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Expression of Fungal Phytase on the Cell Surface of *Saccharomyces cerevisiae*

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Abstract Phytase improves the bioavailability of phytate phosphorus in plant foods to humans and animals, and reduces the phosphorus pollution of animal waste. We have engineered the cell surface of the yeast, *Saccharomyces cerevisiae,* by anchoring active fungal phytase on its cell wall, in order to apply it as a dietary supplement containing bioconversional functions in animal foods and a whole cell bio-catalyst for the treatment of waste. The phytase gene (*phyA*) of *Aspergillus niger* with a signal peptide of rice amylase 1A (*Ramy1A*) was fused with the gene encoding the C-terminal half (320 amino acid residues from the C-terminus) of yeast α-agglutinin, a protein which is involved in mating and is covalently anchored to the cell wall. The resulting fusion construct was introduced into *S. cerevisiae* and expressed under the control of the constitutive glyceraldehydes-3-phosphate dehydrogenase (GPD) promoter. Phytase plate assay revealed that the surface-engineered cell exhibited a catalytically active opaque zone which was restricted to the margin of the colony. Additionally, the phytase activity was detected in the cell fraction, but was not detected in the culture medium when it was grown in liquid. These results indicate that the phytase was successfully anchored to the cell surface of yeast and was displayed as its active form. The amount of recombinant phytase on the surface of yeast cells was estimated to be 16,000 molecules per cell.

Keywords: surface display, *Aspergillus niger*, phytase, *Saccharomyces cerevisiae*

INTRODUCTION

Phytate (myo-inositol hexakisphosphate or myo-inositol 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. It also plays other important roles in plants, including energy storage, competition for ATP, complexation of multivalent cations, and regulation of inorganic phosphate levels. In contrast, as a component of all plant seeds, phytic acid is considered to be an anti-nutrient due to the formation of precipitated complexes that strongly reduce the absorption of essential dietary minerals, such as iron and zinc, in human and animal foods [1].

Phytases (myo-inositol hexakisphosphate 3 and 6-phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) catalyze the hydrolysis of phytate, thereby releasing inorganic phosphate. Since simple-stomached animals, such as swine and poultry, have little phytase activity in their gastrointestinal tracts, nearly all the dietary phytate phosphorus ingested by these species is excreted into the environment, resulting in phosphorus pollution in areas of intensive animal production. Hence, the simple use of inorganic phosphate as an additive to animal feed can no longer be allowed because of its impact on the environment [2]. The enzymatic treatment of phytate has been suggested as an alternative, and Nelson *et al.* [1] demonstrated that when phytase is fed to poultry, the phytin-P presented in soybean meal and corn is made available and is deposited in the bones of the chicks. Furthermore, many countries have approved the use of phytase as a feed additive, and the FDA has also approved a GRAS (Generally Recognized As Safe) petition related to the use of phytase in food [2].

Although plant seeds, such as wheat, beans, rice, corn, and maize have some phytase activity, microorganisms are a more practical source of the enzyme. Several microbial phytases have already been characterized and evaluated for biotechnological purposes. Among these, 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 to 5.5) and a temperature optimum between 55 and 60°C [3,4]. 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 [5].

Because it combines the advantages of both a prokary-

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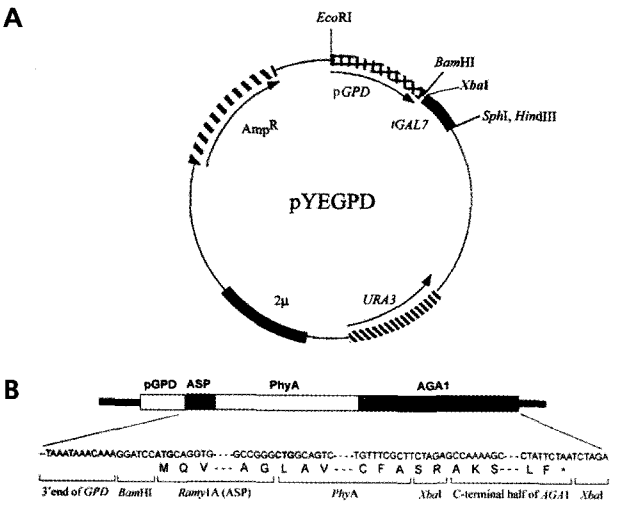

