system that allows proteins of interest to be targeted to the yeast surface. The system not only displays single-subunit proteins, but also hetero-oligomeric multisubunits. In this study, we constructed a novel phytate-degrading yeast strain displaying the phytase on the cell surface. To our knowledge, this is the first report of the phytase expression on the plasmid pYD1, which will be applied as a dietary complement and whole cell bio-catalyst in animal foods and waste. This study also demonstrated the direct hydrolysis of phytate using the recombinant strain.

ACKNOWLEDGEMENTS

The authors would like to thank their advisors who made

them introduce the field of Molecular Biology into this research. They also acknowledge their families and friend who supported them all the time.

REFERENCES

Antipov E, Cho AE, Wittrup KD, Klibanov AM (2008). Highly L and D enantioselective variants of horseradish peroxidase discovered by an ultrahigh-throughput selection method. Proc. Natl. Acad. Sci. USA 105:17694-17699.

Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD (2006). Isolating and engineering human antibodies using yeast surface display. Nat. Protoc. 1:755-768.

Chi, Z., Kohlwein, SD, Paltauf F (1999). Role of phosphatidylinositol (PI) in ethanol production and ethanol tolerance by a high ethanol producing yeast. J. Ind. Microbiol. Biotechnol. 22:58-63.

Colby DW, Kellogg BA, Graff CP, Yeung YA, Swers JS, Wittrup KD (2004). Engineering antibody affinity by yeast surface display. Methods Enzymol. 388:348-358.

Han Y, Wilson DB, Lei XG (1999). Expression of an *Aspergillus niger* phytase gene (phyA) in, Saccharomyces cerevisiae. Appl. Environ. Microbiol. 65:1915-1918.

Kondo A, Uda M (2004) Yeast cell-surface display applications of molecular display. Appl. Microbiol. Biotechnol. 64:28-40.

Lim YY, Park EH, Kim JH, Park SM, Jang HS, Park YJ, Yoon S, Yang MS, Kim DH (2001). Enhanced and targeted expression of fungal phytase in *Saccharomyces cerevisiae*. J. Microbiol. Biotechnol. 11:915-921.

Madzak C, Gaillardin C, Beckerich JM (2004). Heterologous protein expression and secretion in the non-conventional yeast Yarrowia lipolytica: a review. J. Biotechnol. 109:63-81.

Pandey A, Szakacs G, Soccol CR, Rodriguez-Leond JA, Soccol VT (2001). Production, purification and properties of microbial phytases. Bioresour. Technol. 77:203-214.

Segueilha L, Lambrechts C, Boze H, Moulin G, Galzy P (1992). Purification and properties of the phytase from *Schwanniomyces castellii*. J. Ferment. Bioeng. 74:7-11.

Wang XX, Cho YK, Shusta EV (2007). Mining a yeast library for brain endothelial cell-binding antibodies. Nat. Methods 4:143-145.

Wyss M, Pasamontes L, Friedlein A, Remy R, Tessier M, Kronenberger A, Middendorf A, Lehmann M, Schnoebelen L, Rothlisberger U, Kusznir E, Wahl G, Muller F, Lahm HW, Vogel K, van Loon AP (1999). Biophysical characterization of fungal phytases (myoinositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. Appl. Environ. Microbiol. 65:359-366.