Fig. 1. (A) Schematic diagram of the pYEGPD yeast expression vector. The boxes represent genes or their corresponding functional domains. (B) Schematic diagram of the pYEGPAG fusion construct cloned in pYEGPD and the sequence covering the links of *GPD* promoter-*Rice Amy1A* signal peptides-*PhyA-AGA1*. The translation start codon and the first codon of PhyA are shown in bold letters. p*GPD*, promoter of glyceraldehyde-3-phosphate dehydrogenase; ASP, *Rice Amylase1A* signal peptides; *phyA*, phytaseA; *AGA1*, C-terminal half (320 amino acid residues from C terminus) of yeast α-agglutinin anchoring protein; t*GAL7*, terminator of galactose-1-P uridyl transferase.

otic system, such as high expression levels, easy scale-up, and inexpensive growth media, 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, yeast is a GRAS organism, with a long history of application for the production of a rich biomass of high-quality proteins and vitamins [6,7]. 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 [8]. 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 protein to be displayed can be fused to an anchoring motif by N-terminal fusion, C-terminal fusion or sandwich fusion. 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 [9]. In this study, we attempted to achieve the expression of PhyA of *A. niger* on the cell surface of *Saccharomyces cerevisiae* to apply it as a new candidate for dietary yeast supplementation and as a whole cell bio-catalyst which can hydrolyze the phytate in animal feed and waste, respectively.

MATERIALS AND METHODS

Chemicals and Enzymes

Unless otherwise specified, all chemicals, media, and enzymes used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Difco Laboratories (Detroit, MI, USA), or Boehringer Mannheim (Mannheim, Germany), respectively.

Strains and Culture Conditions

The plasmids were maintained and propagated in *E. coli* HB101 or DH5α according to the methods of Sambrook *et al.* [10]. *A. niger* NRRL 3135 was used for the cloning of *phyA*, and *S. cerevisiae* 2805 (*MATαpep4:: HIS3 prb1-δ Can1 GAL2 his3 ura3-52*) was used as the recipient cell for phytase production [11].

S. cerevisiae was maintained in a YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose), and a uracil-deficient selective medium (0.67% yeast nitrogen base without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acid, 2% dextrose, and 2% agar) was used at 30°C to screen the transformants. The primary inoculum was prepared from 5 mL of the uracil selective medium, cultured for 24 h. A total of 1×10^7 cells were inoculated into a 300 mL Erlenmeyer flask containing 40 mL of the YEPD medium. The expression cultures were grown at 30°C with continuous agitation (200 rpm), after which, the culture itself and culture filtrates were assayed for phytase activity.

Construction of the Plasmid and Transformation of Yeast

The amylase 1A (*Ramy1A*) signal peptide (ASP) and the *phyA* gene of *A. niger*, described previously [12-15], were fused by overlap extension PCR [16] to create *Bam*HI and *Xba*I restriction sites at 5′ and 3′ ends, respectively, using primers forward 5′-GGATCCGCATCCA GGTGCTGAAC-3′ and reverse 5′-TCTAGACTTGTAC AGCTCGTCCAT-3′, and overlap-forward 5′-AACTTGA CAGCCGGGCTGGCAGTCCCCGCC-3′ and overlap-reverse 5′-GGCGGGGACTGCCAGCCCGGCTGTCAAG TT-3′. The amplified gene was cloned in pBluescript IIKS (Invitrogen, Carlsbad, CA, USA), analyzed by restriction enzyme digestion, and confirmed by DNA sequencing. To perform cloning in the yeast vector, the ASP-PhyA fusion fragment which had been excised from pBluescript IIKS by digestion with *Bam*HI and *Xba*I was placed in YEGPD vector which had the same restriction enzyme sites between the *GPD* promoter and the galactose-1-P uridyl transferase (*GAL7*) terminator. To anchor the PhyA on the yeast surface, a DNA fragment containing the 3′ half of the α-agglutinin gene (*AGA1*) and encoding C-terminal 320 amino acids, was prepared by PCR using primers 5′-TCTAGAGCCAAAAGCTCTTTTATCTCAA-3′ and 5′-TCTAGATTAGAATAGCAGGTAGGACA-3′ with genomic DNA from *S. cerevisiae* 2805 as a template. This step was followed by digestion by *Xba*I, and the resulting fragment was cloned at the *Xba*I site between

phyA and the *GAL7* terminator. The direction of the fusion construct was confirmed by digestion with *Xba*I and DNA sequencing. The resulting plasmid was named pYEGPAG (Fig. 1).

The constructed recombinant vector was introduced into *S. cerevisiae* 2805 according to the lithium acetate procedure [17]. The stability of the introduced plasmids in yeast was measured as follows: samples grown in the non-selective YEPD medium were serially diluted with sterile H_2O to an expected 50 colony-forming units (CFUs) per plate, and were then plated on a *ura*$^-$ selective plate and a non-selective plate. The relative number of CFUs was subsequently determined.

Northern Blot Analysis

Transformed yeast cells were lysed using glass beads, and the total RNA was extracted according to the previously described procedure [15]. The amount of RNA was measured by UV-spectrophotometry, and the total RNA (30 μg per lane) was separated on a 1% agarose gel (in 2.2 M formaldehyde). Prior to blotting, the gel was stained with ethidium bromide to confirm that a similar amount of RNA had been loaded for each sample. The RNA was transferred onto a Hybond membrane, as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization was performed in church buffer [7% (w/v) SDS, 1% BSA, 1 mM EDTA, 250 mM $NaPO_4$, pH 7.2] at 65°C [15]. The probe was labeled with α-[^{32}P]-dCTP using a random labeling kit (Amersham Pharmacia Biotech).

Enzymatic Assay

The phytase activity was measured according to the modified protocol of Heinonen and Lahti [18], and the corresponding cell density was also estimated using a haemacytometer. In brief, 4 μL of 0.1 M sodium phytate was diluted with 350 μL of a 0.1 M sodium acetate buffer (pH 5.5) and added to a tube containing 50 μL of culture medium or cells, which were separated from a total of 40 mL of culture by centrifugation at 5,000 × g for 10 min. The resulting cell pellet was washed three times with the same amount of sterile water by centrifugation and resuspended in 40 mL of saline solution. The reaction mixture was further incubated at 58°C for 30 min. Next, 100 μL of the reaction mixture was added to 800 μL of an AAM solution (10 mM ammonium molybdate: 5 N H_2SO_4: acetone, 1:1:2, v/v), and the solution was vortexed for 10 sec. Eighty μL of 1 M citric acid was then added. After the reaction, the color change was measured using a Packard Spectra Count™ colorimeter (Packard Instrument, Downers Grove, IL, USA) at a wavelength of 405 nm. The number of phytase molecules on the cell surface was determined according to the calibration curve prepared with purified phytase isolated from *Aspergillus* (Sigma). A standard solution of 15 mM KH_2PO_4 was used as the reference. One unit of phytase activity was defined as the amount of activity that released 1 μmol of phosphate per min at 58°C. The measurements were performed in triplicate. All experiments were carried out at least three times with similar results.

The phytase activity on each plate was measured by the method of Lim *et al.* [4] as follows: equal numbers of cells were dropped onto a plate and grown for 3 days in a modified PSM medium [19] in which all the components were identical, except that 0.3% $(NH_4)_2NO_3$ was added instead of 0.3% $(NH_4)_2SO_4$. After the samples were incubated at 30°C, an opaque precipitation around each colony was observed.

RESULTS AND DISCUSSION

Construction of the Cell Surface Expression Plasmid

The pYEGPAG plasmid was constructed as described in materials and methods (Fig. 1). The pYEGPAG plasmid was a multicopy plasmid for the expression of the phytaseA/α-agglutinin fusion gene containing the secretion signal sequence of amylase 1A (*Ramy1A*) under the control of the GPD promoter. Twenty *S. cerevisiae* transformants were randomly selected on a ura$^-$ medium, and were then used to confirm the presence of the pYEGPAG by plasmid extraction, followed by back transformation into *E. coli*. The plasmid stability of the selected transformants was good enough for more than 80% of the plated cells to harbor the plasmids in the non-selective liquid media up to 72 h after cultivation.

The accumulation level of the recombinant PhyA transcript was measured using Northern blot analysis, which revealed that there were variations in the transcriptional levels of the recombinant *phyA* genes among the strains transformed with the same expression construct. A wide variation in the heterologous gene expression level in *S. cerevisiae* is not unusual when episomal 2 μ ori-based plasmids are used. This variation in gene expression may be due to variations in the plasmid copy number between the different transformants [15]. Thus, the six transformants that showed the highest expression levels were selected and used to confirm the production of the recombinant proteins.

Plate Assay of Phytase Expression

We performed a plate assay to determine whether or not the selected transformants gained phytase activity, which resulted in opaque zones around the colonies on the modified PSM medium [19]. We then compared the phytase activity among different recombinant strains containing the vector only as a control, secreting phytase into a culture medium that was constructed in the previous study [4], and expressing phytase on the cell surface. As shown in Fig. 2, the selected cells which display phytase on the cell surface have a strong, narrow opaque zone strictly around the margin of colonies, while the transformants which secrete phytase into the culture medium have a broad opaque zone. This suggested that phytase, a 50 kDa protein, was correctly anchored to the surface of the cell through the C-terminal half of α-agglutinin.

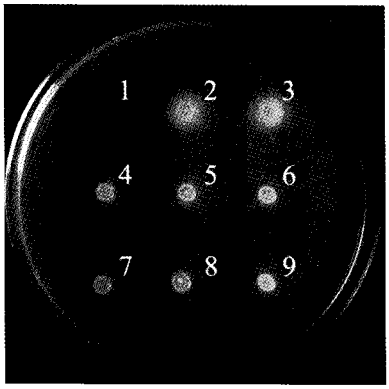

Fig. 2. Comparison of secreted- and surface displayed-phytase activities on an agar plate. 1, nontransformant; 2-3, transformants (*S. cerevisiae* 2805/pGphyA-6) secreting phytase to culture media; 4-9, transformants (*S. cerevisiae* 2805/pYEGPAG) expressing phytase on the cell surface.

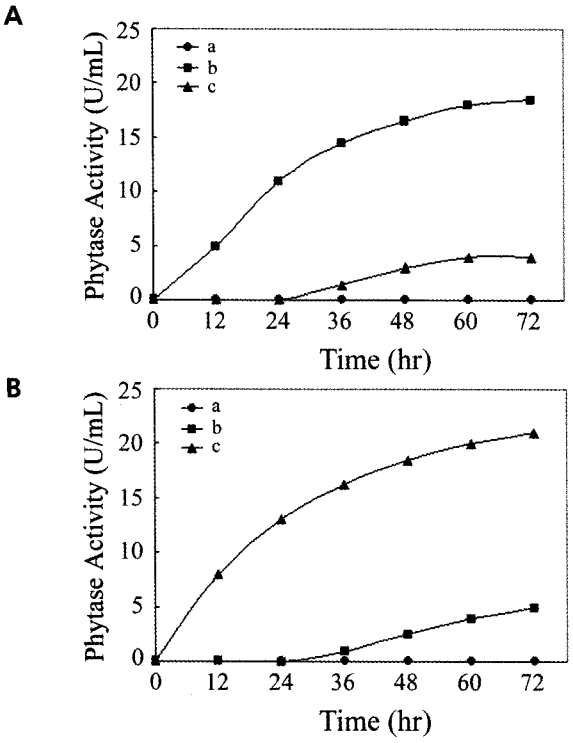

Fig. 3. Comparison of phytase activity (U/mL) in re-suspended cell pellet (A) and culture medium (B). a, control strain (*S. cerevisiae* 2805/pYEGPD); b, phytase-displaying strain (*S. cerevisiae* 2805/ pYEGPAG); c, phytase secreting strain (*S. cerevisiae* 2805/pGphyA-6) [4].

Liquid Assay of Phytase Expression

Cells were cultivated in YEPD medium for 72 h to confirm that phytases were retained on the cell surface. The phytase activities of both fractions, in cells and in the culture medium prepared as described in materials and methods, were measured. As shown in Fig. 3, the strain surface-displaying phytase showed high levels of enzyme activity (18 U/mL) in the re-suspended cell fraction (A), and the strain which secreted phytase to culture medium showed low levels (5 U/mL), and the control plasmid-harboring strain showed no phytase activity. In contrast, the phytase activity in the culture medium was detected highest from the phytase-secreting strain; the phytase activity of this strain was estimated to be 21 U/mL (B). These results correlate well with the plate assay, suggesting that recombinant PhyA functioned enzymatically, and is appropriately anchored and displayed on the cell surface of recombinant yeast.

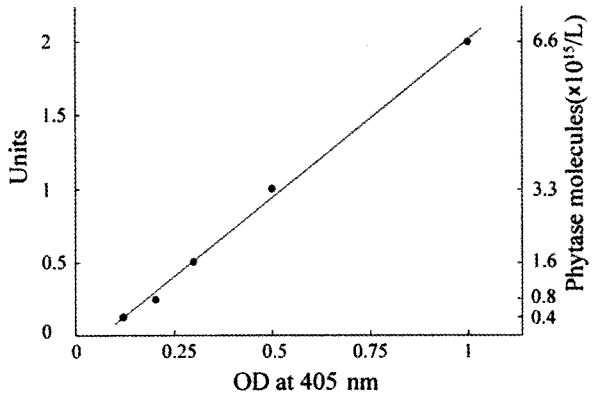

Fig. 4. The standard curve of phytase activity. The known amounts of purified phytase was prepared, and the phytase activity was measured with a colorimeter under the conditions described in materials and methods.

Quantification of Phytase

The number of phytase molecules displayed on the cell surface was calculated by measuring the phytase activity of cell pellet suspended in saline solution by a colorimeter, as similar procedure reported by Shibasaki *et al.* [20]. A standard curve was prepared by using known amounts of purified phytase isolated from *Aspergillus* (Sigma), and shown in Fig. 4. The following formula was obtained from the standard curve and used for calculations: the number of phytase molecules displayed per cell = RU × $3.3 \times 10^{15}/2.1 \times 10^{11}$, where RU (relative unit) is an arbitrary unit measured with a colorimeter under the conditions described in materials and methods, the 3.3×10^{15} indicates the deduced number of standard phytase molecules at $OD_{405} = 0.5$, and 2.1×10^{11} indicates the counted number of yeast cells per liter when the phytase activities showed $OD_{405} = 0.5$. When the RU was calculated as one, the number of phytase molecules displayed on the surface of one cell was determined to be approximately 16,000 molecules. This result almost agrees with that of the yeast strain which displayed EGFP [20], but the number of foreign protein molecules expressed on the cell surface of yeast is known to depend on the copy number of the 2-μ plasmid maintained in the cell and the size of the target protein [20].

Yeast-based systems are known to successfully display large protein molecules; these systems not only display single-subunit proteins, but also hetero-oligomeric multi-subunits. Therefore, these systems are widely applicable for cells displaying enzymes for bioconversion, antibodies and protein receptors for analytical applications and bio-separation, and combinatorial libraries for screening molecules with high binding affinity [21-24]. In addition to these applications, the advantages of the GRAS status of *S. cerevisiae* is very promising for use in bioindustrial processes applied to foods, feed additives, alcoholic beverages, and medicines. 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 cell surface of yeast, 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. Together with other previously studied phytase-producing yeast strains [4], the effects of dietary and waste treatment of the phytate-degrading yeasts in animal feeds and waste, respectively, will be investigated further in future studies.

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REFERENCES

[1] Nelson, T. S., T. R. Shieh, R. J. Wodzinski, and J. H. Ware (1971) Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. *J. Nutr.* 101: 1289-1293.

[2] Wodzinski, R. J. and A. H. Ullah (1996) Phytase. *Adv. Appl. Microbiol.* 42: 263-302.

[3] Han, Y., D. B. Wilson, and X. G. Lei (1999) Expression of an *Aspergillus niger* phytase gene (*phy*A) in, *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 65: 15-18.

[4] Lim Y. Y., E. H. Park, J. H. Kim, S. M. Park, H. S. Jang, Y. J. Park, S. Yoon, M. S. Yang, and D. H. Kim (2001) Enhanced and targeted expression of fungal phytase in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* 11: 915-921.

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

[6] Kim, C. H., K. J. Rao, D. J. Youn, and S. K. Rhee (2003) Scale-up of recombinant hirudin production from *Saccharomyces cerevisiae*. *Biotechnol. Bioprocess Eng.* 8: 303-305.

[7] Cha, K. H., M. D. Kim, T. H. Lee, H. K. Lim, K. H. Jung, and J. H. Seo (2004) Selection of optimum expression system for production of kringle fragment of human apolipoprotein(a) in *Saccharomyces cerevisiae*. *Biotechnol. Bioprocess Eng.* 9: 523-527.

[8] Colby, D. W., B. A. Kellogg, C. P. Graff, Y. A. Yeung, J. S. Swers, and K. D. Wittrup (2004) Engineering antibody affinity by yeast surface display. *Methods Enzymol.* 388: 348-58.

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

[10] Sambrook, J., E. F. Fritsch, and T. Maniatis. (1989) *Molecular Cloning: A Laboratory Manual.* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

[11] Shon, J. H., E. S. Choi, B. H. Chung, D. J. Youn, and J. H. Seo (1995) Process development of the production of recombinant hirudin in *Saccharomyces cerevisiae*: from upstream to downstream. *Proc. Biochem.* 30: 653-660.

[12] Park, E. H., Y. M. Shin, Y. Y. Lim, T. H. Kwon, D. H. Kim, and M. S. Yang (2000) Expression of glucose oxidase by using recombinant yeast. *J. Biotechnol.* 81: 35-44.

[13] Shin, Y. M., T. H. Kwon, K. S. Kim, K. S. Chae, D. H. Kim, J. H. Kim, and M. S. Yang (2001) Enhanced iron uptake of *Saccharomyces cerevisiae* by heterologous expression of a tadpole ferritin gene. *Appl. Environ. Microbiol.* 67: 1280-1283.

[14] Park, S. M., A. Y. Mo, Y. S. Jang, J. H. Lee, M. S. Yang, and D. H. Kim (2004) Expression of a functional human Tumor Necrosis Factor (hTNF)-α in yeast *Saccharomyces cerevisiae*. *Biotechnol. Bioprocess Eng.* 9: 292-296.

[15] Kim, M. J., T. H. Kwon, Y. S. Jang, M. S. Yang, and D. H. Kim (2000) Expression of murine GM-CSF in recombinant *Aspergillus niger*. *J. Microbial. Biotechnol.* 10: 287-292.

[16] Ge, L. and P. Rudolph (1997) Simultaneous introduction of multiple mutations using overlap extension PCR. *Biotechniques* 22: 28-30.

[17] Ito, H., Y. Fukuda, K. Murata, and A. Kimura (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153: 163-168.

[18] Heinonen, J. K. and R. J. Lahti (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal. Biochem.* 113: 313-317.

[19] Yoon, S. J., Y. J. Choi, H. K. Min, K. K. Cho, J. W. Kim, S. C. Lee, and Y. H. Jung (1996) Isolation and identification of phytase-producing bacterium, Enterobacter sp.4, and enzymatic properties of phytase enzyme. *Enzyme. Microb. Technol.* 18: 449-454.

[20] Shibasaki, S., M. Ueda, T. Iizuka, M. Hirayama, Y. Ikeda, N. Kamasawa, M. Osumi, and A. Tanaka (2001) Quantitative evaluation of the enhanced green fluorescent protein displayed on the cell surface of *Saccharomyces cerevisiae* by fluorometric and confocal laser scanning microscopic analyses. *Appl. Microbiol. Biotechnol.* 55: 471-475.

[21] Ueda, M. and A. Tanaka (2000) Cell surface engineering of yeast: construction of arming yeast with biocatalytst. *J.*

Biosci. Bioeng. 90: 125-136.
[22] Kondo, A., H. Shigechi, M. Abe, K. Uyama, T. Matsumoto, S. Takahashi, M. Ueda, A. Tanaka, M. Kishimoto, and H. Fukuda (2002) High level production from starch by a flocculent *Saccharomyces cerevisiae* strain displaying cell surface glucoamylase. *Appl. Microbiol. Biotechnol.* 58: 291-296.
[23] Fujita, Y., S. Takahashi, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda, and A. Kondo (2002) Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. *Appl. Environ. Microbiol.* 68: 5136-5141.
[24] Boder, E. T. and K. D. Wittrup (1997) Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* 15: 553-557.

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